A long-term conserved satellite DNA that remains unexpanded in several genomes of Characiformes fish is actively transcribed

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Abstract

Eukaryotic genomes contain large amounts of repetitive DNA sequences, such as tandemly repeated satellite DNAs (satDNAs). These sequences are highly dynamic and tend to be genus- or species-specific due to their particular evolutionary pathways, although there are few unusual cases of conserved satDNAs over long periods of time. Here, we used multiple approaches to reveal that a satDNA named CharSat01-52 originated in the last common ancestor of Characoidei fish, a superfamily within the Characiformes order, approximately 140–78 million years ago, while its nucleotide composition has remained considerably conserved in several taxa. We show that 14 distantly related species within Characoidei share the presence of this satellite DNA, which is highly amplified and clustered in subtelomeric regions in a single species (Characidium gomesi), while remained organized as small clusters in all the other species. Defying predictions of the molecular drive of satellite evolution, CharSat01-52 shows similar values of intra- and interspecific divergence. Although we did not provide evidence for a specific functional role of CharSat01-52, its transcriptional activity was demonstrated in different species. In addition, we identified short tandem arrays of CharSat01-52 embedded within SMRT long reads of Astyanax paranae (536 bp to 3.1 kb) and A. mexicanus (501 bp to 3.9 kb). Such arrays consisted of head-to-tail repeats and could be found interspersed with other sequences, inverted sequences or neighbored by other satellites. Our results provide a detailed characterization of an old and conserved satDNA, challenging general predictions of satellite DNA evolution.

Key words: repetitive DNA, Neotropical fish, tandem repeats, satDNA
Significance statement

The genomes of eukaryotes are significantly composed by noncoding repeated DNA sequences, known as satellite DNAs (satDNAs). In general, these sequences have no defined function and represent a fast-evolving portion of the genome. For this reason, these sequences are usually species- or genus-specific and the evolutionary persistence of these sequences over a long period is uncommon and not well understood. Here, we found a highly conserved satellite that originated 140–78 million years ago and persisted in the genomes of several fish species in an entire order. By using multiple approaches, we showed that this sequence remained as a typical satellite DNA in all species and is actively transcribed. Here, we provide possible explanations for the long-term maintenance of this satDNA.

Introduction

Satellite DNAs (satDNAs) are noncoding tandemly repeated sequences that constitute large portions of eukaryotic genomes, with head-to-tail arrays reaching up to hundreds of thousands of nucleotides (López-Flores & Garrido-Ramos 2012; Plohl et al. 2012). These sequences are preferably found on the heterochromatin of pericentromeric and subtelomeric regions, although their occurrence in euchromatic areas has been reported (Plohl et al., 2012; Garrido-Ramos, 2015; Ruiz-Ruano et al., 2016; Silva et al., 2017). In general, it is assumed that satDNAs originate \textit{de novo} from random duplication events of a genomic sequence of two or more nucleotides that spread throughout the genome by distinct mechanisms, such as multiple transposable element insertions and/or rolling circle replication and reinsertion (Ruiz-Ruano et al. 2016; Vondrak et al. 2020). Afterwards, stochastic events may lead to the local amplification of those short arrays or to their extinction in the referred locus/genome (Plohl et al. 2012; Ruiz-Ruano et al. 2016; Lower et al. 2018). Remarkably, every satDNA locus within a genome will transcend speciation events and evolve independently in each lineage, giving rise to the library
hypothesis model of satellite evolution, which predicts that related species share a common collection of satDNAs that may be independently amplified or depleted over time (Fry & Salser 1977).

Although highly repetitive and usually spread throughout different chromosomes and/or genomic regions, satDNAs usually exhibit high intraspecific repeat homogeneity and interspecific heterogeneity, which is related to the concerted evolution of these satellite repeats, reached by intraspecific sequence homogenization and fixation (Dover 1982, 1986). In the context of concerted evolution and the general absence of functional selective constraints, satellite DNA sequences are frequently reported as being species- or genus-specific, with few examples of satellite repeats being conserved over a long period of time (e.g., more than 50 Myr) (Plohl et al. 2012; Lorite et al. 2017; Halbach et al. 2020).

The order Characiformes is a species-rich clade in the tree of life, with representatives restricted to freshwater environments of Africa and the Americas (Betancur-R. et al. 2019). This group is split into two well-characterized monophyletic suborders: Citharinoidei, with approximately 110 species in two families, and Characoidei, with almost 2000 species in 22 families (Arcila et al. 2017; Chakrabarty et al. 2017; Dai et al. 2018; Hughes et al. 2018; Betancur-R. et al. 2019). The accumulated cytogenetic data for this group includes great karyotype diversification, distinct sex chromosome systems, independent origins of supernumerary chromosomes and multiple cases of repetitive DNA sequence diversification, notably, multigene families (Oliveira et al. 2009; Cioffi et al. 2011). On the other hand, satDNA information is mainly restricted to unique or few species from the same family (Vicari et al. 2010).

In recent years, powered by the expansion of next-generation sequencing and bioinformatic protocols, entire collections of satellite DNAs have been described for several species, mainly invertebrates and fishes (Ruiz-Ruano et al., 2016; Silva et al., 2017; Palacios-
Remarkably, satellitome analyses performed by us within the Characiformes fish, including distinct species belonging to the Crenuchidae, Anostomidae and Characidae families, revealed the existence of a conserved 52 bp-long satellite DNA (CgomSat02-52, ApaSat29-52 and MmaSat85-52), named here CharSat01-52. Considering that Crenuchidae is a sister group of most Characiformes (Arcila et al. 2017; Betancur-R. et al. 2019), an initial hypothesis of the long-term existence of a satDNA family has been proposed (Utsunomia et al. 2019).

Here, we delimited the origin and assessed the genomic organization of this ancient satellite DNA among Characiformes by analyzing short-read data from 14 species encompassing nine families within this order – Distichodontidae, Crenuchidae, Erythrinidae, Hemiodontidae, Serrasalmidae, Prochilodontidae, Anostomidae, Bryconidae and Characidae – that diverged more than 100 My ago (Arcila et al. 2017; Hughes et al. 2018). Furthermore, fluorescent in situ hybridization (FISH) experiments were performed and corroborated the in-silico analyses, evidencing that the clustered pattern is restricted to a single species. Next, we used long-read data (PacBio sequencing) to decipher the lengths and densities of CharSat01-52 in two species exhibiting a non-clustered pattern and compared them against those of a clustered satellite DNA. The resulting data suggest the long-term maintenance of CharSat01-52, which mainly experienced quantitative changes among species, for dozens of millions of years, corroborating the library hypothesis. However, extreme sequence conservation also defies predictions of concerted evolution patterns.

