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First report on a multiple sex chromosome system ($X_1X_2X_30$) and population variations in the frequency of ring bivalents in Pyrrhocoridae (Hemiptera: Heteroptera)

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Abstract. The family Pyrrhocoridae (Hemiptera: Heteroptera) is characterized by a modal diploid chromosome number of 16 (♂) ranging from 12 to 33 and simple (X0), multiple (X_1X_20) and neo-sex chromosome systems (neoX-neoY). Out of about 340 known species, only 22 species belonging to 7 genera have to date been cytogenetically analysed. In the present study, the chromosome complement and meiotic details of one species, *Odontopus nigricornis* Stål has been revised, whilst that of another species, *Antilochus russus* Stål has been cytogenetically analyzed for the first time. The diploid chromosome complement of *O. nigricornis* is 2n (♂) = 25 = 22A + $X_1X_2X_30$; the first time this particular multiple sex chromosome system has been reported in the Pyrrhocoridae. Three sex chromosomes mostly remain intimately associated during male meiosis and their number was confirmed at diplotene and anaphase II, where they dissociate slightly to become distinct. Meiosis is post-reductional for sex chromosomes. However, unlike other pyrrhocorids with multiple X chromosomes, the X_1, X_2 and X_3 lie outside the autosomal ring on the metaphase plate during both divisions. The male diploid chromosome complement of *A. russus* was found to be 2n = 27 = 26A + X0. Apart from the typical meiotic features of heteropterans, the latter species shows inter- and intra-population variations in frequency of ring bivalents.

INTRODUCTION

The family Pyrrhocoridae (Hemiptera: Heteroptera), which occurs throughout the tropical and subtropical regions of the world with a few species present in temperate areas, contains about 33 genera and 340 species (Schuh & Slater, 1995; Henry, 2009). The supra-generic relationships within the family are poorly understood and there is no accepted subfamily or tribal level classification (Redei et al., 2009). In terms of cytogenetics, only 22 species belonging to 7 genera of Pyrrhocoridae have to date been investigated (Papeschi & Bressa, 2006; Verma & Kurl, 2009; Bardella et al., 2014). This data reveals that the family is characterized by a modal diploid chromosome number of 16 (♂), ranging from 12 to 33, with simple (X0), multiple (X_1X_20) and neo-sex chromosome systems (neoX-neoY), apart from the general features of heteropterans, that is, holokinetic chromosomes and post-reductional meiotic division of sex chromosomes. In the Pyrrhocoridae, X0 is considered the ancestral sex chromosome system, which is represented in 11 out of 22 species (Papeschi & Bressa, 2006; Verma & Kurl, 2009; Bardella et al., 2014). A multiple sex chromosome system (X_1X_20) has been reported in 8 species of *Dysdercus* investigated (Piza, 1947a, 1951; Mendes, 1949; Manna, 1951; Sharma et al., 1957; Banerjee, 1958; Ray-Chaudhuri & Banerjee, 1959; Ruthmann & Dahlberg, 1976; Manna & Deb-Mallick, 1981; Kuznetsova, 1988; Suman, 2010), a species of *Pyrrhopeplus* (Parshad, 1957) and a species of *Odontopus* (Verma & Kurl, 2009), whereas a neo-X neo-Y sex chromosome system was described in another species of *Dysdercus* studied (Bressa et al., 1999; 2009).

The family Pyrrhocoridae is one of the most important families studied in terms of the behavior of the sex chromosomes. In species with an X_1X_20 sex-determining mechanism, the two Xs show a peculiar behaviour. They remain fused during diffuse stage, separate at diplotene to lie apart up to metaphase I, divide equationally during anaphase I, join again at metaphase II, and move to one of the poles together during anaphase II. This phenomenon led Ray-Chaudhuri & Manna (1952) to designate two sex bodies as X and Y and meiosis to be double reductional, which, however, was later contradicted by Battaglia (1956) who designated the sex chromosomes as X_1 and X_2 and proposed the concept of post-reductional meiosis, a proposition which is now widely accepted. The degree of association between X_1 and X_2 is, however, variable in different species. Two Xs simply come close together in *D. cingulatus* (Fabricius) (Sharma, 1956) and *D. mendesi* Blöete (Piza, 1947a); fuse to form a single constricted element in *D. koenigii* (Fabricius) (Battaglia, 1956; Sharma, 1956), and fuse to form a single mass in *P. posthumus* Horváth (Parshad, 1957). Verma & Kurl (2009) reported the diploid number of *O. nigricornis* Stål collected from Forest Research Institute, Dehradun, India to be 2n = 12 = 10A + X_1X_20, a claim which is not clear from their supporting photographic evidence. In contrast, in our study of specimens collected from the same area, the diploid chromosome complement has been found to be different from these earlier findings. Here, we describe the complement and the entire meiotic cycle, both of which are well supported by photographs. In addition, the chromosome number of another species, *A. russus* Stål is given for the first time and meiotic variations in two populations are presented and discussed.

