An Acetocarmine Staining Procedure for Chromosome Banding Studies of Immature Pollen in Triticeae

A. Arzani[1], M. Poursiahbidi[1] and S. E. Mortazavi[1]

ABSTRACT

Studies of postmeiotic chromosomes have been impeded by the thick exine of immature pollen grains. Chromosome staining of immature and adult pollen grains through conventional acetocarmine procedure is tedious and often leads to unsatisfactory results. An acetocarmine stain, used in conjugation with Carnoy's solution as fixative for 72 hrs or until the chlorophyll-containing tissues of spike became colorless, were successfully used to stain chromosome bands and nucleoli of the immature pollen grain in *Aegilops cylindrica*. Mitosis of immature pollen exhibited several remarkable features, one being the distinguishable haploid chromosome set of immature pollen. Only one of two nucleolus organizer regions of *Ae. cylindrica* produced nucleolus at the prophase of first pollen mitosis. An acetocarmine-banded karyotype with only minor variations in the acetocarmine-banding pattern was revealed within and between plants of the accession *of Ae. cylindrica*. Some of banding patterns in the chromosome complement was identical to marker C-bands or accession-specific bands in corresponding genome in *Ae. caudata* (CC) or *Ae. squarrosa* (DD). The reliability of the acetocarmine banding for Triticeae chromosome identification is discussed.

Keywords:Immature pollen mitosis, Acetocarmine, Chromosome banding, *Aegilops cylindrica*.

INTRODUCTION

Chromosome identification has been traditionally dependent on morphological characteristics such as size, arm ratio and secondary constrictions at metaphase of mitosis. However, the identification of somatic chromosomes in some plant species was hindered by their low variation of sizes or high numbers. Duplicate number of each chromosome in the metaphase stage of mitosis increases the restriction of the chromosome identification. In addition, chromosome size and arm ratio data from mitosis or meiosis using an aneuploid series like that utilized in common wheat *(Triticum aestivum* L.) can not be reliably used for the identification of somatic chromosomes (Larson and Kimber, 1973).

In spite of overwhelming evidence of the usefulness of chromosome banding techniques in chromosome identification, their application to plant materials were limited in extent because of the technical procedure required is considered too tedious to be acceptable for large numbers of cells (Sybenga 1992). Furthermore, Zurabishvili *et al*. (1978) claimed that individual chromosome banding patterns cannot be used to deduce homologous and homoeologous chromosome relationship among cultivars and species in the wheat group.

Valuable morphological criteria in pachytene of meiosis are centromere, chromomeres, telomeres, nucleolus organizer region (NOR) with nucleolus attached, knobs and possible distinction into heterchromatin and euchromatin. However, the

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[1] Department of Agronomy and Plant Breeding, College of Agriculture, Isfahan University of Technology, Isfahan-84156, Islamic Republic of Iran.

pachytene analysis is particularly rewarding in species with short chromosomes as well as low number of chromosomes. In pachytene stage of meiotic prophase, each satellited chromosome produces a couple of nucleoli attached to each of the homologous partner (Schulz-Schaeffer 1980).

In several instances, the second pollen mitosis in pollen tubes were reported as favorable material for chromosome analysis, notably because of the haploid chromosome number (Conger 1953; Kwack and Kim 1967). Kindiger and Beckett (1985) reported a hematoxylin procedure for staining chromosomes of maize pollen grain. In addition, the first pollen mitosis at metaphase stage was used in *Scilla sibirica (Liliaceae)* to study the relationship between the duration of mitotic cycle and chromosome length (Fivawo and Rees 1985). However, the use of these procedures has been limited, mainly due to technical complications, insufficient availability of pollen in right stage and sometimes the compactness of metaphase plate (Sybenga 1992).

