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Comparative FISH analysis in five species of Eyprepocnemidine grasshoppers

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The chromosomal localization of ribosomal DNA, and a 180 bp satellite DNA isolated from Spanish *Eyprepocnemis plorans* specimens, has been analysed in five Eyprepocnemidinae species collected in Russia and Central Asia. Caucasian *E. plorans* individuals carried each of the two DNAs, but the rDNA was limited to only two chromosomes (S_9 and S_{11}) in sharp contrast to Spanish specimens that show 4–8 rDNA clusters and to Moroccan specimens which carry rDNA in almost all chromosomes. The four remaining species, however, lacked the 180 bp tandem repeat, and showed rDNA clusters in one (S_9 in *Thisoicetrinus pterostichus*), two (S_9 and S_{10} in *Eyprepocnemis unicolor*, M_8 and S_{11} in *Heteracris adspersa*), or three (S_9, S_{10}, and S_{11} in *Shirakiacris shirakii*) chromosome pairs. The implications of these findings for the evolution of these two chromosome markers in this group of species are discussed.
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Introduction

As the genome era progresses, it is being more apparent that eukaryote genomes contain a significant proportion of repetitive DNA of various types. Two of the tandem repetitive DNA classes, that is, ribosomal (rDNA) and satellite DNA (satDNA), are very different in genetic role (rDNA is very important functionally, but satDNA seems to be nonfunctional) but they share some evolutionary properties (eg concerted evolution).

Ribosomal DNA is arranged in tandem arrays (containing transcriptional units coding for three of the four rRNA types) located at one or more chromosome regions, the so-called nucleolus organizer regions (NORs). NORs are evolutionarily dynamic, which makes them excellent markers for phylogenetic studies (Cabrero and Camacho, 1986; Amemiya and Gold, 1988; Santos and Fox, 1988). Interspecies comparison of NOR number and chromosome location may be a good tool for macroevolutionary studies. Intraspecific variation, moreover, when detected, is indicative of the complex microevolutionary patterns shown by rDNA. The most remarkable conclusion of rDNA research at these two levels is that NORs seem to be able to spread through the genome thus creating new rDNA loci (Castro *et al*, 2001). Several spreading mechanisms have been suggested, for example, transposition (Schubert and Wobus, 1985), insertion of extrachromosomal rDNA amplified during oogenesis (Phillips *et al*, 1988), the presence of repetitive elements facilitating nonhomologous chromosome exchange (Maggini *et al*, 1991), and the amplification of minor rDNA loci (Dubcovsky and Dvörak, 1995).

The presence of satDNA is characteristic of most eukaryote organisms. It is restricted to heterochromatic chromosome regions and is typically noncoding. It usually consists of a tandemly repeated unit of size varying, in most cases, from 10 to 100 bp (Charlesworth *et al*, 1994).

SatDNA constitutes a rapidly evolving marker, which makes it useful for phylogenetic studies. Its presence or absence, and the divergence of sequences shared among species, may be powerful tools, throwing light on the evolutionary relationships among closely related species. Such information has proven to be useful in a variety of organisms, for example, insects (Bachmann and Sperlich, 1993; Juan *et al*, 1993; Bachmann *et al*, 1994; Pons *et al*, 1997), fishes (Garrido-Ramos *et al*, 1995; de la Herrán *et al*, 2001; Lanfredi *et al*, 2001), birds (Madsen *et al*, 1992), mammals (Hamilton *et al*, 1992; Wijers *et al*, 1993; Volobouev *et al*, 1995; Lee *et al*, 1999), and plants (Galasso *et al*, 2001).

