Conservation, divergence and functions of centromeric satellite DNA families in the Bovidae

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Abstract

Repetitive satellite DNA (satDNA) sequences are abundant in eukaryote genomes, with a structural and functional role in centromeric function. We analysed the nucleotide sequence and chromosomal location of the five known cattle (*Bos taurus*) satDNA families in seven species from the tribe Tragelaphini (Bovinae subfamily). One of the families (*SAT1.723*) was present at the chromosomes' centromeres of the Tragelaphini species, as well in two more distantly related bovid species, *Ovis aries* and *Capra hircus*. Analysis of the interaction of *SAT1.723* with centromeric proteins revealed that this satDNA sequence is involved in the centromeric activity in all the species analysed and that it is preserved for at least 15-20 My across Bovidae species. The satDNA sequence similarity among the analysed species reflected different stages of homogeneity/heterogeneity, revealing the evolutionary history of each satDNA family. The *SAT1.723* monomer-flanking regions showed the presence of transposable elements, explaining the extensive shuffling of this satDNA between different genomic regions.

Key words: satellite DNA; centromeric function; *SAT1.723*; Bovinae; Caprinae; Bovidae genomes.

Introduction

Tandemly repeated or satellite DNA (satDNA) represents a major fraction of most eukaryotic genomes, as one of the classes of repetitive sequence (Biscotti et al. 2015a; Biscotti et al. 2015b; Charlesworth et al. 1994; Slamovits and Rossi 2002). Consisting of tandemly repeated DNA motifs, typically arranged in large arrays of hundreds or thousands of copies, satDNA is often (although not exclusively) located in blocks at the heterochromatic regions of chromosomes, at centromeres, near telomeres or in interspersed locations (Garrido-Ramos 2015; Plohl et al. 2012; Vourc'h and Biamonti 2011).

Most eukaryotic species include multiple, unrelated, families of satDNA that differ in sequence, nucleotide composition, monomer unit length, abundance and chromosomal location (Garrido-Ramos 2015; Rojo et al. 2015). Each satellite DNA family consists in a library of monomer variants that can be shared by related species. Expansions and/or elimination of different variants from this library may result in rapid

changes in satDNA distribution and abundance profiles, even among closely related species (Kuhn et al. 2008; Plohl et al. 2012; Rojo et al. 2015). Consequent on the dynamic changes in satellite DNA during an evolutionary period, these sequences can be species- or genus-specific (Garrido-Ramos 2015). Nevertheless, some satDNA sequences seem to have been preserved over long evolutionary periods in some genomes, being considered as "frozen" satDNAs (Biscotti et al. 2015a; Chaves et al. 2017; Kuhn and Heslop-Harrison 2011; Mravinac et al. 2002; Mravinac et al. 2005; Petraccioli et al. 2015). This long-term conservation of ancestral repeats can be explained by the influence of selective constraints imposed on functional motifs, or on structural features of satellites monomers possibly involved in any of the putative roles of satDNAs (Chaves et al. 2017; Plohl et al. 2012; Rojo et al. 2015), including heterochromatin formation and maintenance, chromosome pairing and segregation, chromatin elimination, chromosome rearrangements' promoters, cell cycle control or gene expression regulation (Adega et al. 2009; Enukashvily and Ponomartsev 2013; Ferreira et al. 2015; Louzada et al. 2015; Paço et al. 2013; Pezer et al. 2012; Plohl et al. 2008; Slamovits and Rossi 2002; Ugarkovic 2009). Evolutionary changes in satDNA can drive population and species divergence (Adega et al. 2009; Feliciello et al. 2015; López-Flores and Garrido-Ramos 2012; Paço et al. 2013; Ugarković and Plohl 2002; Vieira-da-Silva et al. 2015). We found that centromeric satDNA was involved in the mechanics of chromosomal fusion in Bovidae (Adega et al. 2006; Adega et al. 2009; Chaves et al. 2003; Chaves et al. 2000; Chaves et al. 2005; Di Meo et al. 2006; Kopecna et al. 2014; Kopecna et al. 2012; Nieddu et al. 2015). SatDNA sequences are also useful phylogenetically where changes in composition, organization and/or physical location can allow inference of evolutionary relationships (Adega et al. 2006; Adega et al. 2009; Chaves et al. 2005; Chaves et al. 2004; Kopecna et al. 2014; Kopecna et al. 2012).

The Bovidae is one of the most important mammalian families in the Cetartiodactyla order, comprising approximately 140 species, whose evolutionary relationships are often obscure (Groves and Grubb 2011; MacEachern et al. 2009). Chromosome evolution studies use the domestic cattle (*Bos taurus*, 2n=60) karyotype as a reference (Gallagher and Womack 1992). In the cattle genome, eight abundant centromeric satDNA families were initially described (Macaya et al. 1978), representing nearly 25% of all the DNA, with interrelated evolutionary histories (Chaves et al. 2000; Chaves et al. 2005; Macaya et al. 1978; Modi et al. 1996; Taparowsky and Gerbi 1982a, b). Some families are ancient and shared by descent in other bovid species (Adega et al.

2006; Chaves et al. 2000; Chaves et al. 2005; Chaves et al. 2004; Kopecna et al. 2014; Kopecna et al. 2012; Modi et al. 1993, 1996; Modi et al. 2004); the bovine *SATI* is present in all Pecoran genomes (Chaves et al. 2000; Chaves et al. 2005; Modi et al. 1993, 1996), unlike families named *SAT1.723*, *SATIV*, *SAT1.711a* and *SAT1.711b* that are not in all Pecora, although results are somewhat equivocal (Escudeiro et al. 2019).

Study of the structure and function of centromeric satDNAs (Adega et al. 2009; Cerutti et al. 2016; Giannuzzi et al. 2012; Plohl et al. 2008; Schmidt and Heslop-Harrison 1998) at the primary constriction (centromere) of mammalian chromosomes shows the sequences interacting with the centromere-specific histone H3 variant (CENP-A) (Aldrup-Macdonald and Sullivan 2014). The ability to bind CENP-A is considered a marker of active centromeres (Cerutti et al. 2016; Henikoff et al. 2015; Plohl et al. 2014; Purgato et al. 2015; Steiner and Henikoff 2015; Talbert et al. 2018; Zhang et al. 2013).

To understand the nature, conservation, evolution and functional role of cattle satDNA, we selected the most abundant families (*SATI*, *SATIV*, *SAT1.723*, *SAT1.711a* and *SAT1.711b*) and studied these in seven species representative of the genera *Tragelaphus* and *Taurotragus*; all were medium- to large-bodied taxa distributed through the savannah and forested regions of sub-Saharan Africa (Groves and Grubb 2011). The orthologous sequences and the differences in sequence similarity, chromosome location, and distribution provide important information on the evolutionary history of these species since their divergence from a common ancestor. We also investigated the association with the CENP-A histone protein to test the involvement of specific sequences in centromeric function.

Material and Methods

Cell culture, chromosome preparation and genomic DNA isolation

Our investigation included representatives of three bovid tribes (Hassanin and Douzery 1999): cattle (*Bos taurus*, BTA, tribe Bovini), the spiral horned antelope species (tribe Tragelaphini), *Tragelaphus angasii* (TAN, Nyala), *Tragelaphus imberbis* (TIM, Lesser kudu), *Tragelaphus scriptus* (TSC, Bushbuck), *Tragelaphus spekii* (TSP, Sitatunga), *Tragelaphus strepsiceros* (TST, Greater kudu), *Taurotragus derbianus* (TDE, Derby Eland) and *Taurotragus oryx* (TOR, Common Eland). The third tribe, the Caprini, was represented by the sheep *Ovis aries* (OAR) and goat *Capra hircus* (CHI). Cell lines

were maintained in DMEM supplemented with 13% AmnioMax C-100 Basal Medium, 2% AmnioMax C-100 supplement, 15% FBS, 100 U/mL and 100 µg/mL of Penicillin/Streptomycin antibiotic mixture, and 200 mM L-Glutamine (all from Gibco, Thermo Fisher Scientific). Chromosome harvesting and metaphase preparations followed routine procedures. Genomic DNA isolation was performed using Quick-Gene DNA Tissue Kit S (Fujifilm Life Science) according to the manufacturer's instructions.

