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Unusual distribution pattern of telomeric repeats in the shrews *Sorex araneus* and *Sorex granarius*

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Abstract

Sorex araneus and *Sorex granarius* are sibling species within the *Sorex araneus* group with karyotypes composed of almost identical chromosome arms. *S. granarius* has a largely acrocentric karyotype, while, in *S. araneus*, various of these acrocentrics have combined together by Robertsonian (Rb) fusions to form metacentrics, with the numbers and types of metacentrics differing between chromosomal races. Our studies on telomeric sequences in *S. araneus* and *S. granarius* revealed differences between chromosomes and between species. In *S. araneus* (the Novosibirsk race), hybridization signals were present on the telomeres of all the chromosomes after FISH with a PCR-generated telomeric probe. In addition, hybridization signals were observed at high frequencies in the pericentric regions of some but not all metacentrics formed by Rb fusion. There were fewer signals on those metacentrics formed earlier in the evolution of *S. araneus*. This suggests that *S. araneus* chromosomes retain at least some telomeric repeats during Rb fusion, but that these repeats are lost or modified over time. These results are critical for the interpretation of the well-studied hybrid zones between chromosomal races of *S. araneus*, given that Rb fission has been postulated in such hybrid zones and that the likelihood of Rb fission will relate to presence/absence of telomeric sequences at the centromeres of metacentrics. In *S. granarius,* there were strong signals at the proximal (centromeric) telomeres of the acrocentrics after FISH with a DNA telomeric probe. FISH with a PNA telomeric probe on *S. granarius* acrocentrics showed that the proximal telomeres were 213 kb on average, while the length of the distal telomeres was 3.8 kb on average. Two-colour FISH, using a telomeric DNA probe and a microdissected probe generated from the pericentric regions of the *S. granarius* chromosomes *a* and *b*, revealed regions on distinct chromatin fibres where telomeric and microdissected probes were colocalized or localized sequentially. The proximal telomeres of *S. granarius* are highly unusual both in their large size and their heterogeneous structure relative to the telomeres of other mammals.

Introduction

Telomeric repeats, $(TTAGGG)_n$, are among the most important sequences with regard to mammalian chromosomal evolution. As their name suggests, the majority of these repeats are localized at the telomeres. It is well known that their dysfunction in this location can give rise to chromosomal rearrange-

ments (Slijepcevic 1998). In addition to the telomeres, $(TTAGGG)_n$ clusters have been detected as interstitial telomeric signals (ITS) away from the ends of the chromosomes. These most often occur in regions of constitutive heterochromatin (Meyne *et al*. 1990) and can represent sites where chromosomes have fused at their telomeres (Ruiz-Herrera *et al*. 2002, Hartmann & Scherthan 2004). It has become clear that ITS may be hot spots for spontaneous and ionizing chromosome breakage and recombination (Mondello *et al*. 2000, Kilburn *et al*. 2001, Rivero *et al*. 2004). Thus, mutations involving telomeric repeat clusters at telomeres or at ITS may lead to chromosomal fusions and fissions, respectively.

Fusion of acrocentrics at their centromeres to form metacentrics (Robertsonian fusion) and the reverse process (Robertsonian fission) are among the most common rearrangements that have taken place during mammalian chromosomal evolution (Searle 1993). To understand Robertsonian (Rb) rearrangements, it is important to study the role and fate of telomeric repeats in the vicinity of the centromeres. This has been studied in detail in the western house mouse, *Mus musculus domesticus* (Garagna *et al*. 1995, Nanda *et al*. 1995) and other species (Metcalfe *et al*. 1998, Dobigny *et al*. 2003). In this paper, we consider a particularly well-characterized model of Robertsonian variation involving shrews.