The grasshopper *Eyprepocnemis plorans* harbours a B chromosome polymorphism which has become a paradigm of the long-term evolution of parasitic B chromosomes, since it has provided clear evidence for an arms race between the coevolving A and B chromosomes (Camacho *et al*, 1997). These B chromosomes are mainly made up of two repetitive DNAs, that is rDNA and a 180 bp satDNA, which are also present in many A chromosomes (López-León *et al*, 1994, 1995b; Cabrero *et al*, 1999). To ascertain the origin of these B chromosomes is, therefore, necessary to investigate the presence and chromosome distribution of these two repetitive DNAs in geographically distant *E. plorans* populations as

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well as in some other closely related species, since such a study might uncover the ancestral patterns and the evolutionary pathways that could explain the present chromosome distribution. In this paper, we analyse the presence and chromosome localization of these two repetitive DNAs in five Eyprepocnemidine species from Russia and Central Asia, including *E. plorans*.

Materials and methods

A total of 24 adult male grasshoppers belonging to five Eyprepocnemidinae species, collected at several localities in Russia and Central Asia (see locations in Table 1 in Bugrov *et al*, 1999), were analysed by fluorescence *in situ* hybridization (FISH) and silver impregnation. Two of the species analysed belonged to the genus *Eyprepocnemis* (*E. plorans*, five males collected at Daghestan, North Caucasus, and *E. unicolor*, six males collected at Tajikistan), the three other species being *Heteracris adspersa* (five males from Daghestan), *Thisoicetrinus pterostichus* (five males from Daghestan), and *Shirakiacris shirakii* (three males from Primorskij kray).

Chromosome preparations were made by squashing two testis follicles in 50% acetic acid. After 10 min, the coverslip was removed with a razor blade after freezing the preparation by immersion in liquid nitrogen for a few seconds. Silver impregnation was performed following the technique described by Rufas *et al* (1982).

For FISH, in order to eliminate cell cytoplasm and thus facilitate probe accessibility, each slide was incubated in 150 µl of pepsin (50 µg/ml in 0.01N HCl) at 37°C in a humid chamber for 10–30 min. After three washes in distilled water, the slides were dehydrated in an ethanol series at 70, 90, and 100% for 3, 3, and 5 min, respectively, and then were air dried. Slides were stored at 60°C overnight before *in situ* hybridization.

FISH was performed with two different DNA probes: pTa71, which contains a 9-kb EcoRI repeat unit of rDNA isolated from *Triticum aestivum* (Gerlach and Bedbrook, 1979), and pEpD15, which contains a 180 pb DraI fragment of a tandemly repetitive DNA isolated from *E. plorans* (López-León *et al*, 1994, 1995b). The probe DNA was labelled by nick translation with Fluorogreen 11-dUTP or Fluorored 11-dUTP, using standard techniques. FISH was performed following the technique described in López-León *et al* (1994). In brief, the two DNA probes were mixed to a final concentration of 5 ng/µl in a solution containing 40% formamide, 10% dextran sulfate, 0.1% sodium dodecyl sulphate (SDS) in 1 × SSC, and 70 ng/µl salmon sperm. Chromosomal DNA was denaturized by incubation with the hybridization mixture (30 µl) in a hot plate at 80°C for 8 min. Hybridization was performed at 37°C overnight. After two washes in 2 × SSC at 37°C, one in 2 × SSC at room temperature and one in 4 × SSC/Tween 20 (5 min. each), slides were counterstained with 2 µg/ml of DAPI (4′6 diamidino-2-phenylindole) and mounted in antifade solution (Vectashield H-1000).

Photographs were taken on Fujichrome 400 Provia colour film. Slides were digitized with a Hewlett-Packard Photo Smart scanner and the figures were composed with Adobe Photoshop and Microsoft Word.

Results

As described elsewhere (Bugrov *et al*, 1999), the five species show $2n = 22+X0♂/XX♀$ acrocentric chromosomes, with two long (L_1 and L_2), six medium (M_3–M_8) and three short (S_9–S_{11}) autosomes, and the X chromosome showing a size similar to that of the longest M autosomes.