Table 1. Geographical and climatic features of Dehradun and Ghumarwin along with species collected.

Place	Elevation (m)	Latitude/ Longitude	Annual average temperature (°C)	Annual average precipitation (mm)	Species collected	No. of individuals cytogenetically analyzed
Dehradun	652	30.316494°N/ 78.032191°E	21.8	1896	*O. nigricornis* *A. russus*	10 12
Ghumarwin	647	31.435840°N/ 76.711865°E	22.3	1317	*A. russus*	6

MATERIAL AND METHODS

Adult male *O. nigricornis* and *A. russus* were collected from wild vegetation on the Forest Research Institute (FRI) campus, Dehradun, which is situated in valley at the foothills of the Himalayas, whilst the later species was also collected at Ghumarwin, a hilly area, both sites lying in the northern part of India at roughly the same elevation, about 300 km apart. Dehradun is termed as the "Rainy City of India" due to continuous and incessant rainfalls, especially during the monsoon season. In contrast, Ghumarwin, has only medium rainfall during the monsoon. The geographical and climatic features of the two places along with number of cytogenetically analyzed specimens of each population of collected species are given in the Table 1.

Testes were dissected out in ~0.7% saline (NaCl in distilled water) and were fixed in freshly prepared Carnoy's fixative (3 : 1, absolute alcohol: glacial acetic acid) for 15 min followed by a second change of the fixative. The fixed material was tapped on clean slides, air dried and stained with carbol-fuchsin stain for 4 h followed by differentiation in n-butanol. The slides were allowed to dry and were mounted in DPX (Carr & Walker, 1961) and examined under a powerful light microscope.

RESULTS

Odontopus nigricornis

The male diploid chromosome number of *O. nigricornis* was found to be 2n = 25 = 22A + $X_1X_2X_3$0, as observed at spermatogonial metaphase (Fig. 1a). Based on size, autosomes fell into three categories: 4 large, 14 medium (with slight size gradation) and 4 small. Sex chromosomes X_1, X_2 and X_3 were seen to be fused as a single element represented by a largest body. During the diffuse stage, three sex chromosomes appeared to be almost fused and condensed, whilst the autosomes appeared to de-condensed (Fig. 1b). The early diplotene revealed two or three autosomal bivalents with two chiasmata, the remainder of the bivalents each with a single terminal, sub-terminal or interstitial chiasma. The sex chromosomes still remained closely associated (Fig. 1c). During late diplotene, three unequal sex chromosomes were seen to slightly fall apart to become distinct for a brief period (Fig. 1d) but in subsequent stages of diakinesis and metaphase I, these again became indistinct due to their close side-by-side association (Fig. 1e, f). During metaphase I, autosomal bivalents were arranged in a ring configuration and fused sex chromosomes were seen to lie outside the ring (Fig. 1f). At anaphase I, the autosomes segregated reductionally, whilst the sex chromosomes divided equationally, and lagged behind (Fig. 1g). During metaphase II, the autosomes were seen to be arranged in a rough ring and the sex chromosomes were associated to form a pseudo-trivalent lying outside the ring (Fig. 1h). During anaphase II, the autosomes divided equationally, whereas the sex chromosomes segregated to one pole, lagging behind the autosomes. In some plates, the X_1, X_2 and X_3 chromosomes remained fused (Fig. 1i) while in others, all three were seen to be distinct but associated terminally (Fig. 1j).