Among the red-toothed shrews (subfamily Soricinae, family Soricidae), the largest genus is *Sorex*, and within that genus there is a complex of sibling species (the *Sorex araneus* group) with a largely Palaearctic distribution characterized by an XX/XY_1Y_2 sex chromosome system (Zima *et al*. 1998). Chromosomally, the ancestral state for that group is believed to be an all-acrocentric karyotype, and the species closest to displaying this is *S. granarius* (Wójcik & Searle 1988, Volobouev & Dutrillaux 1991). Robertsonian fusions have been the primary form of chromosomal rearrangement during the evolution of the group, with different species characterized by different combinations of metacentrics (Zima *et al*. 1998). The chromosome arms that characterize karyotypes within the *Sorex araneus* group are labelled *a–u*, according to decreasing size (Searle *et al*. 1991) and largely represent the ancestral acrocentrics. *S. granarius* has a diploid number of 36 (females) or 37 (males), with acrocentric pairs *a*, *b*, *c*, *f*, *g*, *h*, *i*, *j*, *k*, *l*, *m*, *n*, *o*, *p*, *q* and *r*, an autosomal metacentric pair *tu* and the sex chromosomes *de* (X) in females and *de* (X), *s* (Y_1) and *d* (Y_2) in males. The 'X' in shrews of the *Sorex araneus* group represents a fusion between the true mammalian X (arm *e*) and an autosome (arm *d*) (Sharman 1991, Pack *et al*. 1993). *S. araneus* has a more derived karyotype than *S. granarius*, and is characterized by the autosomal metacentrics *af*, *bc*, *jl* and *tu*, as well as the same sex chromosomes as *S. granarius*. These metacentrics are found throughout the distribution of *S. araneus*, but other metacentrics are found more locally. *S. araneus* is subdivided into at least 68 chromosomal races that are distinguished by combinations of metacentrics formed from chromosome arms *g*, *h*, *i*, *k*, *m*, *n*, *o*, *p*, *q* and *r*, ranging from the Pelister race in the Balkans that has a diploid number of 30 (female)/31 (male) with all these chromosomes as acrocentrics, to a race such as the Novosibirsk race of Siberia with a fully metacentric karyotype consisting of *ik*, *go*, *hn*, *mp* and *qr* (2n = 20/21) (Wójcik *et al*. 2003).

In this paper, we examine the distribution of $(TTAGGG)_n$ repeats in the karyotype of *S. granarius* and of the Novosibirsk chromosomal race of *S. araneus*, in order to make evolutionary inferences in one of the most spectacular systems of Robertsonian variation in any mammal.

Material and methods

Cell culture

The *S. araneus* and *S. granarius* primary fibroblasts were obtained from pieces of intercostal muscle of two wild-caught animals, collected in Novosibirsk (Russia) and in Spain, respectively (the age of the animals is unknown).

Slides for FISH and microdissection

Spreads of methanol–acetic-acid-fixed metaphase chromosomes were prepared by standard methods. Distinct chromatin fibres were obtained from cultured fibroblasts; 1000 live cells in 1 µl PBS were dropped onto the edge of a glass slide inclined at an angle of 45° and air dried. Then cells were subjected to lysis in 10 µl STE1 buffer (0.5% SDS, 0.1 mol/L Tris pH 7.0, 0.05 mol/L EDTA) for 5 min at room temperature, fixed in Carnoy's solution for 2 min, air dried and exposed for 30 min at 60°C.

Generation of DNA probes

The generation of DNA probes from microdissected metaphase chromosomes followed by DOP–PCR has been described previously (Rubtsov *et al*. 2000). The DNA fragments were labelled with biotin-11-dUTP or digoxigenin-11-dUTP over 15 additional PCR cycles. The telomeric DNA probe was generated by PCR using the oligonucleotides $(TTAGGG)_5$ and $(CCCTAA)_5$ (Ijdo *et al*. 1991).

FISH

FISH was performed according to a standard protocol with salmon sperm DNA as a carrier DNA. Biotin- and digoxigenin-labelled probes were visualized with avidin–FITC and mouse antidigoxigenin antibodies conjugated to Cy3, respectively. Two-colour FISH with DNA telomeric and microdissected probes on distinct chromatin fibres was performed. This involved suppression of dispersed repeats with shrew Cot1 DNA so that denaturation of DNA on slides and of the telomeric probe was done at the same time, whereas the denaturated microdissection probe was added 2 h later. Chromosomes and distinct fibres were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and analysed using an Axioskop 2 epifluorescence microscope (Carl Zeiss, Germany) equipped with a CCD camera (CV M300, JAI Corporation, Japan), CHROMA filter set and ISIS4 image-processing package (MetaSystems-Group, Inc., USA).