SatDNA isolation, cloning and sequencing from Bovidae species

We choose the most abundant cattle satellite DNA sequences to study in other Bovinae species. Five satellite DNA families (*SATI*, *SATIV*, *SAT1.723*, *SAT1.711a* and *SAT1.711b*) of the domestic cattle genome and the orthologous satDNAs of seven species from the Tragelaphini were isolated in the current work by PCR amplification of genomic DNA obtained from cryopreserved cells of these species using specific primers (Supplementary Table S1) designed following Nijman and Lenstra (2001). Between 100 and 300 ng of genomic DNA from each species was used as template with an initial denaturing step at 94ºC for 10 min, followed by 30 cycles of 94ºC for 1 min (denaturation), 55/57/59ºC for 45 s (annealing) and 72ºC for 45 s (extension). The reaction terminates with a final extension at 72ºC for 10 min. The annealing temperature was optimized for each set of primers used: 55ºC for the *SATI* and *SATIV* primers, 57ºC for the *SAT1.723* and *SAT1.711b* primers and 59ºC for the *SAT1.711a* primers. PCR products were cloned and 20 clones of each SatDNA from each species were sequenced and submitted to GenBank with the references MK499473 to MK499615.

SatDNAs clones sequences analysis

Sequence analysis was performed using BLAST in the NCBI database. Multiple alignments were obtained with the CLUSTALW cost matrix in Geneious R9 version 9.1.2 (Biomatters); parameters were set to default values. For the *in silico* analysis we used the *B. taurus* satellite sequences with NCBI accession numbers: *SATI* – AJ293510.1; *SATIV* – AF446392.1; *SAT1.711a* – AF162491.1; *SAT1.711b* – AF162499.1; *SAT1.723* – M36668.1. The GC content and distribution of the satDNAs monomers was calculated using the Biologicscorp facility (https://www.biologicscorp.com/tools/GCContent/). Bendability/curvature propensity plots were determined using the bend.it server, applying

the DNase I based bendability parameters (Brukner et al. 1995) and the consensus bendability scale (Gabrielian et al. 1996). Monomer-flanking regions of the satDNAs sequences clones from each Tragelaphini species were screened for the presence of repetitive elements in the Eukaryota Repbase using the Censor software, and the repetitive elements found were mapped in the satDNAs clones sequences using Geneious program. The presence of the mammalian CENP-B box motif (17 bp - YTTCGTTGGAAACGGGA) in these satDNA sequences was also investigated using Geneious tools.

Fluorescent *in situ* hybridization with SatDNA clones

Physical mapping of Bovidae SatDNA sequences (*SATI, SATIV, SAT1.723*, *SAT1.711a* and *SAT1.711b*) onto the chromosomes used standard *in situ* hybridization methods (Schwarzacher and Heslop-Harrison 2000). Metaphases from BTA and the Tragelaphini species were hybridized with cloned sequences isolated from the same species except OAR and CHI were hybridized *in situ* with *SAT1.723* clone isolated from BTA. The sequences corresponding to the SatDNAs analysed were labelled with digoxigenin-11-dUTP or biotin-16-dUTP (both from Roche Biochemical reagents, Sigma-Aldrich) by PCR. The most stringent post-hybridization wash was 50% formamide/2×SSC at 42°C. Digoxigenin-labelled probes were detected with antidigoxigenin-5'-TAMRA (Roche Biochemical reagents, Sigma-Aldrich) and biotin-labelled probes were detected with FITC-conjugated avidin (Vector Laboratories). Preparations were mounted with Vectashield containing 4'-6-diamidino-2-phenylindole (DAPI) (VectorLaboratories) to counterstain chromosomes.

CENP-A immunolocalization and FISH

Immunostaining on metaphase chromosomes from BTA, the Tragelaphini species, CHI and OAR was performed as described by Piras et al. (2010) with slight modifications. Cells were incubated overnight with 40 ng/ml Colcemid (Gibco, Thermo Fisher Scientific). The cells were harvested, washed once with phosphate-buffered saline and re-suspended in 0.075M KCl for 20 minutes at 37ºC following which 200 µl of cell suspension was cyto-spun (Hettich rotofix 32A Benchtop) onto slides at 1400 rpm for 10 minutes. Slides were incubated in KCM (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, 0.5 mM NaEDTA, 0.1% (v/v) Triton X-100) for 10 minutes at room temperature. A cross-

linking treatment was performed with UVs radiant exposure of 150 mJ/cm^3. The primary antibody, mouse anti-human centromere protein A (CENP-A) monoclonal antibody (ab13939, Abcam) was added at a concentration of 1:100 and the slides were incubated at 37ºC for 1 hour. Slides were then washed in KCM for 10 minutes at room temperature. The secondary antibody, anti-mouse monoclonal FITC (81-6511, Zymed) was added at a concentration of 1:200 and the slides incubated for a further hour at 37ºC. Following another wash in KCM, slides were fixed in 4% formaldehyde for 10 minutes at room temperature and washed again in KCM. Chromosomes were mounted with coverslips and counterstained with DAPI (Vector laboratories). For combined immunofluorescence/FISH, the slides were washed in 4xSSC, 0.05% Tween 20 at room temperature for 4 hours with agitation and equilibrated in 50% formamide/2×SSC for 7 days at 4ºC. Collocalization analysis was performed with AutoQuant X3 software (Media Cybernetics) using Pearson's Correlation and Manders' Overlap Coefficients.

Image Capture and Processing

FISH images were observed using a Zeiss ImagerZ microscope coupled to an Axiocam digital camera using AxioVision software (version Rel. 4.5, Zeiss). Digitized photos were prepared for printing in Adobe Photoshop (version 7.0).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed for all the species using the Pierce Agarose ChIP Kit (Thermo scientific), following the manufacturer's recommendations. Immunoprecipitation was carried out using 2 µg of mouse anti-human CENP-A monoclonal antibody (ab13939, Abcam) and normal rabbit IgG to control non-specific binding. One-tenth of starting material was reserved as input DNA control. DNA immunoprecipitated (IP) and input samples were analysed by a PCR amplification with specific primers for the several satellite DNA sequences (Supplementary Table S1). The input/IP ratio was quantified using Image J software.

Statistical analysis

GraphPad Prism 6 (version 6.01) was used in the statistical analysis. The Pearson's correlation and Manders' overlap test was performed to determine the presence of collocalization between CENP-A antibody signals and the studied satDNA sequences

in each species' centromeres. As the samples did not present a Gaussian distribution, the values were transformed with the log function in order to normalize the values distribution.

