Cytogenetic Analysis of Red Rust Flour Beetle *Tribolium castaneum*, Herbst (Coleoptera: Tenebrionidae)

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Abstract- Chromosomal information of species are essential since species are considered to be the objective reality of some particular genetic continuity and karyological studies has come to recognized as an important tool in understanding the problem of taxonomy and biology. In the present investigation *T. castaneum* has been studied with the view to determine the diploid (2n) number, morphology, centromeric index, sex determination mechanism, C-banding pattern and NOR localization which are essential to obtain a complete knowledge of cytogenetics of any animal. The observations revealed that karyotype of *T. castaneum*, showed a diploid number of 2n = 20 chromosome and sex determining mechanism was of "parachute type" due to the X and y chromosome (9AA + Xy_p). C- banded preparations showed positive staining in centromeric regions of all autosomes. In sex chromosomes only Xp chromosome indicated heterochromatised block. Nucleolar organizing regions were also localized.

Key words- chromosomes, sex determination, autosomes, heterochromatised, NOR (Nuclear organizing regions)

INTRODUCTION

The red rust flour beetle *Tribolium castaneum* (Herbst) is a word wide distributed stored grain pest which contributes to maximum spoilage of the stored grains at the larval as well as the adult stages. It belongs to order Coleoptera and Family Tenebrionidae. The order Coleoptera has approximately 350,000 known species; the total number may be ten times higher. With the advances of technique s for observing animal's chromosomes, an increasing number of investigations on coleopteran karyotypes have been reported by many authors. Integration of the earlier list of chromosome number of Coleoptera (Smith, 1960) and that of the events available data (Due and Kacker, 1980) make general karyotypic comparison possible and promise a fruitful future.

The family Tenebrionidae comprises about 6% of this order (Costa, 1999). According to Smith and Virkki (1978) and Juan and Petitpierre (1989), 200 species of Tenebrionidae have well characterized karyotypes, i.e. diploid number, the chromosome morphology and the type of sex chromosome determination system are known.

Inconsistent reports on the cytogenetics of Tenebrionids in India are available. Some efforts have been made by (Dutt, 1953; Agarwal 1960; Juneja, 1960; Sharma et al. 1977and Dua and Kacker, 1980) are probably the only workers who had paid some attention towards this problem. The main reason for the lack of attention toward Tenebrionidae for Cytogenetical studies could have been its small sized body and also the chromosomes. In the last few decades significant vigor have been observed in the chromosome compliments and karyotypes of *Tribolium* due to the perennial problem of control of stored grain pests.

According to Smith (1950, 1951, 1952, 1953, 1960 and 1962) the primitive number in this order is nine pairs of autosomes, and X about the size of autosome and minute Y. This formula is characteristic of Tenebrionidae as a whole but a good number of variation

has also been observed. The sex chromosome appears to be V shape and associated during division at two terminal contact points in the form of a "parachute". An additional symbol have been introduced by Dua and Kacker (1980) for comparison the association of X and y, in meiotic metaphase by calling it either like parachute (Xyp) or end to end (Xyr). When the association is not known between the sex chromosome then the symbol Xy is resumed. A fourth category of the Xy system in which y is larger than X was also reported by John and Lewis (1960).

In Tenebrionidae the diploid number of chromosomes varies from 14 to 36 with 20 as the model number (Juan and Petitpierre, 1990). *Tribolium* has been also observed to provide the total number 20 in their diploid set. The nomenclature of chromosomes was standard followed on the pattern of the nomenclature of the vertebrates (Levan *et al.*,1964). According to which the chromosome categories depending upon their centromeric position. Both acro and metacentric types of chromosomes are met with in the karyotypes of Tenebrionids. The relative average length is variable among the species, ranging from 3.25µ-0.75µ. The variation is brought about by the structure of sex chromosomes and autosomes.

The question whether there are morphologically differentiated single mechanism for *T. castaneum* is still unresolved. Sokoloff (1972) stated that the male and female presented identical number of karyotype. Constitutive effects of banding are essential help in this regard because they provide differentiated labeling of heterochromatised area. Advanced techniques are now showing tremendous potential for providing valuable cytogenetical information on *Tribolium*.

In the present investigation *T. castaneum* has been studied with the view to determine the diploid (2n) number, morphology, centromeric index, sex determination mechanism, C-banding pattern and NOR localization which are essential to obtain a complete knowledge of cytogenetics of any animal. These information's would add to the reportoir of the cytogenetics of the *Tribolium* on the whole.