Antilochus russus

A. russus was found to have a male diploid chromosome complement of 2n = 27 = 26A + X0. The single X was the largest chromosome of the complement (Fig. 2a) and was represented by a positively heteropycnotic body during the diffuse stage (Fig. 2b). Variations with respect to the number and location of chiasmata at diplotene/diakinesis and metaphase I were recorded in specimens collected from Ghumarwin and Dehradun. In all specimens collected from Ghumarwin, all the bivalents each showed a single terminal/subterminal or interstitial chiasma and the mean chiasma frequency of each individual was found to be 13 per cell (Fig. 2c, d). However, in specimens from Dehradun, all the cells showed a few bivalents with two chiasmata, while the remainder of the bivalents each possessed a single terminal/sub-terminal or interstitial chiasma (Fig. 2e–i). The number of ring bivalents was seen to be highly variable, varying from one to six, with three as the commonest number (Table 2). The mean chiasma frequency of the whole population was 16.5 (± 1.7) at diakinesis and 15.1 (± 1.5) at metaphase I (Table 3). The architecture of metaphase karyotype was the same in both populations, i.e. autosomal bivalents arrange in a random manner on the plate with the X a little away in all the specimens (Fig.

Table 2. Relative frequency of cells with different number of chiasmata at diplotene/diakinesis in *A. russus* from Dehradun (total cells analysed = 79; the most frequent situation in bold).

Number of ring bivalents per cell	Number of chiasmata per cell	Number of cells with a particular number of chiasmata at diplotene/diakinesis	Relative frequency of cells with a particular number of chiasmata
1	14	13	0.16
2	15	9	0.11
3	**16**	**21**	**0.27**
4	17	13	0.16
5	18	9	0.11
6	19	14	0.18