Results

Repeat identification and intra- and interspecific abundance and divergence values

To delimit the occurrence of CharSat01-52, we searched for this satDNA in the genomes of several fish species by using multiple approaches. BLAT searches followed by graph
clustering with RepeatExplorer generated sphere-shaped graphs for all the Characoidei genomes analyzed, except that of *H. malabaricus*. This indicates that CharSat01-52 is consistently present as a typical satellite DNA in the referred species (Figure 1A). We retrieved 52 bp-long monomers from all the species and calculated the A + T content of the consensus sequences. This was biased towards A + T richness, varying from 59.3% to 71.5%, with a median value of 65.2%.

The CharSat01-52 CNV profiles indicated that this satellite shows a higher abundance in *C. gomesi* (average coverage = 2,697 copies, with a peak at 12,000 copies) than in the other species, in which CharSat01-52 abundance seems to be lower (mean = 181 copies, SD = 712.8; Table 1, Figure 1B, Figure S1). All the obtained scaled profiles showed high correlation values among each other (*r* = 0.99), pointing to a conserved, tandemly arrayed monomer structure in all the species, except *H. malabaricus*. Notably, a 3 bp valley (monomer positions 22–24) was observed in the graphs of *B. orbignyanus* and *H. gracilis*, indicating a deletion of these bases in approximately half of the copies of CharSat01-52 in both genomes (Figure 1B). In general, the variant profile graphs were similar among species belonging to the same families (Figure 1C), consistent with the phylogenetic relationships. Additionally, some recurrent variants were observed, such as position 32 of the CharSat01-52 monomers, which seems to be prone to variation in all the analyzed species (Figure 1C).

The repeat landscapes also pointed to a higher abundance of CharSat01-52 in *C. gomesi* and evidenced some distinctive species-specific or family-specific landscape shapes (Table 2, Figure S2), corroborating the variant profiles results. For example, the landscapes obtained from *C. gomesi, M. macrocephalus, P. lineatus, B. orbignyanus* and *M. sanctaefilomenae* each exhibited a different peak of abundance in particular Kimura divergence values, pointing to a differential amplification of variants in each species (Figure S2). Notably, very similar landscape patterns, with small intraspecific deviations, were retrieved by analyzing multiple
individuals of *C. gomesi* (Figure S2; Table 2), indicating a low degree of interindividual variation. Remarkably, none of the approaches applied here were capable of detecting signals of CharSat01-52 presence in the genomes of the non-Characoidei species *D. sexfasciatus*, *G. sylvius* and *P. corruscans*. On the other hand, although we were not able to collect CharSat01-52 monomers from the *H. malabaricus* genome, two reads were isolated using RepeatMasker and RepeatProfiler (Figure 1B), suggesting the residual existence of this satDNA in this species.

Direct short read-derived monomer extraction was performed for all the species and the resulting data were aligned to generate separate sequence logos, which indicated a general intra- and interspecific conservation of this satDNA, with particular positions exhibiting polymorphisms (Table 2, Figure 2; Figure S3), as evidenced in the analysis of the variant profile coverage. After a global alignment, we produced a minimum spanning tree (MST) that depicted an interesting scenario for CharSat01-52, since general species-specific groups of haplotypes were not a general rule, except for some grouped haplotypes of *C. gomesi* (Figure 2; Figure S3). Additionally, several variants (haplotypes) were shared among distantly related species, including two variants that were common to three and five species. Notably, these shared variants do not reflect the phylogenetic proximity between the referred species (Figure 2; Figure S3). The interspecific Kimura divergence value obtained here was similar or even higher than the intraspecific values (Table 2), corroborating the MST results. Quantification of relative copy number of CharSat01-52 was investigated by qPCR and results obtained confirmed a higher abundance of this sequence in *C. gomesi*, as expected (Figure 2).

BLAST searches of the CharSat01-52 consensus sequence against the nucleotide collection of the NCBI produced different significant alignments. As expected, low *e*-values (max *e*-value= 1e-10) were observed for the previously described variants (MmaSat085-52, MelSat49-52, ApaSat29-52 and CgomSat02-52). In addition, significant matches (*e*-value=2e-08) with transcript variants of the PTPRF interacting protein alpha 1 (*ppfia1*) from *Astyanax*.
were also obtained. Downstream analyses of the assembled genomes of several Ostariophysi species (*Astyanax mexicanus, Pygocentrus nattereri, Pangasianodon hypophthalmus, Electrophorus electricus* and *Danio rerio*) revealed the occurrence of a CharSat01-52 array (34 imperfect monomers reaching approximately 1,809 bp) near the end of the 3’ UTR region of this gene in *A. mexicanus*. After that, we manually searched the same region in the *P. nattereri* genome and obtained similar results, with the occurrence of an array of 23 imperfect monomers of CharSat01-52 reaching approximately 1,263 bp located 637 bp downstream of the corresponding exon (Figure S4). Although the position of the CharSat01-52 array is similar in both species, the *ppfia1* gene has an additional exon in *P. nattereri* in comparison with that in *A. mexicanus* (Figure S4). For this reason, the satDNA array is located within the last intron in *P. nattereri*. These results elucidate the positive BLAST search for CharSat01-52 for only the *ppfia1* of *A. mexicanus*, since one perfect monomer appears to be transcribed in this species as a part of the 3’ UTR, which is not the case for *P. nattereri*. Considering the other analyzed species belonging to distinct orders, we could not find any tandemly repeated pattern of sequences in the corresponding regions of this gene (Figure S4).

**CharSat01-52 is tandemly repeated in several genomes – PCR and FISH**

PCR amplification of the referred satDNA in eight species corroborated the in-silico analyses and yielded a ladder-like pattern of bands in all the species, except *H. malabaricus* (Figure 1D). After that, the FISH probes labeled with digoxigenin-dUTP were hybridized against the chromosomes of eight species within the Characiformes, which yielded visible FISH signals only in *C. gomesi*, in which it displays intense signals on subtelomeric regions of all chromosomes (Figure 3). All the other analyzed species did not show primarily any visible signals (Figure S5), probably as a result of the CharSat01-52 sequences being organized in short arrays, i.e., less than 10 kb, the boundary of the sensitivity of the FISH technique, in these
species, as we further confirmed with long-read data (see below). After enhancing the FISH signals of CharSat01-52 using conjugated anti-avidin-biotin, we confirmed that this satellite DNA is organized as short tandem arrays in all species (Figure 3, 5).