Results

Bos taurus satDNA families and their chromosomal locations in the Bovidae

In the present study, five previously characterized satDNA families, *SATI, SATIV, SAT1.723, SAT1.711a* and *SAT1.711b* (Macaya et al. 1978), were isolated by PCR (Figure 1a; Supplementary table S1), cloned, sequenced, and physically mapped by *in situ* hybridization. The five satDNA sequences showed that all bovine satDNAs analysed display a pericentromeric to centromeric location in BTA cattle autosomes (Figure 1b). The orthologous *SATI, SATIV, SAT1.723, SAT1.711a* and *SAT1.711b* sequences were amplified from seven species of the Tragelaphini using the same PCR primers (Figure 1a; Supplementary table S1). The amplicons were subsequently cloned and sequenced. All showed high sequence similarity to the corresponding BTA satellites. The satellite clones were mapped by *in situ* hybridization to the chromosomes of the seven Tragelaphini species (Figure 1c-i). All species shared the (peri)centromeric location of *SATI* and *SAT1.723* families with BTA chromosomes. *SATI* was found in all the chromosomes from *T. angasii* (2n=55/56) (Figure 1c), *T. strepsiceros* (2n=31/32) (Figure 1g), *T.derbianus* (2n=31/32) (Figure 1h) and *T. oryx* (2n=31/32) (Figure 1i), and it was also found in some of the chromosomes from *T. spekii* (2n=30) (Figure 1f), a species with a large block of *SATI* in a submetacentric chromosome (Figure 1f). *SAT1.723* was found at the (peri)centromeric regions of all the chromosomes in *T. angasii, T. imberbis* (2n=38) (Figure 1d), *T. spekii, T. strepsiceros, T.derbianus* and *T. oryx*. In *T. scriptus* (2n=33/34) (Figure 1e), the *SAT1.723* was present only in about half of the chromosomes. In contrast to the cattle genome, where no satellite signals were detected on either sex chromosomes, the other seven Tragelaphini species carried *SATI* and *SAT1.723* sequences on the X chromosome. Two species of *Taurotragus* similarly presented *SATIV* at the (peri)centromeric regions (Figure 1h, i). In *T. derbianus* and *T. oryx*, *SATIV* orthologous sequences were on more than half of the chromosomes. After finding *SAT1.723* in all seven Tragelaphini species, we tested its presence in *Capra hircus* (CHI) and *Ovis aries* (OAR) (Figure 1j, l). *SAT1.723* present in these distant species, exhibiting also a (peri)centromeric location.

SAT variation across species

An analysis of the intrinsic features of the nucleotide sequences of the satDNA families isolated in each species was performed. The GC distribution in *SAT1.723, SATI* and *SATIV* monomers showed substantial differences (Figure 2a, b, c): *SAT1.723* from 57 to 67%; *SATI* from 51 to 54% and *SatIV* from 40 to 45%. In fact, the GC distribution is significantly higher and more constant across *SAT1.723* monomer length (Figure 2a), in comparison with the other two satDNAs (Figure 2b,c). Moreover, the GC periodicity across the *SAT1.723* monomer seems to be approximately 10-bp, which is in agreement with the nucleosomal organization (Kaplan et al. 2009; Kogan and Trifonov 2005; Zhang et al. 2013).

Differences were also detected in the curvature-propensity and bendability of *SAT1.723, SATI* and *SATIV* across the monomers (Figure 2d, e, f). *SAT1.723* monomer presents the higher values of bendability (Figure. 2d), while those of *SATIV* were lower (Figure 2f). Examination of the curvature-propensity plot calculated with DNase I-based trinucleotide parameters, reveals only one peak of a potential curvature around the 120 bp position in *SAT1723* monomer (Figure 2d). In *SATI* (Figure 2e) and *SATIV* (Figure 2f) monomers, at least two peaks with similar curvature propensity were detected.

A pairwise alignment of the cloned satDNA sequences isolated from each Tragelaphini species was performed with the related BTA sequences deposited in Genbank (Figure 3a, b, c and Figure S1, S2 and S3). *SATI* is conserved among the species analysed, revealing low intra- and intersequence variability overall (Figure 3 a; light and medium blue coloring showing sequence similarity >70%; Supplementary table S2). *SAT1.723* sequences show higher discrepancies in similarity values when comparing the clones from all the species (Figure 3b; dark blue, dark green and yellow showing a sequence similarity range of 100-40%; Supplementary table S2). This color palette shows that this sequence is much more conserved in some of the species. *SATIV* monomers (Figure 3c) revealed to be highly different between TDE and TOR (39-20% similarity; Supplementary table S2). Finally, *SATIV* from BTA is more similar to the monomer from TDE than from the one of TOR.

***SAT1.723* is associated with CENP A protein across the Bovidae family**

We used immunofluorescence (IF) with an anti-CENP-A antibody combined with *in situ* hybridization using probes for each isolated satDNA family to characterize the association of sequences and CENP-A in metaphase chromosomes (Figure 4a). *SAT1.723* colocalized more closely with CENP-A antibody signals compared to the hybridization signals of the other satDNA families (Figure S4). These results are similar both for BTA and Tragelaphini species' genomes analysed. The phylogenetically more distant species CHI and OAR genomes were also analysed and revealed analogous results. Pearson's correlation coefficient (Figure 4b) and Manders' overlap coefficient (Figure S5) confirmed the existence of a strong colocalization between *SAT1.723* and CENP-A in the combined IF-FISH experiment.

To further confirm the association of *SAT1.723* with centromeric function, chromatin from all the species analysed was immunoprecipitated with the anti-CENP-A antibody. The CHIP assay showed that the satDNA sequence was able to form DNA-protein complexes with CENP-A in living cells (Cerutti et al. 2016; Hayden and Willard 2012; Henikoff et al. 2015; Khademi 2017; Melters et al. 2013; Piras et al. 2010; Talbert et al. 2018; Zhang et al. 2013). The input DNA and the immunoprecipitated sample (IP CENP-A) from each species was analysed by PCR using specific primers for each satDNA family in our study (Figure S6). As shown in Figure4c, the ratio between IP CENP-A and input values for *SAT1.723* ranged between 5.0 and 16.6, confirming that this satDNA is enriched in CENP-A bound chromatin for all taxa. On the contrary, no enrichment of the other satDNA families was observed, as reflected by the IP CENP-A/Input ratio values which ranged from 0.1 to 0.4.

In order to identify features of centromeric activity, an *in silico* search for the CENP-B box-like motifs (a conserved short sequence acting as the binding site for CENP-B, which directly interacts with CENP-A to maintain kinetochore nucleation) (Dumont and Fachinetti 2017; Schalch and Steiner 2017) was also performed in *SAT1.723* sequence monomers from BTA and the seven Tragelaphini species. In fact, this analysis revealed the presence of a CENP-B box-like motif in the monomers of this satDNA family in all species examined (Figure4d). The *in silico* search was performed allowing the occurrence of a maximum of 4 mismatches within the 17 bp CENP-B box motif used.

***SAT1.723* monomer-flanking regions are enriched in transposable elements**

The conservation of *SAT1.723* in the Bovidae family suggests an essential function, so a detailed analysis of the genomic context of ends of satellite sequences was made. An *in silico* analysis of *SAT1.723* was performed on the assembled genomes of BTA (Btau_5.0.1 assembly, Genbank assembly accession GCA_000003205.6), CHI (ARS1 assembly, GenBank assembly accession: GCA_001704415.1) and OAR (Oar_V4.0, GenBank assembly accession: GCA_000298735.2) as there are no Tragelaphini genome sequence assemblies currently available. This analysis showed an interspersed presence of *SAT1.723* in the three species' genomes (~300, 30 and 50 BLAST hits distributed on all the autosomes and on the X chromosome from BTA, CHI and OAR, respectively) (Supplementary table S3, S4, S5). The *SAT1.723* neighbor sequences revealed that the monomers from this satDNA family present in the genome assemblies are flanked by transposable elements, both in the isolated and clustered *SAT1.723* BLAST hits (Figure 5a). The global annotation revealed that non-LTR sequences are the most represented TEs in all the *SAT1.723* BLAST hits-flanking regions of all the chromosomes of the three species (Figure 5b). An additional BLASTN search for *SATI, SATIV, SAT1.711a* and *SAT1.711b* was also performed using BTA, CHI and OAR sequencing data, and this revealed that these satDNAs present an interspersed distribution pattern in these genomes (Supplementary table S3, S4, S5), being similarly flanked by TEs (Figure S7).

