CompCytogen 12(1): 1–12 (2018)
doi: 10.3897/CompCytogen.v12i1.22052
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Studies in two allopatric populations of *Hypostomus affinis* (Steindachner, 1877): the role of mapping the ribosomal genes to understand the chromosome evolution of the group

Karina de Oliveira Brandão[1], Dinaíza Abadia Rocha-Reis[2], Caroline Garcia[3], Rubens Pazza[2], Lurdes Foresti de Almeida-Toledo[4], Karine Frehner Kavalco[2]

1 *Leiden University Medical Center, Department of Anatomy and Embryology, S-1-P, P.O. Box 9600, 2300 RC Leiden, The Netherlands* **2** *Universidade Federal de Viçosa, Campus Rio Paranaíba, Institute of Biological and Health Sciences, Laboratory of Ecological and Evolutionary Genetics, BR 354 - km 310, PO Box 22, ZIP 38810-000, Rio Paranaíba, MG, Brazil* **3** *Universidade Estadual do Sudoeste da Bahia, Campus Jequié, Department of Biological Sciences, Av. José Moreira Sobrinho s/n, Jequiezinho, ZIP 45206-190, Jequié, BA, Brazil* **4** *Universidade de São Paulo. Institute of Biosciences, Department of Genetics and Evolutionary Biology, Rua do Matão, 277 – Edifício André Dreyfus, Cidade Universitária, ZIP 05508090, São Paulo, SP, Brazil*

Corresponding author: *Karine Frehner Kavalco* (kavalco@ufv.br)

Academic editor: *I. Kuznetsova* | Received 3 November 2017 | Accepted 5 December 2017 | Published 9 January 2018

http://zoobank.org/B9E398FD-39E3-4843-A4AF-C36FF47E7DA7

Citation: Brandão KO, Rocha-Reis DA, Garcia C, Pazza R, Almeida-Toledo LF, Kavalco KF (2018) Studies in two allopatric populations of *Hypostomus affinis* (Steindachner, 1877): the role of mapping the ribosomal genes to understand the chromosome evolution of the group. Comparative Cytogenetics 12(1): 1–12. https://doi.org/10.3897/CompCytogen.v12i1.22052

Abstract

Several cytogenetic markers show chromosomal diversity in the fish such as "armoured catfish". Although studies have characterized many species in the major genera representing these Siluridae, particularly in the genus *Hypostomus* Lacépède, 1803, trends in chromosome evolution of this group remain unclear. The Paraíba do Sul river basin contains the armoured catfish *Hypostomus affinis* Steindachner, 1877, which is unique because of its distribution of repetitive DNAs, the 5S and 18S rDNA. Identified samples and registered collections in Brazilian museums were identified as the same typological species, while we observed wide variations in the physical location of this gene in the karyotype based on fluorescent in situ hybridization results. In this study, we propose that these species can represent evolutionarily independent units, as these fish frequently undergo processes such as dispersion and vicariance and that the rDNA is associated with DNA that spreads in the genome, such as transposons. Additionally, the absence of gene flow due to

the distance of the sample location could intensify evolutionary processes. The phenotypes found for the 18S rDNA showed minor changes in relation to the number of sites between the lower and upper drainage regions of Paraíba do Sul. The large difference in the number of sites found for the 5S rDNA entered the same region (upper drainage of the basin) and the literature data could represent a population dynamics where an expansion of the 5S rDNA sites provides an extinct or non-sampled cytotype in this work.

Keywords
Biodiversity, Catfish, Cytogenetics, Hypostominae, Teleostei

Introduction

With a wide geographic distribution in nearly all of the Neotropical region from Costa Rica to Argentina, Loricariidae is considered one of the largest Neotropical fish families and the largest Family of catfishes (Siluriformes) (Nelson et al. 2016), with more than 1100 species described to date (Eschmeyer and Fong 2017).

The great diversity of armored catfish is also reflected in the available cytogenetic data of the group. Loricariidae exhibits large variations in diploid number, ranging from 2n = 36 chromosomes in *Loricaria latirostris* Boulenger, 1900 (Giuliano-Caetano 1998) to 2n = 96 in *Hemipsilichthys gobio* Lütken, 1874 (Kavalco et al. 2005, previously identified as *Upsilodus* sp.). This group shows several structural differences (Mariotto et al. 2009), numerous polymorphisms (Giuliano-Caetano 1998, Cereali et al. 2008), and morphologically differentiated sex chromosome systems (Alves et al. 2006, Oliveira et al. 2007, Konerat et al. 2015, Oliveira et al. 2015a, Rocha-Reis et al. unpublished data), which nearly always correspond to unique chromosomal features.

Most of this great diversity is related to the genus *Hypostomus* Lacépède, 1803, which contains approximately 200 valid species (Eschmeyer and Fong 2017), only some of which have their taxonomic resolution fully understood and resolved (Armbruster 2004). *Hypostomus* is considered one of the most diverse genus of Neotropical fish, and many genetic studies have examined their complex karyotype evolution (Rubert et al. 2008, Bitencourt et al. 2012, Endo et al. 2012, Pansonato-Alves et al. 2013, Traldi et al. 2013); studies have also been conducted to identify different species and detect phylogenetic relationships within the genus (Montoya-Burgos et al. 2002, Armbruster 2004, Lujan et al. 2015).

Fluorescent in situ hybridization (FISH) for localization of the 18S ribosomal RNA (18S rRNA) gene was one of the first cytogenetic-molecular markers applied in Neotropical fish (Hatanaka and Galetti Jr 2004), which revealed phenotypic variations in different groups. Although potentially interesting for gene expression studies, silver nitrate localization of Ag-NORs has not been widely used and is routinely applied only for comparison. Because not every 18S ribosomal DNA (18S rDNA) site is correctly identified using this technique (Dobigny et al. 2002), it is thought that the evolution of ribosomal genes can be determined from FISH data. These data, however, are rare for most fish, although some trends have been observed in smaller groups and have been examined in detail. In *Hypostomus*, through the efforts of different research

groups, 18S gene localization data are available for approximately 30 species/populations (for review, see Rubert et al. 2016).

In contrast, data for 5S ribosomal DNA (5S rDNA) are limited. This marker has been defined in only approximately a dozen species of the genus for some Neotropical populations (Kavalco et al. 2004a, Mendes-Neto et al. 2011, Traldi et al. 2012, Pansonato-Alves et al. 2013, Traldi et al. 2013, Baumgärtner et al. 2014, Bueno et al. 2014, Rocha-Reis et al. unpublished data). Similar results were observed for the distribution of constitutive heterochromatin, although this type of highly compacted DNA requires further examination. The numerous chromosomes and their small sizes may be the main reason for the low prevalence of cytogenetic studies of armored catfish, despite their great species diversity and relative abundance in Brazilian rivers.

Although *Hypostomus affinis* Steindachner, 1877 was found in the Mucuri and Doce river basin, most of the records are related to the Paraíba do Sul river, indicating a wide distribution of this species in this river basin (Mazzoni et al. 1994). In this study, two populations of *H. affinis*, both upstream and downstream in the Paraíba do Sul River, were analyzed. Data for the evolution of ribosomal sequences were compared with polymorphisms observed in the populations presented here and those reported in the literature for the genus *Hypostomus*.

Material and methods

Two populations of *H. affinis* were collected from Jacuí creek, Cunha/SP (-23.04052/-44.93408, Fig. 1 – point a; one male/seven juvenile fish) and the Paraíba do Sul River, in Itaocara/RJ (-21.66141/-42.07454, Fig. 1 – point b; one female/five juvenile fish). Both collections were carried out in the year 2005. These samples were analyzed by classical and molecular cytogenetic techniques. First, the samples were processed, fixed in 10% formaldehyde, and stored in 70% ethanol. Finally, samples were sent to the Museum of Science and Technology of the Pontifical Catholic University of Rio Grande do Sul – MCP, where they were identified and deposited in the ichthyologic collection under vouchers MCP 43299 and MCP 43301 (populations from Cunha/SP and Itaocara/RJ, respectively).

The chromosomal preparations were obtained from kidney cells of the animals as described by Gold et al. (1990) with some modifications. Silver nitrate (Ag-NOR) staining to detect nuclear organizing regions (NORs) was performed according to Howell and Black (1980) and Kavalco and Pazza (2004), and C-banding followed a protocol adapted from Sumner (1972).

The physical location of the ribosomal genes was detected via FISH (Pinkel et al. 1986 modified by Pazza et al. 2006), using 18S ribosomal DNA (18S rDNA) and 5S ribosomal DNA (5S rDNA) probes obtained from the genome of *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka and Galetti Jr 2004) and *Megaleporinus elongatus* Valenciennes, 1850 (Martins and Galetti Jr 1999), respectively. The 18S and 5S rDNA probes were labeled with biotin-14-dATP by nick translation using BioNick Labeling System according to manufacturer instructions (Invitrogen).

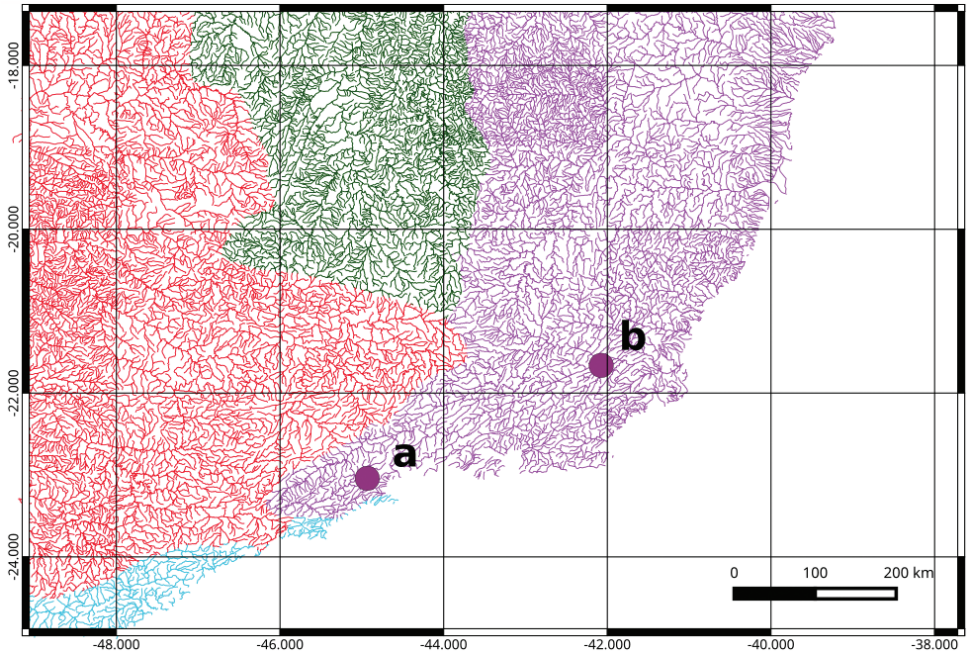

Figure 1. Hydrographic map of the southeast coast of Brazil with the collection points of *Hypostomus affinis*. Point "a" corresponds to Cunha/SP and point "b" corresponds to Itaocara/RJ. Hydrographic basins: Paraíba do Sul (in purple), São Francisco (in green), Upper Paraná (in red) and Rios Costeiros (in blue).