**Transcription of CharSat01-52**

The expression analysis revealed that CharSat01-52 is expressed in the muscle and ovaries of *A. paranae* as well as in the muscle of *P. mesopotamicus* (Figure 4). Importantly, the read counts of CharSat01-52 were directly affected by and associated with the protocol applied to generate the RNA sequencing libraries (i.e., the lncRNA or mRNA libraries). The expression of CharSat01-52 in the lncRNA libraries was approximately 15.4 times higher in the muscle than in the ovaries of *A. paranae* (*p* = 0.0022, *t* = 5.71 df = 5.035) (Figure 4). In the mRNA libraries, the expression was 4.5 times higher in the muscle than in the ovaries (*p* = 0.0001, *t* = 6.171, df = 10) (Figure S6).

The generated MST from monomers derived from DNA- and RNA-seq libraries revealed an interesting divergence of transcribed monomers in different tissues of *A. paranae* and *P. mesopotamicus*. Notably, the most abundant RNA-seq-derived monomer of *A. paranae*, which is a monomer shared with *C. gomesi* and *P. lineatus*, is the most abundant variant in the gDNA-derived sequences (Figures 2 and 4). We also performed RT-qPCR in different tissues of *A. paranae* and *C. gomesi*. Results obtained for *A. paranae* corroborated the RNA-seq data, with a higher expression of CharSat01-52 in the muscle compared with the ovaries. For *C. gomesi*, we observed that this satellite DNA is also transcribed in both tissues, with a higher expression in the muscle (Figure 4).

**Estimating CharSat01-52 repeat abundance and array sizes using PacBio SMRT reads**

http://mc.manuscriptcentral.com/gbe
Overall, the throughput of the PacBio sequencing subreads of *A. paranae* was 3.04 Gb, while the downloaded data for *A. mexicanus* totaled 28.5 Gb (Figure 5). The calculated repeat densities for each satDNA in the SMRT reads showed that the AmeSat02-179/ApaSat10-179 (clustered satDNA) density was 27.2- and 174.4-fold higher than the CharSat01-52 (non-clustered satDNA) density in *A. paranae* and *A. mexicanus*, respectively, corroborating the in-silico and FISH analyses. The lengths of the arrays were also consistent with the FISH results (Figure 5). Thus, the longest repeat arrays we recovered for the highly clustered satellites, i.e. those detected by the FISH experiments without signal enhancement (AmeSat02-179/ApaSat10-179), were over 32.8 kb and 12.6 kb in *A. mexicanus* and *A. paranae*, respectively. Conversely, the FISH signals for CharSat01-52 were visible exclusively after signal enhancement (see Material and Methods section), corroborating the in-silico analyses that evidenced that the longest arrays of this satDNA were 3.9 kb and 3.1 kb. Finally, our two approaches to identify the recurrent association of sequences with CharSat01-52 arrays did not return any associated sequence with this satellite DNA in our libraries. Thus, although specific and isolated cases of association between CharSat01-52 and other described satellite DNAs were found, we did not find a recurrent association between CharSat01-52 with other sequence.

**Discussion**

In this study, we identified and characterized a conserved satellite DNA in 14 Characoidea species using multiple approaches and dissected the array organization of this satDNA by using long reads from two species. CharSat01-52 exhibits the main features of an authentic tandem repeat in almost all the sampled Characoidea species, as evidenced by the ladder-like pattern of PCR amplification and the tandem-repeated structure of RepeatExplorer contigs (Novák et al. 2013). Given the occurrence and distribution of CharSat01-52, we suggest that this satDNA originated in the last common ancestor species of Characoidea, before the
split of the Crenuchidae (*C. gomesi*), which lived approximately 140–78 million years ago (mya) (Burns & Sidlauskas 2019; Melo personal communication). To our knowledge, this is one of the oldest satDNA sequences described so far, along with APSP-I in ants (80–74 mya; Lorite et al. 2017), PRAT in coleopterans (60–50 mya; Mravinac et al. 2002), PstI in sturgeon fishes (100 mya; Robles et al. 2004) and the three most ancient satDNA sequences ever reported: BIV160 and PjHaal in molluscs (540 mya; Plohl et al. 2010; Petraccioli et al. 2015) and tapiR in *Drosophila* (200 mya; Halbach et al. 2020).

We also combined different sequencing technologies to enhance our knowledge about satDNA array organization, since the genomic analyses of long reads applied to satDNAs have been restricted to highly clustered satellites to date (Khost et al. 2017; Cechova et al. 2019; Heitkam et al. 2020; Vondrak et al. 2020). Here, by analyzing SMRT reads with NCRF software (Harris et al. 2019), an algorithm that tackles the noisy error profiles of PacBio and Nanopore reads, we were able to recover several tandemly arrayed CharSat01-52 sequences in two species that did not primarily produce conspicuous cluster-type signals after FISH, unless the signals are enhanced (*A. paranae* and *A. mexicanus*). These arrays did not sum up to 5 kb long in both species, explaining the requirement of signal enhancement to detect FISH signals (as this method has a sensitivity of approximately 10 kb). In fact, the array sizes found for a known highly clustered satellite DNA were much longer in both species (up to 32.8 kb in *A. mexicanus*).

Recent results related to satellite DNA organization have revealed that, in general, satDNA arrays are usually composed of a mix of perfect and incomplete repeats interspersed by and/or adjacent to different kinds of sequences, including different transposable element families, which usually participate in the spreading of satellites (Khost et al. 2017; Cechova et al. 2019; Heitkam et al. 2020; Vondrak et al. 2020). Our data indicated that CharSat01-52 constitutes small tandem arrays but can also be interspersed by other sequences, as well as
adjacent to different known and unknown repetitive DNA sequences, including tandem repeats (Figure 5). However, we could not identify a recurrent common pattern of association between CharSat01-52 and other elements, indicating that specific transposable elements do not seem to actively participate in the intragenomic diversification of this satDNA.