Therefore, any technique which would readily permit the identification of individual chromosomes would greatly advance cytogenetics of the plant species. This stimulated the authors of the present study to attempt to use immature pollen-mitosis for characterization of 14 single chromosomes (haploid set) of *Aegilops cylindrica.*

MATERIALS AND METHODS

The optimum procedure for acetocarmine staining of immature anthers at mitosis stage only is described here. An accession of *Ae. cylindrica* from West Iran was used in the study. The spikes with anthers in immature pollen stage were harvested, and fixed in Carnoy's solution (6:3:1 of ethanol: chloroform: glacial acetic acid, respectively) for 72 hrs or until the chlorophyll-containing tissues of spike became colorless. The anthers were then placed in acetocarmine stain for 72 hrs. One or two anthers were then placed in a drop of acetic acid on a slide, and a nick

made in anther wall with a laboratory needle. Coverslip was placed on the immature pollen released through rolling the rod of needle on the anther, and after eliminating anther debris. Eventually, the preparation was smeared by placing between filter paper and exerting a slight press by fingers and observed under light microscope. Chromosome images were then taken using an Olympus BH2-RFCA microscope equipped with a C35 AD4 Olympus camera. The acetocarmine staining was carried out in 10 plants of the accession of *Ae. cylindrica.* Chromosome measurements were made on 20 complete mitotic prophases for establishing an idiogram of *Ae. cylindrica.*

Measurment of mitotic chromosomes in immature pollens followed Friebe (1992a). The position of the primary constriction (centromere) was estimated using long arm to short arm ratio (r = L/S). The relative chromosome length, arm ratio and C-banding patterns of C genome *of Ae. caudata* (Friebe *et al*. 1992a), and D genome of *Ae. squarrosa* (Friebe *et al*. 1992b) were used to differentiate C and D genomes of *Ae. cylindrica,* respectively. The relative chromosome length and arm ratio were used to compare the results with those reported earlier by Friebe *et al*. (1992a) for C-genome, Friebe *et al.* (1992b) and Gill (1987) for D-genome in *T. aestivum* and *Ae. squarrosa,* respectively. Since homeologous relationship of chromosomes in C genome has not been established yet, these chromosomes were lettered from A to G, following the nomenclature system used by Teoh and Hutchinson (1983).

RESULTS

Mitosis of immature pollen exhibited several remarkable features. The distinguishable haploid chromosome set of immature pollen was the most outstanding. Figure 1 shows 14 single chromosome from the chromosome complement of *Ae. cylindrica* in metaphase stage of the first pollen-mitosis. *Aegilops cylindrica* possesses a chromosome number
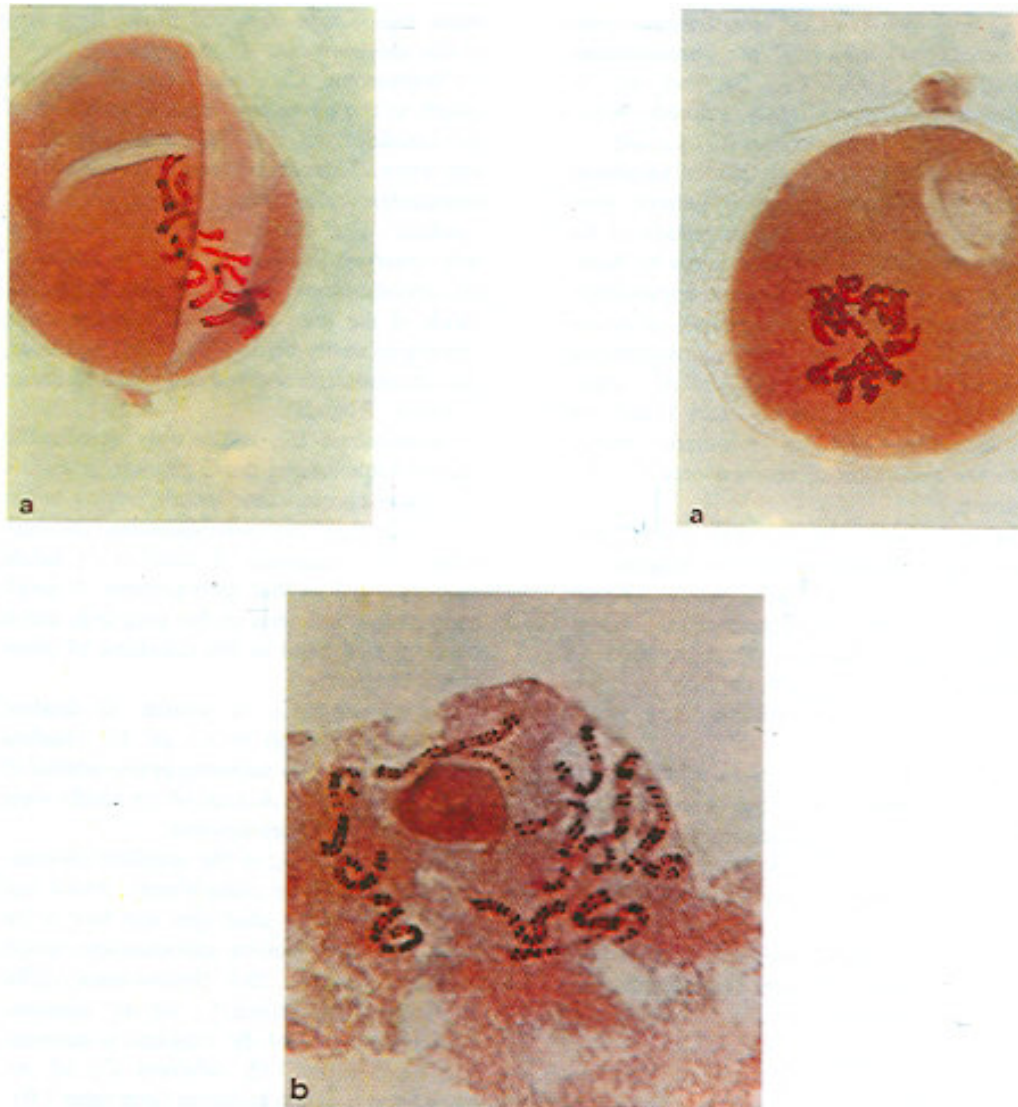
Figure 1. Haploid set of chromosomes in immature pollen mitosis of *Ae.cylindrica* (a) metaphase stage, (b) acetocarmine- banded prophase stage.