MATERIAL AND METHODS

For cytogenetic analysis chromosomes were prepared from fourth instar larvae. Chromosome preparations have been done according to the procedure given by Wolf in 1997 with some modifications. Larvae were immersed in hypotonic solution i.e. KCl (0.075M) having 0.05% colchicine for 10 minutes which is followed by fixation for 10 minutes in fixative (ethanol : chloroform : acetic acid) in the ratio of 6:3:1. Then larvae was minced in a droplet of 70% acetic acid on a clean glass slide and this cell suspension was covered with the cover slip and placed on a hot plate for about 1 minute allowing part of the droplet of acetic acid to evaporate. Remaining acetic acid was discarded and the coverslips and slides were air dried. Then these slides and coverslips having chromosome preparation were utilized for Giemsa staining, C-banding and NOR staining. For Giemsa staining the procedure as described by Trivedi (1993) was followed with slight modification.

For C – banding , technique of Sumner *et al.*,(1972) was utilized with some modifications. C- banding is almost invariably induced by treatment with hydrochloric acid followed by either sodium hydroxide or, more commonly barium hydroxide treatment and incubation in a warm salt wash and finally Giemsa staining. The one step method of silver staining was used (Howell and Black, 1980) for NOR localization. The chromosome preparations were analysed with Olympus microscope with oil immersion objective at 100 x and photographed with Canon Power Shot A1100IS Digital Camera (12.1 megapixels).Chromosome terminology of Levan *et al.*,1964 has been followed.

RESULTS

Observations of cytogenetical investigation are presented in Fig.1. The total cell count index made from the metaphase stages of both mitotic and meiotic cells was shown in the Table-1 which shows that the diploid number of chromosomes per cell varied from 16 to 20. Out of 395 counts 2n is equal to 20 has been observed 207 times and referred to as model number. This confirms the uniformity in 2n values in the Tenebrionids.

The total arm length as depicted in the Table- 2 of *T. castaneum* has been found to be 6 to 16mm with metacentric, submetacentric, subtelocentric and telocentric chromosomes. The metaphases shows that the diploid complement is formed by 9 pairs of autosomes and 1 pair of sex chromosomes (Fig. 2) out of which 7 pairs were metacentric, 1pair submetacentric, 1 pair subtelocentric and in sex chromosomes X is metacentric while y is telocentric.

Autosomal metacentric chromosomes range between 0.05 to 0.14 where 1^{st} pair of metacentric chromosome have 0.14, 2^{nd} pair have 0.13, 3^{rd} pair have 0.12 and 4^{th}, 5^{th} and 6^{th} pair have 0.08 relative length. 7^{th} pair is submetacentric with 0.07 relative length, 8^{th} pair is again metacentric with 0.06 relative length and the 9^{th} pair is subtelocentric with 0.07 relative length. X chromosome is metacentric with 0.12 relative length while y chromosome is telocentric with 0.05 relative length. The shape of the chromosome is rod like and take deep stain. The ratio of the total length of autosomes (TA) and the length of "X", (TA/X) is 14.46.

Sex determining mechanism is of "parachute type" in which the y represents the aviator and the X resembling the open canopy of the parachute (X denoted as X_p and y chromosome as $y_{p)}$. The X_p chromosome is metacentric of medium size and usually is difficult to be identified due to its similarity with autosomal chromosomes of the same size. The y_p is telocentric and is characterized as the smallest chromosome of the karyotype (Fig.3).

The metaphase of *T. castaneum* cells showed a diploid number of 2n = 20 chromosome (Fig. 1A). The chromosome meioformula 9 + X y was observed in all cells in metaphase I. The haploid complement n = 9 + X or n = 9 + y was observed, indicating that chromosomes segregate normally during anaphase I. The nomenclature of chromosomes was the same standard based on centromeric position was followed on the pattern of the vertebrates (Levan *et al.*, 1964).

The karyotype of *T. castaneum* metaphases shows that the diploid complement is formed by metacentric, submetacentric, subtelocentric and telocentric chromosomes. The X chromosome is metacentric of medium size and usually is difficult to be identified due to its similarity with autosomal chromosomes of the same size. The y is telocentric and is characterized as the smallest chromosome of the karyotype.