The occurrence of a CharSat01-52 array in different noncoding regions of \textit{ppfia1} (e.g., the intron and 3’ UTR) in two Characiformes species is notable but should not be taken as evidence of its origin, as in the CapA satDNA, present in Platyrrhini mammals, which is suggested to have originated from the intron of the NOS1AP gene (Valeri et al. 2018). In the referred case, the authors found that CapA satDNA is arranged in a single copy fashion in several eutherian genomes, while it is amplified and tandemly arrayed in only the Platyrrhini clade. Here, we did not find any sign of CharSat01-52 presence in other non-Characiformes species or any similarity between this satDNA and other sequences, such as transposable elements or other noncoding sequences. For this reason, we suggest that CharSat01-52 sequences were inserted into the noncoding regions of \textit{ppfia1} after it originated as a satellite DNA and that this occurred at least before the split of the Characidae (\textit{A. mexicanus}) and Serrasalmidae (\textit{P. nattereri}).

Current ideas of satellite DNA evolution include the library hypothesis and concerted evolution of repeats (Fry & Salser 1977; Dover 1982). Together, both models can explain the evolution of the great majority of satDNAs described so far, which include high chromosomal and nucleotide dynamics, the occurrence of species- or genus-specific sequences, high levels of intraspecies sequence homogeneity and low rates of evolutionary persistence (Dover, 1982; Garrido-Ramos, 2015, 2017). For this reason, the long-term conservation of satDNAs is unexpected and not yet well understood. After its origin, CharSat01-52 experienced array amplification (e.g., \textit{C. gomesi}) and depletion (e.g., \textit{H. malabaricus}) events, which is consistent with the library hypothesis. Such quantitative changes may be attributed to events like unequal
crossing over, as well as loop deletions and reinsertion of resulting extrachromosomal circles (Smith 1976; Walsh et al. 1987; Plohl et al. 2008; Lower et al. 2018). Remarkably, the complete depletion of satDNA arrays is a dead end and neutrally evolving arrays will eventually reach this state and become extinct (Charlesworth et al. 1986; Lower et al. 2018).

Previous studies indicated that the rate of recombination in short arrays would be too low to fully homogenize the repeats (Dover 1982; Ambrose and Crease 2011; Pavlek et al. 2015). Here, our data revealed that homogenization of CharSat01-52 repeats is taking place in all the species, regardless of their genomic organization (highly clustered or not). Our data also defy the expectations of molecular drive, since the interspecific Kimura divergence values were not higher than the intraspecific values (Table 2) and several monomer sequences are shared among distantly related species, including one variant present in at least five of them, which does not reflect their phylogenetic relationships.

The satellite landscape profile of a given genome is a multifactorial feature that depends on several components, such as genomic organization and homogenization patterns, population and reproductive issues and even functional constraints (Dover 1982; Mravinac et al. 2002; Meštrović et al. 2006; Kuhn et al. 2008; Chaves et al. 2017; Smalec et al. 2019). In this context, the existence of long-term conserved satDNA in sturgeon fishes, for example, was explained by a low mutation and homogenization rate (de la Herrán et al. 2001). Slow rates of evolution are not restricted to this single satellite DNA, but sturgeon genomes as a whole tend to evolve more slowly than those of other teleosts (Du et al. 2020). Here, it does not seem that a general slow evolution could explain the conservation of CharSat01-52, since this is the only satellite DNA common to all four species from three distinct families within the Characiformes (from a sample of more than 200 satDNA families) (Silva et al., 2017; Utsunomia et al., 2019; Serrano-Freitas et al., 2020; Crepaldi & Parise-Maltempi, 2020). However, considering that satellite DNA families evolve independently within a genome (Kuhn et al. 2008), slow rates of
concerted evolution in CharSat01-52 could explain its conservation across millions of years. In fact, a general similarity among samples from the same family in the shapes of the repeat landscapes is observed. Another explanation would be the particular combinations of nucleotides and structural features of the DNA molecule that are favored by homogenization mechanisms or their functional potential, characterizing a selective constraint (Plohl et al. 2008, 2012).

Although the transcriptional activity of satellites can be viewed as a failure of normal transcription termination (e.g., the “read-through” hypothesis, Varley et al. 1980; Epstein et al. 1986; Deryusheva et al. 2007), recent studies have revealed that satDNA transcripts might be involved in several cellular functions and could act as noncoding RNAs, which could explain their evolutionary persistence (Pezer et al. 2012; Petraccioli et al. 2015; Ferreira et al. 2019; Halbach et al. 2020; Louzada et al. 2020). Here, we detected CharSat01-52 transcripts in different tissues of three distantly-related species (140–78 mya) and, whether this satellite DNA has been actively conserved in Characoidei species through selective constraints or due to other unknown mechanisms remains to be investigated in the near future. Importantly, one must note that the lncRNA libraries retained many more CharSat01-52 fragments than enriched poly-A fragments, suggesting that satDNA transcription should be analyzed from rRNA-depleted total RNA libraries.

In the present study, by using multiple approaches, we delimited the occurrence and origin of a conserved satellite DNA that remains unexpanded as short arrays in several genomes of Characiformes fish, while it became highly abundant in Characidium gomesi. Although intragenomic homogenization was observed, an unusual case of interspecific homogenization was also found, which might be explained by functional constraints, since CharSat01-52 monomers are actively transcribed in distinct tissues of Astyanax paranae and Characidium gomesi. Moreover, by analyzing the long reads of two species, we corroborated the recent view
that satDNA loci are not homogeneous head-to-tail arrays, as we found several small arrays interspersed with other sequences; however, we did not find evidence of recurrent association with transposable elements, for example. Thus, despite the high error rates (approximately 15% for subreads), a growing interest in workflows directed at the analysis of satellite DNAs on raw long reads in the next few years is expected. By combining different technologies, we call attention to the importance of analyzing the genomic structure of repetitive sequences using multiple layers of information.

**Materials and Methods**

**Ethics**

The animals were collected in accordance with Brazilian environmental protection legislation (Collection Permission MMA/IBAMA/SISBIO—number 3245) and the procedures for the sampling, maintenance and analysis of the fishes were performed in compliance with the Brazilian College of Animal Experimentation (COBEA) and approved (protocol 504) by the BIOSCIENCE INSTITUTE/UNESP ETHICS COMMITTEE ON THE USE OF ANIMALS (CEUA).

