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STUDIES OF THE KINETOCHORE IN *ELEOCHARIS MACROSTACHYA* BRITT.[1]

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INTRODUCTION

Historically the first observations of apparently "non-localized" or "diffuse" kinetochores or centromeres were made in plants. In the work of Horne (1930) on the fungus *Spongospora* sp. (Phycomycete) obtained from potato, he reported at metaphase of mitosis the chromosomes showed a peculiar disposition with respect to the spindle axis and at anaphase there was a parallel disjunction of the chromosomes. Geitler (1930), who investigated the green alga *Spirogyra crassa* (Conjugatae), observed in the somatic divisions a parallel anaphasic disjunction of the chromosomes (also cf. Godward, 1950). King (1953) also observed this phenomenon in Desmids. Other workers, (literature reviewed by Lima-de-Faria, 1949), have observed in the animal kingdom (Hemiptera, Lepidoptera, Odinata, Acari and Scorpinidae) the existence of "non-localized" or "diffuse" kinetochores.

In the case of *Ascaris megalocephala* it should be mentioned that the cells which eventually give rise to gonial tissue have undiminutive chromosomes (Boveri, 1904). Bonnevie (1913) observed "traction cones" on the poleward surfaces of these long metaphase chromosomes. Lima-de-Faria (1949) and White (1936) considered these chromosomes to have multiple kinetochores. Schrader (1935), however considers the long chromosomes to be composed of many small localized kinetochoric chromosomes joined end to end.

Prior to the investigations of Malheiros, Castro and Camara (1947), the existence of a non-localized kinetochoric system was unknown in higher plants. During their routine investigations on the chromosome numbers of species in the Juncaceae from Portugal, they obtained a very low chromosome count in *Luzula purpurea* Link, (2n=6). This low chromosome count enabled these workers to observe the chromosomes with minimal obstruction. Several peculiarities were noticed with regard to chromosome morphology and be-

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havior during mitosis and meiosis. The chromosomes were of irregular contour and no constrictions could be found. Examination of different metaphase plate configurations revealed the chromosomes were variable in width and length. it was also noticed here that the chromosomes were closely associated with each other with special reference to their extremities. These observations led the workers to hypothesize the existence of a "matrix" substance that not only surrounds the chromosomes but which is fluid enough to flow over them, from one chromosome to another, even when they are slightly separated.

Malheiros, et al, also report that when examining longitudinal sections of cells undergoing karyogomy metaphase chromosomes appear as a straight colored band. At anaphase the daughter chromosomes migrate parallel to each other towards the poles. When these chromosomes approach the poles they become cresent-shaped; viz. their extremities bend slightly in the direction of their respective pole.

During meiosis, Malheiros, et al, reported that equational division precedes reduction division (="post reduction division"). The formation of quartet of microspore nuclei within a common exine substantiates the findings of Wulff (1939) in other plants of the Juncaceae.

In order to test the hypothesis of a non-localized kinetochoric system Castro, Camara and Malheiros (1949) exposed living material of *Luzula purpurea* to X-rays. Some small fragments resulting from this X-ray exposure did not migrate to the poles with the other larger chromosomal fragments. These small bodies were interpreted to be pieces of "matrix" substance and the normal migration of the larger fragments supported their belief of a non-localized or "diffuse" kinetochore chromosomal system.

La Cour (1953) also exposed the spikelets of *Luzula purpurea* to X-rays, and considered the small non-migrating fragments as chromatic substance. He suggests then that a "diffuse" kinetochoric system is non-existent in this species, but is instead "polycentric" (=multiple kinetochores or centromeres). LaCour's findings, with the exception of this one feature, are in accord with those recorded by Malheiros, et al, and Castro, et al.

Håkansson (1954) analyzed X-rayed and untreated spikelets of *Eleocharis palustris* subsp. *vulgaris* in order to investigate the possibility of a "diffuse" kinetochoric system in the family Cyperaceae. He suggests that either a "diffuse centromere" or "polycentric" condition is present in the chromosomes of this species. He was unable, however, to demonstrate with reasonable certainty "post reduction division" in meiosis. In a recent publication Håkansson (1958) has supplemented his previous X-irradiation investigations on *Eleocharis palustris* and an additional species, *E. mamillata,* and considers the chromosomes to be "holocentric" or diffuse centromeric in nature on the basis of dividing and migrating chromosomal fragments.