In-situ hybridization with a telomere Cy-3-conjugated $(CCCTAA)_3$ peptide nucleic acid (PNA) probe (PBIO/Biosearch Product, Bedford, MA) was performed as described previously (Lansdorp *et al*. 1996). FISH and image analysis followed Martens *et al*. (1998). Chromosomes were counterstained with DAPI. Slides were analysed on an Axioskop 2 microscope equipped with a quintet-band pass filter for Cy3/FITC/DAPI/Texas Red/DEAC (Axioplan 2 Imaging, Carl Zeiss, Germany). The images were captured by CCD camera (AxioCam MRm, Carl Zeiss, Germany) and analysed with an ISIS4 image-processing package. Two levels of calibration were used to ensure a reliable quantitative estimation of telomere length. First, to correct for daily variations in lamp intensity and alignment, images of fluorescent beads (orange beads, size 0.2 μm, Molecular Probes) were acquired and similarly analysed with the 'TFLTelo version 2' computer program. Second, relative telomere fluorescence units (TFU) were extrapolated from plasmid calibration data. Plasmids with variable size inserts (0.15, 0.4, 0.8 and 1.6 kb) of $(TTAGGG)_n$ repeats (Hanish *et al*. 1994) were sequenced. Inserts of 0.8 and 1.6 kb were found to contain many telomere-like repeats, whereas plasmids with smaller inserts contained, respectively, 144 bp and 330 bp of 'pure' TTAGGG repeats suitable for calibration purposes. DNA (0.1 μg) from these plasmids was denatured (5 min, 80°C) on a Theromixer 5436 (Eppendorf) in the presence of a Cy3-labelled PNA probe, followed by hybridization for 30 min at room temperature. After the hybridization mixture (100 μl) had been dropped on a microscope slide, it was processed in the same way as slides with metaphase spreads.

Results

Localization of the telomeric repeats on S. araneus *chromosomes*

FISH with the telomeric DNA probe gave distinct signals on the telomeres and fainter ones on interstitial regions of all the *S. araneus* chromosomes (Figure 1a, Table 1). Data for 102 metaphases are given in Table 1. ITS were most frequently observed in the pericentric regions of chromosomes *ik*, *go*, *jl*, *hn*, *mp* and *qr*. The frequency of occurrence of the signals on these chromosomes of this group ranged from 29% for chromosome *jl* to 81% for chromosome *mp*. In the pericentric regions of the other metacentric chromosomes, *af*, *bc*, *de* (X) and *tu*, the percentage of hybridization signals did not exceed 10%. In addition, ITS were detected on arms *a* and *b* of chromosomes *af* and *bc* with frequencies of 19% and 12%, respectively.

Localization of the telomeric repeats on S. granarius *chromosomes*

FISH with the telomeric DNA probe on *S. granarius* chromosomes showed strong signals only on the proximal telomeres of acrocentrics (Figure 1b; where 'proximal' telomeres are defined as those close to the