**Sampling**

Here, we analyzed several Characiformes species for distinct purposes. Cell suspensions of some species containing mitotic metaphase plates were already available in our laboratory from previous studies (Scacchetti et al. 2015; Silva et al. 2013, 2014, 2016; Utsunomia et al. 2016) (Table S1).

Genomic DNA was extracted from the muscle, liver or blood of several species and preserved in 100% ethanol using the Wizard Genomic DNA Purification Kit (Promega) following the manufacturer’s instructions, including a step for RNA removal with RNase A
The samples were run on 1% agarose gel to check the DNA integrity. Total RNA extraction was performed using the TRIzol® Kit (Invitrogen) following the manufacturer’s instructions. Then, the samples were treated with DNase I (Thermo Fisher Scientific) and checked on 1% agarose gel and with 2100 Bioanalyzer® (Agilent) equipment. Only RNA samples with RIN > 7 were used for the subsequent analysis. Information regarding the sampling and methods applied for each specimen is detailed in Table S1.

**Sequencing data**

To uncover the extension and presence of CharSat01-52, we analyzed short-read sequencing data from species comprising three different fish orders within Otophyya, namely, Characiformes, Gymnotiformes and Siluriformes (Table 2). Some libraries had already been sequenced by us or other research groups, and data were downloaded from the sequence read archive (SRA-NCBI), totaling six libraries (Table S1). To include five superfamilies within Characiformes (Betancur-R. et al. 2019), we sequenced ten additional species on the BGISEQ-500, Illumina HiSeq or Illumina MiSeq platforms at BGI (BGI Shenzhen Corporation, Shenzhen, China) or at the Center of Functional Genomics (ESALQ/USP, Brazil) (Table S1). Several studies have already demonstrated that sequencing data obtained from the BGISEQ-500 and Illumina platforms are largely comparable (Mak et al. 2017; Zhu et al. 2018; Natarajan et al. 2019; Senabouth et al. 2020), so we did not consider any possibility of platform bias. Quality checks and trimming of the adapters was performed using Trimmomatic software (Bolger et al. 2014) to remove adapter sequences and select read pairs with Q > 20 for all nucleotides. In total, we analyzed 16 species distributed in three orders and 11 families (Figure 1; Table S1).

To reveal the transcription of CharSat01-52, we searched for CharSat01-52 transcripts in different samples using RNA-seq data. Here, we sequenced depleted rRNA samples from
the muscle and ovaries of *A. paranae* individuals. For this experiment, all the biological samples were collected on the same day. After dissection, the tissues were immediately frozen in liquid nitrogen and stored at -70 °C. Then, RNA was extracted using the TRizol® Kit (Invitrogen) following the manufacturer’s instructions. Subsequently, the samples were treated with DNAse I and checked on 1% agarose gel with 2100 Bioanalyzer® (Agilent) equipment. Only RNA samples with an A260/280 ratio of 1.8–2.0, an A260/230 ratio > 2.0, and a RIN > 7 were used for the subsequent analysis. The samples were sent to BGI (BGI Shenzhen Corporation, Shenzhen, China) and depleted with rRNA with the MGIEasy rDNA Depletion Kit before use for directional RNA-seq library preparation. Then, the samples were sequenced with the BGISEQ-500 platform (Table S1). Furthermore, we also downloaded mRNA polyadenylated RNA-seq data (SRA-NCBI) from the same sequenced tissues of *A. paranae* described above (muscle and ovaries) (Silva in prep.) and muscle of *Piaractus mesopotamicus* (Table S1).

We evaluated the densities and lengths of CharSat01-52 arrays in two PacBio SMRT (single-molecule real-time sequencing) libraries for *A. paranae* and *A. mexicanus*. For the first species, we extracted DNA and checked its integrity using the HS Large Fragment 50 kb kit (Agilent). Subsequently, library preparation and sequencing on a PacBio Sequel I platform (movie time = 600 minutes) were performed by RTL Genomics (Research and Testing Laboratory, Lubbock, TX, United States). In addition, we analyzed several PacBio RS II libraries of *A. mexicanus* gDNA available in the SRA of the NCBI (Table S1) with the kind permission of Dr. Wesley Warren.

**Bioinformatic protocols – short read sequencing data**

We applied distinct pipelines to determine CharSat01-52 abundance, diversity and organization in Characiformes genomes. We subsampled 5 million read pairs (2 x 101 bp) per species for the subsequent downstream analyses. For those libraries with different read lengths,
we trimmed all of the reads using Seqtk software. To investigate the tandemly repeated nature of CharSat01-52 and possible structural variations in the analyzed species, we selected pairs of reads showing homology with this satDNA by using BLAT (Kent 2002) and then created cluster graphs using RepeatExplorer (Novák et al. 2013) with at least 2 × 2,500 reads as the input.

We examined patterns in the copy number variation (CNV) profiles of CharSat01-52 by applying the RepeatProfiler workflow (Negm et al. 2020; https://github.com/johnssproul/RepeatProfiler). We first mapped our subsampled libraries with 5 million reads to a 208 bp-concatenated consensus monomer fragment of CharSat01-52 (MmaSat85-52, NCBI accession number MG819078.1). We also provided 10 single-copy fish genes to be mapped for single-copy normalization of the read coverage [(ppfa1 (XM_022685633.1), foxl2 (XM_007232295.3), prospero (XM_017708821.1), msh4 (XM_017711771.1), zdhdc22 (XM_017711775.1), coq6 (XM_017711829.1), znf106 (XM_017711848.1), lactamase (XM_022682177.1), gastrula zinc finger (XM_022685636.1) and tubulin-kinase (XM_017711762.1)]. The mapping was performed with Bowtie2 (Langmead & Salzberg 2012); the preset values for the --sensitive and --no-mixed parameters were used. After this step, the pipeline generates color-enhanced profiles to provide a visual indication of read depth at each site of a reference sequence and allows us to test the degree of correlation in profile shape within and among groups (in our case, the within-group comparison was performed only for C. gomesi). The pipeline automatically applied a color ramp such that the color of all the CharSat01-52 profiles shown here indicates the copy number relative to the maximum value observed (11,435 copies in C. gomesi) throughout all the profiles. Furthermore, RepeatProfiler also generates variant-enhanced profiles, providing a visual summary of variant sites relative to the reference sequence.
Intra- and interspecies abundance and divergence for CharSat01-52 were also determined by RepeatMasker (Smit et al. 2017) with a cross_match search engine. After that, Kimura 2-parameter divergence values between CharSat01-52 and each of the analyzed genomes were calculated using the calcDivergenceFromAlign.pl module within the RepeatMasker suite and plotted as a repeat landscape per species (Smit et al. 2017).