of 2n=28 (CCDD) and carries two satellited chromosomes of which one belongs to each of C and D genomes. Only one of the NORs produced nucleolus at prophase of first pollen mitosis (Figure 1b). The observation of nucleolus and chromosome associated with nucleolus was another feature of the first pollen mitosis. This kind of mitosis also produced prophasic chromosome bands

(Figure 1b and Figure 2). The chromosome complement consists of six more or less submetacentric chromosomes, one acrocentric chromosome belonging to C genome, as well as one metacentric chromosome, and six submetacentric chromosomes belonging to D genome. Primary constrictions (centromeres) of majority of chromosomes including C_A, C_B, C_C, C_F and all D-

chromosomes were lightly stained. While the remainder chromosomes (C_D, C_E and C_G) were stained darkly. Only minor variations in the acetocarmine-banding pattern were observed within and between plants of the accession *of Ae. cylindrica.* Some of banding patterns in the chromosome complement were identical to marker C-bands or accession-specific bands in corresponding genome in *Ae. caudata* (CC) or *Ae. squarrosa* (DD). All of the C- and D-genome chromosomes revealed telomeric bands, with the exception of chromosome C of C-genome.

Table 1 shows chromosome lengths, arm ratios, and relative chromosome lengths of C and D genomes *of Ae. cylindrica,* and Figure 3 shows an idiogram of the banded chromosomes *of Ae. cylindrica,* which summarizes the results of acetocarmine late prophase stage of first pollen mitosis used in the study.

Chromosome C_A, differs in relative chromosome length to the corresponding chromosome of the C genome in *Ae. caudata,* but it is similar with respect to arm ratio. A secondary constriction and a satellite were present in the distal region of the short arm. Two bands are present on each of the satellite and short arm. Nine bands were observed on the long arm.

Chromosome C_B, differs in relative chromosome length, being longer in the long arm than the corresponding chromosome of *Ae. caudata.* Chromosome C_B of *Ae. cylindrica* is much more submetacentric (arm ratio 4) than C_B of *Ae. caudata* (arm ratio 2.9). Chromosome is the largest in total of 17 bands.

Chromosome Cc, is almost similar in relative chromosome length and arm ratio to that of *Ae. caudata,* a submetacenrtic chromosome with five bands at the short arm and nine bands at the long arm. This was the only chromosome in the complement that did not show a band at the telomere of short arm. A relatively large interstitial euchromatic region was observed in the long arm of this chromosome.