C- banded preparations of *T. castaneum* showed positive staining in the centromeric regions of all the autosomes (Fig 1A,1B). The X chromosome also shows the blocks of constitutive heterochromatin in the centromeric region while in y chromosome no constitutive heterochromatin blocks were detected.

Silver nitrate staining has been used to study nucleolar activity and the distribution and position of NORs. Amorphous masses corresponding to nucleolar remnants were observed by silver nitrate staining (Fig. 1C). Silver nitrate also labeled the constitutive heterochromatin blocks and the sex chromosomes showed affinity for silver and continued to be labeled during different phases of meiosis.

The sex chromosomes of the observed *T. castaneum* appear to be primitive type with the X about the size of autosomes while the Y is minute (Fig.1A) and appears to be a total heterochromatised body. The association of X and Y is by two terminal contact points the shape appears like a “parachute”. Although this type of association is characteristic of Tenebrionids as a whole but in the present study has been also observed by C- banding and NOR. This is the possible confirmation of the existing primitive association of the sex chromosomes. The presence of small Y chromosome rubs out the possibility of neo-X and neo-Y mechanism in this group.

The bivalents as observed during the meiotic study consists of rod shaped, condensed heavily stained chromosomal segments with occasional patches of diffused stained portions in centromeric position. These diffused areas could be the sight of ongoing functions of the euchromatin chromosomes. The Xy bivalents differ from the other bivalents in stainability with only small unstained area at the centromeric position of X chromosome while y chromosome is deeply stained. During meiosis, the associated X and y observed to be oriented on the spindle with the y more closer to the “equatorial plate”.