Caucasian males of *E. plorans* show two rDNA clusters located on the S_9 and S_{11} autosomes (Figure 1a). The 180 bp tandem DNA repeat, however, is located on paracentromeric regions of the X chromosome and eight autosomal bivalents (excluding M_8, S_9, and S_{10}) (Figure 1a,b). Note that S_{11} is the only standard chromosome carrying the two repetitive DNAs. None of the four other Eyprepocnemidinae species carried the *E. plorans* repetitive DNA, suggesting that it is species specific. In consistency with rDNA location, silver impregnation showed active NORs only in bivalents S_9 and S_{11} (Figure 2a).

In the remaining species, the rDNA was located on a variable number of bivalents: one in *Thisoicetrinus pterostichus* (S_9) (Figure 1e), two in *Eyprepocnemis unicolor* (S_9 and S_{10}) (Figure 1c) and *Heteracris adspersa* (M_8 and S_{11}) (data not shown), and three in *Shirakiacris shirakii* (S_9, S_{10}, and S_{11}) (Figure 1d). Silver impregnation showed that all rDNA clusters were active in all four species (Figure 2; data not shown for *T. pterostichus*).

Discussion

Species-specific satDNA has been found in some cases, for example, the cave cricket *Dolichopoda schiavazzii* (Bachmann *et al*, 1994). In these cases, this marker provides very useful information for microevolutionary studies. When the same satDNA is shared by other species, however, it becomes a valuable marker for phylogenetic studies. For example, it may be shared by part of a genus (eg in *Tribolium* flour-beetles, Juan *et al*, 1993), a complete genus (eg *Peromyscus* rodents, Hamilton *et al*, 1992, *Pimelia* beetles, Pons *et al*, 1997, and *Lens* legumes, Galasso *et al*, 2001), several genera in a same family (eg the *RBMII* sequences in the avian Anatidae family, Madsen *et al*, 1992, and the DraI satDNA in the Sparidae fish family, de la Herrán *et al*, 2001), and even a whole family (eg the EcoRI satDNA in the Sparidae fish family, de la Herrán *et al*, 2001) or order (eg the human gamma-X centromeric satellite DNA, which seems to be widespread among primates, Lee *et al*, 1999).

The 180 bp tandem DNA repeat isolated from Spanish specimens of *E. plorans* (López-León *et al*, 1995b) is present in Caucasian males of this species, but not in the four remaining Eyprepocnemidinae species analysed. It was absent from *E. unicolor* suggesting that this satDNA is specific for *E. plorans* and is not a characteristic of the genus. Chromosomal location of this satDNA, in paracentromeric regions of most chromosomes, is roughly similar in *E. plorans* populations from such distant places as Spain (López-León *et al*, 1994), Morocco (Bakkali, 2001), and the Caucasus (this paper). In a recent analysis of 12 Spanish populations, we have found the presence of this satDNA in seven to 10 out of the 12 chromosomes of *E. plorans*, with the S_{10} chromosome lacking satDNA in all populations (as in our present study) and the S_9 carrying it only in populations from southern Spain