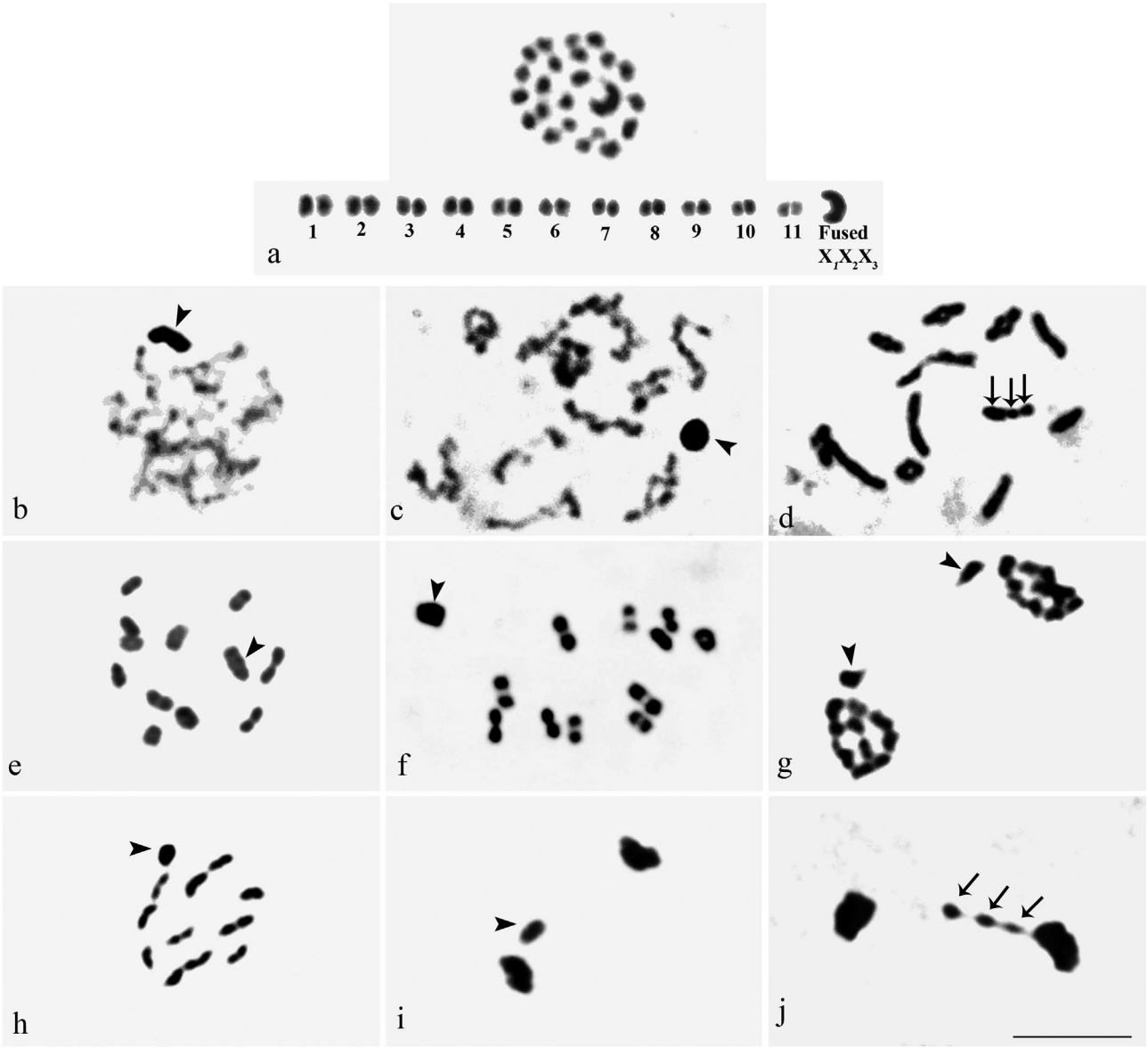

Fig. 1. Spermatogenesis of *O. nigricornis*. a – spermatogonial metaphase along with karyotype; b – diffuse stage; c – early diplotene; d – late diplotene; e – diakinesis; f – metaphase I; g – anaphase I; h – metaphase II; i–j – anaphase II. Arrowheads indicate fused sex chromosomes ($X_1X_2X_3$). Arrows indicate end to end associated sex chromosomes ($X_1X_2X_3$). Bar = 10 μm.

2j, k). At metaphase II, the autosomes were arranged in a rough ring with the X lying outside the ring (Fig. 2l). During anaphase II, the autosomes were seen to divide equationally, whereas the sex chromosome segregated to one of the daughter cells lagging behind the autosomes. This yielded telophase II nuclei with thirteen and fourteen chromosomes (13A + X, 13A) (Fig. 2m).

TABLE 3. Mean chiasma frequency in each individual and whole population of *A. russus* from Dehradun.

Serial number of individual	Mean chiasma frequency at diplotene/diakinesis (± S.D.)	Number of cells	Mean chiasma frequency at metaphase I	Number of cells
1	17.8 (± 1.4)	17	17.4 (± 1.3)	7
2	16.8 (± 1.3)	9	16.7 (± 2.6)	4
3	16.5 (± 1.4)	12	Not found	–
4	15.0 (± 1.1)	19	14.6 (± 0.8)	18
5	15.0 (± 1.0)	5	14.7 (± 1.0)	6
6	17.2 (± 0.9)	10	Not found	–
7	17.8 (± 2.2)	5	Not found	–
8	15.0 (± 0.0)	2	14.3 (± 0.7)	17
Mean chiasma frequency of population	16.5 (± 1.7)		15.1 (± 1.5)	