To provide more direct estimates of intra- and interspecies monomer abundance and similarity, we generated a minimum spanning tree (MST) from CharSat01-52 monomers. First, we subsampled the short-read sequencing libraries according to the genome sizes available for each of the analyzed species (Table S1) (Carvalho et al. 1998); _Megaleporinus macrocephalus_ was used as a starting point for selecting 1,000,000 paired reads (genome size of 1.38 Gb). Then, we extracted complete CharSat01-52 monomer sequences directly from the short-read data, discarded those sequence variants found only once (singletons) using a custom python script (https://github.com/fjruizruano/ngs-protocols/blob/master/cd_hit_filter_size.py) and aligned the resulting data using the Muscle algorithm (Edgar 2004) under default parameters. Subsequently, this alignment file was used as input in the PHYLOViZ software (Nascimento et al. 2016) to generate an MST, as described in Utsunomia et al. (2019). In addition, these aligned monomers were displayed as separate sequence logos using WebLogo 3.3 software (Crooks et al. 2004).

BLASTn searches (Altschul et al. 1990) were also carried out using consensus sequences of CharSat01-52 monomers against the nucleotide collection of the NCBI (nr database). Subsequently, we retrieved results with _e-values_ lower than 1e-10. Significant alignments were produced against CharSat01-52 variants (ApaSat29-52, CgomSat02-52, MmaSat85-52 and MelSat49-52) and against PTPRF interacting protein alpha 1 (_ppfia1_) (transcript variants X1, X4, X6, X8, X10, X14 and X15). To better understand the cause of this alignment, we retrieved the genomic region of _ppfia1_ from the assembled genomes of the
Characiformes species *Astyanax mexicanus* (Unplaced_Scaffold 2658 from Astyanax mexicanus-2.0) and *Pygocentrus nattereri* (Scaffold 361 from Pygocentrus_nattereri-1.0.2), the Gymnotiformes species *Electrophorus electricus* (scaffold184 from Ee_SOAP_WITH_SSPACE), the Siluriformes species *Pangasianodon hypophthalmus* (chromosome 6 from GENO_Phyp_1.0) and the Cypriniformes species *Danio rerio* (chromosome 18 from GRCz11). After that, we manually searched for the presence of CharSat01-52.

RNA-seq reads were mapped to a 208 bp-concatenated consensus monomer fragment of CharSat01-52 and also to four endogenous genes, namely: 1) *rpl13a* (accession number: XM_007244599.3); 2) *rpl32* (accession number: XM_007251493.2); 3) *rpl8* (accession number: XM007227850.3); and 4) *hprt* (accession number: XM_022684242.1) using Bowtie2 (Langmead & Salzberg 2012) with the preset values for the --sensitive and --no-mixed parameters. Then, the mapping data were converted into a sorted binary format using SAMtools (Li et al. 2009). Subsequently, we extracted the number of mapped reads with a custom script (https://github.com/fjruizruano/ngs-protocols/blob/master/bam_coverage_join.py) and estimated their transcription level as FPKM (fragments per kilo-base of transcript per million reads mapped). The values are presented as the mean ± standard deviation (SD). Furthermore, we subsampled the RNA-seq libraries (32,000,000 paired-end reads), isolated monomers directly from raw reads and constructed an MST together with extracted monomers from the genomic libraries of *A. paranae* (subsample of 32,000,000 paired-end reads) and *P. mesopotamicus* (sample of 4,914,670 paired-end reads) (Table S2).

**Bioinformatic protocols – long-read sequencing data**

Short-read data can only provide information regarding total repeat abundances and the tandemly repeated nature of satellite DNAs. Therefore, we used long reads in conjunction with
FISH analyses to provide a broader genomic panorama of *A. paranae* and *A. mexicanus*. Considering the error-prone nature of PacBio CLR technology (approximately 15% error rates; Rhoads and Au 2015), repeated motifs were identified in PacBio subreads using NoiseCancellingRepeatFinder (NCRF) version 1.01.00 (Harris et al. 2019). The `--maxnoise` parameter was set to 20% to retain long reads with noisy repeat arrays, as described in Cechova et al. (2019). To test the reliability of our data, we searched in the PacBio libraries for the sequences of two satDNAs with different FISH patterns: i) CharSat01-52, which is organized as small tandem arrays in *A. paranae* and *A. mexicanus*; and ii) AmeSat02-179 and ApaSat10-179 (NCBI accession number: MF044776.1), which are homologous and consistently clustered in both species in the pericentromeric regions, as demonstrated in a previous study (Utsunomia et al. 2017). Subsequently, the CharSat01-52 and AmeSat02-179/ApaSat10-179 repeat densities were calculated as the total number of kilobases annotated per million sequenced bases (kb/Mb).

We also applied distinct pipelines to search for the recurrent association of CharSat01-52 arrays with other repeats, such as transposable elements and satellite DNAs. First, we applied a custom python script ([https://github.com/MilanCalegari/FlankerExtractor](https://github.com/MilanCalegari/FlankerExtractor)) to select 10 kb regions upstream and downstream of every CharSat01-52 locus identified with NCRF; when the corresponding adjacent region of the read did not reach 10 kb, we analyzed the read up to the end. At this point, we applied two distinct pipelines to this subset of sequences. 1) We constructed a custom database composed of the transposable elements identified in the genome of *A. mexicanus* ([http://www.fishtedb.org/project/download?species=Astyanax+mexicanus](http://www.fishtedb.org/project/download?species=Astyanax+mexicanus)) and the satellitome of *A. paranae* (Silva et al. 2017). Then, we performed a search of these sequences with LASTZ (Harris 2007) in our subset and summarized the frequencies of the distinct types of TEs/satellite DNAs detected within the 10 kb window (Vondrak et al. 2019). 2) Considering that the results obtained by applying the abovementioned approach are biased
to sequences present in our database, we also clustered our subset of sequences using CD-HIT (Li & Godzik 2006) with a minimum cluster size of 3 and a similarity threshold of 0.8. Such a method would cluster recurrent sequences associated with CharSat01-52 arrays that could be searched against different databases (the nr database of the NCBI and the giri REPBASE, for example). Finally, we used FlexiDot (Seibt et al. 2018) to generate dotplots for studying the structure of the CharSat01-52 arrays in the PacBio reads. In these dotplots, we highlighted the presence of *A. paranae* satDNAs (Silva et al. 2017).