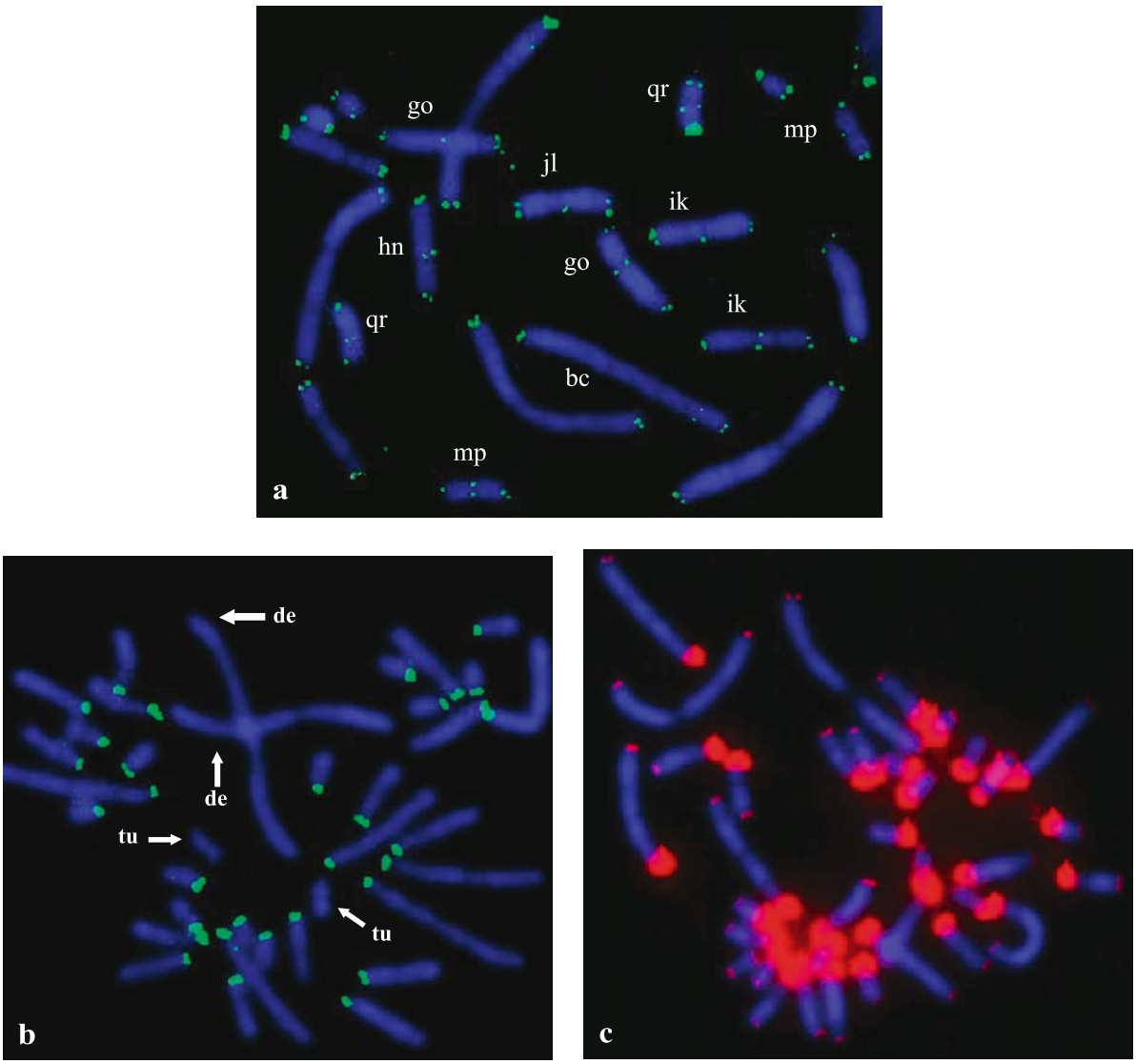

Figure 1. FISH on *S. araneus* (**a**) and *S. granarius* (**b**) chromosomes using a biotinylated telomeric probe generated by PCR. Chromosomes counterstained with DAPI. The *S. araneus* chromosomes on which ITS were detected and chromosomes of *S. granarius* which are metacentric, are indicated. (**c**) FISH on *S. granarius* chromosomes, using a telomeric PNA probe.

centromeres and 'distal' telomeres are those at the ends of the long arms of chromosomes). This was observed in all 30 metaphases examined.

The $(TTAGGG)_n$ sequences were detected on all the telomeres of all the *S. granarius* chromosomes only when using the telomeric PNA probe (Figure 1c), which provided a quantitative estimate, based on all chromosomes in six metaphases. The mean (± SE) length of the proximal telomeres was 213 ± 5.8 kb (maximally 300 kb) and the telomeres of the metacentrics and the distal telomeres of the acrocentrics were a hundred-fold shorter (3.8 ± 0.2 kb).

FISH with telomeric and microdissected DNA probes on S. granarius *chromosomes and distinct chromatin fibres*

The microdissected DNA probe generated from the pericentric regions of six copies of the *S. granarius* chromosomes *a* and *b* painted the proximal telo-

meres of all the *S. granarius* acrocentrics at the $(TTAGGG)_n$ signal sites. In *S. araneus,* the probe painted only the pericentric regions of arms *a* and *b* of chromosomes *af* and *bc*. These results were obtained consistently in 30 metaphases each for the two species (Figure 2a–c). The microdissected DNA probe gave an intense signal on the proximal telomeres of the acrocentric chromosomes of *S. granarius* presumably because of the very high copy number of the TTAGGG sequence or because, along with this sequence, the probe contained other DNA repeats located at the proximal telomeres of the *S. granarius* acrocentrics and in the pericentric regions of arms *a* and *b* of the *S. araneus* chromosomes.

To analyse the organization of the proximal telomeres in the *S. granarius* acrocentrics, we performed two-colour FISH on distinct chromatin fibres, using microdissected and PCR generated telomeric probes. In the case of fibre FISH, regions painted with the two probes were seen interspersed among those painted with one (Figure 2d).