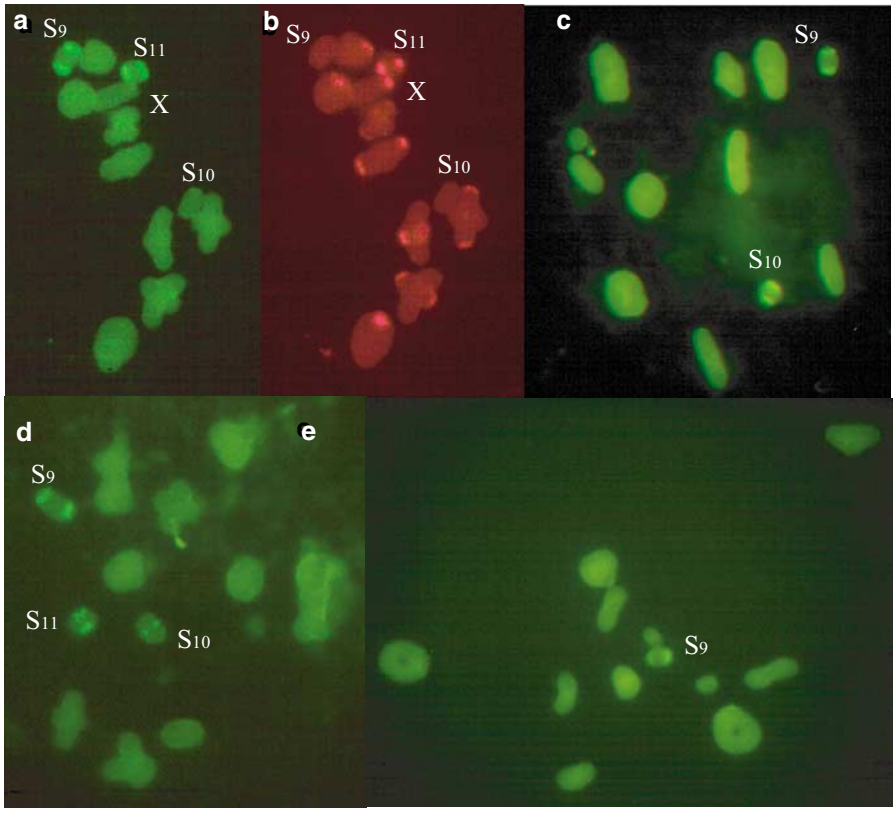

Figure 1 Fluorescence *in situ* hybridization with the rDNA (**a, c–e**) and the 180 bp satDNA (**b**) in Eyprepocnemidinae species: *E. plorans* (**a,b**), *E. unicolor* (**c**), *Shirakiacris shirakii* (**d**), and *Thisoicetrinus pterostichus* (**e**).

(Cabrero *et al*, 2003). This interpopulational resemblance suggests that this satDNA appeared soon in the evolution of *E. plorans*, most likely in the ancestral African populations before the colonization of the remaining regions, since *Eyprepocnemis* is a genus of African origin (Dirsh, 1958). It would be, however, interesting, to compare the DNA sequence of this repeat among specimens from the three geographic regions. In addition, the analysis of presence, chromosome localization, and sequence in the four *E. plorans* subspecies that have been described in Africa (Dirsh, 1958) could be very informative for the evolution of this species.

The rDNA is restricted to one to three of the smallest chromosomes (M_8–S_{11}) in the five Eyprepocnemidinae species analysed here. This suggests that the ancestral number of rDNA clusters in the Eyprepocnemidinae is restricted to one to three of the four smallest autosomes (M_8–S_{11}). In some animal groups, for example, fishes, the existence of a single rDNA cluster is considered a plesiomorphic character (see Jankun *et al*, 2001), although many examples of multichromosomal location of rDNA have been described (see Castro *et al*, 2001). In the Eyprepocnemidinae grasshoppers analysed here, the most conserved rDNA cluster is that located on the S_9 chromosome, which is present in four of the five species analysed. This chromosome constitutes the so-called 'megameric' bivalent during male meiosis, which shows an allocyclic behaviour similar to the X univalent, with coincident changes in heteropycnosis (Hewitt, 1979). In a study of chromosome NOR location in 21 species of grasshoppers, by means of silver impregnation, Rufas *et al* (1985) suggested the megameric chromosome to be one of the ancestral NOR locations within the family Acrididae. It is thus possible that the monochromosomal ancestral location of rDNA in Eyprepocnemidinae grasshoppers was the megameric autosome pair, a condition that is still conserved in *Thisoicetrinus pterostichus*. In the remaining species, the number of rDNA clusters has increased by the presence of additional rDNA clusters in one (S_{11} in *E. plorans* and S_{10} *E. unicolor*) or two small chromosomes (S_{10} and S_{11} in *Shirakiacris shirakii*). In *Heteracris adspersa*, however, the ancestral S_9 cluster has been lost and the rDNA is located on M_8 and S_{11} chromosomes.