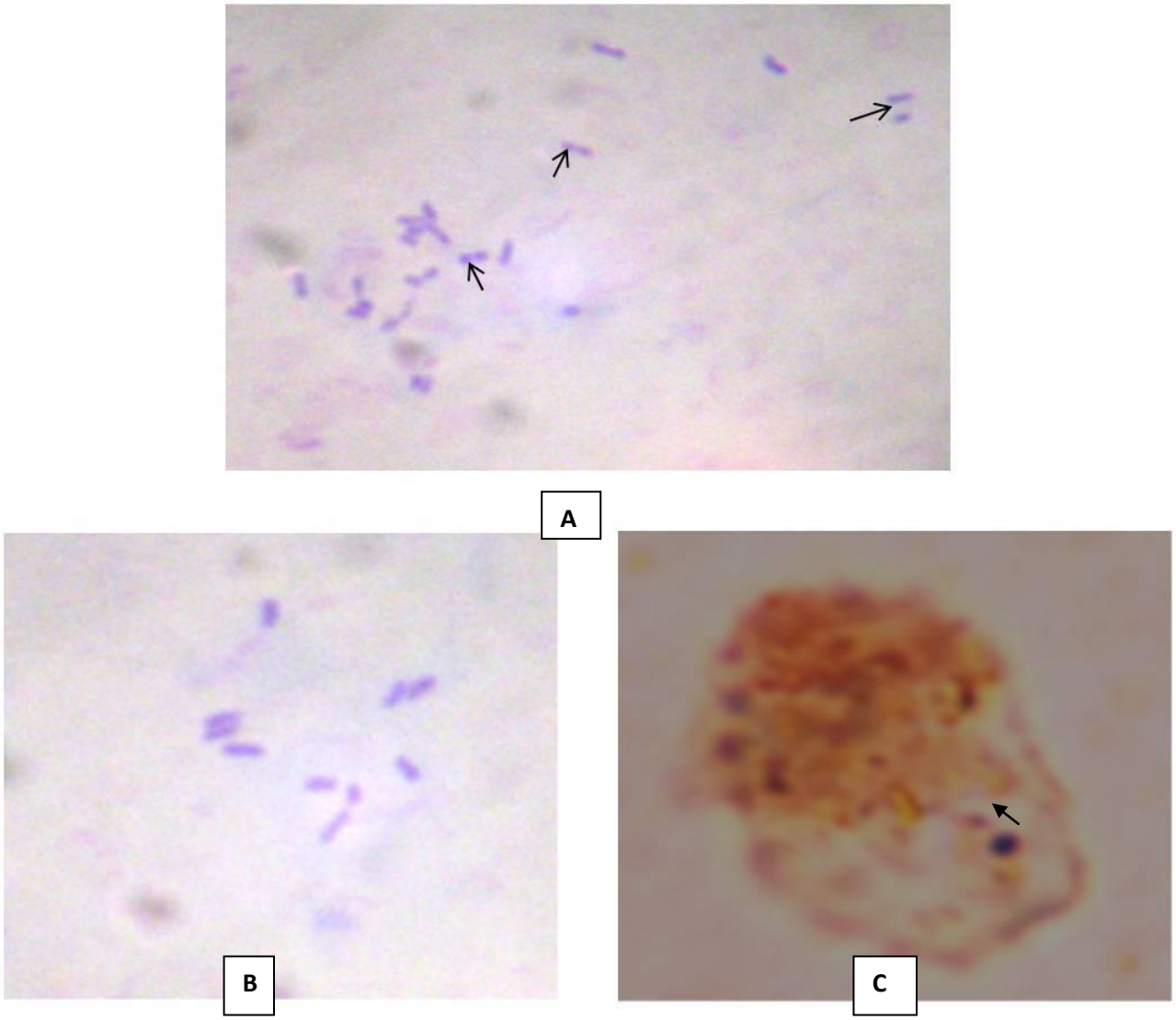

Fig.1 A. Metaphase plate showing - diploid no. (2n=20) of chromosomes, C- banding in centromeric region of chromosome and Xy_p chromosomes. B. Metaphase showing haploid (n=10).chromosome. C. Silver impregnated micronuclei in interphase