To evaluate the validity of the fibre FISH results, we performed hybridization on chromatin fibres of *S. araneus* and *Mus musculus* (mouse strain C57BL/6). It is known that the telomere is up to 50 kb long in the mouse of inbred strains (Zijlmans *et al*. 1997). In the two species, hybridization signals were seen as long painted tracks denser and significantly shorter than those in *S. granarius*, when using the telomeric DNA probe. No specific signals were observed after hybridization with the use of the microdissected DNA probe.

Thus, the FISH results indicate that, besides $(TTAGGG)_n$ sequences, other repeats appear to be located in the proximal telomeres of the *S. granarius* acrocentrics, producing complex interspersion patterns.

Discussion

It is now evident that the terminal regions of the mammalian chromosomes are standardly composed of $(TTAGGG)_n$ clusters and telomere-associated repetitive sequences. These sequences are likely to be important in chromosomal rearrangements, most notably Rb fusions, which occur frequently in mammals. Clearly, it is of interest to know whether metacentrics produced by Rb fusion retain proximal telomeric repeats; this would suggest the involvement of the repeats in the Rb fusion mutation and would increase the chance of reverse mutation; i.e. for the fusion product to undergo Rb fission.

The involvement of telomeres in Rb fusion has been considered by Slijepcevic (1998). He demonstrated that inactivation or structural disruption of the telomeres may lead to chromosomal fusion in which the telomeric repeats are retained. This leads to ITS at the centromeres of Rb fusion products, as have been demonstrated in humans (Azzalin *et al*. 2001), *Muntiacus muntjak* (Hartmann & Scherthan 2004), *Wallabia bicolor* (Metcalfe *et al*. 1998), *Mus minutoides/musculoides* (Castiglia *et al*. 2002), *Taterillus* (Dobigny *et al*. 2003), *Miciureus demerarae* (Pagnozzi *et al*. 2000) and other examples. The absence of $(TTAGGG)_n$ tracts at the centromeres of Rb fusion products may indicate alternative mechanisms of Rb fusion. In the latter case, fusion presumably results from breakpoints in the subtelomeric regions, as can be implicated in the formation of metacentrics in chromosomal races of the western house mouse *Mus musculus domesticus* (Garagna *et al*. 1995, 2002, Nanda *et al*. 1995). The pericentric regions of mouse telocentric chromosomes contain large blocks of the major satDNA fraction. They are next to blocks of the minor satDNA which in turn are adjacent to the $(TTAGGG)_n$ repeats. In the house mouse, the entire telomeric DNA, as well as much of the minor satDNA fraction, is lost from the pericentromeric regions of metacentrics. ITS are also absent from the centromeres of Rb fusion products in *Suncus murinus* (Rogatcheva *et al*. 2000).

In *S. araneus* there is a more complex situation than described in the above species. Some metacentrics clearly have a substantial amount of telomeric material at their centromeres, while others have very little. The reason for this becomes clear when the two types of metacentric are compared. The metacentrics with few proximal $(TTAGGG)_n$ repeats are *af*, *bc*, *de* (X) and *tu*. These are metacentrics that are found in all *S. araneus* and therefore are relatively old metacentrics. Not only were they present in the common ancestor of all *S. araneus*, these metacentrics were formed earlier than that. Metacentric *de* (X) is found in all members of the *S. araneus* group, *tu* is also present in *S. granarius*, all the chromosomes are found in *S. antinorii* (the closest relative of *S. araneus*: Brünner *et al*. 2002) and *af* and *tu* are present in *S. coronatus* (the second closest relative to *S. araneus* in terms of chromosome complement: Hausser *et al*. 1998). Therefore, *af*, *bc*, *de* (X) and *tu* were all formed

Table 1. Frequency of ITS in *S. araneus* (Novosibirsk race) chromosomes (based on 102 metaphases) after FISH with PCR-generated telomeric DNA probe (ITS either positioned at centromeres ['cen'] or within a chromosome arm [e.g., '*a*']).