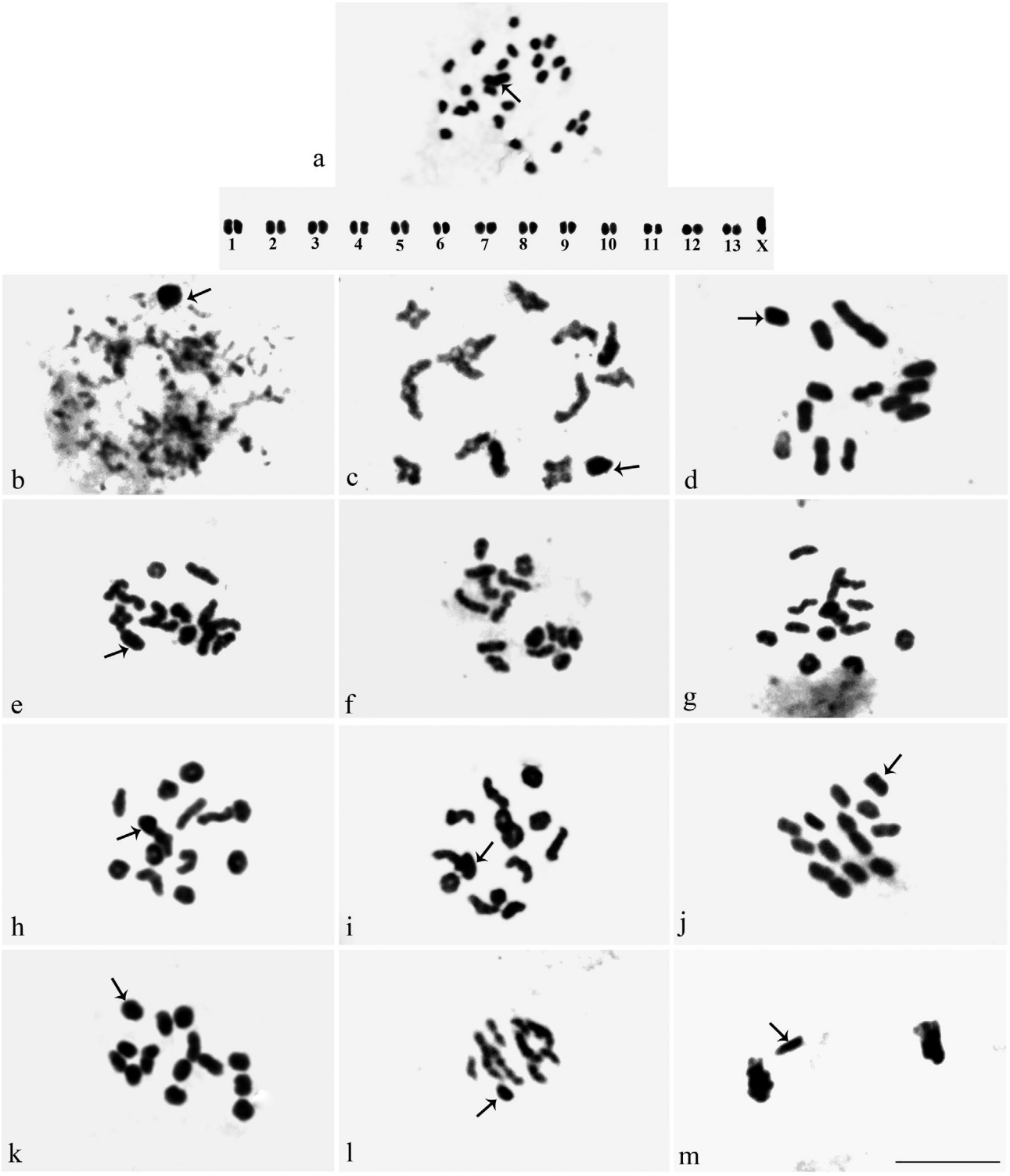

Fig. 2. Spermatogenesis of *A. russus*. a – spermatogonial metaphase along with karyotype; b – diffuse stage; c – diplotene in individuals of Ghumarwin; d – diakinesis in individuals of Ghumarwin; e–i – diplotene/diakinesis with variable no. of ring bivalents in individuals of Dehradun; j – metaphase I in individuals of Ghumarwin; k – metaphase I showing retention of ring bivalents in individuals of Dehradun; l – metaphase II; m – anaphase II. Arrows indicate sex chromosome. Bar = 10 μm.

DISCUSSION

The chromosomal karyotype of *O. nigricornis* was earlier studied by Verma & Kurl (2009) who reported the male diploid number to be $2n = 12 = 10A + X_1X_20$. However, their account, including the photographs presented by these authors, do not match with each other. In their paper, figure 1, labelled as "mitotic metaphase" and used to present the male karyotype, is actually metaphase II. Similarly, figure 7, is labelled as "metaphase I" in which five dumbbell shaped autosomal bivalents and one pair of sex chromosomes are shown arranged in a circle. In this figure, eleven autosomal bivalents forming a ring with the

Xs lying outside of it are clearly visible. The number of Xs is not clear in their figure. Furthermore, they have wrongly labelled their figure 12 as "metaphase I", which is actually metaphase II, in which 11 chromosomes form a rough ring with fused Xs lying outside the ring. It is unclear why these previous authors concluded that the male chromosome complement was 10A + X_1X_20, and it is hence pertinent to revise the chromosomal details of this species. In the present paper, we have revised the male karyotype and meiotic details of *O. nigricornis*, our new analysis being well supported by photographs. It is noteworthy that the specimens analyzed in this study were obtained from the same area as those of Verma & Kurl (2009).