The writer of this paper follows the terminology pertaining to the kinetochore as outlined by Lima-de-Faria (1949). There are three basic categories; viz. 1. the localized kinetochore known in most

plants and animals; 2. the diffuse kinetochore known among some of the organisms listed by him; and 3. the apparent multiple kinetochore in *Ascaris megalocephala*. The term "non-localized" kinetochore is used by Lima-de-Faria to denote a kinetochore which is not localized but may or may not be of the diffuse type.

The objective of this investigation was to elucidate the nature and if possible the location of the kinetochore in chromosomes of *Eleocharis macrostachya* Britton.

MATERIALS AND METHODS:

Living rhizomes of *Eleocharis macrostachya* Britton were collected in San Diego County, California during the month of August 1957. They were forwarded by mail in polyethylene plastic bags to the University of Minnesota greenhouse, Minneapolis, Minnesota. Herbarium specimens were not collected in the field. Instead, clones were cultivated in the greenhouse under incandescent lamps during the winter months, and upon flowering herbarium sheets were prepared for deposition in the University of Minnesota herbarium (my label, No. 57-15).

Root tips prepared for sectioning were killed and fixed for 24 to 48 hours in Karpechenko's modification of Navashin's fluid. The root tips were then washed in gently running tap water for fifteen minutes and were then dehydrated by passing through a butyl alcohol series, embedded in "Tissuemat" (56-58.5° C.) and sectioned at 6μ or 10μ. Sections were stained with Safranin "O" with and without a Fast Green counterstain.

Root tips harvested for squashing were killed and fixed in propionic acid absolute alcohol (1:3) for 18 to 24 hours at 0° to 4° C. The root tips were briefly rinsed in two changes of distilled water prior to staining in order to wash away any reaction products formed by the two reagents in the fixative which might interfere with the staining of the chromosomes. Three staining schedules were investigated in order to establish which would yield the most favorable preparations.

Root tips were hydrolyzed in lN HCl for 25 minutes at 60° C. and then rinsed in distilled water before being stained in Schiff's reagent for three hours. The Feulgen stained material was transferred to a vial containing 45% acetic acid, stored here if necessary in the dark, and then finely chopped with a razor blade and squashed under a No. 1 cover glass on microscope slides. The Feulgen technique yielded the most favorable preparations with regard to well separated and spread cells having excellent chromsomal stain.

Although aceto-carmin was also employed for staining chromosomes in the root tips it was found that these preparations were of inferior quality with respect to consistent chromosomal stainability, cellular separation and spreading as compared to those prepared by the Feulgen technique. The difficulty encountered with the aceto-carmin preparations involved obtaining the correct time of treating the root tips in Warmke's fluid for the purpose of loosening the mid-

dle lamella without unduly interferring with the staining reaction of the chromosomes.

Aceto-orceine preparations were made of root tips according to Tjio and Levan (1950), and the reaction of this stain was more favorable and consistent than those prepared with aceto-carmin. However, in making these orceine squashes difficulty was likewise encountered with respect to obtaining well separated and spread cells. A manipulation of this staining schedule followed by the longitudinal slicing of the root tip in order to separate the meristem from the epidermis by teasing should prove to yield more favorable results.

In order to study microspore mitosis flowering heads were harvested between 2:00 p.m. and 4:00 p.m. and killed and fixed in propionic acid alcohol (1 pt. propionic acid to 3 pts. 95% ethyl alcohol) for 24 to 48 hours at 0° C. to 4° C. and prepared for microscopic examination according to Ehrlich (1958). Treatments with propionic acid absolute alcohol (1:3) were compared with the above mentioned fluid concentration, but no apparent difference in the fixation quality was recognized. The microspore chromosomes were stained with aceto-carmin.

The penetration of fixing fluids were in all cases facilitated by means of mechanical evacuation.