Chromosome	Number of chromosomes with the signals on two chromatids	Number of chromosomes with the signals on one chromatid	Frequencies of chromosomes with ITS (%)
af (cen)	4	1	2
af (*a*)	7	33	19
bc (cen)	11	8	9
bc (*b*)	10	15	12
ik (cen)	53	51	50
go (cen)	46	45	44
jl (cen)	25	35	29
hn (cen)	42	53	46
mp (cen)	112	57	81
qr (cen)	120	34	74
tu (cen)	11	3	7
de (cen)	13	2	7
Y_1	5	1	3
Y_2	0	14	7

earlier in the evolution of *S. araneus* than those metacentrics that do have substantial quantities of telomeric repeats at their centromeres: *ik*, *go*, *jl*, *hn*, *mp* and *qr*. Of this second set of chromosomes, *jl* is the only one that is found throughout the distribution of *S. araneus*, and interestingly it has the fewest proximal $(TTAGGG)_n$ repeats of this second set of metacentrics. Nevertheless, *jl* has more telomeric material at their centromeres than *af*, *bc*, *de* (X) and *tu*, and is a more recent metacentric; although it is found throughout the distribution of *S. araneus*, it is limited to that species. So, there is a perfect negative relationship between age of the metacentrics in *S. araneus* and amount of telomeric material at their centromeres. This is most easily explained by the retention of $(TTAGGG)_n$ repeats on Rb fusion in *S. araneus*, although this telomeric material has become lost or modified through time, such that it is virtually absent from older metacentrics.

Interestingly, there is also evidence of modification through time of pericentric constitutive heterochromatin in *S. araneus*. Data for other races (Aberdeen and Hermitage, in Britain), show an absence of centromeric C-banding on those metacentrics found throughout the species: *af*, *bc*, *de* (X), *jl* and *tu*, moderate centromeric C-banding on those chromosomes that characterize all races in Britain and Western Europe: *gm* and *hi*, and strong centromeric C-banding on acrocentrics and metacentrics specific to particular races (Searle 1983).

The occurrence of telomeric sequences at the centromeres of many of the chromosomes of *S. araneus* is very important with respect to the population genetics of this species. There has been particular interest in the hybrid zones between chromosomal races of the common shrew. Some of these hybrid zones between metacentric races are dominated by acrocentric chromosomes, which are apparently favoured by natural selection (Searle 1993). One possible explanation for the occurrence of these acrocentrics is that they arose by Rb fission (Searle 1993). Clearly, because the races involved in such hybrid zones differ by metacentrics of the 'young' type, those metacentrics would have telomeric sequences at their centromeres. The presence of such sequences makes Rb fission a possibility. A fission event could generate acrocentrics that already have telomeres.

The metacentric chromosomes *de* (X) and *tu* are found in *S. granarius* as well as *S. araneus* and are, of course, of the same age. As in *S. araneus*, proximal telomeric sequences are absent or in very small quantities for these chromosomes in *S. granarius*. The situation with the acrocentric chromosomes of *S. granarius* is quite different. To our knowledge, telomeres as long as 300 kb have not hitherto been described in any species of mammal. The evolutionary basis of such extremely long telomeres is intriguing and worthy of more study. In recent years, there has been an interest in the genetic factors that determine differences in telomere