From our analysis, *O. nigricornis* has a chromosomal complement of 2n = 25 = 22A + $X_1X_2X_30$ with four large, 14 medium with slight size gradation and four small autosomes and three fused sex chromosomes at spermatogonial metaphase. Banerjee (1958) investigated another species of *Odontopus*, *O. sexpunctatus* Laporte and reported 2n = 27 = 26A + X0 with six large, 18 medium and two small autosomes and the largest X chromosome. In Pyrrhocoridae, the diploid number ranges from 12 to 33 with 14 autosomes and an X0 sex mechanism as the ancestral complement. Fusions and fragmentations are considered to be the main mechanisms of chromosome evolution (Piza, 1947a, b, 1951; Mendes, 1949; Mola & Papeschi, 1997; Bressa et al., 2002a, 2003).The finding of an increased number of autosomes in two species of *Odontopus* might reflect its origin from ancestral number of 14 autosomes through different number and patterns of fragmentations. In Pyrrhocoridae, X0 (52.38% of 21 cytologically described species), X_1X_20 (42.86%) and neo-XY (4.76%) sex mechanisms have been reported (Papeschi & Bressa, 2006; Suman, 2010; Bardella et al., 2014), excluding *O. nigricornis* data given by Verma & Kurl (2009). *O. nigricornis* is the first record of a multiple sex chromosome system in Pyrrhocoridae comprising three X chromosomes. The simple sex chromosome system X0 is probably the ancestral one from which $X_1X_2X_30$ derived by fragmentation of the original X. It is clear that during karyotype evolution of *O. nigricornis*, the X could have fragmented, while it has been retained intact in *O. sexpunctatus*.

In heteropteran species with a X_n0 sex chromosome system, the multiple Xs tend to fuse to form a single element throughout or for a greater part of meiosis (Manna, 1951; Dutt, 1957; Parshad, 1957; Fossey & Liebenberg, 1995; Grozeva et al., 2006; Suman, 2010; Bansal, 2012; Kaur & Bansal, 2012 a, b; Bansal & Kaur, 2013), except in some cases like *Dysdercus* (Manna, 1957). Manna (1984) suggested that in species with a X_n0 multiple sex chromosome system, the number of X chromosomes could be confirmed in the spermatogonial complement because all the chromosomes lie as separate entities. In *O. nigricornis,* however, the three sex chromosomes remain intimately associated for most of the meiotic cycle including the gonial stage. Only for a brief period during diplotene and anaphase II, do they become distinct and identifiable, and we could determine the male sex system as $X_1X_2X_30$. The presence of multiple X chromosomes is not uncommon in Pyrrhocoridae (Mendes, 1947; Ray-Chaudhuri & Manna, 1952; Parshad, 1957; Sharma et al., 1957; Banerjee, 1958; Ray-Chaudhuri & Banerjee, 1959; Suman, 2010), and the Xs usually remain associated closely or fused during the diffuse stage but become well separated during diplotene and metaphase I, and thus are distinct. The intimate association of the X chromosomes almost throughout meiosis in *O. nigricornis* is the first record for Pyrrhocoridae, although this condition has earlier been recorded in two coreid species, *Spartocera fusca* (Thunberg) and *Spartocera batatas* (Fabricius) by Cattani & Papeschi (2004) and Franco et al. (2006), respectively. In fact, Cattani & Papeschi (2004) described a single X chromosome showing a particular morphology (butterfly-shaped) in *S. fusca.* In their paper, the authors failed to differentiate both X chromosomes, but Franco et al. (2006) re-examined the male and female gonial prometaphases of *S. fusca* and confirmed the sex chromosome constitution, i.e. $X_1X_20/X_1X_1X_2X_2$, male/female.

In both *O. nigricornis* and *A. russus*, the $X_1X_2X_3$ in the former and the single X in the latter lie outside the autosomal group at metaphase I as well as at metaphase II. This kind of sex chromosome behavior has earlier been reported in pyrrhocorid species with a single X such as *O. sexpunctatus* and *Antilochus conqueberti* (Fabricius) (Banerjee, 1958). However, in other species of Pyrrhocoridae with multiple Xs, i.e. *Dysdercus koenigii* (Fabricius), *D. fasciatus* Signoret, *D. intermedius* Distant, *D. superstitiosus* (Fabricius) and *Pyrrhopeplus posthumus* Horváth, all Xs lie within the autosomal ring at metaphase I but outside of it at metaphase II (Ray-Chaudhuri & Manna, 1952; Parshad, 1957; Sharma et al., 1957; Banerjee, 1958; Ray-Chaudhuri & Banerjee, 1959; Ruthmann & Dahlberg, 1976; Suman, 2010). It thus appears that *O. nigricornis* is the first pyrrhocorid species with this type of metaphase architecture in both meiotic divisions, which, otherwise, is commonly observed in species with both X and multiple Xs in the family Coreidae (Manna, 1951; Sands, 1982; Suja et al., 2000; Cattani & Papeschi 2004; Franco et al., 2006; Souza et al., 2009; Bansal, 2012; Kaur & Bansal, 2012 a, b; Bansal & Kaur, 2013).