Hybridization was detected with avidin and fluorescein isothiocyanate for 18S rDNA probes and Cy3 for 5S rDNA probes. Blade assembly was performed with antifade and propidium iodide, and antifade and DAPI for 18S rDNA and rDNA 5S probes, respectively. High-stringency washes with >75% (20% formamide/0.1× SSC) were performed for 15 min, and the signals were amplified using biotin-conjugated anti-avidin solution and incubated in non-fat dry milk buffer. Images were acquired with a camera coupled to an OLYMPUS BX41 microscope (Olympus Inc., Tokyo, Japan) using QCapture 6.0 (QImaging Surrey, BC, Canada) software.

To assemble the karyotypes, chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a) according to the arm ratio proposed by Levan et al. (1964). All chromosomes were measured to avoid identification errors.

Results

Male and female fish in both populations showed a diploid number of 2n = 66 chromosomes, karyotype composed of 12m+12m+14st+28a, and fundamental number FN = 104 (Fig. 2A, B).

C-banding staining of both populations revealed subtle pericentromeric markers on several chromosomes, as well as conspicuous terminal blocks on two pairs of acrocentric

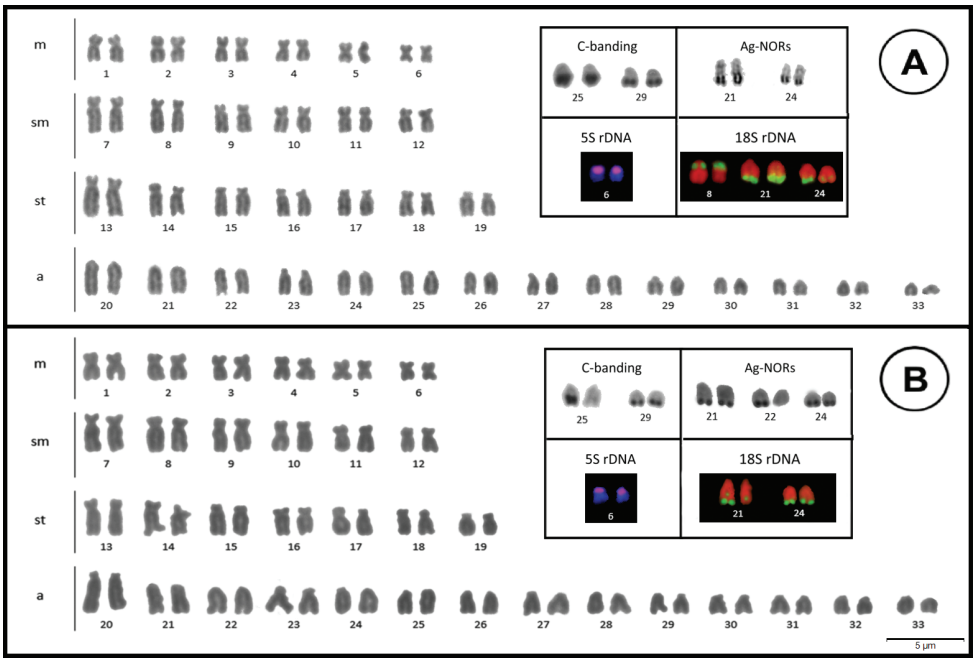

Figure 2. Karyotypes found for the populations of Cunha/SP (**A**) and Itaocara/RJ (**B**). In the boxes are the phenotypes for C banding, Ag-NORs and FISH with 5S and 18S rDNA probes.

chromosomes (a) (25, 29), although a size heteromorphism was found in one of the pairs in the population of Itaocara (25) (Fig. 2A, B, box). These markers did not correspond to the location of the major Ag-NORs (Fig. 2A, B, box).

Silver nitrate staining of both populations revealed the existence of multiple systems of NORs. In Cunha/SP specimens, two pairs of chromosome a (21, 24) exhibited large markers on their long arms (Fig. 2A, B). According to FISH, the 18S rDNA contained these four and two other sites located on the short arm of a small submetacentric (sm) pair, for a total of six gene sites (Fig. 2A, box). In Itaocara/RJ specimens, Ag-NORs analysis revealed five sites marked by silver nitrate: two located in the long arms of pairs a (21, 24) and another located in the terminal position of the short arm of chromosome a (22) (Fig. 2B, box). However, only four markers were detected on the 18S rDNA probe using FISH, corresponding to markers obtained from silver nitrate staining on chromosomes a (Fig. 2B, box).

Hybridization of the 5S rDNA probe revealed two sites marked in the lowest metacentric pair of the complement in both populations (pair 6) (Fig. 2A, B, box).

Discussion

From a cytogenetic perspective, only one sample of *H. affinis* from the Jacuí creek Cunha/SP has been previously studied (Kavalco et al. 2004a, 2004b, 2005). In this

study, we evaluated populations from the upper and lower Paraíba do Sul River. We first sought to expand sampling from the Jacuí creek to further analyze the heterochromatin polymorphism described previously (Kavalco et al. 2004b). Unexpectedly, we observed the conservation of chromosomal characteristics between the two populations analyzed in this study, as well as large variations, particularly with respect to the 5S rDNA sites compared to the previously described sample. Although geographically close, both populations from Cunha/SP showed large differences in their chromosomes. These populations showed relatively higher karyotypic divergence than geographic divergence, as they are only approximately 100 m away in geodesic distance and are part of the same drainage.

Although the chromosome number is the same and the karyotypic formula observed in the populations studied slightly differs from the previously sampled population, other cytogenetic features revealed differentiated evolutionary units. The difference in karyotype symmetry observed between the chromosomes of both samplings from Cunha/SP were clear; this was also clear when the relative size of the chromosomes was organized based on type, even when the same measurement and classification criterion proposed by Levan et al. (1964) and same magnification scale were used. In the previously analyzed sample from Jacuí creek, karyotypic asymmetry was clearly observed, even within each chromosomal group (Kavalco et al. 2005). In addition, the distribution of constitutive heterochromatin and existence of conspicuous blocks (Kavalco et al. 2004b) differed completely from the patterns observed in this study.

The difference among the observed chromosomal sites in the populations cannot be attributed to the use of 18S and 5S ribosomal DNA probes isolated from different species. The rRNA in eukaryotes presents as two subunits (one formed by 28S, 18S and 5.8S and another one formed by 5S) and their DNA sequences vary very slowly due to selective pressure, being considered highly conserved (Long and Dawid 1980). This allows the interspecific hybridization of the mentioned probes (obtained from *Prochilodus argenteus* and *Megaleporinus elongatus*), with chromosomes of a wide variety of fishes, like Characiformes (de Marco Ferro et al. 2001, Pazza et al. 2006, da Silva et al. 2016), Gymnotiformes (Fernandes et al. 2017a, 2017b) Perciformes (Jacobina et al. 2014, Argôlo and Affonso 2015, Oliveira et al. 2015b), Siluriformes (Blanco et al. 2014, Kantek et al. 2015, Ribeiro et al. 2015), including other species of *Hypostomus* (Kavalco et al 2004a, 2005, Traldi et al. 2013, Baumgärtner et al. 2014, Oliveira et al. 2015a, Lara Kamei et al. 2017).

For the location of 18S rDNA, we observed conservation of the number and position of sites in samples of the upper drainage region (Kavalco et al. 2005, this study), as well as chromosome differentiation in the lower Paraíba do Sul population, which showed the lowest number of sites. In addition to chromosome number, this is the only characteristic shared between samples from Jacuí creek.

The existence of different chromosomal formulas in close groups of different organisms, or nominally similar species, is attributed to chromosomal rearrangements. In armored catfish, two major types of chromosomal rearrangements appear to be involved in karyotype differences, depending on fixation of the diploid number (non-

Robertsonian) or their variation (Robertsonian) (Artoni and Bertollo 1996, 2001, Kavalco et al. 2005). However, other factors should be considered in the chromosome evolution of the group, such as the dispersion trends of repetitive sequences such as ribosomal genes (Kavalco et al. 2004a). Because the presence of a pair of chromosomes carrying the rDNA in fish is thought to be a plesiomorphic condition (Martins and Galetti Jr 1999, Oliveira and Gosztonyi 2000), even for Loricariidae (Kavalco et al. 2004a, Alves et al. 2012), the genus *Hypostomus* may contain lines with contrasting tendencies (Pansonato-Alves et al. 2013) and possibly an ancestral phenotype with a site in a chromosomal pair (Traldi et al. 2013). Dispersion of ribosome cistrons may be related to not only species-specific events but also populational events, as in armored catfish in which the formation of isolated populations typically occurs because of low vagility (Artoni and Bertollo 2001, Bitencourt et al. 2012). In fact, variations in the distribution of 18S rDNA sites in the genus *Hypostomus* were clear, and it was difficult to establish evolutionary tendencies for the character, as observed among different populations of the Paraíba do Sul river. In addition, their co-location with DNAs similar to transposons (Pansonato-Alves et al. 2013) is unfavorable for observing macroevolutionary tendencies.

The divergent phenotype observed by Kavalco et al. (2004a) for the 5S rDNA cistrons in *Hypostomus* reflects well-known characteristics of genomic evolution in repetitive DNA. The evolutionary dynamics of this gene are related not only to variations in non-transcribed spacers, but also to synteny with long and short interspersed nuclear elements, non-long terminal repeat retrotransposons, U-snRNA families, and microsatellite polymorphisms (Rebordinos et al. 2013). According to these authors, polymorphisms in non-transcribed regions are observed in fish and polymorphisms in transcribed regions do not appear to interfere with the cellular activity of 5S rDNA. Although in some species, the molecular diversity of the 5S rDNA gene families is greater than the chromosome diversity (Rebordinos et al. 2013), this rule may not be applied for Neotropical ichthyofauna biodiversity. In several respects, the genus *Hypostomus*, as well as others, show various chromosomal evolutionary novelties at several levels, potentially reflecting recent adaptive radiation.

The speciation by allopatry can be an important source of diversity in Neotropics and could be responsible for the biodiversity of fishes from Brazilian rivers and it is possible that very short time periods can produce new phenotypes on *Hypostomus* chromosomes. At the same time, the extensive chromosomal variation observed in the sample of *H. affinis* analyzed previously by Kavalco et al. (2004a, 2004b, 2005; collected in the year 2001 - personal communication) could be related with an event that today represents a "dead end" in the evolutionary history of the population, highlighting sympatric evolutionary processes. Since the great number of 5S rDNA spread in the karyotype is an uncommon feature to the catfishes and it can increase chromosomal rearrangements, to consider the karyotype shown in this paper as the resident cytotype of the drainage is the most parsimonious idea. It is possible that the phenotype of the 18S rDNA disposition in the chromosomes shared between the individuals from Cunha/SP represents an evidence of introgression between a variant extinct cytotype

and the ancient one, stated in this work. In this case, the variant form probably had lower adaptive value and was not able to fixation, or we do not sample the variant cytotype, just the ancient one.

Conclusion

Minor chromosome changes were found between the two sampled populations, especially regarded to an extra chromosome pair bearing 18S rDNA in population from Cunha. In addition, 18S rDNA distribution in Cunha was the same as previously sample. However, the remarkable difference in the 5S rDNA distribution between two sampling at Cunha, separated by four years between the collections, could represent a population dynamic where an expansion of the 5S rDNA sites provide a phenotype furtherly extinct or not sampled in this work.

Acknowledgments

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, 484626/2013-2) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES - Fellowship for M.Sc. degree).