**Relative quantification of genomic abundance and transcription analysis of CharSat01-52**

Quantification of relative copy number of CharSat01-52 was carried out in the referred species in Table S3 through quantitative PCR (qPCR). The relative quantification (RQ) of CharSat01-52 was assessed by using the 2^−ΔCt method (Bel et al., 2011) using the single-copy gene hypoxanthine phosphoribosyltransferase (*hprt1*) as reference. Primers for this gene were designed with Primer3 (Untergasser et al. 2012). The reactions were performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific). Target and reference sequences were simultaneously analyzed in triplicate for three independent samples. The specificity of the PCR products was confirmed by dissociation curve analysis. The values are presented as the mean ± standard deviation (SD).

Transcription of CharSat01-52 was separately analyzed in the muscle and gonads of *A. paranae* and *C. gomesi*. In this case, cDNA of each sample was firstly synthetized using the High-Capacity cDNA Reverse Transcription Kit® (Thermo Fisher Scientific) with 100 μg per sample of total RNA, following the manufacturer’s instructions. After that, the RT-qPCR followed the same parameters as the qPCR detailed above, except for using cDNA instead of gDNA. Here, we also chose the level of *hprt* expression as reference mRNA control. Target
and reference sequences were simultaneously analyzed in multiple replicates (Figure 4; Table S3). Relative gene expression profiles were calculated using the 2^{-\Delta\Delta Ct} method (Livak et al. 2001).

**Molecular and cytogenetic analyses**

A primer pair was previously designed for CharSat01-52 in *A. paranae* (ApaSat29-52F and ApaSat29-52R primers, see Silva et al. 2017). We verified that this primer pair anchors in a conserved region of the monomers (Figure 2) and then used them to amplify CharSat01-52 using PCR in all our Characiformes species. The PCRs contained 1x PCR buffer, 1.5 mM of MgCl2, 200 μM of each dNTP, 0.1 μM of each primer, 2-100 ng of genomic DNA and 0.5 U of Taq polymerase in a total volume of 25 μL. The PCR program consisted of an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles at 95 °C for 10 s, 56 °C for 15 s, 72 °C for 10 s and a final extension at 72 °C for 15 min. The PCR products were checked in 2% agarose gels. Next, we generated DNA probes for CharSat01-52 using these PCR products for all the species, except *H. malabaricus* (for which PCR failed), and labeled the probes with digoxigenin-11-dUTP or biotin-16-dUTP. This procedure allowed us to perform FISH for distinct species using probes obtained from their own genomes. In addition, we produced biotin-labeled probes of AmeSat02-179/ApaSat10-179 (NCBI accession number: MF044776.1), because this satellite DNA is highly clustered in the genomes of *A. mexicanus* and *A. paranae* (Figure 5C) (Utsunomia et al. 2017). For this reason, it could be used as a parameter to the array sizes of CharSat01-52. FISH was performed under high-stringency conditions using the method described by Pinkel et al. (1986) with small modifications that were described in Utsunomia et al. (2017). Since FISH signals were not detected in several species (Fig. S5), we performed two rounds of signal amplification using conjugated anti-avidin-biotin. Each round consisted of incubating the slides for 30 minutes in a moist chamber at 37°C with the amplification mix,
containing 2.5% anti-avidin-biotin conjugate in blocking buffer (5% non-fat dry milk in 4xSSC), washing the slides three times in 4xSSC, 0.5% Triton for 3 minutes each, then incubating the slides for 30 minutes in a moist chamber at 37°C in the avidin-FITC solution (containing 0.07% avidin-FITC conjugate in blocking buffer). From each individual, a minimum of ten cells was analyzed for FISH.

**Data availability statement**

The data underlying this article are available in the GenBank Nucleotide Database and can be accessed with the following accession numbers: MmaSat85-52 (MG819078.1), ppfia1 (XM_022685633.1), foxl2 (XM_007232295.3), prospero (XM_017708821.1), msh4 (XM_017711771.1), zdhhc22 (XM_017711775.1), coq6 (XM_017711829.1), znf106 (XM_017711848.1), lactamase (XM_022682177.1), gastrula zinc finger (XM_022685636.1), tubulin-kinase (XM_017711762.1), rpl13a (XM_007244599.3), rpl32 (XM_007251493.2), rpl8 (XM007227850.3), hprt (XM_022684242.1). Whole genome sequencing data are also available in the short read archive (SRA) and the accession numbers for all the analyzed libraries are indicated in Table S1.

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**Figure captions**
Fig. 1: A head-to-tail tandem repeat organization of CharSat01-52 occur widely in several Characiformes species. 

A) Phylogenetic tree adapted from Betancur-R et al. (2019) showing the relationships between the Characiformes species analyzed here. Colors in the clade indicate distinct families within this order (Distichodontidae, Crenuchidae, Bryconidae, Characidae, Erythrinidae, Hemiodontidae, Serrasalmidae, Prochilodontidae and Anostomidae). On the right side of each species, the outputted graphs after clustering reads with circular shapes. Red asterisk denotes the proposed origin of CharSat01-52, restricted to Characoidea species. 

B) CNV profiles for CharSat01-52. Note the higher abundance of the referred satDNA in C. gomesi. 

C) Variant profiles for CharSat01-52 against a consensus sequence. Note the similarity of profiles in the within-family level. 

D) Agarose gel electrophoresis after PCR amplification in several species. M: Molecular marker; 1: Astyanax paranae, 2: Moenkhausia sanctaefilomenae, 3: Brycon orbignyanus, 4: Leporinus friderici, 5: Megaleporinus macrocephalus, 6: Prochilodus lineatus, 7: Characidium gomesi, 8: Hoplias malabaricus. B: negative control.

Fig. 2: A) CharSat01-52 sequence logos denoting a considerable sequence conservation of this satellite DNA. Green arrows indicate the anchoring regions of primers previously designed by Silva et al. 2017. 

B) Minimum spanning tree (MST) showing the relationships between the isolated monomers obtained from distinct species. Colored circles represent monomers retrieved from Illumina reads and the diameter of the circles is proportional (log scale) to their abundance. Each black dot represents a mutational step. Note the multiple occurrences of shared variants, including one common to five species. 