The diploid chromosome complement of *A. russus* was 2n = 27 = 26A + X0 in all the individuals from Ghumarwin and Dehradun, which has also been reported in *A. conqueberti* (Parshad, 1957; Banerjee, 1958; Suman, 2010). However, variations have been observed in the number of chiasmata in diplotene/diakinesis and metaphase I between the two populations and within the single population from Dehradun. Individuals from Ghumarwin show a chiasma frequency of 13 per cell with complete absence of ring bivalents in all the cells. Contrastingly individuals from Dehradun show one to six ring bivalents at diplotene/diakinesis in all the studied cells, which increases the expected frequency of 13 to 16.5 with a range from 14 to 19. Maximum cells were found to show three ring bivalents (16 chiasmata per cell) with a relative frequency of 0.27 (Table 2). The mean chiasma frequency is found to decrease to 15.1 at metaphase I (Table 3), which may be

due to release of one chiasma in a few bivalents to become free to attach to the spindle as is evident from the presence of some V-shaped bivalents which finally adopt a rod shape at metaphase I (Fig. 2g–i). A high mean chiasma frequency of 15.1 at metaphase I versus the expected frequency of 13 suggests a high retention of chiasmata, even up to metaphase I. Genetic determinants and environmental factors, both internal and external to the organism, are known to cause chiasma variations, and temperature is one such environmental factor that has been observed to cause variations in chiasma frequency during meiosis (Henderson, 1962; Jones, 1987). It is evident from the Table 1 that both Dehradun and Ghumarwin lie almost at the same elevation and annual temperature is also not very different. However, Dehradun receives continuous and incessant rains, while Ghumarwin receives only moderate rains during the monsoon season. Therefore, humidity could perhaps be one of the environmental factors responsible for inducing the observed variations in chiasma frequency, although we could not find any reference in support of this contention. Mola & Papeschi (1993) observed heterogeneity in terms of chiasma frequency in a single population of *Largus rufipennis* Laporte (Heteroptera: Pyrrhocoroidea: Largidae) from Argentina. They, however, could not assess influence of the genetic background and role of internal and external environment on chiasma frequency, and considered external conditions to play the major role. Jacobs & Liebenberg (2001) also reported significant differences in the number of chiasmata between populations and also among individuals of the same population in *Adamanotus uncotibialis* Jacobs (Heteroptera: Aradidae) but were unable to give reasons.

It has been generally accepted that most heteropterans possess, as a rule, only one chiasma per bivalent (Ueshima, 1979; Manna, 1984). However, the present observations on *A. russus* together with previous findings obtained in some other heteropteran species indicate that the frequency of ring shaped bivalents could be much higher than originally suggested (Camacho et al., 1985; Mola & Papeschi, 1993; Bressa et al., 1998, 1999, 2001, 2002b, 2003; Jacobs & Liebenberg, 2001; Rebagliati et al., 2001, 2003; Papeschi et al., 2003; Lanzone & Souza, 2006).

CONCLUSIONS

Cytogenetic analysis of *Odontopus nigricornis* and *Antilochus russus* (Pyrrhocoridae) has yielded certain peculiar features. A novel multiple sex chromosome system $X_1X_2X_30$) has been recorded for the first time in Pyrrhocoridae, i.e. in *O. nigricornis*. The sex chromosomes remain intimately associated almost throughout meiosis, including during the gonial stage. Unlike other pyrrhocorids, multiple Xs ($X_1X_2X_3$) lie outside the autosomal ring on the metaphase plate during both the divisions. In addition, *A.russus* showed inter- and intra-population variations in frequency of the ring bivalents.

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