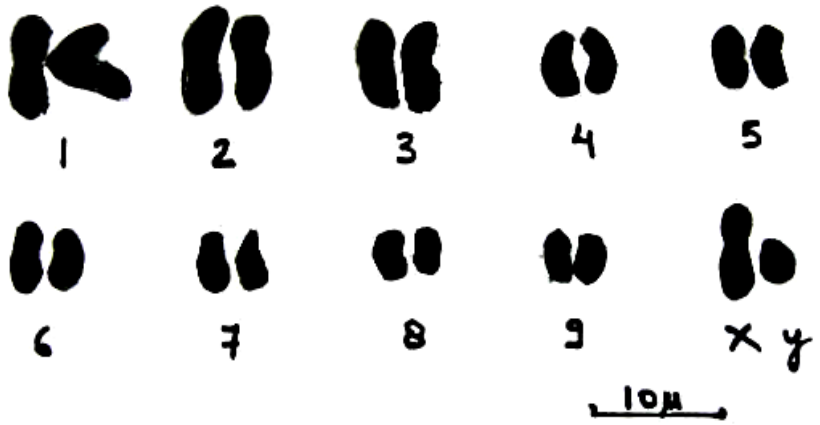

Fig.2 Karyotype of *Tribolium castaneum*

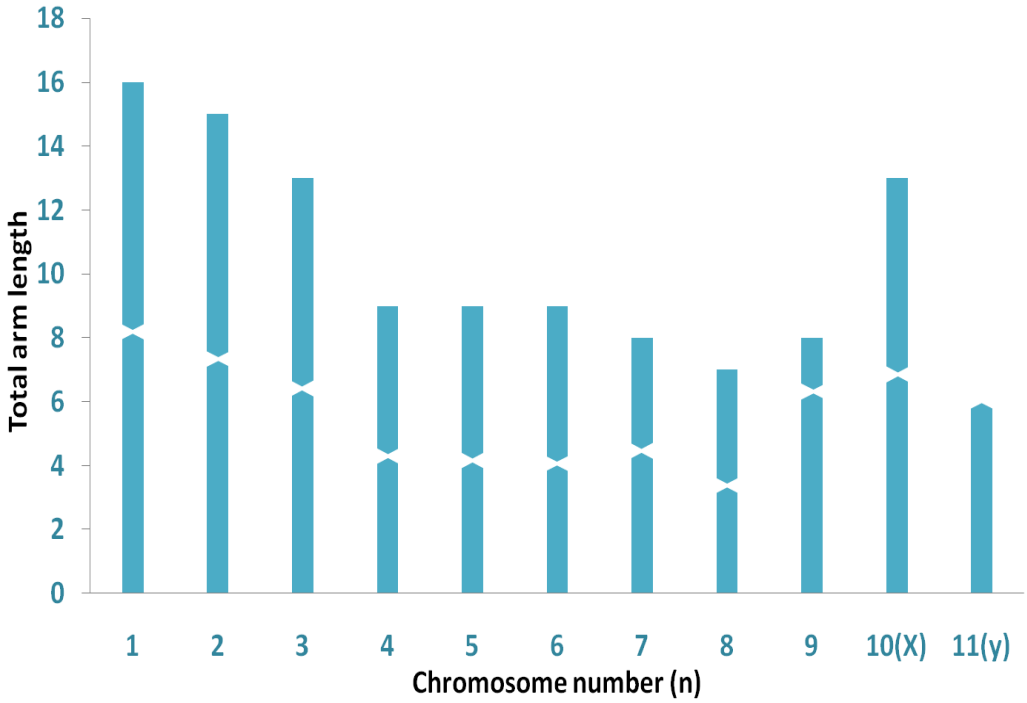

Fig-3 Idiogram of *T. castaneum* karyotype

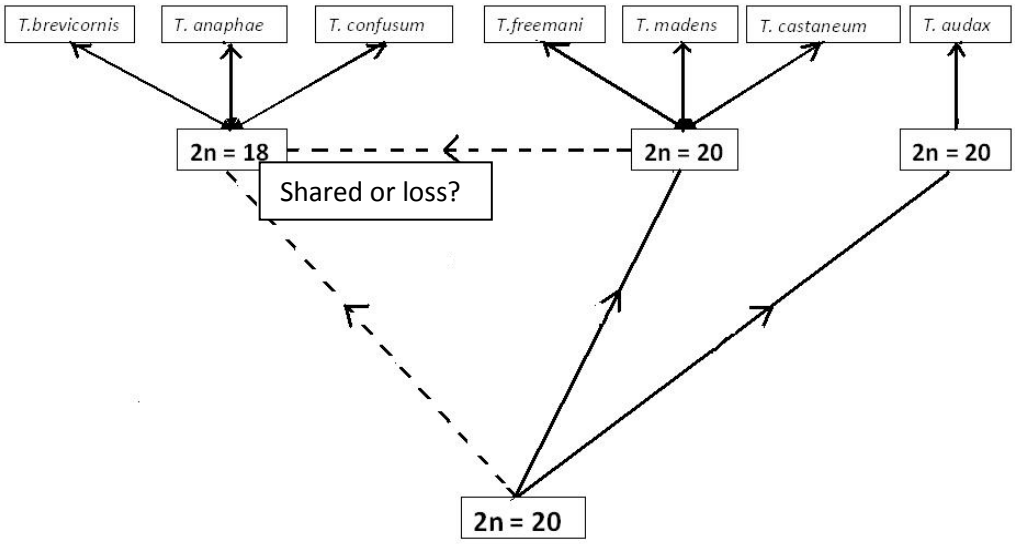

Fig. 4 Probable path of cytotaxonomical status of *Tribolium*

Table - 1 Cell count index

STAGES	CELL COUNT	CHROMOSOME FREQUENCY COUNT		
		16	18	20
Mitotic Metaphase	132	12	38	82
Meiotic Metaphase	263	48	90	125
Total	395	60	128	207

Table-2 Karyometrical analysis

Chromosome pair	Short arm length (mm)	Long arm length (mm)	Total arm length (mm)	Relative length (%)	Arm ratio (r)	Chromosome Type
1	8	8	16	0.14	1	M
2	8	8	16	0.14	1	M
3	7	8	15	0.13	1.14	M
4	7	8	15	0.13	1.14	M
5	6	7	13	0.12	1.16	M
6	6	7	13	0.12	1.16	M
7	4	5	9	0.08	1.25	M
8	4	5	9	0.08	1.25	M
9	4	5	9	0.08	1.25	M
10	4	5	9	0.08	1.25	M
11	4	5	9	0.08	1.25	M
12	4	5	9	0.08	1.25	M
13	3	5	8	0.07	1.66	Sm
14	3	5	8	0.07	1.66	Sm
15	3	4	7	0.06	1.33	M
16	3	4	7	0.06	1.33	M
17	2	6	8	0.07	3	St
18	2	6	8	0.07	3	St
19(X)	5	8	13	0.12	1.6	M
20(y)	0	6	6	0.05	∞	t

DISCUSSION

Karyotypic status **-** Advances of techniques has brought about increasing number of investigations on coleopteran karyotypes and integration of the earlier list of chromosome number of Coleoptera (Rozek and Lachowska, 2001) and that of the events available data (Costa, 2003) make general karyotypic comparison possible and promise a fruitful future. Currently inconsistent reports on the

cytogenetics of Tenebrionids in India are available. Some efforts have been made by (Dutt, 1953; Agarwal 1960; Juneja, 1960; Sharma *et al*. 1977and Dua and Kacker, 1980) are probably the only workers who had paid some attention towards this problem.