References

Alves AL, Oliveira C, Nirchio M, Granado A, Foresti F (2006) Karyotypic relationships among the tribes of Hypostominae (Siluriformes: Loricariidae) with description of XO sex chromosome system in a Neotropical fish species. Genetica 128: 1–9. https://doi.org/10.1007/s10709-005-0715-1

Alves AL, Borba RS, Pozzobon APB, Oliveira C, Nirchio M, Granado A, Foresti F (2012) Localization of 18S ribosomal genes in suckermouth armored catfishes Loricariidae (Teleostei, Siluriformes) with discussion on the Ag-NOR evolution. Comparative Cytogenetics 6(3): 315–321. https://doi.org/10.3897/compcytogen.v6i3.2667

Argôlo LA, Affonso PRAM (2015) First cytogenetic report in *Cichlasoma sanctifranciscense* Kullander, 1983 (Perciformes, Cichlidae) from northeastern Brazil with inferences on chromosomal evolution of Cichlasomatini. Comparative Cytogenetics 9(4): 671–681. https://doi.org/10.3897/CompCytogen.v9i4.5562

Armbruster JW (2004) Phylogenetic relationships of the suckermouth armored catfishes (Loricariidae) with emphasis on the Hypostominae and the Ancistrinae. Zoological Journal of the Linnean Society 141: 1–80. https://doi.org/10.1111/j.1096-3642.2004.00109.x

Artoni RF, Bertollo LAC (1996) Cytogenetic studies on Hypostominae (Pisces, Siluriformes, Loricariidae). Considerations on karyotype evolution in the genus *Hypostomus*. Caryologia 49(1): 81–90. https://doi.org/10.1080/00087114.1996.10797353

Artoni RF, Bertollo LAC (2001) Trends in the karyotype evolution of Loricariidae fish (Siluriformes). Hereditas 134: 201–210. https://doi.org/10.1111/j.1601-5223.2001.00201.x

Baumgärtner L, Paiz LM, Zawadzki CH, Margarido VP, Portela Castro ALDB (2014) Heterochromatin polymorphism and physical mapping of 5S and 18S ribosomal DNA in four populations of *Hypostomus strigaticeps* (Regan, 1907) from the Paraná River Basin, Brazil: evolutionary and environmental correlation. Zebrafish 11(5): 479–487. https://doi.org/10.1089/zeb.2014.1028

Bitencourt JA, Affonso PRAM, Giuliano-Caetano L, Carneiro PLS, Dias AL (2012) Population divergence and peculiar karyoevolutionary trends in the loricariid fish *Hypostomus* aff. *unae* from northeastern Brazil. Genetics and Molecular Research 11(2): 933–943. https://doi.org/10.4238/2012.April.13.1

Blanco DR, Vicari MR, Lui RL, Artoni RF, de Almeida MC, Traldi JB, Margarido VP, Moreira-Filho O (2014) Origin of the $X_1X_1X_2X_2/X_1X_2Y$ sex chromosome system of *Harttia punctata* (Siluriformes, Loricariidae) inferred from chromosome painting and FISH with ribosomal DNA markers. Genetica 142(2): 119–126. https://doi.org/10.1007/s10709-014-9759-4

Bueno V, Venere PC, Konerat JT, Zawadzki CH, Vicari MR, Margarido VP (2014) Physical mapping of the 5S and 18S rDNA in ten species of *Hypostomus* Lacépède (Siluriformes: Loricariidae): evolutionary tendencies in the genus. The Scientific World Journal ID 943825: 8 pp. https://doi.org/10.1155/2014/943825

Cereali SS, Pomini E, Rosa R, Zawadzki CH, Froehlich O, Giuliano-Caetano L (2008) Karyotype description of two species of *Hypostomus* (Siluriformes, Loricariidae) of the Planalto da Bodoquena, Brazil. Genetics and Molecular Research 7(3): 583–591. https://doi.org/10.4238/vol7-3gmr404

Dobigny G, Ozouf-Costaz C, Bonillo C, Volobouev V (2002) "Ag-NORs" are not always true NORs: new evidence in mammals. Cytogenetic and Genome Research 98(1): 75–77. https://doi.org/10.1159/000068541

Endo KS, Martinez ERM, Zawadzki CH, Paiva LRSP, Júlio Júnior HF (2012) Karyotype description of possible new species of the *Hypostomus ancistroides* complex (Teleostei: Loricariidae) and other Hypostominae. Acta Scientiarum Biological Sciences 34(2): 181–189. https://doi.org/10.4025/actascibiolsci.v34i2.9318

Eschmeyer W, Fong JD (2017) Catalog of Fishes: Species by Family/Subfamily. California Academy of Sciences. http://www.researcharchive.calacademy.org/research/ichthyology/catalog/speciesbyfamily.asp [accessed 10. October 2017]

Fernandes CA, Baumgärtner L, Paiz LM, Margarido VP, Portela Castro ALB (2017a) Chromosomal characteristics of rDNA in a conserved karyotype of two *Sternopygus macrurus* (Gymnotiformes: Sternopygidae) populations from upper Paraná River basin. Biologia 72(6): 680–685. https://doi.org/10.1515/biolog-2017-0071

Fernandes CA, Paiz LM, Baumgärtner L, Margarido VP, Vieira MMR (2017b) Comparative cytogenetics of the black ghost knifefish (Gymnotiformes: Apteronotidae): evidence of chromosomal fusion and pericentric inversions in karyotypes of two *Apteronotus* species. Zebrafish 14(5): 471–476. https://doi.org/10.1089/zeb.2017.1432

Giuliano-Caetano L (1998) Polimorfismo cromossômico Robertsoniano em populações de *Rineloricaria latirostris* (Pisces, Loricariinae). PhD Thesis, Universidade Federal de São Carlos, São Carlos, São Paulo, 78 pp. [In Portuguese]

Gold JR, Li C, Shipley N, Powers PK (1990) Improved methods for working with fish chromosomes with a review of metaphase chromosome banding. Journal of Fish Biology 37(4): 563–575. https://doi.org/10.1111/j.1095-8649.1990.tb05889.x

Hatanaka T, Galetti Jr PM (2004) Mapping of the 18S and 5S ribosomal RNA genes in the fish *Prochilodus argenteus* Agassiz, 1829 (Characiformes, Prochilodontidae). Genetica 122(3): 239–244. https://doi.org/10.1007/s10709-004-2039-y

Howell WMT, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Cell and Molecular Life Sciences 36(8): 1014–1015. https://doi.org/10.1007/BF01953855

Jacobina UP, Bertollo LAC, Cioffi MB, Molina WF (2014) Physical mapping of 18S and 5S genes in pelagic species of the genera *Caranx* and *Carangoides* (Carangidae). Genetics and Molecular Research 13(4): 9628–9635. https://doi.org/10.4238/2014.November.14.7

Kantek DL, Peres WAM, Moreira-Filho O (2015) Cytogenetics of *Trichomycterus brasiliensis* (Siluriformes: Trichomycteridae) from the Upper São Francisco River Basin (MG). Cytologia 80(1): 25–29. https://doi.org/10.1508/cytologia.80.25

Kavalco KF, Pazza R (2004) A rapid alternative technique for obtaining silver-positive patterns in chromosomes. Genetics and Molecular Biology 27(2): 196–198. https://doi.org/10.1590/S1415-47572004000200012

Kavalco KF, Pazza R, Bertollo LAC, Moreira-Filho O (2004a) Gene mapping of 5S rDNA sites in eight fish species from the Paraíba do Sul river basin, Brazil. Cytogenetic and Genome Research 106(1): 107–110. https://doi.org/10.1159/000078567

Kavalco KF, Pazza R, Bertollo LAC, Moreira-Filho O (2004b) Heterochromatin characterization of four fish species of the family Loricariidae (Siluriformes). Hereditas 141(3): 237–242. https://doi.org/10.1111/j.1601-5223.2004.01850.x

Kavalco KF, Pazza R, Bertollo LAC, Moreira-Filho O (2005) Karyotypic diversity and evolution of Loricariidae (Pisces, Siluriformes). Heredity 94(2): 180–186. https://doi.org/10.1038/sj.hdy.6800595

Konerat JT, Bueno V, Margarido VP, Portela-Castro ALB, Martins-Santos IC (2015) Diversity of sex chromosome systems in Ancistrini (Loricariidae, Hypostominae): ZZ/ZW in *Ancistrus taunayi* Miranda Ribeiro, 1918. Cytogenetic and Genome Research 146: 306–310. https://doi.org/10.1159/000441431

Lara Kamei MCS, Baumgärtner L, Paiva S, Zawadzki CH, Martins-Santos IC, Portela-Castro ALB (2017) Chromosomal diversity of three species of *Hypostomus* Lacépède, 1803 (Siluriformes, Loricariidae), from the Paraná River Basin, Brazil: a species complex in *Hypostomus ancistroides* reinforced by a ZZ/ZW sex chromosome system. Zebrafish 14(4): 357–363. https://doi.org/10.1089/zeb.2017.1429

Levan A, Fredga K, Sandberg AA (1964) Nomenclature of centromeric position on chromosomes. Heredity 52(2): 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Long EO, Dawid IB (1980) Repeated genes in eukaryotes. Annual Review of Biochemistry 49: 727–764. https://doi.org/10.1146/annurev.bi.49.070180.003455

Lujan NK, Armbruster JW, Lovejoy NR, López-Fernández H (2015) Multilocus molecular phylogeny of the suckermouth armored catfishes (Siluriformes: Loricariidae) with a focus on subfamily Hypostominae. Molecular Phylogenetics and Evolution 82: 269–288. https://doi.org/10.1016/j.ympev.2014.08.020

de Marco Ferro DA, Néo DM, Moreira-Filho O, Bertollo LAC (2001) Nucleolar organizing regions, 18S and 5S rDNA in *Astyanax scabripinnis* (Pisces, Characidae): populations distribution and functional diversity. Genetica 110: 55–62. https://doi.org/10.1023/A:1017963217795

Mariotto S, Centofante L, Miyazawa CS, Bertollo LAC, Moreira-Filho O (2009) Chromosome polymorphism in *Ancistrus cuiabae* Knaack, 1999 (Siluriformes: Loricariidae: Ancistrini). Neotropical Ichthyology 7(4): 595–600. https://doi.org/10.1590/S1679-62252009000400006

Martins C, Galetti Jr PM (1999) Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Research 7(5): 363–367. https://doi.org/10.1023/A:1009216030316

Mazzoni R, Caramaschi U, Weber C (1994) Taxonomical revision of the species of *Hypostomus* Lacépède, 1803 (Siluriformes, Loricariidae) from the Lower rio Paraíba do Sul, State of Rio de Janeiro, Brazil. Revue Suisse de Zoologie 101(1): 3–18. https://doi.org/10.5962/bhl.part.79897

Mendes-Neto EO, Vicari MR, Artoni RF, Moreira-Filho O (2011) Description of karyotype in *Hypostomus regani* (Ihering, 1905) (Teleostei, oricariidae) from the Piumhi river in Brazil with comments on karyotype variation found in *Hypostomus*. Comparative Cytogenetics 5(2): 133–142. https://doi.org/10.3897/compcytogen.v5i2.964

Montoya-Burgos JI, Weber C, Le Bail PY (2002) Phylogenetic relationships within *Hypostomus* (Siluriformes: Loricariidae) and related genera based on mitochondrial D-loop sequences. Revue Suisse de Zoologie 109(2): 369–382. https://doi.org/10.5962/bhl.part.79596

Nelson JS, Grande TC, Wilson MVH (2016) Fishes of the world. Fifth Edition. New Jersey, 752 pp. https://doi.org/10.1002/9781119174844

Oliveira C, Gosztonyi AE (2000) A cytogenetic study of *Diplotnystes mesembrinus* (Teleostei, Siluriformes, Diplomystidae) with a discussion of chromosome evolution in siluriforms. Caryologia 53(1): 31–37. https://doi.org/10.1080/00087114.2000.10589178