The *SAT1.723* monomer-flanking regions were also analysed in the sequenced clones of the seven Tragelaphini species. Only the flanking regions of the *SAT 1.723* repeats with centromeric location were analysed, as these were the ones isolated by PCR due to their higher abundance. *SAT1.723* clone sequences from each species were screened for the presence of repetitive elements in the Eukaryota Repbase using *Censor* software. This revealed that a sequence of 26 bp from a specific LTR, the TCR1_LTR was present in almost all *SAT1.723* clones from TAN, TSC, TSP, TDE and TOR (Figure 5c, d). The LTR sequence was found in the terminal region of *SAT1.723* monomers and the 26 bp of the LTR corresponded to the last 26 bp of the complete sequence of TCR1_LTR (164 bp) (Figure 5e). No significant sequence similarity was found between the primers used for isolating *SAT1.723* sequences with the sequence of TCR1_LTR.

The presence of the TCR1_LTR in the monomer flanking regions of *SAT 1.723* clone sequences in almost all Tragelaphini species (except for TIM and TST), may be due to the biased character of the PCR technique which may not have allowed the isolation of

terminal monomers in these two genomes. No transposable elements were found in the monomers-flanking regions of the other satDNAs sequence clones (*SATI* and *SATIV*).

Discussion

SatDNA families and their chromosomal location

We present an analysis of the five most abundant satDNAs families in bovid species not restricted to *Bos*. Orthologous bovine satDNA sequences were isolated from seven Tragelaphini genomes, molecularly characterized and mapped (Figure 1a-i). *SAT1.723* has not previously been analysed outside BTA; the presence of bovine SATI (Kopecna et al. 2014; Kopecna et al. 2012) and *SATIV* (Adega et al. 2006) in species from the Tragelaphini has previously been reported. Nevertheless the *SATI* isolated from cattle and used for FISH analysis by Kopecna et al. (2012) produced only a weak hybridization signal on the Tragelaphini chromosomes, and later the same authors reported that *SATI* sequences isolated specifically from Tragelaphini genomes were present in all the acrocentric chromosomes of those species (Kopecna et al. 2014), but not the bi-armed chromosomes in *T. spekii* and *T. strepsiceros*. This contrasts with the results obtained from the newly isolated sequences from these species (Figure 1f, g). Although of the same satDNA family, different sequence variants were probably isolated in each study, suggesting that *SATI* family is composed of subfamilies/variants of sequences that are not necessarily identical to each other: studies on the Bovinae initially suggested that *SATIV* was specific for Bovini genomes (Modi et al. 2004) but Adega et al. (2006) reported the presence of this sequence in Tragelaphini indicating that the ancestral sequence most likely predates the divergence of the Bovini and Tragelaphini. In addition to the expected presence of *SATI* (considered the oldest bovine satDNA and present in all pecorans), and the confirmation of a *SATIV* presence in *Taurotragus*, we found *SAT1.723* sequences at the (peri)centromeric region in all the genomes exhibiting typical satellite DNA sequence features i.e., characterized by a tandemly repetitive pattern at the constitutive heterochromatin regions (Figure 1). This suggests that this satDNA family was likely preserved in other subfamilies of Bovidae (including Caprini, subfamily Caprinae, as well as Bovini and Tragelaphini, subfamily Bovinae). Modi et al. (1996) dated the origin of *SATI* family to 20–40 Mya, while Adega et al. (2006) reported the origin of *SATIV* at 10 Mya. We now propose that *SAT1.723* family predated the separation

of the Bovidae subfamilies Bovinae and Caprinae (Chaves et al. 2005) by at least 15-20 Mya.

SatDNA evolution on Bovidae reflects the different stages of the library model

The sequence similarity among the satDNAs isolated sequences from BTA (Bovini) and the Tragelaphini revealed significant differences in the homogeneity/heterogeneity of each satDNA family, probably reflecting different stages of in their evolution (Figure 6). The high sequence conservation among *SATI* clones (Figure 3a) suggested that this sequence is the oldest bovine satDNA (Figure 5) and is moving into the 'homogenization' stage. The multiple alignment of *SAT1.723* clone-sequences (Figure 3b) showed that this family has not yet reached the homogenization stage, being much more similar between some species than in others. The high discrepancy in the similarity values when comparing the clones from all the species strongly suggests that this satDNA family is in a 'degeneration' stage of the satDNA nucleotide sequence evolutionary process (Figure 6).

The nucleotide sequence variability of *SATIV* monomers (Figure 3c), even between phylogenetically related species, reflects its dynamic evolution and mutation rate which generally characterizes the initial, 'amplification', stage of satDNA evolution (López-Flores and Garrido-Ramos 2012). Bovine *SATIV* is considered the evolutionarily youngest satDNA family in the Bovinae (Adega et al. 2006; Jobse et al. 1995; Modi et al. 1996; Modi et al. 2004), and being in the amplification/contraction stage has probably undergone independent amplification events from the other bovine satDNA sequences (Lenstra et al. 1993) (Figure 6).

Despite the overall level of homogeneity, we found some intra- and interspecific variability in the clones of the three satDNAs (Supplementary table S2), a finding that is consistent with the existence of different satDNA sequence variants as allowed for in the library model (Fry and Salser 1977; Mestrović et al. 1998; Ugarković and Plohl 2002). Each satDNA family consists in a library of monomer variants shared by related species, and each species presents a specific repeats' profile shaped by expansions and/or elimination of different variants from the library (Fry and Salser 1977; Mestrović et al. 1998; Ugarković and Plohl 2002). In agreement with this model, the three satDNA families analysed presented distinct subfamilies differing by sequence length and composition (Supplementary figure S1, S2, S3). Moreover, different turnover rates of

each satellite repeat, even among closely related species, can result in profound differences in overall sequence homogeneity. Genomic constraints such as karyotype architecture as well as the evolutionary age of a satDNA family may influence the turnover rates of satellite DNA sequences (Louzada et al. 2015; Paço et al. 2013; Plohl et al. 2010).

SAT1.723 has a centromeric function in the Bovidae family

The localization of *SAT1.723* at the centromeres in all the species analysed suggested an involvement in centromeric function. It is accepted that centromeres are defined by epigenetic factors and through interactions between centromeric satDNA sequences and proteins (Heslop-Harrison and Schwarzacher 2013; Plohl et al. 2014; Purgato et al. 2015; Rocchi and Archidiacono 2006). In the majority of eukaryotes, the centromere identity is defined epigenetically by the presence of the histone H3 variant centromere protein A, CENP-A in the centromeric nucleosomes (McKinley and Cheeseman 2016; Plohl et al. 2014; Steiner and Henikoff 2015; Talbert et al. 2018), and genetically by the presence of satDNA sequences containing CENP-B box motifs (Dumont and Fachinetti 2017). Our analysis of the interaction of *SAT1.723* sequences with CENP-A (Figure 4a, b, c) and CENP-B (Figure 4d) shows centromeric activity of this satDNA in BTA (Bovini), the seven Tragelaphini species and the two Caprini species suggesting that this satDNA may have been retained (conserved) due to functional constrains in bovid evolution. The CENP-B boxes found in the *SAT1.723* monomers were demonstrated to be functional as these monomers interact with CENP-A (see IF-FISH and CHIP experiments). Despite the existence of different satDNA families at the centromeric and pericentromeric regions of these species' chromosomes, only *SAT1.723* seems to be involved with centromeric function. Although the finding that only one specific satDNA family is capable of binding CENP-A has been described in other species (humans - (Plohl et al. 2014) and horses - (Cerutti et al. 2016)), ours is the first report of this functional satDNA sequence in the centromeres of bovids. Additionally, since *SAT1.723* seems to be associated with centromere function, this satDNA is most probably located at the centromeric region (at least in some of the monomers). Thus, only a fraction of *SAT1.723* monomers may be associated with CENP-A, similar to the alpha-satellite in the human genome, where only a few sequences are associated to CENP-A (Sullivan et al. 2011).