Observations were made with a Bausch & Lomb microscope fitted with a 95X "Fluorite" oil immersion objective (N.A.=1.30) and a 10X "Hyperplane" ocular. The microscope was fitted with a B. and L. "Achromat" substage condenser (N.A.=1.40). Critical observations were made with the microscope slides oiled to the condenser. Illumination was obtained with a Leitz "Microlux" microscope lamp.

OBSERVATIONS:

Root Tip Mitosis: Prophase chromosomes in squashed cells were observed to have definite Feulgen negative regions. In most cases immediately on both sides of these Feulgen negative regions there was observed a Feulgen positive reaction. The Feulgen positive reaction at these points was more intense than the remaining positively stained regions of the chromosomes. The Feulgen negative regions, depending on the chromosome involved, were located at various points along the chromosome. These points ranged from median, or nearly median, to subterminal in position.

Some of the Feulgen negative regions observed in prophase may represent potential kinetochores. The intense Feulgen positive chromomere-like structures immediately on either side of these Feulgen negative regions may represent heterochromatin. On one prophase chromosome three such Feulgen negative regions were observed (cf. Fig. 2). It is possible that more of these regions could have existed but were not observed or recognized.

One well spread cell was diagnosed as containing late prophase-prometaphase chromosomes. Many of the sister chromatids were visibly separated from each other. The sister chromatids were held tenuously together at various points along the inner surfaces giving the chromatids a definite "zigzag" appearance. Occasional projections or

delicate processes essentially transverse to the main chromosomal axis were observed.

Metaphase chromosomes in squashed cells were observed to have intense Feulgen positive chromomere-like structures opposite each other at the periphery of each chromatid. Each chromomere-like structure was approximately 0.5μ in diameter. These structures did not change markedly in their position during careful optical sectioning with the microscope. By chromosomal contraction the Feulgen negative regions and their associated intensely staining chromatin observed in prophase may be represented as the chromomere-like structures found on metaphase chromosomes. In the populations of chromosomes analyzed at metaphase there were two to five chromomere-like structures per chromosome.

Chromosome counts were determined at metaphase by the squashing and sectioning techniques. Both methods enabled 20 somatic chromosomes to be observed and measured. In their most contracted condition the chromosomal length categories from sectioned material were found to be as follows: one extra long pair, ca. 4 to 4.5μ; two long pairs, ca. 3 to 3.5μ; two medium pairs, ca. 2 to 2.5μ; three small pairs, ca. 1.75μ; and two extra small pairs, ca. 1.5μ (cf. Fig. 1a, 1b). Chromosome lengths determined from squashed cells were approximately one to two micra longer than those from sectioned material, but the same categorical lengths were recognized. The variation in chromosomal lengths encountered between the two techniques has been attributed to the characteristics of the two fixing fluids utilized plus the effects of infiltration, heat and embedding, etc.

No primary or secondary constrictions were found on the chromosomes. It was possible, however, in some instances to detect what appeared to be light staining bands on some of the chromosomes. It was not possible to identify with certainty the actual chromosomes involved except as to chromosomal size. There was one such band located medially or sub-medially on a small chromosome. Another small chromosome was seen to have a band situated sub-terminally at each end. There were two bands on a medium sized chromosome; one band was sub-terminal and the other median or sub-median. Similar observations have been made by Håkansson (1954) on chromosomes of *Eleocharis palustris subsp. vulgaris.*

The chromomere-like structures of metaphase chromosomes were observed to be slightly raised from each chromatid surface. The chromatids were visibly separated from each other in the regions opposite each chromomere-like structure in chromosomes diagnosed as entering anaphase. The above evidence is interpreted as being suggestive of polar forces acting upon these structures and it would appear then that these chromomere-like structures represent the attachment regions for spindle fibers.

In squashed cells equatorial views of what appeared to be metaphase configurations revealed in some instances the existence of small Feulgen positive hair-like projections on the poleward surface of the chromatids. These projections were approximately 0.5μ to 1.0μ long and ca. 0.25μ wide. It was possible in some instances to detect a small

chromomere-like structure on the order of ca. 0.25μ in diameter positioned directly off the tip of each projection. Most of the chromosomes were squashed on top of each other, but it was possible, however, to observe from two to four of these projections on chromosomes either isolated from the main chromosomal mass or at its periphery. These projections have also been observed to stain with both aceto-carmin and aceto-orceine (cf. Fig. 3).