The diploid complement in the Tenebrionids varies from 14 to 36 numbers of chromosomes with a model number being 20. *T. castaneum*, as observed during the present study, confirms similar patterns of chromosomes, although the range varies between 16 to 20. The total frequency count of the chromosome number is fixed at 20 (Table 1). Overview of the earlier result reasserts the hypothesis according to which the pest among the tenebrionid have the basic karyotype of 10 bivalent chromosomes (White, 1979).

Karyological studies often are able to distinguish between closely related taxa and played some role in the evolution of karyotype of the species. Metaphase count reveals the diploid chromosome number as 20 in *T. castaneum*. Yosida (1958) studied that in the order Coleoptera, there exists a relative constancy in the ratio of the total length of the autosomes (TA) and length of the 'X' chromosome. Robertson (1959) also studied seven species of Cucujidae and observed different TA/X values. In the present study the TA/X value is 14.46.

On comparing the chromosome morphology of *T. castaneum* with other *Tribolium*, one is struck by apparent uniformity of relative chromosome size and centromeric position but differences in chromosome number. The *T. confusum* and *T. destructor* have only 18 chromosome in their diploid set. It is believed that the reduction in number in the latter two *Tribolium* is probably due to pericentric inversion /deletions during evolution (Sharma *et al.*,1973; White, 1979; Kacker, 1980). Since *T. castaneum* has the basic karyotype, it is highly probably that it belong to the primitive stock in the genus.

Karyometrical relationships with other Tenebrionids - On the available data on the Tenebrionids, the diploid number of chromosomes varies from 14 to 36 as observed in *T. castaneum, T. destructor, T. confusum, Alphitobius diaperinus, Sphenariopsis impolita* (Dutt, 1953; Sharma, 1954; White, 1979; Dua and Kacker, 1980; Rich and Bell, 1980). Of the three species of *Tribolium* , *T. castaneum*, has 20 in both sexes while *T. destructor* and *T. confusum* have only 18 chromosomes in their diploid sets. *Alphitobius* has 2n is equal to 19 and *Sphenariopsis* has 20. The average mean length of chromosome varies, for example the length of *A. diaperinus* varies from 2.5 to 1.25µ whereas in *T. castaneum* and *S. impolita* it varies from 3.25- 0.75µ.

Hinton (1948) classified the extent species of *Tribolium* into five groups based on its morphological characters. Considering our species of *Tribolium castaneum* and other seven species, as described by Juan *et al.,* (1993), they can be grousped under Hinton's criteria as follows : *confusum* species group with *T. confusum* and *T. anaphe*, *castaneum* species group *T. castaneum, T. audax* and *T. brevicornis* . The latter is considered most primitive and well differentiated from others. Based on cytogenetic data of Smith (1952) claimed that *T. confusum* with 2n = 18 chromosomes were derived from similar ancestors of *Tribolium* 2n = 20 by translocation of one autosome to the X chromosome.Samollow *et al.*,(1983) provided genetic support of this hypothesis.. The data derived from dot –blot hybridization of DNA also supports this view (Juan and Petitpierre, 1990) based on the comparison of phylogenetic model can be prepared by the occurrences and relationship between the *Tribolium* can be made. Fig.4 depicts that the ancestral chromosome number to be the 20 which forms the base. The *T. freemani, T. castaneum* and *T. madens* appear to be closely related. The earliar concept of confusum is now not valid because *T. anaphe, T. confusum* and *T. brevicornis* form a separate species group with 2n=18.*T. audax* although has 2n=20 chromosome but yet differ in chromosome structure and hence, depicted in Fig.4 as having arisen from the common ancestral stem.

Sex determining mechanism - A diversity of opinion exists for the sex chromosomes of Coleopterans among the investigators. According to Smith (1963) there are three different type of sex chromosome mechanism available in this group. The first category of sex chromosome is the XO system but this mechanism has been restricted to some selected families (Lycidae, Lampyridae, Cantharidae). This reflects the idea that Y is not an essentially male determining mechanism. Probably it becomes in a similar manner to the Y of *Drosophila.* However, generalization of this category for the order Coleoptera is rather doubtful.