C) Quantification of relative copy number of CharSat01-52 in several species.
**Fig. 3:** Distribution of CharSat01-52 on the metaphase chromosomes of several Characoidea species. Note that two rounds of signal enhancement were carried out in all species, except *C. gomesi*, indicating that large clusters are only present in *C. gomesi*. Bar =10 μm.

**Fig. 4:** Transcription analysis of CharSat01-52. **A)** Transcription levels of CharSat01-52 and several other endogenous genes in different lncRNA-seq libraries from *A. paranae*, measured as FPKM. **B)** Gardner-Altman estimation plots showing CharSat01-52 transcription levels in gonads and muscle tissues of *A. paranae* and *C. gomesi* individuals, analyzed by RT-qPCR. Both groups are plotted on the left axes and the mean difference (effect size) is plotted on a floating axe on the right as a bootstrap sampling distribution. The mean difference is depicted as a black dot, and the 95% confidence interval is indicated by the ends of the vertical error bar. **C)** Minimum spanning trees showing the relationships between the isolated monomers from gDNA-seq and RNA-seq libraries. Note that the most abundant variant in the genome of *A. paranae* is also the most transcribed variant.

**Fig. 5:** **A)** Raincloud plots of lengths of reads, AmeSat02-179/ApaSat10-179 and CharSat01-52, recovered from PacBio data of *A. paranae* and *A. mexicanus*. **B)** Overall repeat density of CharSat01 and AmeSat02-179/ApaSat10-179. **C)** Metaphase plates after FISH with distinct probes, as indicated in the figure. Metaphases bordered in blue are from *A. mexicanus*, while metaphases bordered in red are from *A. paranae*. **D)** Annotated dotplots showing isolated cases of CharSat01-52 arrays interspersed with other sequences, inverted sequences or neighbored by other satellites, revealing that ChatSat01-52 arrays are not always consisted of perfect head-to-tail monomers. Bar =10 μm.
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119x109mm (600 x 600 DPI)
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Fig. 5: A) Raincloud plots of lengths of reads, AmeSat02-179/ApaSat10-179 and CharSat01-52, recovered from PacBio data of A. paranae and A. mexicanus. B) Overall repeat density of CharSat01 and AmeSat02-179/ApaSat10-179. C) Metaphase plates after FISH with distinct probes, as indicated in the figure. Metaphases bordered in blue are from A. mexicanus, while metaphases bordered in red are from A. paranae. D) Annotated dotplots showing isolated cases of CharSat01-52 arrays interspersed with other sequences, inverted sequences or neighbored by other satellites, revealing that ChatSat01-52 arrays are not always consisted of perfect head-to-tail monomers. Bar =10 μm.
### Table 1: Main results of CNV profile of CharSat01-52 in multiple Characiformes species

| Species              | Total reads | Proportion of bases with coverage | Normalized average coverage |
|----------------------|-------------|----------------------------------|-----------------------------|
| *D. sexfasciatus*    | 5,000,000   | 0                                | 0                           |
| *C. gomesi*          | 5,000,000   | 0.975961538                      | 2822.325499                 |
| *C. gomesi*          | 5,000,000   | 1                                | 2876.667544                 |
| *C. gomesi*          | 5,000,000   | 0.975961538                      | 2697.887615                 |
| *B. orbignyanus*     | 5,000,000   | 1                                | 554.1420943                 |
| *M. sanctaefilomenae*| 5,000,000   | 1                                | 326.909913                  |
| *A. paranae*         | 5,000,000   | 1                                | 205.7046328                 |
| *A. mexicanus*       | 5,000,000   | 1                                | 245.5193184                 |
| *H. malabaricus*     | 5,000,000   | 0.533653846                      | 1.536351898                 |
| *H. gracilis*        | 4,173,860   | 1                                | 95.39636771                 |
| *P. mesopotamicus*   | 4,914,670   | 0.9375                           | 43.55158902                 |
| *M. asterias*        | 5,000,000   | 1                                | 68.52400619                 |
| *P. nattereri*       | 5,000,000   | 0.995192308                      | 129.9237403                 |
| *P. lineatus*        | 5,000,000   | 1                                | 178.0744825                 |
| *M. macrocephalus*   | 2,318,964   | 1                                | 202.8145728                 |
| *L. friderici*       | 2,235,104   | 0.990384615                      | 131.5511865                 |
| Species                     | N   | Monomer Size (bp) | A + T (%) | Abundance (%) | Intraspecific KD (%) | Interespecific KD (%) |
|-----------------------------|-----|-------------------|-----------|---------------|----------------------|------------------------|
| C. gomesi                  | 2145| 52                | 71.5      | 3.53E-03 ±    | 15.43 ± 0.03         |
| H. malabaricus             | -   | 52                | 3.03917E-05 | 2.15E+01     |
| H. gracilis                | 116 | 52                | 66        | 0.000923      | 1.61E+01              |
| P. mesopotamicus           | 6   | 52                | 67.4      | 0.000124      | 1.94E+01              |
| M. asterias                | 12  | 52                | 66.2      | 0.000155      | 1.82E+01              |
| P. nattereri               | 63  | 52                | 65.5      | 0.000182849   | 1.86E+01              |
| P. lineatus                | 90  | 52                | 59.3      | 0.000287      | 1.44E+01              |
| L. friderici               | 69  | 52                | 67.4      | 0.000196      | 1.45E+01              |
| M. macrocephalus           | 187 | 52                | 67.6      | 0.000315      | 1.35E+01              |
| B. orbignyanus             | 63  | 52                | 64.6      | 0.000191      | 8.16E+00              |
| A. mexicanus               | 33  | 52                | 68.2      | 0.000179      | 1.32E+01              |
| A. paranae                 | 65  | 52                | 67.7      | 0.000137      | 1.13E+01              |
| M. sanctaefilomenae        | 104 | 52                | 67.9      | 0.00074       | 9.48E+00              |
| D. sexfasciatus            | -   | -                 | -         | -             | 0                     |
| Total                      | 2953|                   |           |               | 1.37E+01              |

Mean | 65.2 | 0.000499303 | 1.37E+01 |
S.D. | 4.020779361 | 0.000909406 | 5.47E+00 |
C.V. | 6.16683951 | 182.135036 | 39.51418598 |