It is suggested that a correlation exists between these projections and the previously mentioned chromomere-like paired structures. If five such projections were to have been observed this correlation would have a firmer foundation. Perhaps chromosomes with five projections were concealed in the clumped chromosomal mass. The tips of these projections may likewise represent the location of spindle fiber attachments. The projections themselves are interpreted as being chromonemata stretched under tension towards the poles and may be termed "traction cones." Chromosomal configurations such as these are interpreted as being in a state of transition between late metaphase and early anaphase.

In longitudinally sectioned material chromosomes were observed to be aligned parallel to the equatorial plate. Spindles were mostly broad and truncate. Evidence of chromosome stickiness was suggested in some anaphasic disjunctions (cf. Fig. 6). Daughter chromosomes appeared to separate parallel from each other in most instances. Some chromosomes appeared to separate with their ends slightly bent in the direction of their respective poles. This condition could be more readily detected in some chromosomes at middle anaphase (cf. Fig. 4). Two small pairs of chromosomes were observed to migrate towards the poles from oblique to somewhat parallel to the spindle fibers (cf. Fig. 5).

Metaphase chromosomes appeared very much contracted and stained intensely. No definite morphological characters like those observed in chromosomes from squashed cells were detected. It was possible, however, in some late prophase chromosomal configurations to observe the presence of dark staining paired chromomere-like structures near the surface of the chromatids. A difference in staining intensity was also noticed along segments of the chromosomes having the same light staining bands characterized on chromosomes from squashed cells.

More than one spindle fiber was apparently attached to any one visible chromosome of a complement. Because of the large number of chromosomes present it was not possible to readily determine with any degree of accuracy the exact number of spindle fibers involved per chromosome.

In the populations of cells analyzed, in both squashed and sectioned material, various numbers of small nucleoli were observed. Generally there were four per cell, but as few as two and as many as five were readily visible. In some instances six nucleolar structures appeared to be distinguishable. In any given nucleus the nucleoli were not always the same size. Regardless of the number of small nucleoli present it appears that they all coalesce to form one large nucleolus prior to its

disappearance. Chromosomes lie in close proximity to the peripheral surface of this large nucleolus, and it was not possible to detect with certainty any satellites on the chromosomes. At telophase four daughter nucleolar structures were observed to be reorganized.

Microspore Mother Cells: Most cells observed containing pachytene chromosomes were of little value, because most of the chromosomes were hopelessly entangled. One well spread cell containing fairly well separated chromosomes was observed. The synapsed chromosomes were observed to have deeply staining chromomere-like structures situated along the chromosomes at different points. Some of these structures were paired linearly along the chromosomal axis. In another cell a chromosome extending out in the cytoplasm was observed to have what appeared as a kinetochoric structure located approximately 4μ in from the tip of the chromosome. Its architecture was morphologically similar to the pachytene kinetochore of *Agapanthus umbellatus* as recorded by Lima-de-Faria (1950, cf. Fig. 1), in that a single pair of chromomeres were bound on each side by localized dark-staining regions. One large nucleolus was observed associated with one synapsed pachytene chromosome. A small chromosomal segment extending beyond the nucleolar organizer region was interpreted as being a potential satellite.

Chronological stages of meiosis were investigated, but sufficient data concerning chromosomal morphology and segregation were not obtained.

Microspore Mitosis: The quartet of microspore nuclei were formed within the microspore mother cell wall. The nucleus nearest the anther wall undergoes mitosis to form the generative and tube nucleus. The chromosomes in the other three nuclei progress up to metaphase with the above mentioned nucleus. However, the chromosomes of these three nuclei were much smaller and they soon disappeared.