Oliveira RR, Feldberg E, dos Anjos MB, Zuanon J (2007) Karyotype characterization and ZZ/ZW sex chromosome heteromorphism in two species of the catfish genus *Ancistrus* Kner, 1854 (Siluriformes: Loricariidae) from the Amazon basin. Neotropical Ichthyology 5(3): 301–306. https://doi.org/10.1590/S1679-62252007000300010

Oliveira LC, Ribeiro MO, Dutra ES, Zawadzki CH, Portela-Castro ALB, Martins-Santos IC (2015a) Karyotype structure of *Hypostomus* cf. *plecostomus* (Linnaeus, 1758) from Tapajós River basin, Southern Amazon: occurrence of sex chromosomes (ZZ/ZW) and their evolutionary implications. Genetics and Molecular Research 14(2): 6625–6634. https://doi.org/10.4238/2015.June.18.5

Oliveira IA, Argôlo LA, Bitencourt JA, Diniz D, Vicari MR, Affonso PRAM (2015b) Cryptic chromosomal diversity in the complex "*Geophagus*" *brasiliensis* (Perciformes, Cichlidae). Zebrafish 13(1): 33–44. https://doi.org/10.1089/zeb.2015.1169

Pansonato-Alves JC, Serrano EA, Utsunomia R, Scacchetti PC, Oliveira C, Foresti F (2013) Mapping five repetitive DNA classes in sympatric species of *Hypostomus* (Teleostei: Siluriformes: Loricariidae): analysis of chromosomal variability. Reviews in Fish Biology and Fisheries 23(4): 477–489. https://doi.org/10.1007/s11160-013-9303-0

Pazza R, Kavalco KF, Bertollo LAC (2006) Chromosome polymorphism in *Astyanax fasciatus* (Teleostei, Characidae). 1. Karyotype analysis, Ag-NORs and mapping of the 18S and 5S

ribosomal genes in sympatric karyotypes and their possible hybrid forms. Cytogenetic and Genome Research 112: 313–319. https://doi.org/10.1159/000089886

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proceedings of the National Academy of Sciences of the United States of America 83(9): 2934–2938. https://doi.org/10.1073/pnas.83.9.2934

Rebordinos L, Cross I, Merlo A (2013) High evolutionary dynamism in 5S rDNA of fish: state of the art. Cytogenetic and Genome Research 141: 103–113. https://doi.org/10.1159/000354871

Ribeiro MO, Noleto RB, Lorscheider CA, Porto FE, Prizon AC, Zawadzki CH, Oliveira LC, Portela Castro ALB (2015) Cytogenetic description of *Ancistrus abilhoai* (Siluriformes: Loricariidae) from Iguaçu River basin, southern Brazil. Genetics and Molecular Research 14(2): 4051–4057. https://doi.org/10.4238/2015.April.27.20

Rubert M, Zawadzki CH, Giuliano-Caetano L (2008) Cytogenetic characterization of *Hypostomus nigromaculatus* (Siluriformes: Loricariidae). Neotropical Ichthyology 6(1): 93–100. https://doi.org/10.1590/S1679-62252008000100011

Rubert M, Rosa R, Zawadzki CH, Mariotto S, Moreira-Filho O, Giuliano-Caetano L (2016) Chromosome mapping of 18S Ribosomal RNA genes in eleven *Hypostomus* species (Siluriformes, Loricariidae): diversity analysis of the sites. Zebrafish 13(4): 360–368. https://doi.org/10.1089/zeb.2016.1279

da Silva LLL, dos Santos AR, Giuliano-Caetano L, Dias AL (2015) Chromosomal characterization in two species of na *Asryanax bimaculatus* complex (Characidae, Characiformes) using different techniques of chromosome banding. Cytotechnology 68(4): 1277-1286. https://doi.org/10.1007/s10616-015-9888-3

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75(1): 304–306. https://doi.org/10.1016/0014-4827(72)90558-7

Traldi J, Vicari M, Blanco D, Martinez J, Artoni RF, Moreira-Filho O (2012) First karyotype description of *Hypostomus iheringii* (Regan, 1908): a case of heterochromatic polymorphism. Comparative Cytogenetics 6(2): 115–125. https://doi.org/10.3897/CompCytogen.v6i2.2595

Traldi JB, Blanco DR, Vicari MR, Martinez JF, Lui RL, Barros AV, Artoni RF, Moreira-Filho O (2013) Chromosomal diversity in *Hypostomus* (Siluriformes, Loricariidae) with emphasis on physical mapping of 18S and 5S rDNA sites. Genetics and Molecular Research 12(1): 463–471. https://doi.org/10.4238/2013.February.8.11

CompCytogen 12(1): 13–26 (2018)
doi: 10.3897/CompCytogen.v12i1.21799
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Molecular phylogenetic reconstruction and localization of the (TTAGG)n telomeric repeats in the chromosomes of *Acromyrmex striatus* (Roger, 1863) suggests a lower ancestral karyotype for leafcutter ants (Hymenoptera)

Tássia Tatiane Pontes Pereira[1,2], Ana Caroline Coelho Corrêa dos Reis[1], Danon Clemes Cardoso[1,2], Maykon Passos Cristiano[1]

1 *Departamento de Biodiversidade, Evolução e Meio Ambiente, Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil* **2** *Programa de Pós-graduação em Genética, Universidade Federal do Paraná, Curitiba, PR, Brazil*

Corresponding authors: *Danon Clemes Cardoso* (danon@iceb.ufop.br; danonclemes@gmail.com); *Maykon Passos Cristiano* (maykon@iceb.ufop.br; maykonpcristiano@gmail.com)

Academic editor: *V. Gokhman* | Received 22 October 2017 | Accepted 27 November 2017 | Published 9 January 2018

http://zoobank.org/7DEA8B94-51CE-40A8-9693-0DC9DD108205

Citation: Pereira TTP, Reis ACCC, Cardoso DC, Cristiano MP (2018) Molecular phylogenetic reconstruction and localization of the (TTAGG)n telomeric repeats in the chromosomes of *Acromyrmex striatus* (Roger, 1863) suggests a lower ancestral karyotype for leafcutter ants (Hymenoptera). Comparative Cytogenetics 12(1): 13–26. https://doi.org/10.3897/CompCytogen.v12i1.21799

Abstract

Chromosome counts and karyotype characterization have proved to be important features of a genome. Chromosome changes during the diversification of ants might play an important role, given the diversity and success of Formicidae. Comparative karyotype analyses on ants have enriched and helped ant systematics. Among leafcutter ants, two major chromosome counts have been described, one frequent in *Atta* Fabricius, 1804 (2n = 22 in all *Atta* spp. whose karyotype is known) and the other frequent in *Acromyrmex* Mayr, 1865 (2n = 38 in the majority of species whose karyotype is known). The main exception is *Acromyrmex striatus* (Roger, 1863), which harbors a diploid chromosome set of 22. Here we describe the use of fluorescence in situ hybridization (FISH) with telomeric probes with $(TTAGG)_6$ repeats to describe the telomere composition of *A. striatus* and to recover potential interstitial non-telomeric signals that may reflect fusion events during the evolution of leafcutter lineage from 38 to 22 chromosomes. Further, we reconstruct the ancestral chromosome numbers of the leafcutter clade based on a recently proposed molecular phylogenetic hypothesis and phylogenomic tree. Distinct signals have been observed in both extremities on the telomere chromosomes of *A. striatus*. Non-telomeric signals have not been retrieved

in our analysis. It could be supposed that the low-numbered karyotype indeed represents the ancestral chromosome number of leafcutters. The phylogenetic reconstruction also recovered a low chromosome number from the diverse approaches implemented, suggesting that n = 11 is the most likely ancestral karyotype of the leafcutter ants and is a plesiomorphic feature shared between *A. striatus* and *Atta* spp.

Keywords
fluorescence in situ hybridization (FISH), telomere, phylogenetic reconstruction, chromosome evolution, Formicidae

Introduction

The nuclear genome of any eukaryote is confined within the chromosomes, which vary in number, size, and shape. In turn, macromolecular structures, such as centromeres and telomeres, can be cytologically distinguished on each chromosome (Lysak and Schubert 2013). These latter terminal structures on the chromosomes are composed of tandem repeats that usually prevent the loss of DNA during replication, thereby promoting their stability. The telomere sequences are conserved across the species of a particular group. Among insects, the most common telomeric sequence reported is $(TTAGG)_n$, but that is not a general consensus (Frydrychová et al. 2004). Among ants, it has been confirmed by fluorescence in situ hybridization (FISH) and by the Southern blotting technique as being the most frequent repeat in Dolichoderinae, Formicinae, and Myrmicinae subfamilies (Lorite et al. 2002).

Several studies have correlated the presence of non-telomeric signals or interstitial telomeric signals as evidence that the chromosomes have undergone structural and/or numerical rearrangements. For instance, after a Robertsonian chromosome fusion, the telomeric sequences might remain in interstitial sites of this new fused chromosome and can be detected today. Interstitial telomeric signals have been detected on the chromosomes of different animal groups, such as mammals (Ventura et al. 2006), fishes (Bitencourt et al. 2014), and insects (Šíchová et al. 2016). Among ants, the localization of $(TTAGG)_n$ telomeric repeats on the chromosomes was carried out only with the well-known bulldog ants from the genus *Myrmecia* Fabricius, 1804 (Meyne et al. 1995), and on the chromosome set (2n = 18) of the ant species *Tapinoma nigerrimum* (Nylander, 1856) (Lorite et al. 2002). In both studies, positive hybridization signals were observed on the telomeres of the chromosomes. Lorite et al. (2002) suggested that the telomeric repeat $(TTAGG)_n$ is conserved among the ant lineages. It has been recently suggested that this telomere repeat was likely lost in the ancestor of Apocrita and is putatively regained in Formicidae and Apidae, since only they comprise species in which this motif was detected (Menezes et al. 2017).

Ants comprise a natural and diverse group consisting of more than 16,000 species (Bolton 2017). In the Nearctic and Neotropics, but mainly in the latter, the leafcutter ants of the genera *Atta* Fabricius, 1804 and *Acromyrmex* Mayr, 1865 stand out, which, together, with all other 15 genera of fungus-farming ants, cultivate crops of symbiotic Basidiomycete fungi inside their nests. Cytogenetic data for leafcutter ants are avail-

able, so far, for 21 species comprising *Atta* (five spp.) and *Acromyrmex* (16 spp. including subspecies and/or varieties). All *Atta* species (five cytogenetically studied out of 17 valid species) display karyotype uniformity with a diploid set of 22 chromosomes and karyotypic formula of 2n = 18M + 4A, except *Atta robusta* Borgmeier, 1939, whose karyotypic formula is 2n = 18M + 2SM + 2ST (Barros et al. 2015). Yet, *Acromyrmex* (14 species and subspecies cytogenetically studied out of 62) display a slightly higher uniformity in the chromosome counts, with a diploid set of 38 chromosomes, but a very large variability in its chromosome morphology, bearing distinct karyotypic structures (Barros et al. 2016), suggesting a structural dynamic nature of the genome of *Acromyrmex* lineages. Two chromosome counts diverge from the 2n = 38 common chromosome number, the first from *Ac. ameliae* de Souza, Soares & Della Lucia, 2007 (2n = 36) (Barros 2010), and the second much more pronounced from *Ac. striatus* (Roger, 1863) (2n = 22) (Cristiano et al. 2010), the same chromosome number as *Atta* spp. (Cristiano et al. 2013). It has been proved by molecular phylogenetic analysis that *A. striatus* is a sister group of the remaining leafcutter ants (Cristiano et al. 2013), which was later confirmed by phylogenomic analysis (Branstetter et al. 2017). Although *A. striatus* and *Atta* spp. shared some morphological characteristics and the same number of chromosomes, they differ in some karyotype features (Cristiano et al. 2013, Barros et al. 2015).