Chromosome C_D, is similar in relative length to the corresponding chromosome of *Ae. caudata,* but it differs with respect to arm ratio. This chromosome is more submetacentric (arm ratio 4) than D of *Ae. caudata.* The largest euchromatin region was observed adjacent to the centromere of this chromosome. The distal two large dark bands in the long arm of this chromosome could also easily be distinguished. However, this chromosome showed the lowest number of bands (7 bands).

Chromosome C_E, is the only acrocentric chromosome among the C genome. It differs in relative chromosome length, having larger long arm than the corresponding chromosome of *Ae. caudata.* A total of 14 bands was observed in this chromosome. A small band at the telomere of the long arm and a medium size band at the telomere of short arm was observed.

Chromosome C_F, is similar in relative chromosome length to C_F of *Ae. caudata* which, however, is submetacentric instead of being acrocentric. A total of 16 bands were observed in this chromosome.

Chromosome C_G, is the smallest chromosome of the whole complement, which had three bands in the short arm and five in the long arm. The relative chromosome length and arm ratio of this chromosome differ from the chromosome C_G *of Ae. caudata.* Chromosome C_G of *Ae. caudata* is acrocentric (arm ratio 7.0), whereas C_G of *Ae. cylindrica* is submetacentric (arm ratio 1.9).

Chromosome 1D, differs in relative length and arm ratio from 1D of hexaploid wheat and *Ae. squarrosa.* A total of 13 bands were present at several interstitial regions and at the telomeres of both arms.

Chromosome 2D, differes in relative chromosome lengh from the corresponding chromosome of cultivated wheat, but it is identical to the corresponding chromosome of *Ae. squarrosa.* In *Ae. cylindrica,* this chromosome is slightly more submetacentric (arm ratio 1.6) than both *Ae. squarrosa* and *T. aestivum.* The largest euchromatic region was observed in the middle of the long arm.

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Table 1. Chromosome lengths, arm ratio (L/S) and relative lengths of mitotic prophase chromosomes of C- and D-genomes of *Aegilops cylindrica*.

C-genome: Chromosome	Length μm	Arm ratio	Relative length	D-genome: Chromosome	Length μm	Arm ratio	Relative length
C_A	10.2	1.5	13.1	1D	11.7	2.2	16.7
C_B	14.1	4.0	18.1	2D	11.7	1.6	16.7
Cc	12.4	2.2	16.0	3D	10.2	1.5	14.5
C_D	11.6	4.0	14.9	4D	8.5	1.9	12.1
C_E	12.4	6.8	16.0	5D	10.4	2.0	14.8
C_F	10.9	2.9	14.0	6D	7.8	1.2	11.1
C_G	6.1	1.9	7.8	7D	9.9	3.3	14.1

A total of 11 bands were detected in this chromosome.

Chromosome 3D, is identical in relative chromosome length to the corresponding chromosome of *Ae. squarrosa,* but it is smaller than 3D of *T. aestivum.* Chromosome 3D of *Ae. cylindrica* is slightly more submetacentric (arm ratio 1.5) than 3D in both bread wheat and *Ae. squarrosa* (arm ratio 1.4). The largest band was observed in both sides of the centromere in this chromosome.

Chromosome 4D, is shorter than chromosome 4D of both *T. aestivum* and *Ae. squarrosa,* but it is identical *to T. aestivum* with respect to arm ratio, chromosome 4D of *T. aestivum* being much more submetacentric than 4D of *Ae. squarrosa.* Three bands in the short arm and six bands in the long arm were observed in this chromosome.

Chromosome 5D, is identical in relative chromosome length to the corresponding one in cultivated wheat (*T. aestivum)* and *Ae. squarrosa,* but however, is slightly more submetacentric. This chromosome was the only satellited one in the complement that produced nucleolus (Fig. 1 c). This chromosome has a distally located secondary constriction and a small satellite in the short arm. A total of 13 bands were observed in this chromosome.

Chromosome 6D*,* is identical in relative chromosome length and arm ratio to chromosome 6D of *Ae. squarrosa,* but it differs from cultivated wheat with respect to being smaller and slightly more metacentric. This chromosome showed a total of nine bands.

Chromosome 7D, is similar in relative chromosome length to that of cultivated wheat, but it differs with respect to arm ratio. This chromosome revealed three bands in the short arm and ten bands in the long arm.