The mechanism responsible for the activity of the *SAT1.723* centromeric sequence is unknown. In humans, alpha-satellite is the preferred component of the active centromeres (Aldrup-MacDonald et al. 2016). Recent studies on the architecture of centromeres have reported the presence of specific secondary structures such as DNA loops suggesting that active centromeric sequences were selected for their ability to form secondary structures, rather than for the nucleotide sequence itself (Aze et al. 2016; Kasinathan and Henikoff 2017). In fact, a bioinformatic analysis on the prediction of secondary structures showed that *SAT1.723* can indeed form DNA loops and G-quadruplexes (data not shown; see (Kejnovsky et al. 2015). Moreover, the high GC content of *SAT1.723* is in agreement with recent reports that GC richness is compatible with the centromeric function (Cerutti et al. 2016; Melters et al. 2013).

Models for predicting nucleosomes have been developed using DNA sequence properties, such as dinucleotide periodicity (Ioshikhes et al. 2011; Kaplan et al. 2009; Segal et al. 2006; Zhang et al. 2013) and curvature pattern (Liu et al. 2011; Liu et al. 2008). The observation of a 10-bp periodicity of GC dinucleotides (in agreement to the DNA helical repeat ~10.4 bp) across the *SAT1.723* monomer (Figure 2a) could be considered as a facilitator of DNA bendability and nucleosome formation (Ioshikhes et al. 2011; Kaplan et al. 2009; Kogan and Trifonov 2005). Moreover, the predicted curvature for the *SAT1.723* monomer (Figure 2d) resembles the "curvature pattern" for the nucleosomal DNA helix proposed by Liu et al. (2008). In this satDNA monomer, the two ends have a large curvature, whereas the middle has a small curvature which provides powerful evidence for a periodicity characteristic of core DNA (Liu et al. 2011; Liu et al. 2008). The less variable and higher bendability values detected across the *SAT1.723* monomer (Figure 2d), in contrast with the other two satDNAs (Figure 2e,f), suggest this monomer to comprise the more bendable and flexible sequence, a factor which could facilitate CENP-A nucleosomal organization (Heslop-Harrison and Schwarzacher 2013; Steiner and Henikoff 2015; Zhang et al. 2013). The differences in curvature/bendability patterns of the three satDNA sequences could potentially reflect different modes of nucleosomes packaging, suggesting a more relaxed conformation of the *SAT1.723* monomer. We propose that the *SAT1.723* sequence intrinsically favours the translational and rotational phasing of the CENP-A nucleosomes in bovids, similar to that proposed for CentO on rice by Zhang et al (2013).

SAT1.723 monomer-flanking regions

Current whole genome assemblies collapse most copies of the satellite sequences and do not show the long arrays present at the centromeres of chromosomes. However, assembly algorithms would be expected to assemble regions flanking satellite monomer fragments correctly, regardless of the ability to assemble either satellite arrays, or small contigs including satellite fragments into larger scaffolds. Thus the analysis of satDNA monomer-flanking regions can provide important insights on the organization and mode of evolution of these sequences (Chaves et al. 2017; Satović et al. 2016). An analysis of the flanking regions of the *SAT1.723* monomers identified the frequent presence of transposable elements with both non-LTR and LTR sequences (Figure 5). Moreover, the *in silico* mapping of the bovine satDNAs (Supplementary table S3, S4, S5) showed that these sequences are present not only at the (peri)centromeric regions, but they also occur in an interspersed fashion on the chromosomes of BTA, CHI and OAR (although in too low copy numbers and density to be detected by *in situ* hybridization). These findings point to an intense intragenomic reshuffling of satDNAs mediated by the TEs found in the satDNAs monomer-flanks. Several studies have reported a physical association between satDNAs and TEs (Chaves et al. 2017; Heslop-Harrison and Schwarzacher 2011; Louzada et al. 2015; Petraccioli et al. 2015), suggesting a role for these elements in satDNA evolution probably by promoting their intragenomic movement and expansion in the genomes (Biscotti et al. 2008; Chaves et al. 2017; Kuhn et al. 2008; López-Flores et al. 2004; Macas et al. 2009; Petraccioli et al. 2015; Šatović and Plohl 2013; Satović et al. 2016; Scalvenzi and Pollet 2014). Our analysis reveals that the TE association seems to be the rule for all the bovine satDNAs analysed, as all are embedded in TEs, with a particular emphasis for non-LTR elements (Figure S7). The presence of the same class of TEs in the monomer-flanking regions of all the five satDNAs suggests that their movement may occur by the same transposition mechanism.

The presence of LTR sequences flanking the *SAT1.723* monomers (Figure 5c) reinforces the centromeric activity of this sequence. It has been hypothesized that retrotransposons, particularly LTRs, may accumulate at active centromeres because of their favored integration into an epigenetically modified centromeric "environment" or, alternatively, due to the preferred association with CENP-A nucleosomes in both animals and plants (Plohl et al. 2014; Wolfgruber et al. 2009). Similarly, as reported for maize and wheat (both of which present species-specific centromeric retrotransposons), the TCR1_LTR sequence found in the *SAT1.723* monomer-flanking regions could be a

specific centromeric retrotransposon in several of the Tragelaphini species (Figure 5d, e). However, additional work is needed to disclose if there are any specific centromeric retrotransposon.

It is important to highlight that the different classes of TEs associated with the dispersed *SAT1.723* hits mapped in the BTA, OAR and CHI genomes (Figure 5a, b), and at the flanking regions of the *SAT1.723* centromeric monomers in Tragelaphini (Figure 5c), could reflect their different chromosome locations. Alternatively, these differences could be due to the limited length of flanking sequences present in the *SAT1.723* cloned monomers. Our data for transcriptional activity of *SAT1.723* agrees with reports of transcription of other centromeric repetitive sequences (Carone et al. 2009; Gent and Dawe 2012; Hall et al. 2012; Quénet and Dalal 2014), potentially having a role in kinetochore assembly and maintenance. Centromeric transcripts have been shown to be required for CENP-A loading in humans, as depletion of these transcripts leads to mitotic defects (Quénet and Dalal 2014).

Bovids are ecologically, economically, and biologically important animals. Whole genome sequencing generally gives information on low copy sequence evolution, but the data here show the value of understanding the evolution of repetitive DNA copy number, sequence motif, and chromosomal location, on both autosomes and sex chromosomes from different Bovidae species.

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Conflict of interest The authors declare that they have no conflict of interest.

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Figure Captions

Fig 1. SatDNA isolation and mapping onto *Bos taurus* (BTA), Tragelaphini, *Capra hircus* (CHI) and *Ovis aries* (OAR) chromosomes. (a) satDNAs amplicons obtained by PCR from the genomic DNA of the species analysed. PCR amplicons were: *SATI*- 400 bp, *SATIV* – 604 bp, *SAT1.723* – 680 bp, *SAT1.711a* – 400 bp, *SAT1.711b* – 975 bp *SATI* amplicons from the Tragelaphini genomes are 500 bp long. *SAT1.723* amplicons from TAN, TIM, TDE and TOR are approximately 600 bp in length, and those of the *SAT1.723* amplicons from TSC, TSP and TST are approximately 750 bp. TOR and TDE *SATIV* sequences revealed an amplicon size of 700 bp. **(b-l)** Physical mapping of the satDNAs present at pericentromeric and centromeric regions by *in situ* hybridization (red or green) in the respective species chromosomes (blue, DAPI). The name and color of each probe were indicated within each metaphase. Scale bar represents 10 μm.