Given the phylogenetic position of *A. striatus* and chromosome evolution based on a phylogenetic approach, it has been supposed that n = 11 is the ancestral chromosome number of leafcutter ants (Cristiano et al. 2013). Here, we investigate the position of telomeric signals by means of FISH with telomeric probes $(TTAGG)_n$ in order to detect telomeric and/or non-telomeric hybridization signals on the chromosomes of *A. striatus*, in order to dismiss the alternative hypothesis that *A. striatus* karyotype arose by chromosome fusion due to the uniform chromosome number in the genus. We tested the hypothesis that no interstitial markers would be observed given that the haploid chromosome number of 11 chromosomes of *A. striatus* is the ancestral karyotype of the leafcutter ants. Further, we tested once more the ancestral chromosome number of all leafcutter ants by using the renewed phylogenetic approach to ancestral reconstruction CHROMEVOL 2.0 (Glick and Mayrose 2014) to estimate the potential ancestral chromosome number of leafcutter ants based on a phylogenetic tree with a more comprehensive phylogenetic and cytogenetic matrix.

Material and methods

Sampling, chromosome preparations and fluorescence *in situ* hybridization

Colonies were sampled from restinga environments of Morro dos Conventos, Araranguá–Santa Catarina, Brazil (S28°56'08.2', W49°21'28") (permit SISBIO-ICMBio 45464-1), transferred to the Laboratório de Genética Evolutiva e de Populações of the Universidade Federal de Ouro Preto–MG, and maintained as described by Cardoso et

al. (2011) until obtaining a brood. Metaphase chromosomes were obtained from 20 individuals according to the protocol established by Imai et al. (1988), with modifications described in Cardoso et al. (2012), using the ganglia of prepupae. Metaphase spreads were conventionally stained with Giemsa and with Fluoroshield with DAPI (Sigma-Aldrich, St. Louis, Missouri, USA) for chromosome counting and karyomorphometry as described by Cristiano et al. (2017).

Telomeric FISH was carried out according to the procedure described by Cioffi et al. (2011) on seven slides where the best metaphase spreads and an average number of ten metaphases were analyzed. The telomeric probe $(TTAGG)_6$ was directly labeled with Cy3 on the 5' end. Briefly, metaphase spreads were denatured for 5 min in 70%/2xSSC formamide at 75 °C. Probes were hybridized with chromosomes in 20 µl of hybridization mix containing the following: 200 ng of labeled probe, 50% formamide, 2xSSC, and 10% dextran and sulfate 20xSSC. This hybridization mix was heated for 10 min at 85 °C; slides were kept in a moist chamber at 37 °C overnight. Then, slides were washed in 4xSSC/Tween and dehydrated in a series of alcohol solutions. Slides were mounted in antifade solution with DAPI (DAPI Fluoroshield, Sigma-Aldrich). The DAPI-stained slides were then analyzed under an Olympus BX53 epifluorescence microscope coupled to an MX10 digital camera using cellSens imaging software.

Phylogenetic analysis and ancestral state inference

The ant DNA sequences were obtained from GenBank, representing the matrix data from Cristiano et al. (2013) and Cardoso et al. (2014a, b), with some sequences originally obtained from Schultz and Brady (2008). Four genomic fragments were included: *wingless*, *long-wavelength rhodopsin*, *elongation factor 1-alpha paralog F1* and *paralog F2*. The sequences were aligned visually in MEGA v7 (Kumar et al. 2016). Ambiguously aligned sites were excluded (i.e., the *long-wavelength* intronic region) and the alignment was confirmed by translation to amino acids, whereas the missing data were coded as "?".

The phylogenetic inference was carried out by using Bayesian methods with Markov Chain Monte Carlo (MCMC) methods. In order to select the substitution model of DNA evolution that fits best to each potential partition under Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC), we used PARTITIONFINDER2 (Lanfear et al. 2014, 2017). Taking into account the estimated parameters, we carried out a Bayesian analysis in MRBAYES v3.2.6 (Ronquist and Huelsenbeck 2003), which consisted of two independent runs of twenty million generations each, sampled every 1000 generations, and the convergence between runs was determined using TRACER v1.4 (Rambaut and Drummond 2007). A burn-in period, in which the initial 25% of the trees were discarded, was adopted to produce a consensus topology that was visualized using the FIGTREE V1.4 program (Rambaut 2009). The consensus topologies inferred were implemented in the inference of ancestral chromosome numbers at internal nodes.

In order to estimate the ancestral haploid chromosome number of the leafcutter ants and all remaining internal nodes, we carried out three independent analyses using CHROMEVOL 2.0 (Glick and Mayrose 2014). We used this integrative cytogenetic and molecular phylogeny approach by using two probabilistic methods, maximum likelihood (ML) and Bayesian inference (BI), to infer the chromosome evolution model and haploid ancestral states (haploid chromosome number at internal nodes), relying on a phylogenetic hypothesis estimated using the matrix and models described above. The new data matrix included about 50% (20 spp.) more species whose chromosome counts are available.

CHROMEVOL 2.0, under ML and BI inference, evaluates ten chromosome evolution models and different transitions between chromosome numbers. Basically, models evaluate dysploidy (decrease or increase by a single chromosome number in the haploid set of chromosomes, constant or linear, the latter being dependent on the current chromosome number), polyploidy (duplication of whole chromosome complement), and demi-polyploidy (the process that allows karyotypes with multiples of a haploid karyotype). The latter mechanism allows the transition from a haploid karyotype (n) to 1.5n, which could be possible in ants if related species hybridize due to the haplodiploid genetic system. Yet, polyploidy could be more unlikely. Although it is widespread and common in plants, polyploidization occurs very rarely in animals due to various incompatibility problems, so models with this parameter were not evaluated. All parameters were adjusted for the data, as described by Mayrose et al. (2010) and Glick and Mayrose (2014), and as performed by Cristiano et al. (2013) and Cardoso et al. (2014). The model that, to date, fits best, and the null hypothesis of no duplication, were analyzed with 10,000 simulations under the AIC. Further, to check for possible inconsistencies in the ancestral reconstruction due to the topologies recovered in our phylogenetic analysis, an additional run was performed by using the phylogenomic tree of Branstetter et al. (2017). Then, the same chromosome number matrix was implemented by adding the information from the new taxa when available, to meet the taxa comprising the phylogenomic tree.

Results

The chromosome counts for all individuals of *A. striatus* analyzed here were 2n = 22 (Figure 1a). The karyotype of this species consists of 10 metacentric (M) pairs and one submetacentric (SM) pair. Thus, the karyotypic formula of the diploid set was 2K = 20M + 2SM and the fundamental number was FN = 44. Morphometric data for chromosomes were confirmed and are presented in Table 1. We were able to detect the telomere sequences $(TTAGG)_6$ by FISH (Figure 1b–h). Positive signals were recovered at both ends of the chromosomes of *A. striatus*; however, the size and intensity of the signals varied among the terminal telomeric portions of the chromosomes, and between metaphase spreads (Figure 1b–h). No signals for interstitial telomeric sites were detected.

Table 1. Karyomorphometric analyses of the chromosomes of *Acromyrmex striatus* from ten well-spread metaphases.

Chromosome	TL	L	S	RL	*r*	Classification
1(a)	4.34±0.62	2.58±0.41	1.67±0.21	7.01±0.34	1.55±0.16	Metacentric
2(a)	3.98±0.65	2.33±0.45	1.59±0.23	6.42±0.45	1.46±0.17	Metacentric
3(b)	3.66±0.64	2.1±0.46	1.52±0.23	5.9±0.53	1.37±0.17	Metacentric
4(b)	3.43±0.52	1.89±0.31	1.47±0.19	5.54±0.37	1.28±0.11	Metacentric
5(c)	3.17±0.5	1.73±0.31	1.4±0.22	5.11±0.27	1.24±0.18	Metacentric
6(c)	2.98±0.39	1.6±0.23	1.36±0.17	4.82±0.1	1.18±0.1	Metacentric
7(d)	2.94±0.38	1.56±0.22	1.31±0.18	4.76±0.1	1.19±0.07	Metacentric
8(d)	2.87±0.37	1.57±0.23	1.28±0.18	4.63±0.12	1.25±0.19	Metacentric
9(e)	2.82±0.33	1.56±0.2	1.18±0.19	4.56±0.12	1.35±0.21	Metacentric
10(e)	2.76±0.32	1.5±0.21	1.24±0.15	4.47±0.13	1.22±0.14	Metacentric
11(f)	2.72±0.31	1.54±0.19	1.17±0.17	4.4±0.1	1.33±0.15	Metacentric
12(f)	2.66±0.29	1.44±0.21	1.23±0.12	4.32±0.1	1.17±0.09	Metacentric
13(g)	2.62±0.3	1.49±0.27	1.14±0.11	4.24±0.11	1.31±0.15	Metacentric
14(g)	2.56±0.31	1.41±0.18	1.13±0.13	4.14±0.09	1.25±0.13	Metacentric
15(h)	2.45±0.35	1.35±0.22	1.06±0.14	3.95±0.18	1.27±0.12	Metacentric
16(h)	2.33±0.32	1.25±0.21	1.02±0.16	3.76±0.16	1.23±0.09	Metacentric
17(i)	2.07±0.17	1.09±0.14	0.92±0.13	3.37±0.18	1.2±0.17	Metacentric
18(i)	1.95±0.13	1.06±0.1	0.88±0.12	3.17±0.19	1.21±0.12	Metacentric
19(j)	1.75±0.16	0.9±0.11	0.75±0.07	2.84±0.15	1.2±0.09	Metacentric
20(j)	1.59±0.18	0.83±0.11	0.69±0.09	2.57±0.17	1.21±0.07	Metacentric
21(k)	3.2±0.55	2.16±0.35	0.96±0.18	5.23±1.1	2.27±0.27	Submetacentric
22(k)	2.94±0.43	2.02±0.3	0.93±0.16	4.8±0.74	2.18±0.25	Submetacentric
Σ	61.79					

TL: total length; ***L***: long arm length; ***S***: short arm length; ***RL***: relative length; ***r***: arm ratio (= *L*/*S*).

Table 2. Models of molecular evolution by genes and codons implemented in the Bayesian analyses to infer the molecular phylogeny of fungus-growing ants. This tree was the topology inputted in CHROMEVOL 2.0 to estimate the ancestral chromosome numbers.