DISCUSSION

A method for identifying the C and D genome donors of *Ae. cylindrica* (CCDD) from *Ae. caudata* (CC) and *Ae. squarrosa* (DD) diploid progenitors was used by Johnson (1967). Similarly, in the present study, relative chromosome length, arm ratio and C-banding patterns of C genome of *Ae. caudata* (Friebe *et al.* 1992a), and D genome *Ae. squarrosa* (Friebe *et al*. 1992b) were used to differentiate C and D genomes of *Ae. cylindrica.* The karyotype and idiogram of the banded chromosomes of *Ae. cylindrica* (Figure 2 and Figure 3) clearly shows many bands are produced in the *Ae. cylindrica* chromosomes in prophase stage of the immature pollen mitosis.

The results clearly show that by using the simple aceto-carmine technique at prophase and prometaphase of immature pollen-mitosis, many bands are produced in the haploid-set of chromosomes of *Ae. cylindrica.* Likewise, Wang and Kao (1988) using *Vicia hajastana* plant material reported that G-banding in prophase and prometaphase chromosomes was of better
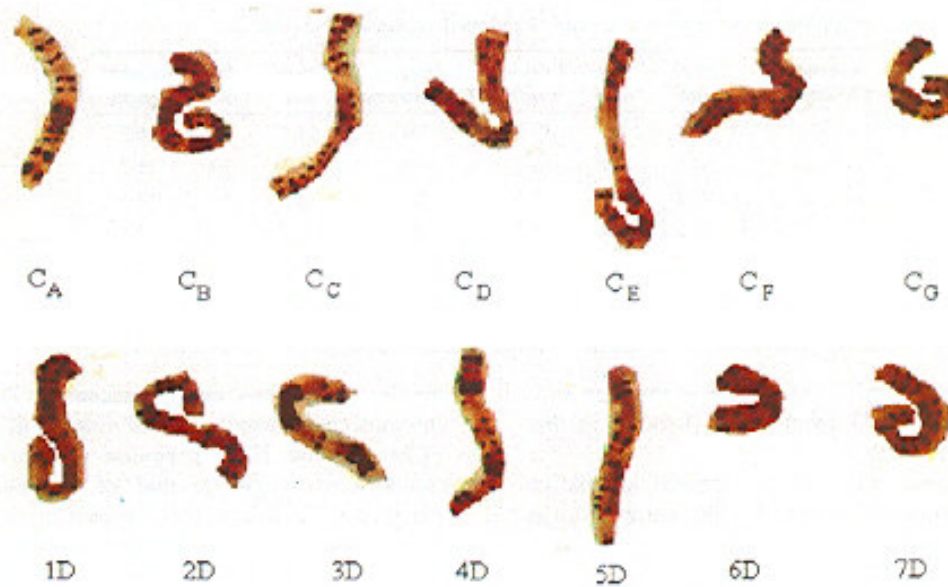
Figure 2. Acetocarmine-banded karyotype of *Ae. cylindrica,* C - genome and D-genome.

quality and more consistent than in metaphase chromosomes. They also observed the banding in metaphase chromosomes, after appearing as dot-like structures of different sizes , to be much more difficult to produce than in prophase chromosomes. Nevertheless, as Sybenga noted, the typical G-banding is not possible in plants. Wang and Kao (1988) and Yang and Zhang (1988) only observed a beaded appearance in prophase stage by using normal G-banding procedure. Yang and Zhang (1988) found a relationship between chromosome spirals and G-bands, and induced both G-band and macrocoils with the same procedures. Yang and Zhang (1988) have reported up to 20 bands per chromosome in several model plant species for chromosome studies viz *Secale cereale, Hordeum vulgare,* and *Vicia faba.* However, no reproducible method is available for G-banding analysis in plants (Gill *et al.* 1991). The authors suggest that more or less similar chromosome banding as the G-banding can readily be prepared by the simple aceto-carmine procedure applied to first pollen mitosis as in the present study.