Fig 2. Intrinsic features of satDNAs monomers. GC content distribution across *SAT1.723* **(a)**, *SATI* **(b)** and *SATIV* **(c)** monomers. Curvature/bendability propensity plots of *SAT1.723* **(d)**, *SATI* **(e)** and *SATIV* **(f)** monomers. The sequences used in these analyses were M36668, AJ293510 and AF446392.

Fig 3. Orthologous bovine satDNA sequence identity. Distance matrix of pairwise alignments of *SATI* **(a)**, *SAT1.723* **(b)** and *SATIV* **(c)** clones from BTA and the Tragelaphini species analysed in the present study. Cells showing nucleotide identities of 90-100% are in dark blue; 80–89%, medium blue; 70–79%, light blue, 60-69%, dark green; 50-59%, light green; 40-49%, yellow; 30-39%, orange; and <30% in red. The multiple alignment of all the clones is shown in Supplementary Figure S1, S2, S3.

Fig 4. The centromeric function of *SAT1.723* in BTA, Tragelaphini, CHI and OAR genomes. (a) Representative images of IF with CENP-A antibody (green; DNA DAPI blue) followed by DNA-FISH with *SAT1.723* (red) in BTA, Tragelaphini, CHI and OAR species. A colocalization spot was amplified 300% (top, right). Scale bar represents 10 μm. **(b)** Graphic validation of the co-localization of the CENP-A antibody signals with the satDNA sequence signals in BTA, the Tragelaphini species, CHI and OAR. Each colocalization spot in each cell was analysed by Pearson's correlation coefficient. A minimum of 15 spots per cell in at least 10 images of each species and satDNA FISH experiment were analysed (a minimum of 150 spots per variable). As the samples did not present a Gaussian distribution, the values were transformed with the log function in order to normalize the values 'distribution. The correlogram was made with GraphPad Prism 6 (version 6.01). All values are expressed as mean ± SD (standard deviation). **(c)** Relative quantification of band intensity from CHIP sample analysis by PCR with specific primers for the satDNA sequences isolated in each species' (peri)centromeric regions. This analysis was performed using the software Image J. The area of each band was determined, and the value of each IP sample was compared with the value

of Input band. **(d)** *In silico* search for the CENP-B box-like motif in the *SAT1.723* monomer from BTA and the seven Tragelaphini species.

Fig 5. *In silico* analysis of the flanking regions of *SAT1.723* hits mapped in BTA, CHI and OAR genomes and the Tragelaphini clones. (**a**) Representative image of a BTA, CHI and OAR chromosome showing the annotation of *SAT1.723* BLAST hits (in red) on the top line and the annotation of the repetitive sequences found in the flanking sequences of *SAT1.723* on the bottom bar (Repbase). (**b**) Quantification of the different classes of repetitive sequences flanking the *SAT1.723* monomers along the BTA, CHI and OAR genomes. The flanking regions of all the BLAST hits of *SAT1.723* mapped onto BTA, CHI and OAR chromosomes were screened for the presence of repetitive elements in the *Eukaryota Repbase* using the *Censor* software. **(c)** Representative *SAT1.723* clones from TAN, TDE, TOR, TSC and TSP with the TCR1_LTR motif mapped in the monomer sequence. The *SAT1.723* clones' sequences from each species were screened for the presence of repetitive elements in the Eukaryota Repbase using the *Censor* software, and the TCR1_LTR elements found were mapped in clone sequences. **(d)** Multiple alignment of the TCR1_LTR motifs found in the *SAT1.723* clones analyzed. **(e**) Location of the TCR1_LTR motif in the representative *SAT1.723* monomers from TAN, TDE, TOR, TSC and TSP.

Fig 6. Schematic representation of the different stages of satDNA evolutionary process – Library model. *SATI*, *SAT1.723* and *SATIV* are represented in the scheme as satDNAs families in different stages of the evolutionary process. The origin of each family was inferred considering the presence/absence in Bovinae and Caprinae analysed species.