Gene (number of base pairs)	Position	Model
wingless (411bp)	1st – first position	K81+G
	2nd – second position	TIM+I+G
	3rd – third position	GTR+G
elongation factor-1 alpha F1 (402 bp)	1st – first position	TIM+I+G
	2nd – second position	GTR+G
	3rd – third position	GTR+G
elongation factor-1 alpha F2 (519 bp)	1st – first position	TIM+I+G
	2nd – second position	GTR+G
	3rd – third position	HKY+G
long-wavelength rhodopsin (464 bp)	1st – first position	SYM+I+G
	2nd – second position	GTR+I+G
	3rd – third position	TVM+G

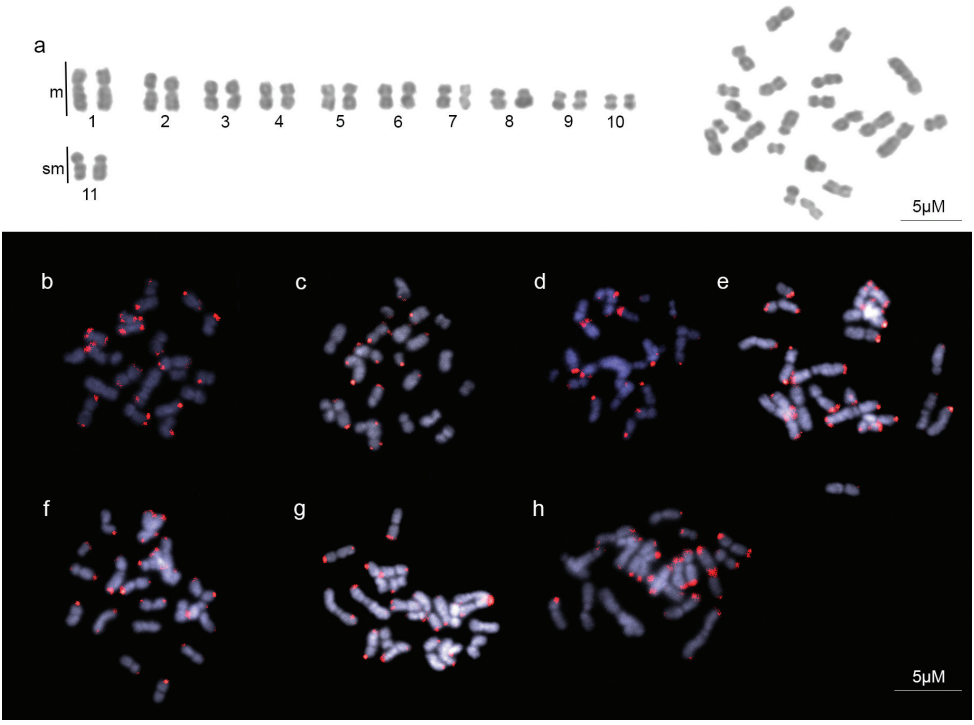

Figure 1. Metaphase and karyotype of *A. striatus* and metaphase spreads after FISH with the telomeric probe $(TTAGG)_6$. **a** Metaphase and karyotype stained with Giemsa **b–h** Best metaphase spreads stained with DAPI (uniform blue) and the telomeric probes with Cy3-dUTP in red.

An alignment of 1796 base pairs was obtained for the four concatenated nuclear genes comprising 49 sequences of fungus-growing ants, whose species from the genera *Apterostigma* Mayr, 1865, *Mycocepurus* Forel, 1893 and *Mycetarotes* Emery, 1913 were placed as outgroups. Nine different substitution models were estimated by PARTITIONFINDER2 for each gene codon position (see Table 2 for details on the partition scheme) and were employed in the Bayesian analysis. As expected, all species of the *Acromyrmex* and *Atta* genera formed a well-supported clade that fell as a sister group of *A. striatus*. The results obtained in the analysis of chromosome evolution suggested that the best-supported model of the process underpinning chromosome change was the hypothesis with constant gain, loss, and duplication (likelihood = –58.53, AIC = 123.1). These results revealed the possible occurrence of duplication events or an increase of the chromosome number by the whole genome duplication in the chromosome evolution of these species. However, the main events inferred were loss (fusion) and gain (fission), which showed PP > 0.5. In the Bayesian analysis, the haploid chromosome number at the most recent common ancestor (MRCA) of leafcutter ants with the highest posterior probability (PP) was n = 11. Likewise, in the ML analysis the most likely haploid number was n = 11 (Figure 2). The same results were observed in the analysis based on the phylogenomic tree, under both estimation approaches (Suppl. material 1: Figure S1).

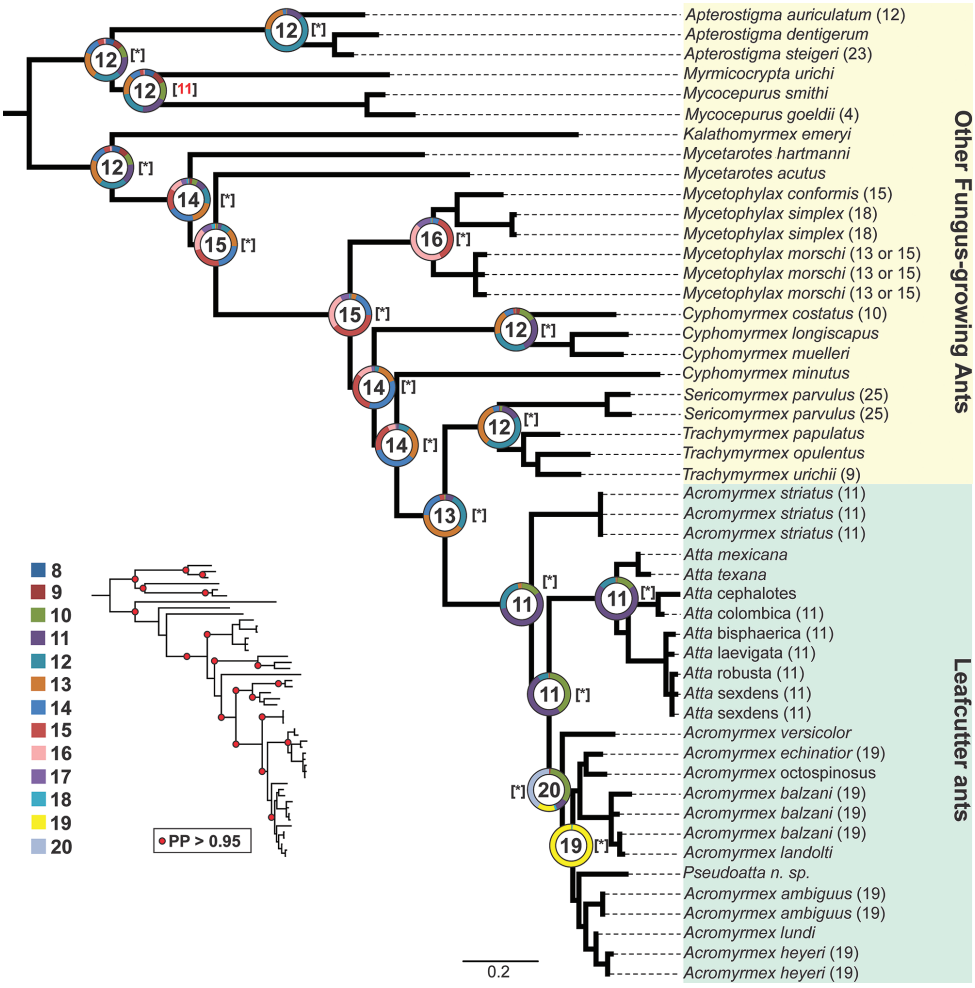

Figure 2. Ancestral haploid chromosome state reconstruction inferred under Bayesian Inference and Maximum Likelihood methods. The ancestral chromosome number with the highest probability is given inside the circle and pie charts at the main nodes. The colors on the pie charts represent the proportional probability of each given chromosome number according to the legend. The known karyotypes of species are given at the tip. The haploid ancestral chromosome numbers with the best likelihood are given in brackets. * represent the same estimated haploid number in BI.

Discussion

All individuals of *A. striatus* from the population evaluated here had chromosome counts of 2n = 22. The karyotype of this species consists of 20 metacentric (M) and two submetacentric (SM) chromosomes, as reported by Cristiano et al. (2013). No difference in karyotype was expected since the samples analyzed here belong to closely related populations analyzed previously. However, differences in karyotype number within the same species are fairly likely among ants (Meyne et al. 1995, Imai et al.

1988, Cardoso et al. 2014b). No variation in the karyotypic formula was observed and the diploid set was 2K = 20M + 2SM, therefore, the fundamental number that corresponded to the number of chromosome arms in the diploid karyotype was FN = 44.

The $(TTAGG)_6$ probe hybridized to both ends of chromosomes of *A. striatus*. This reveals the composition of the telomeric portions on chromosomes of the leafcutter ant *A. striatus*. The presence of the repeat $(TTAGG)_6$ at the telomeres has already been reported in Apidae and Formicidae (Frydrychová et al. 2004) including ants *Tapinoma nigerrimum* and *Myrmecia* spp. (Lorite et al. 2002, Meyne et al. 1995). However, the TTAGG telomere motif was not detected in many other Hymenoptera, suggesting that it may have been lost and only regained in Apidae and Formicidae (Menezes et al. 2017). Nevertheless, the authors do not discard multiple losses along hymenopteran evolutionary history. Within Formicidae, the TTAGG motif was confirmed by Southern blot hybridization against digested genomic DNA in ant species from the Dolichoderinae, Formicinae, and Myrmicinae subfamilies (Lorite et al. 2002), suggesting that the telomeres of ants may be mainly comprised of $(TTAGG)_n$. However, positive signals using the common vertebrate repeat $(TTAGGG)_n$ were also detected by Meyne et al. (1995) on the chromosomes of *Myrmecia* spp. under low stringency (reduced percentage of formamide, less than 50%). Thus, the authors did not reject that both repeats might occur on ant chromosomes (Meyne et al. 1995). In fact, Lorite et al. (2002) emphasize that the main telomere sequence in ants is $(TTAGG)_n$ instead of $(TTAGGG)_n$, and that the latter should be present in very low copy number considering the Southern blot hybridization results. Here, we reported for the first time telomeric sequences by FISH in a neotropical leafcutter ant, adding to cytogenetic knowledge on this important insect group, and helping us to identify trends in ant chromosome evolution.

The size and intensity of $(TTAGG)_6$ probe signals varied along the termini of the metaphase spreads of *A. striatus* (see Figure 1b-h). This was also observed in both studies that have performed telomeric FISH on ant chromosomes. Meyne et al. (1995) detected differential signals among termini of *Myrmecia* spp. by using both repeats mentioned above; this pattern is quite evident in *M. croslandi* Taylor, 1991 and *M. haskinsorum* Taylor, 2015. This variation in the hybridization signals was also observed on the chromosomes of *T. nigerrimum* (Lorite et al. 2002). We assume that this variation may be the result of two non-excluding processes. The first can be attributed to the difference in the number of telomeric repeats comprising the chromosomes, the second to the impairing of the probe hybridization as a result of protocol limitations in the chromosome preparation. In fact, when different metaphase spreads are evaluated, positive signals are detected over the negative signals on the chromosome of the next spread. Further, if positive signals have never been observed on dozens of metaphases, as recently reported for parasitoid Hymenoptera (Gokhman et al. 2014), this may represent an absence of signal of the evaluated telomeric repeat. Moreover, a negative signal on all chromosomes, as well as on all metaphases analyzed, was also reported in the neotropical wasp *Metapolybia decorata* (Gribodo, 1896) of the family Vespidae (Menezes et al. 2013), but this was not the case here.