Microspore metaphase chromosomes are larger than those observed in somatic cells. Aceto-carmin yielded a haploid complement of ten well differentially stained chromosomes (cf. Fig. 7). There were three long chromosomes, ca. 8μ to 9μ; two medium, ca. 4μ to 5.5μ; three small, ca. 3μ to 3.5μ; and two extra small, ca. 2.5μ. Sometimes one of the large chromosomes would be slightly longer than the other two, and occasionally this situation would be reversed. On one occasion a small satellite structure was observed on the end of each chromatid of one of the large chromosomes (cf. Fig. 7).

Definite deep staining chromomere-like paired structures were observed on these chromosomes similar in size and location to those found on chromosomes from somatic cells. These structures were observed to occupy a position near the poleward surface of the chromatids. In the most favorable preparations some of these structures were pulled slightly away from the chromatid surface suggesting the activity of polar forces. Some chromatids were separated from each other directly between these paired structures. A configuration such as this was interpreted as being in a state of late metaphase-early anaphase transition. A comparison of both somatic and microspore chromosomes indicated great similarities between the two with

respect to location of these chromomeric-like structures. Also light staining chromosomal segments were recognized in the shape of bands similar to those found in somatic chromosomes (cf. Fig. 8). A pair of chromomeric-like structures were observed in the center of a light staining region of one medium sized chromosome. Each structure was raised above the surface to its respective chromatid (cf. Fig. 9). This feature was also observed in one small somatic chromosome, but the structures were at the chromatid periphery and not extending beyond.

DISCUSSION

This investigation has established a qualitative correlation between paired dark staining chromomeric-like structures occurring in chromosomes of the pro-,meta- and anaphases of diploid nuclei and metaphase of haploid nuclei. These structures are interpreted as being intimately associated with the attachment of the spindle fibers to the chromosomes. It is suggested that the chromosomes of this species have multiple kinetochores.

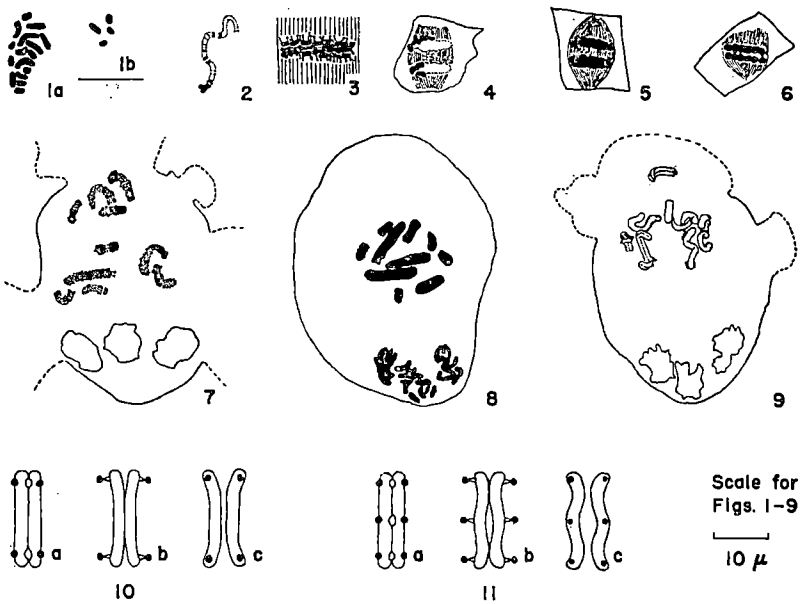
The "zigzag" configurations of chromatids of prometaphase chromosomes later followed by the formation of traction cones during the metaphase-anaphase transition suggests the classical anaphasic disjunction of chromosomes in *Ascaris megalocephala*. Östergren's (1949) "kinetic stripe" hypothesis of *Luzula purpurea* chromosomes does not seem applicable to those of *Eleocharis macrostachya*. However, his alternative scheme based on chromomeres with "one-sided" kinetic properties may be involved. In order to incorporate such a system in chromosomes of *Eleocharis macrostachya* it would be desirable to separate kinetic chromomeres from non-kinetic (or akinetic) chromomeres.

Since no special cycle of division was recognized between the dark paired chromomere-like structures and chromatids of chromosomes during this investigation the diagram of Lewis and Scudder (1950, Fig. 15(i)) appears applicable to this species. For a discussion of the special division cycle of kinetochores consult Lima-de-Faria (1953, 1955).