The second category is an XY system in which the minute y is associated by both its arms with X. This two terminal contact points result in the form of "parachute" bivalent (terminology according to Smith) in which the y represents the aviator and the X resembling the open canopy of the parachute. This is by far the commonest type of sex mechanism in Coleopteran this formula is characteristic of Tenebrionidae as a whole.

There had been two attempted interpretations of these parachute bivalents. One is that are two terminal pairing segments in each sex chromosome, that is, the parachute is really ring bivalent composed of two metacentric chromosomes of quite different sizes held together by a terminalised chiasmata (Smith, 1960). The other interpretation (John and Lewis, 1960) assumes that there are no chiasmata, the X and y being held together by a nucleolar formation which is suppose to occupy the space between them.

Our observation with the *T. castaneum* indicate the existence of the parachute association of the X and y chromosomes (9AA + Xy). *T. confusum* and *T. destructor* on this basis have been considered to be the derived species, the first having eight pairs of autosomes, a large X and large Y (Neo X and neo-Y). In *T. destructor* the size of the Neo- X and Neo- Y is reduced. According to Smith (1952) *T. castaneum* following fusion of the X chromosome with one pair of autosome. The homologue of which took over the mechanism function of the minute y chromosome. The purpose for using the statement here is to indicate the primitiveness of *T. castaneum* in comparison to the other *Tribolium.*

The third category is a Xy system in which the y is quite large and about the same size as one limb of X chromosome. Such mechanism are reffered to as Neo- XY mechanism by Manna (1969). These mechanism are interpreted to have arisen from XO system in the same way as in the Orthoptera. Such mechanism have been reported in *T. destructor* and the *T. confusum* but it has never been seen in the *T. castaneum* during our investigation. The another hypothesis for the above fact may be that the minute y chromosome of *T. castaneum* increases in size by duplication of some reason without the incorporation of the autosomal material into the sex chromosome pair. Throughout our studies we have always found that y to be minute and associated in parachute form. This gives a feeling that the *T. castaneum* are a conservative group in terms of the sex determining mechanism.

C- banding studies- C-banding of chromosomes used to define regions of constitutive heterochromatin. This type of DNA often contains highly repetitious sequences, such as satellite DNA, and is thought to be genetically inert. C- banding is very species specific or even individual specific.

The order Coleoptera has the highest species diversity within the animal kingdom, yet cytogenetic data using specific banding techniques are still scarce. C- banding data have revealed a preferential localization of autosomal constitutive heterochromatin (CH) in the centromeric area and less so observed in interstitial and telomeric areas. Sex chromosomes also show a variable CH distribution, as it has been observed in the pericentromeric region or along the entire chromosome (Ennis, 1974; Vidal *et al.*,1977; Angus, 1983; Drets *et al.*,1983; Virkki, 1983; Juan and Petitpierre, 1989).

Another phenomenon which has been observed during the C-banding studies is that chromosomes are better observed by this method then by routine giemsa stain method. It is true that many species of Coleopterans are chromosomaly conservative in terms of lacking gross xx chromosomal changes. If the karyotype in an evolutionary series consists of degree of completely biarmed chromosome than the probability that *Tribolium* species are in the process of undifferentiation.

The C-banding results on the mitotic chromosomes of the *T. castaneum* (Fig. 1A) shows positive staining in the centromeric regions of all the autosomes. The X_p chromosome also shows the blocks of constitutive heterochromatin in the centromeric region while in y_p chromosome no constitutive heterochromatin blocks were detected.

NOR studies- Silver nitrate staining of both mitotic and meiotic chromosomes of eukaryotic species has been a very useful approach for the analysis of the structure and variability of nucleoli, nucleolar organizer regions (NORs) and kinetochores (Goodpasture and Bloom, 1975; Virkki and Denton, 1987; Virkki *et al.*,1991). Although in Coleoptera this technique has been considered unsuitable to identify active rDNA clusters (Colomba *et al.*,2000). It is generally accepted that silver impregnation is indicative of rRNA synthesis and that it stains functionally active NORs (Miller *et al.*,1976; Hubbell, 1985).

Silver nitrate staining has been used to study nucleolar activity and the distribution and position of NORs. Amorphous masses corresponding to nucleolar remnants were observed by silver nitrate staining. Silver nitrate also labeled the constitutive heterochromatin blocks and the sex chromosomes showed affinity for silver and continued to be labeled during different phases of meiosis.

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