In several species, the presence of interstitial telomeric signals on chromosomes has been observed (Meyne et al. 1990). These have been correlated with chromosome rearrangements and then used as markers of karyotype evolution and, consequently, may be used to evaluate the phylogenetic relationship of species, and even populations (Meyne et al. 1995, Quing et al. 2013). No interstitial telomere sequences were detected on the chromosomes of *A. striatus*. Thus, it could be supposed that the chromosome number observed in this species may represent the ancestral chromosome number of leafcutter ants. We do not ignore the fact that, due to the limitation of the FISH technique's resolution, small interstitial telomeric signals originated by chromosomal fusions were not detected. However, the results of the cyto-phylogenetic approach to estimate the ancestral chromosome number have recovered the haploid number of 11 chromosomes in ML and BI (see Figures 2 and Suppl. material 1: Figure S1), in accordance with the previous estimate by Cristiano et al. (2013). This new estimate is based on 20 cytogenetic data values of fungus-growing ants instead of 12. Here, more cytogenetic and molecular data were added. However, a more detailed chromosome evolution hypothesis for fungus-growing ants, instead of just estimating the ancestral karyotype, will be possible when more simultaneous cytogenetic and molecular data are available. Chromosome evolution hypotheses based on cytogenetics coupled with molecular phylogenetic data were already drawn on the fungus-growing ant lineage from the genus *Mycetophylax* Emery, 1913, where fusion events were evidenced as having taken place during its karyotype evolution (Cardoso et al. 2014).

Likewise, the ancestral haploid chromosome number of 11 was recovered based on the phylogenomic tree of fungus-growing ants from Branstetter et al. (2017). On this tree, several non-fungus-growing outgroups were included, as well as other fungus-growing ants, but comprising the species included in the matrix of four nuclear genes used in the molecular phylogeny presented here. Together, telomeric FISH analysis of the karyotype of *A. striatus* and chromosomal reconstruction under two phylogenetic hypotheses based on independent data reinforce a low chromosome number as the putative ancestral karyotype for leafcutter ants. In fact, known karyotypes of *Trachymyrmex* Forel, 1893, the sister group to leafcutter ants, show as few as nine and 10 chromosomes in the haploid chromosome set (Murakami et al. 1998, Barros et al 2013).

Overall, in light of the results reported here, it is important to note that the evolution of the remaining *Acromyrmex* species, in contrast to the *Atta* spp. and *A. striatus* lineages, was mainly driven by the increase of chromosome number by centric fission. This could be followed by structural events, which, based on chromosome banding techniques, was suggested by Barros et al. (2016). Thus, the diploid number of 38 chromosomes, and likely 36 chromosomes in *A. ameliae*, represents a derived feature of the lineage leading to all other *Acromyrmex* species. Yet, in the lineage leading to *A. striatus* and *Atta* spp., the maintained number of 22 chromosomes may represent a plesiomorphic feature of their karyotypes.

Acknowledgements

The present study was supported by a research grant to MPC (PPM-00126-15) from the Fundação de Amparo a Pesquisa do Estado de Minas Gerais—FAPEMIG. ACRC was awarded a grant by FAPEMIG in the undergraduate program of initiation in science and TTPP by Coordenação de Pessoal de Nível Superior (CAPES). We thank Natália Travenzoli, Denilce Menezes Lopes, Jorge Dergam, and many others that made this study possible.

References

Barros LAC (2010) Citogenética de espécies de Attini (Formicidae: Myrmicinae) [Dissertation]. Programa de Pós-graduação em Genética e Melhoramento. Viçosa: Universidade Federal de Viçosa.

Barros LAC, de Aguiar H, Mariano CDF, Andrade-Souza V, Costa MA, Delabie JHC, Pompolo SD (2016) Cytogenetic data on six leafcutter ants of the genus *Acromyrmex* Mayr, 1865 (Hymenoptera, Formicidae, Myrmicinae): insights into chromosome evolution and taxonomic implications. Comparative Cytogenetics 10(2): 229–243. https://doi.org/10.3897/CompCytogen.v10i2.7612

Barros LAC, de Aguiar H, Mariano CDF, Delabie JHC, Pompolo SD (2013) Cytogenetic characterization of the ant *Trachymyrmex fuscus* Emery, 1934 (Formicidae: Myrmicinae: Attini) with the description of a chromosomal polymorphism. Annales de la Societe Entomologique de France 49(4): 367–373. http://dx.doi.org/10.1080/00379271.2013.856201

Barros LAC, de Aguiar H, Teixeira GA, Mariano CDF, Teixeira MD, Delabie JHC, Pompolo SD (2015) Cytogenetic data on the threatened leafcutter ant *Atta robusta* Borgmeier, 1939 (Formicidae: Myrmicinae: Attini). Comptes Rendus Biologies 338(10): 660–665. https://doi.org/10.1016/j.crvi.2015.07.006

Bitencourt JA, Sampaio I, Ramos RTC, Affonso P (2014) Chromosomal fusion in Brazilian populations of *Trinectes inscriptus* Gosse, 1851 (Pleuronectiformes; Achiridae) as revealed by internal telomere sequences (ITS). Journal of Experimental Marine Biology and Ecology 452: 101–104. https://doi.org/10.1016/j.jembe.2013.12.012

Bolton B (2017) AntWeb: Ants of All AntWeb. https://www.antweb.org [Accessed 30 September 2017]

Branstetter MG, Jesovnik A, Sosa-Calvo J, Lloyd MW, Faircloth BC, Brady SG, Schultz TR (2017) Dry habitats were crucibles of domestication in the evolution of agriculture in ants. Proceedings of the Royal Society B-Biological Sciences 284(1852): 10. https://doi.org/10.1098/rspb.2017.0095

Cardoso DC, Cristiano MP, Barros LAC, Lopes DM, Pompolo SD (2012) First cytogenetic characterization of a species of the arboreal ant genus *Azteca* Forel, 1978 (Dolichoderinae, Formicidae). Comparative Cytogenetics 6(2): 107–114. http://doi.org/10.3897/CompCytogen.v6i2.2397

Cardoso DC, Cristiano MP, Heinze J, Tavares MG (2014a) A nuclear DNA based phylogeny of endemic sand dune ants of the genus *Mycetophylax* (Emery, 1913): How morphology is

reflected in molecular data. Molecular Phylogenetics and Evolution 70: 378–382. https://doi.org/10.1016/j.ympev.2013.10.012

Cardoso DC, Cristiano MP, Tavares MG (2011) Methodological remarks on rearing basal Attini ants in the laboratory for biological and evolutionary studies: overview of the genus *Mycetophylax*. Insectes Sociaux 58(3): 427–430. https://doi.org/10.1007/s00040-011-0160-3

Cardoso DC, Pompolo SD, Cristiano MP, Tavares MG (2014b) The role of fusion in ant chromosome evolution: insights from cytogenetic analysis using a molecular phylogenetic approach in the genus *Mycetophylax*. PloS ONE 9(1): e87473. https://doi.org/10.1371/journal.pone.0087473

Cioffi MB, Kejnovsky E, Bertollo LAC (2011) The chromosomal distribution of microsatellite repeats in the genome of the wolf fish *Hoplias malabaricus*, focusing on the sex chromosomes. Cytogenetic and Genome Research 132(4): 289–296. https://doi.org/10.1159/000322058

Cristiano MP, Cardoso DC, Fernandes-Salomao TM (2013) Cytogenetic and molecular analyses reveal a divergence between *Acromyrmex striatus* (Roger, 1863) and other congeneric species: taxonomic implications. PloS ONE 8(3): 9. https://doi.org/10.1371/journal.pone.0059784

Cristiano MP, Pereira TTP, Simões LP, Sandoval-Gómez VE, Cardoso DC. (2017) Reassessing the chromosome number and morphology of the turtle ant *Cephalotes pusillus* (Klug, 1824) using karyomorphometrical analysis and observations of new nesting behavior. Insects 8: 114. https:// doi.org/10.3390/insects8040114

Frydrychová R, Grossmann P, Trubac P, Vitkova M, Marec FE (2004) Phylogenetic distribution of TTAGG telomeric repeats in insects. Genome 47(1): 163–178. https://doi.org/10.1139/g03-100

Glick L, Mayrose I (2014) ChromEvol: Assessing the pattern of chromosome number evolution and the inference of polyploidy along a phylogeny. Molecular Biology and Evolution 31(7): 1914–1922. https://doi.org/10.1093/molbev/msu122

Gokhman VE, Anokhin BA, Kuznetsova VG (2014) Distribution of 18S rDNA sites and absence of the canonical TTAGG insect telomeric repeat in parasitoid Hymenoptera. Genetica 142(4): 317–322. https://doi.org/10.1007/s10709-014-9776-3

Imai HT, Taylor RW, Crosland MW, Crozier RH (1988) Modes of spontaneous chromosomal mutation and karyotype evolution in ants with reference to the minimum interaction hypothesis Japanese journal of genetics 63(2):159–185. http://doi.org/10.1266/jjg.63.159

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33(7): 1870–1874. https://doi.org/10.1093/molbev/msw054

Lanfear R, Calcott B, Kainer D, Mayer C, Stamatakis A (2014) Selecting optimal partitioning schemes for phylogenomic datasets. BMC Evolutionary Biology 14: 14. https://doi.org/10.1186/1471-2148-14-82

Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B (2017) PartitionFinder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. Molecular Biology and Evolution 34(3): 772–773. https://doi.org/10.1093/molbev/msw260

Lorite P, Carrillo JA, Palomeque T (2002) Conservation of (TTAGG)(n) telomeric sequences among ants (Hymenoptera, Formicidae). Journal of Heredity 93(4): 282–285. https://doi.org/10.1093/jhered/93.4.282

Lysak MA, Schubert I (2013) Mechanisms of chromosome rearrangements. In: Greilhuber J, Dolezel J, Wendel JF (Eds) Plant genome diversity volume 2: Physical structure, behaviour and evolution of plant genomes. Springer, Vienna. 2(1)137–147. https://doi.org/10.1007/978-3-7091-1160-4_9

Mayrose I, Barker MS, Otto SP (2010) Probabilistic models of chromosome number evolution and the inference of polyploidy. Systematic Biology 59(2): 132–144. https://doi.org/10.1093/sysbio/syp083

Menezes RST, Silva TM, Carvalho AF, Andrade-Souza V, Silva JG, Costa MA (2013) Numerical and structural chromosome variation in the swarm-founding wasp *Metapolybia decorata* Gribodo 1896 (Hymenoptera, Vespidae). Genetica 141(7/9): 273–280. https://doi.org/10.1007/s10709-013-9726-5

Menezes RST, Bardella VB, Cabral-de-Mello DC, Lucena DAA, Almeida EAB (2017) Are the TTAGG and TTAGGG telomeric repeats phylogenetically conserved in aculeate Hymenoptera? The Science of Nature 104: 85. https://doi.org/10.1007/s00114-017-1507-z

Meyne J, Baker RJ, Hobart HH, Hsu TC, Ryder OA, Ward OG, Wiley JE, Wursterhill DH, Yates TL, Moyzis RK (1990) Distribution of nontelomeric sites of the $(TTAGGG)_n$ telomeric sequence in vertebrate chromosomes. Chromosoma 99(1): 3–10. https://doi.org/10.1007/BF01737283

Meyne J, Hirai H, Imai HT (1995) FISH analysis of the telomere sequences of bulldog ants (*Myrmecia*, Formicidae). Chromosoma 104: 14–18.

Murakami T, Fujiwara A, Yoshida MC (1998) Cytogenetics of ten ant species of the tribe Attini (Hymenoptera, Formicidae) in Barro Colorado Island, Panama: 135–139. Chromosome Science 2: 135–139.