Figures

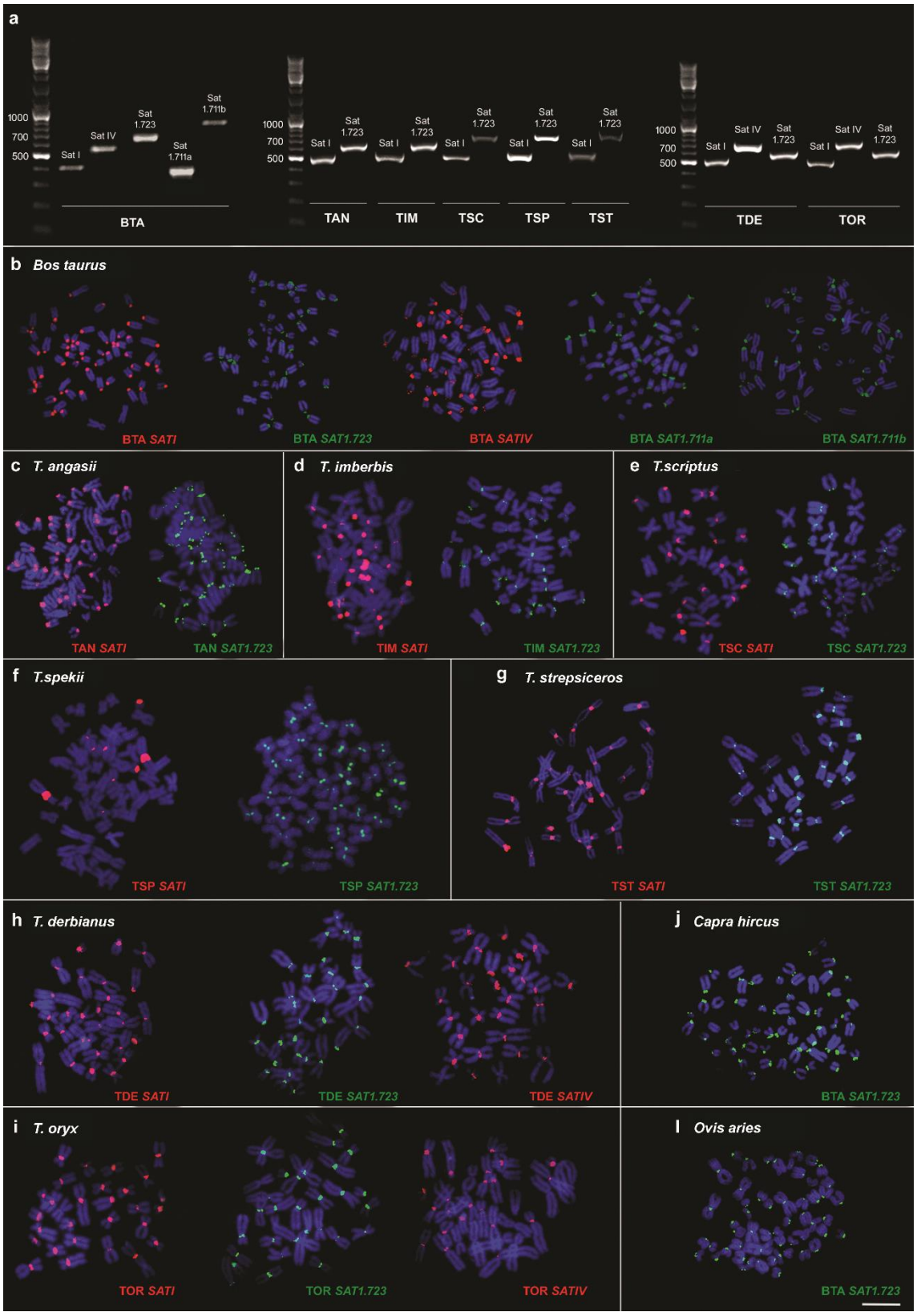

Figure 1

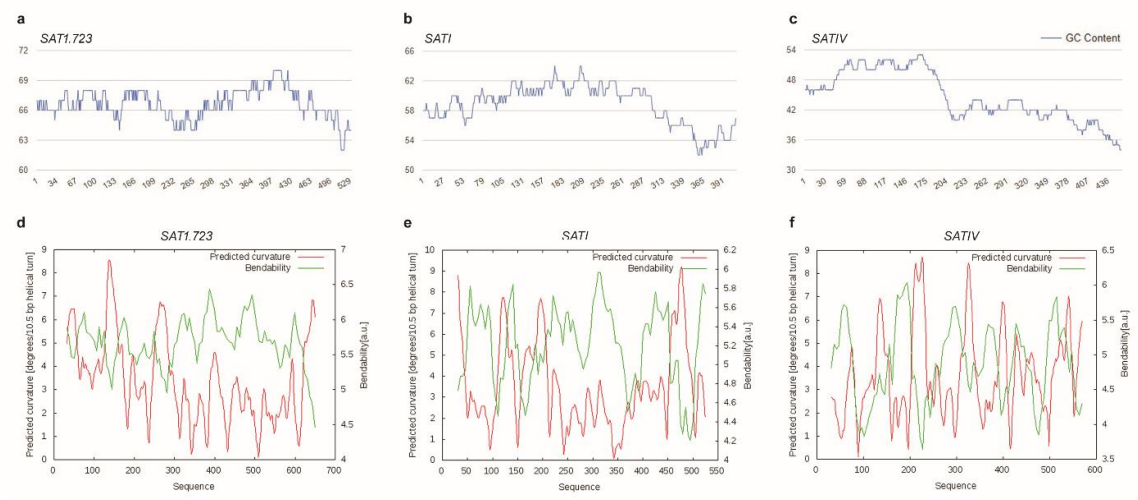

Figure 2

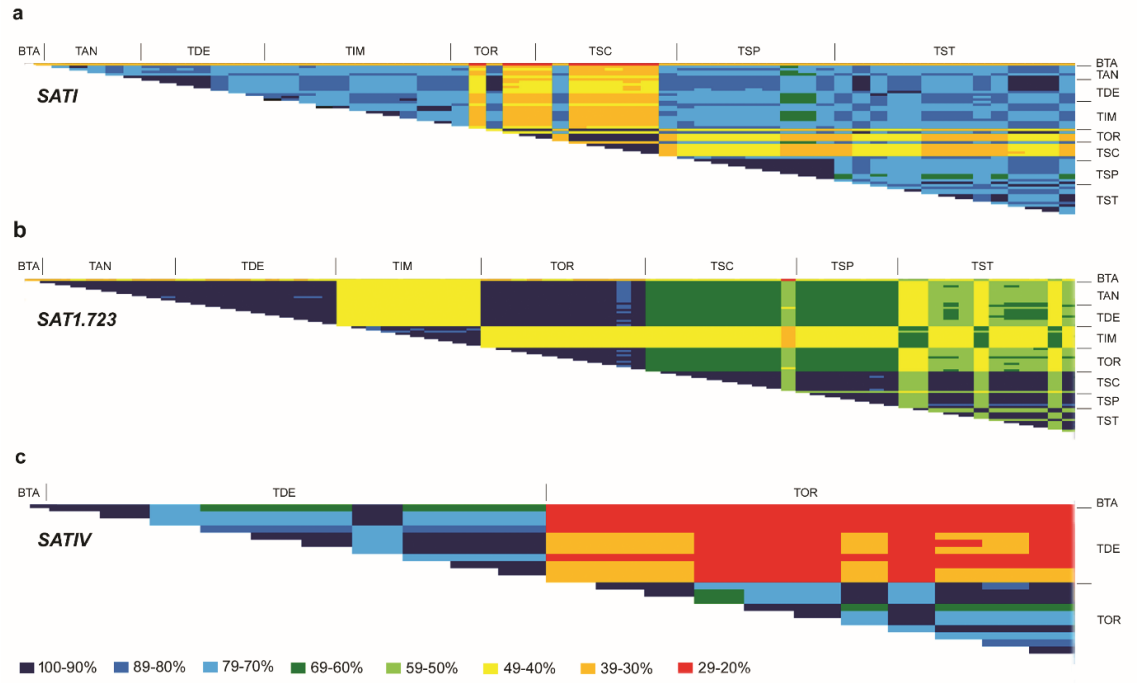