Chromosomes with their ends bent towards the poles suggest subterminal or mostly sub-terminal kinetochoric loci. Prakken and Müntzing (1942) and Östergren and Prakken (1946) observed the terminal segments of a pair of meiotic chromosomes from an inbred line of rye (*Secale cereale*) to exhibit the property of active mobility common to normal kinetochores. The bending of chromosomes in *Eleocharis macrostachya* with kinetochores involving more chromosomal segments than their extremities may perhaps be due to two suggested factors. First, all kinetochores may not be attracted to the poles with equal force at the same time. This may involve the differences in the intrinsic property of mobility of each kinetochore and/or the influences of polar forces acting upon these kinetochores. Second, the chromosomes might bend in the center because of resistance brought about by their forward motion through the cytoplasm. It would be theoretically possible to find chromosomes with their middle regions bent

towards the poles. This would be brought about by chromosomes having stronger mobile kinetochores occupying the middle region of the chromosome. At present no such chromosomal configurations have been observed to support this hypothesis in chromosomes of *Eleocharis macrostachya* during the course of this investigation.

Chromosomes of *Eleocharis macrostachya* harboring chromomere-like structures in their light staining regions are highly suggestive of the "spindle spherules" of Schrader (1936, 1939). They are also comparable to the Feulgen positive "centromeric chromomeres" de-



Figs. 1-6. *Eleocharis macrostachya* root tip mitosis. Fig. 1a-1b. Polar view of metaphase plate with 20 chromosomes drawn from serial sections. Fig. 2. Prophase chromosome from squashed cell. Fig. 3. Equatorial view of late metaphase-early anaphase transition chromosomes from squashed cell. Figs. 4-5. Equatorial view of sectioned cells depicting middle anaphase chromosomes at periphery of main chromosomal mass. Fig. 6. Equatorial view from sectioned cell indicating stickiness of chromosomes at anaphasic disjunction.

Figs. 7-9. *Eleocharis macrostachya* microspore mitosis. Fig. 7. Microspore squash containing genome of 10 chromosomes indicating relative position of dark staining chromomere-like structures. Fig. 8. Microspore squash with complete genome indicating relative position of light staining bands. Fig. 9. Microspore squash with one large chromosome with projections perpendicular to chromosomal axis and a small chromosome with chromomere-like structures slightly pulled away from the surface of each chromatid.

Figs. 10-11. Interpretation of chromosomes with two and three kinetochores respectively during late metaphase-early anaphase transition by semidiagrammatic representation. Figs 10–11a. Late metaphase chromosomes with chromomere-like structures slightly raised from the surface of the chromatids. Figs. 10b-11b. Metaphase-anaphase transition chromosomes with "traction cones" bearing spindle spherules. Figs. 10c-11c. Anaphasic disjunction of daughter chromosomes. The spindle spherules are no longer separated from the main body of the chromosome.

scribed in *Agapanthus umbellatus* pachytene chromosomes by Lima-de-Faria (1950, cf. Fig. 1).

The chromosomal projections ("traction cones") observed on chromosomes in metaphase plates may represent the first visible evidence of active mobility initiating anaphasic disjunction of the daughter chromosomes. Their presence is all too frequent to be interpreted as undesirable fixation artifacts. They stain with all the nuclear reagents thus far employed in this investigation. They are interpreted as evidence of anaphasic stretch of chromonemata bearing apically a spindle spherule. The number of kinetochores per chromosome is based on the resolution attained in this study. It is possible that the actual number per chromosome is greater.

SUMMARY

Chromosome numbers of 2n=20 and n=10 have been observed from the nuclei of root tips and microspores respectively in *Eleocharis macrostachya* Britton. Dark staining chromomere-like structures located at the periphery of chromatid surfaces have been interpreted as being intimately associated with the attachment of spindle fibers to the chromosomes. Root tip chromosomes in the late metaphase-early anaphase transition were observed to have chromosomal projections interpreted as "traction cones" bearing spindle spherules directed towards their respective pole. On the basis of the material analyzed it is suggested that the chromosomes of this species have multiple kinetochores.

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