This concept is being further examined, by comparing an acetocarmine banding procedure in root tip meristems, recently employed in our laboratory, with G-banding procedure in *Secale cereale.*

In the present study from two satellited chromosomes C_A and 5D, only the NOR of chromosome 5D produced nucleolus. Sybenga (1992) claimed that it is a very general characteristic of nucleoli to fuse when more than one per nucleus present, and in many cases the maximum number of nucleoli observed may be an indication of the ploidy level. Friebe *et al*. (1992a) found two NORs in *Ae. caudata* (CC), whereas, in our study only one NOR was observed in the C genome of *Ae. cylindrica.* Two hypotheses might be taken into account to explain this: the first is the differences from a phylogenetical point of view and the second is the suppression effect of D genome. Panayotov and Tsujimoto (1997) reported that chromosomes of *Ae. mutica* strongly suppressed the NORs in wheat chromosomes lB and 6B.

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Figure 3. Idiogram of *Ae. cylindrica*, (genomes C and D with acetocarmine bands shown in black).

For a reliable karyotype analysis, the cells must be flat, in a single layer, but yet complete, and often the last need cannot be realized with the first two having been met. Here a serious problem arises when the cell is flattened, the chromosomes are stretched, and the stretching is not homogeneous. Sybenga (1992) noted that some chromosome arms stretch relatively more than others, which causes intrinsic differences between non-homologous chromosomes as well as intrinsic differences between homologous ones. He also mentioned that extended stretches of chromosome with orcein leads to some chromosomal breakage.

On the other hand, the relatively simple acetocarmine technique reported here does not encounter the above mentioned problems, since only a slight pressure is required for spreading the microspores (immature

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pollens). The recommended acetocarmine technique is simple and reproducible. In prophase chromosomes, many bands are clearly visible (Figure 1 and Figure 2). The reproducibility of this procedure was confirmed using 'Chinese Spring' cultivar of common wheat (T. *aestivum)* (data not shown). In addition, it has several advantages as compared with the available techniques for chromosome identification, the advantages being:

1. Observation of only a single copy from the somatic chromosome complement, and hence the reduction of the chromosome complement to half, which in turn readily enables the identification of plant chromosomes.

2. Prophase-mitosis analysis of chromosomes can be used not only as identification of chromosomes but also as studies of polymorphism criteria of chromosome band and nucleolus activated-chromosome, within species as well as among species.

3. Selection of desirable adult plants (i.e. cytogenetic stocks such as aneuploids) based on identification of chromosome number of the immature pollen-mitosis.

4. Mitosis and meiosis studies can be conducted using same material, but in different stages.

5. Simultaneous discrimination between meiotic restituted microspores and non-meiotic restituted microspores in interspecific and intergeneric hybrids.

6. Molecular cytogenetic studies, using *in situ* hybridization, will be facilitated.

7. Study of B chromosomes can be accomplished.

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روش رنگ آمیزی استوکارمن برای مطالعه نواربندی کروموزومی دانه گرده نارس در تریتیسه

چکیده

مطالعات کروموزومی پس میوزی به لحاظ وجود لایه ضخیم بیرونی دانه های گرده نارس با مشکل مواجه بوده است. رنگ آمیزی کروموزوم های دانه گرده نارس یا بالغ با روش استوکارمن که به همراه محلول تثبیت کننده کارنوی به مدت ۷۲ ساعت و یا تا زمانی که بافتهای کلروفیل دار سنبله بیرنگ شوند، به طور موفقیت آمیزی برای رنگ آمیزی نوارهای کروموزومی و هستک های دانه گرده نارس در گیاه *Aegilops cylindrica* مورد استفاده قرار گرفت. میتوز دانه های گرده نارس چندین ساختار قابل توجه را نشان داد. فقط یکی از دو NOR ایژیلوپس سیلندریکا در پروفاز اولین تقسیم دانه گرده تولید هستک نمود. کاریوتیپ نواربندی استوکارمنی تغییرات جزئی در داخل بوته و میان بوته های مختلف ایژیلوپس سیلندریکا ایجاد نمود. برخی از الگوهای نواربندی مجموعه کروموزومی این گیاه با نوارهای نشانگر و یا نوارهای ویژه نمونه ای در ژنوم های ایژیلوپس کائودانا *(Ae. Caudata)* (CC) یا ایژیلوپس اسکواروزا *(Ae. Squarrosa)* (DD) مشابه بودند. کاربرد این نواربندی استوکارمنی برای کروموزوم های قبیله تریتیسه نیز در اینجا مورد بحث قرار می گیرد.