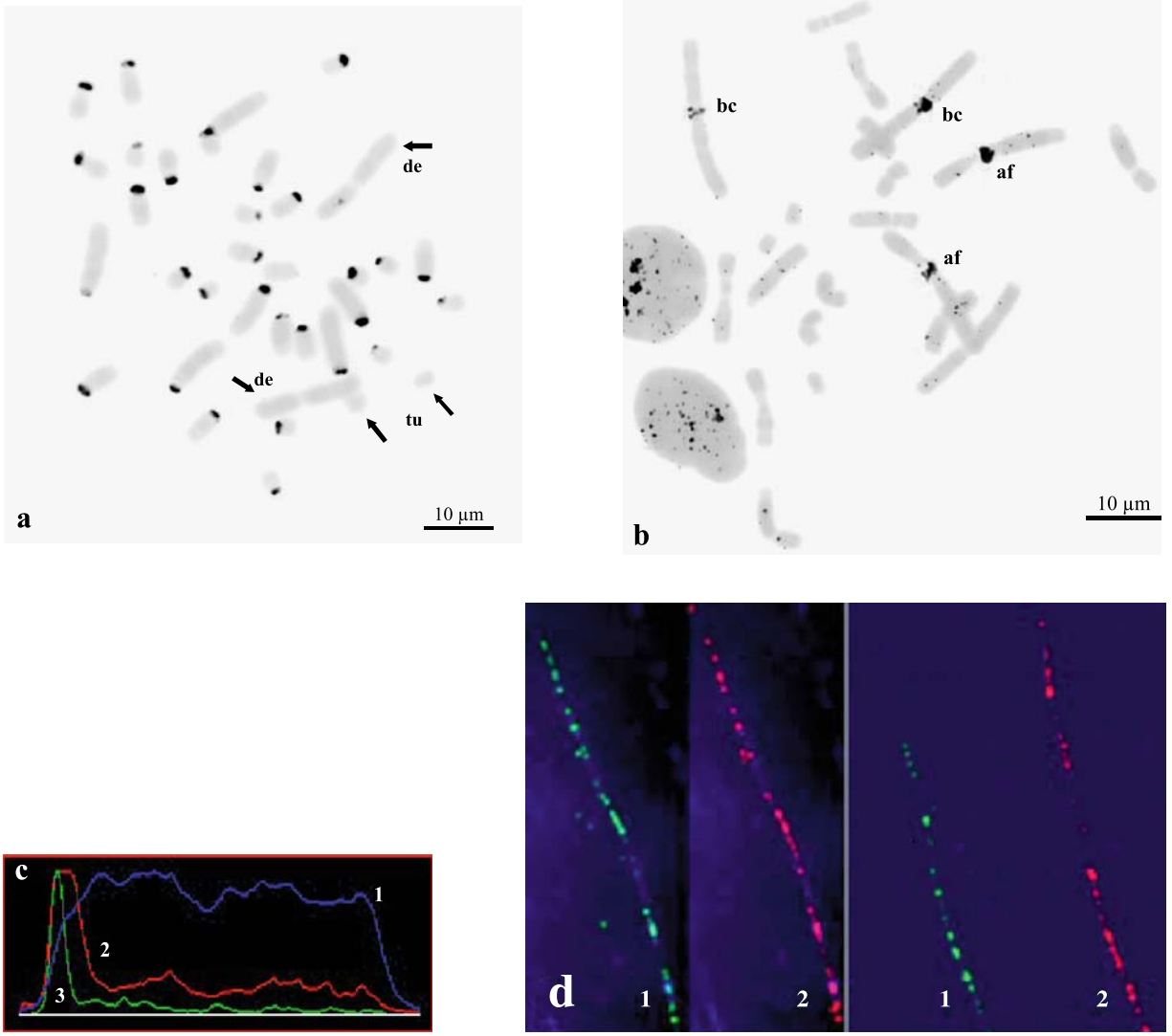

Figure 2. FISH on *S. granarius* (**a**) and *S. araneus* (**b**) chromosomes, using a biotinylated microdissected probe generated from the pericentric regions of six copies of the *S. granarius* chromosomes *a* and *b*. Chromosomes counterstained with DAPI. The *S. araneus* chromosomes on which signals were detected and chromosomes of *S. granarius* that are metacentric, are indicated. (**c**) Typical profiles of relative signal intensities along acrocentric chromosomes of *S. granarius* from the proximal telomere (left) to the distal telomere (right). The maximum signal in the analysed image is given as 100%. (1) DAPI staining of chromosome; (2) digoxigenin-11-dUTP-labelled microdissected probe generated from pericentric regions of six copies of the *S. granarius* chromosomes *a* and *b*; (3) biotinylated telomeric probe generated by PCR. (**d**) Two-colour FISH on *S. granarius* distinct chromatin fibres. The same fibres painted by biotinylated telomeric probe generated by PCR (1) and digoxigenin-11-dUTP-labelled microdissected probe generated from the pericentric regions of six copies of *S. granarius* chromosomes *a* and *b* (2).

length within and between species, as analysed in the mice, *Mus musculus* and *M. spretus* (Zijlmans *et al*. 1997, Zhu *et al*. 1998, Manning *et al*. 2002).

Another salient feature of the telomeres of the *S. granarius* acrocentrics is the almost hundred-fold difference in telomere size between the proximal and distal telomeres on the same chromosome. Discrepancies have been observed before in acrocentrics but not to the same degree as in *S. granarius* and with the distal telomeres longer than the proximal (Zijlmans *et al*. 1997, Slijepcevic 1998). Not only are the proximal centromeres of *S. granarius* unusually large but,

based on the painting results, they have a more complex molecular organization than the telomeres in previously studied species of mammal. Further analysis is required to characterize these unexpected structures.

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