There is no clear explanation for interspecies differences in the number of rDNA clusters in the genome. Some authors have explored the possibility of a relation with anatomical differences, but the number of rDNA sites does not show any consistency with morphologically based phylogeny in carabid beetles (Sánchez-Gea *et al*, 2000). Other authors have found a weak but positive correlation between chromosome number and the number of rDNA loci (Hirai *et al*, 1994). But this is not applicable to the Eyprepocnemidinae grasshoppers analysed here, because the observed variation in the number of rDNA clusters was found in species with the same chromosome number.

The presence of rDNA only in the S_9 and S_{11} chromosomes of Caucasian specimens of *E. plorans* is in sharp contrast with its presence in most chromosomes of Spanish and Moroccan specimens (López-León *et al*, 1994; Bakkali *et al*, 2001; Cabrero *et al*, 2003). This shows

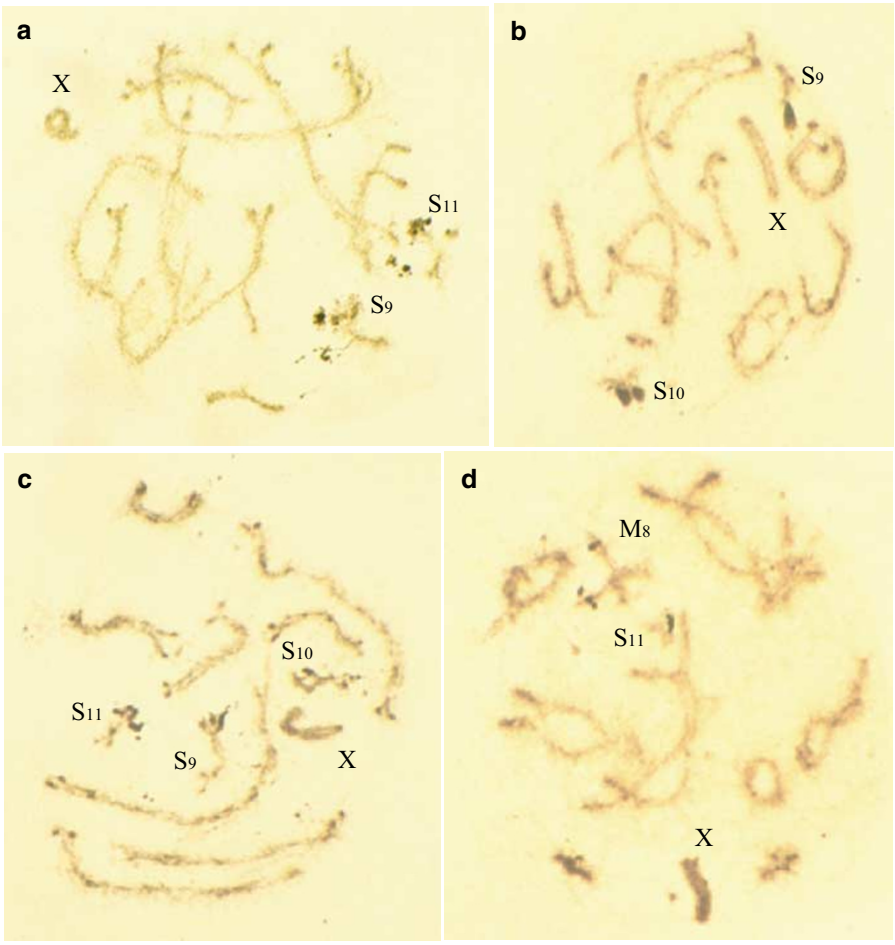

Figure 2 Silver impregnation in Eyprepocnemidinae species: *E. plorans* (**a**), *E. unicolor* (**b**), *Shirakiacris shirakii* (**c**), and *Heteracris adspersa* (**d**).

the occurrence of a dramatic spread of rDNA over the whole genome in western populations of *E. plorans*. Especially remarkable is the absence of rDNA from the X chromosome in Caucasian *E. plorans* and the four other Eyprepocnemidinae species analysed, a chromosomal location which constitutes one of the principal active NORs in western individuals (López-León *et al*, 1995a; Cabrero *et al*, 1987; Bakkali *et al*, 2001) which suggests that this absence is ancestral.

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