Figure 3

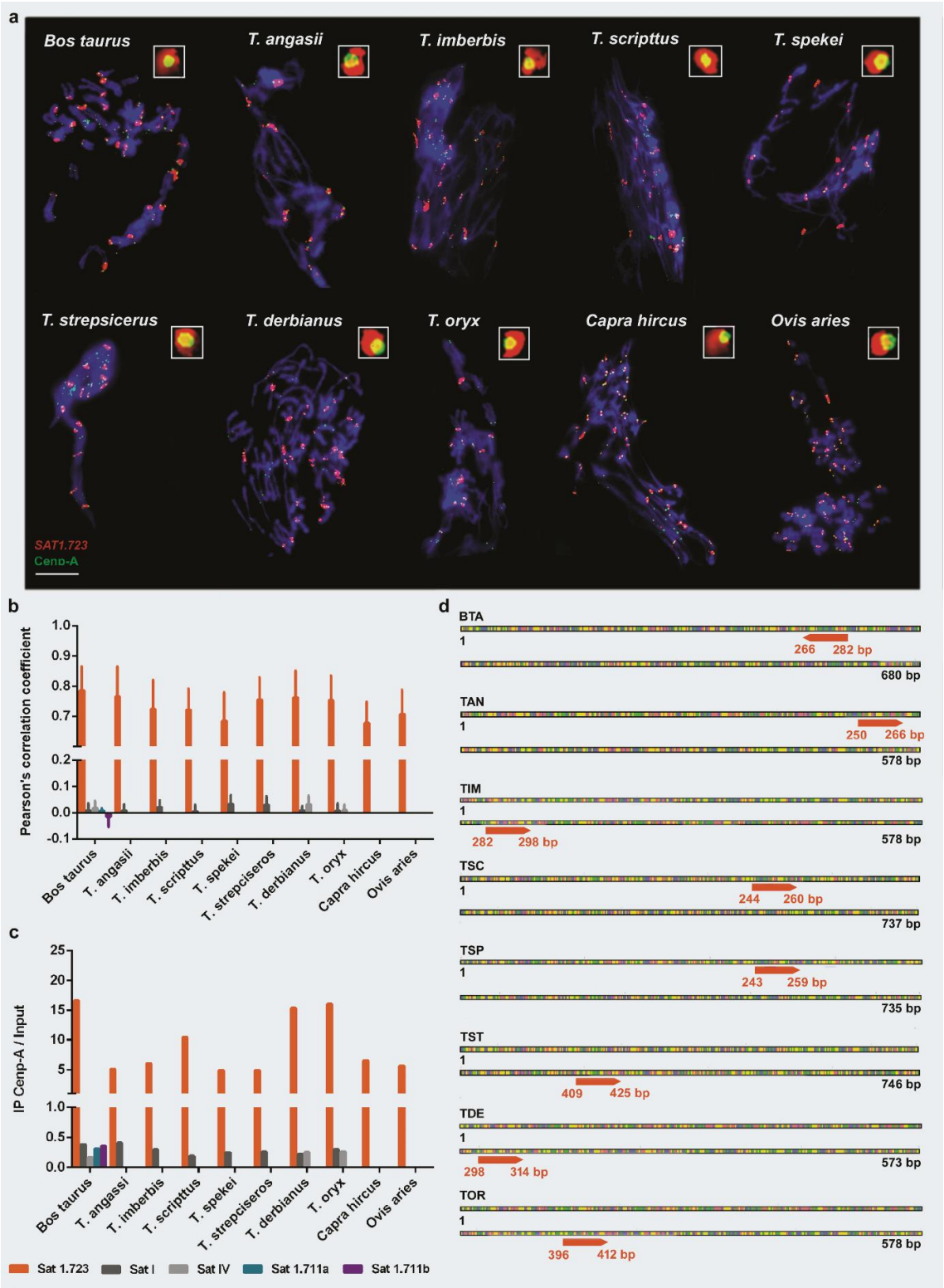

Figure 4

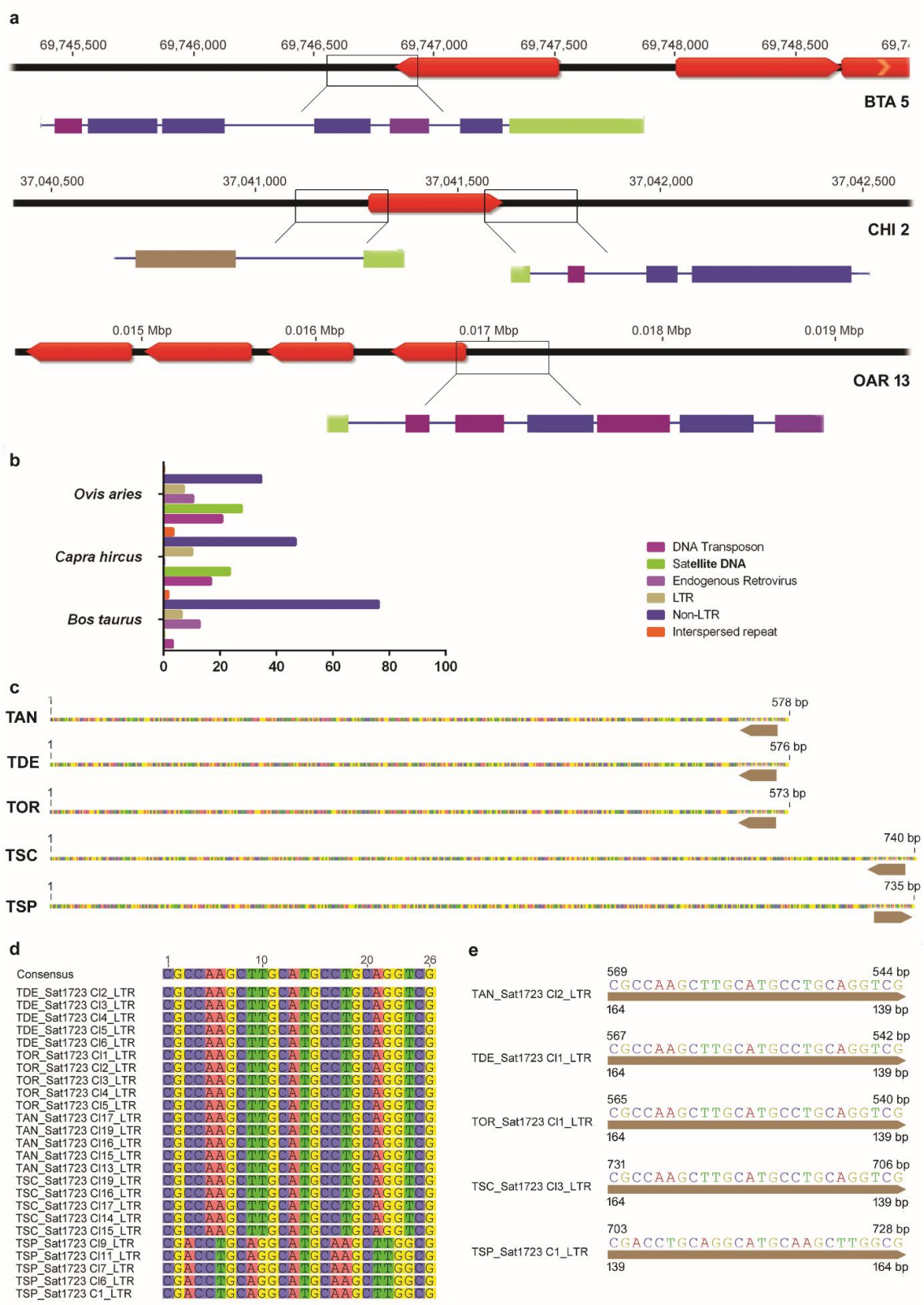

Figure 5

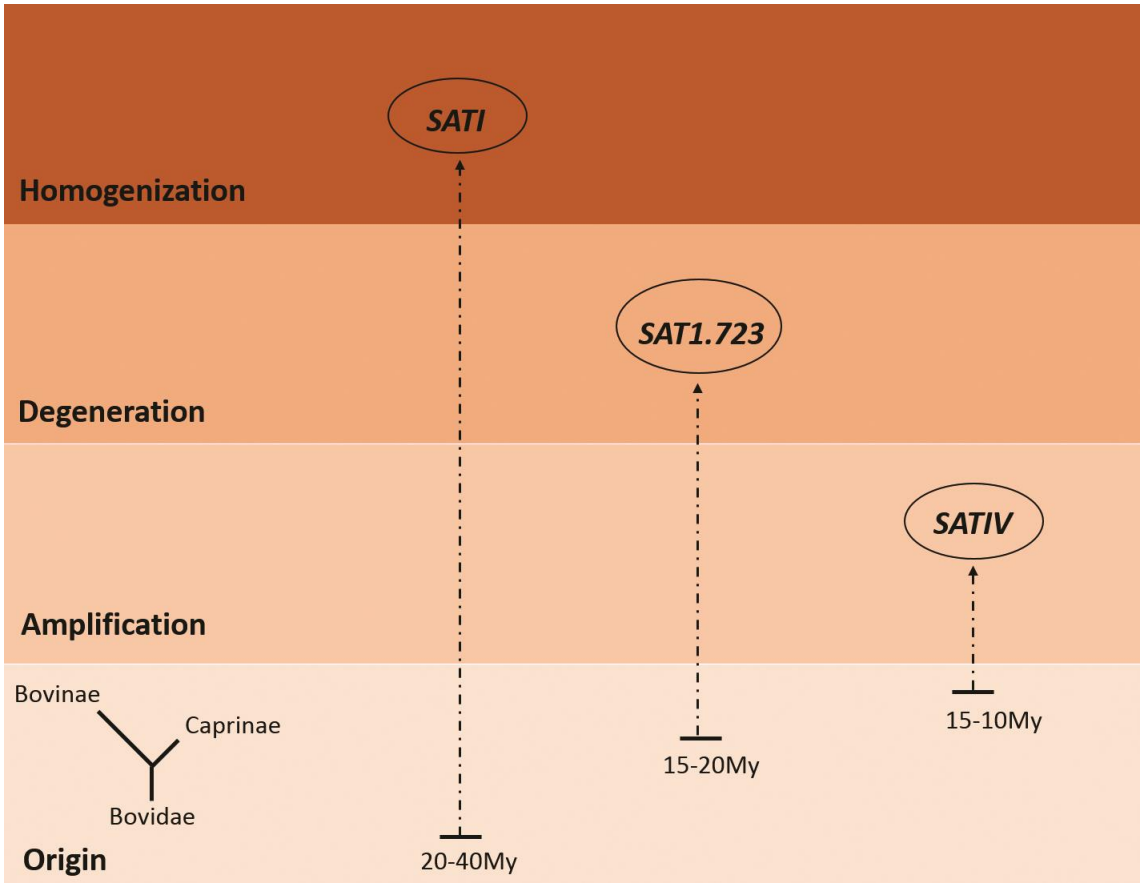

Figure 6