Qing LY, Xia Y, Zheng YC, Zeng XM (2013) FISH of 5S rDNA and telomeric $(TTAGGG)_n$ repeats in normal and translocated populations of the frog *Quasipaa boulengeri* (Anura, Ranidae). Chinese Science Bulletin 58(18): 2168–2173. https://doi.org/10.1007/s11434-013-5690-9

Rambaut A, Drummond AJ (2007) Tracer v1.4. http://tree.bio.ed.ac.uk/software/tracer [Accessed 25 November 2017]

Rambaut AJ (2009) Figtree v1.3.1. http://tree.bio.ed.ac.uk/software/figtree/ [Accessed 25 November 2017]

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12): 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. Proceedings of the National Academy of Sciences of the USA 105(14): 5435–5440. https://doi.org/10.1073/pnas.0711024105

Šíchová J, Ohno M, Dinca V, Watanabe M, Sahara K, Marec F (2016) Fissions, fusions, and translocations shaped the karyotype and multiple sex chromosome constitution of the northeast-Asian wood white butterfly, *Leptidea amurensis*. Biological Journal of the Linnean Society 118(3): 457–471. https://doi.org/10.1111/bij.12756

Ventura K, Silva MJJ, Fagundes V, Christoff AU, Yonenaga-Yassuda Y (2006) Non-telomeric sites as evidence of chromosomal rearrangement and repetitive $(TTAGGG)_n$ arrays in heterochromatic and euchromatic regions in four species of *Akodon* (Rodentia, Muridae). Cytogenetic and Genome Research 115(2): 169–175. https://doi.org/10.1159/000095238

Supplementary material 1

Figure S1. Phylogenomic tree used to estimate the ancestral chromosome number.
Authors: Tássia Tatiane Pontes Pereira, Ana Caroline Coelho Corrêa dos Reis, Danon Clemes Cardoso, Maykon Passos Cristiano
Data type: species data
Explanation note: Numbers at nodes represent the first and second most likely haploid chromosome number followed by posterior support values under Bayesian optimization and the ancestral haploid chromosome number with best likelihood under maximum likelihood optimization, as follows: [first haploid state (P.P.%)// second haploid state (P.P.%)// ML haploid state].

Link: https://doi.org/10.3897/CompCytogen.v12i1.21799.suppl1

CompCytogen 12(1): 27–40 (2018)
doi: 10.3897/CompCytogen.v12i1.19381
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Evolutionary insight on localization of 18S, 28S rDNA genes on homologous chromosomes in Primates genomes

Sofia Mazzoleni[1,2], Michail Rovatsos[2], Odessa Schillaci[1], Francesca Dumas[1]

1 *Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università degli Studi di Palermo, via Archirafi 18, Palermo, Italy* **2** *Faculty of Science, Department of Ecology, Viničná 7, Charles University, Pragha 2, Czech Republic*

Corresponding author: *Francesca Dumas* (francesca.dumas@unipa.it)

Academic editor: *N. Bulatova* | Received 24 July 2017 | Accepted 24 November 2017 | Published 24 January 2018

http://zoobank.org/09A6D6D6-410C-4C1C-BA2C-650FC7E88878

Citation: Mazzoleni S, Rovatsos M, Schillaci O, Dumas F (2018) Evolutionary insight on localization of 18S, 28S rDNA genes on homologous chromosomes in Primates genomes. Comparative Cytogenetics 12(1): 27–40. https://doi.org/10.3897/CompCytogen.v12i1.19381

Abstract

We explored the topology of 18S and 28S rDNA units by fluorescence *in situ* hybridization (FISH) in the karyotypes of thirteen species representatives from major groups of Primates and *Tupaia minor* (Günther, 1876) (Scandentia), in order to expand our knowledge of Primate genome reshuffling and to identify the possible dispersion mechanisms of rDNA sequences. We documented that rDNA probe signals were identified on one to six pairs of chromosomes, both acrocentric and metacentric ones. In addition, we examined the potential homology of chromosomes bearing rDNA genes across different species and in a wide phylogenetic perspective, based on the DAPI-inverted pattern and their synteny to human. Our analysis revealed an extensive variability in the topology of the rDNA signals across studied species. In some cases, closely related species show signals on homologous chromosomes, thus representing synapomorphies, while in other cases, signal was detected on distinct chromosomes, leading to species specific patterns. These results led us to support the hypothesis that different mechanisms are responsible for the distribution of the ribosomal DNA cluster in Primates.

Keywords

Fluorescence *in situ* hybridization, repetitive DNAs, synapomorphy, Primates, tree shrew

Introduction

Repetitive DNA elements make up a large portion of eukaryotic genomes and include tandem arrays and dispersed repeats. These genomic components are able to change the molecular composition of chromosomes and their study will contribute to the knowledge of karyotype differentiation (Cioffi et al. 2010, Dumas et al. 2017). A prominent repetitive DNA element organized in tandem repetition consists of ribosomal DNA (rDNA) encoding the ribosomal RNA, essential for cell function. The rDNA region is divided into two families: the 5.8S (minor) and the second one the 45S (major) comprising 18S and 28S loci. The chromosome regions with transcriptionally active 45S loci, referred as the Nucleolus Organizer Regions (NORs), can be identified either by silver staining (Ag-NOR) or, more accurately, by fluorescence in situ hybridization (FISH) which permits researchers to identify both inactive and active NORs. rDNA probes have been cytogenetically mapped by FISH in the karyotypes of several vertebrate species, representatives of fishes (Srikulnath et al. 2009, 2011, Sember et al. 2015), reptiles (Rovatsos et al. 2015a, 2015b, 2016), and Artiodactyla (Nguyen et al. 2008, Degrandi et al. 2014), rodents (Gornung et al. 2011, Cazaux et al. 2011, Britton-Davidian et al. 2012) and bats (Calixto et al. 2014) in mammals in order to clarify their chromosomal location and mechanisms of dispersion. The topology of rDNA loci is widely used as marker for comparative cytogenetic studies and to explore evolutionary relationships, since such loci often show species-specific patterns (Srikulnath et al. 2009, 2010, Cazaux et al. 2011, Bulatova and Pavlova 2016). Furthermore, the variation in number and topology of rDNA genes has been shown at inter- and intra-species levels, explained as consequence of chromosomal rearrangements, ectopic recombination through association of rDNA with other chromosomal segments during meiotic division or transposition events (Hirai et al. 1996, Eickbush and Eickbush 2007, Baicharoen et al. 2016).

Concerted evolution of rDNA clusters caused by unequal cross over is a well-documented process; rDNA gene copies within an individual and within a species remain identical in sequence, while between closely related species the sequence can vary widely (Averbeck and Eickbush 2005). In humans, it has been demonstrated that the dynamic length variation occurring at rDNA clusters, is the direct result of unequal cross over occurring both inter- and intrachromosomally (Stults et al. 2008). Recently it has been showed that highly degraded, but near full length, rDNA units can be found at multiple sites in the human genome chromosomes. These sequences tend to accumulate close to centromeres and to change from canonical rDNA to pseudogenes, representing different stages in the evolution of the rDNA sequences (Robicheau et al. 2017).

rDNA distribution especially of the 18S and 28S loci has been investigated in many species of Primates either by FISH (Henderson et al. 1974a,b, 1976, 1977, 1979, Hirai et al. 1999, 2007, Guillén et al. 2004, Baicharoen et al. 2016) or silver staining (Tantravahi et al. 1976, Bedard et al. 1978, Masters et al. 1987, Nagamachi et al. 1992, Hirai et al. 2007, Tanomtong et al. 2009), including *Homo sapiens* Linnaeus, 1758. In humans, NORs have been identified on the secondary constriction of five pairs of acrocentric chromosomes: 13, 14, 15, 21 and 22 (Henderson et al. 1972, Tantravahi et al. 1976).

In pioneering comparative studies on Primates, it was assumed that there is no homology between chromosomes bearing rDNA (Henderson et al. 1977). Furthermore, exchanges among rDNA genes on non-homologous chromosomes (Arnheim et al. 1980) and a multiple topologies of rDNA sites with species-specific variations (Hirai et al. 1999) have been shown in Hominoidea. Later, intra-species polymorphisms have also been described in Primates such as *Pan troglodytes* (Blumenbach, 1775) (Guillén et al. 2004), *Hylobates lar* (Linnaeus, 1771), (Tanomtong et al. 2009) and *Nycticebus bengalensis* (Geoffroy, 1812) (Baicharoen et al. 2016), possibly related to unequal crossing over or to transcriptional inactivation by methylation of NORs.

Therefore, we tried to explore the chromosomal distribution of rDNA loci in Primate genomes, by mapping the 18S and 28S probe in thirteen species of Primates and in *Tupaia minor* (Günther, 1876), the representative of the order Scandentia, as outgroup (Lin et al. 2014, Zhou et al. 2015). The chromosome topology of rDNA genes by FISH has been analyzed in a wide phylogenetic framework taking in consideration previous literature.

Material and methods

The Primates species analyzed through rDNA probes mapping are listed in Table 1. In the present work, rDNA distribution is documented by FISH analysis for the first time in ten species and hybridization was repeated for *Hylobates lar, Lemur catta* (Linnaeus, 1758) and *Symphalangus syndactylus* (Raffles, 1821) formerly studied (Warburton et al. 1975, Henderson et al. 1977, Hirai et al. 1999). Metaphases for all species have been obtained following the standard protocol (Sineo et al. 2007, Small et al. 1985), from primary cultures of fibroblast cell lines treated and fixed at the National Cancer Institute, USA by F. Dumas and R. Stanyon. All karyotypes have been analyzed after DAPI inverted banding. The probe for the rDNA sequence was prepared from a plasmid (pDmr.a 51#1) with a 11.5-kb insert encoding the 18S and 28S ribosomal units of *Drosophila melanogaster* (Meigen, 1830) (Endow 1982), and it was subsequently labelled with biotin-dUTP using a Nick Translation Kit (Abbott). In situ hybridization of the probe with the chromosomal spreads was performed overnight according to standard protocol and the probe signal was enhanced and detected using an avidin-FITC/biotinylated anti-avidin system (Vector Laboratories) (Rovatsos et al. 2015a). Probe signals have been pseudocolored in red for better contrast. The chromosomes were counterstained with DAPI, and the slides were mounted with antifade medium Fluroshield (Sigma-Aldrich).

Karyotypes were examined by inverted DAPI method, as previously performed (Dumas et al. 2016, Mazzoleni et al. 2017); the human homology between chromosomes with rDNA signal was identified based on painting data from previous projects (Table 1). Our data have been compared with previous literature data on rDNA mapping in Primates (Table 2). The results of distribution of rDNA loci on the chromosomes of all analyzed species are illustrated in a graphical reconstruction of the primate phylogenetic tree, following Perelman and colleagues (2011) with some modification, created by MESQUITE v.2.75 (Maddison and Maddison 2011).

Table 1. List of species (Primates, Scandentia) studied cytogenetically with rDNA probes mapped by FISH; the chromosomes pairs bearing rDNA probe signals and the human homologies (HSA) identified through the analysis of the painting references are reported. A- acrocentric, SM - submetacentric, C - centromere. * - FISH markers position in human synteny association. HSA homology was extrapolated for *Otolemur garnettii* (OGR#) from *O. crassicaudatus* Géoffroy, 1812 G-banding data (Masters et al. 1987) since they showed close karyotypes.

Species	rDNA mapping				HSA homologs	Painting References
	Chr.	Chromosome type	Position	2ndary constriction		
Strepsirrhini						
Lemur catta LCA (Linnaeus, 1758)	21	Acrocentric	Centromere	No	22/12	Cardone et al. 2002
	25	Acrocentric	Centromere	No	8	
Otolemur garnetti OGR (Ogilby, 1838)	19	Acrocentric	Centromere	No	17	Stanyon et al. 2002*
Platyrrhini						
Callithrix jacchus CJA (Linnaeus, 1758)	15	Acrocentric	Centromere	No	3	Neusser et al. 2001
	17	Acrocentric	Centromere		3	
	19	Acrocentric	Centromere		1	
Callimico goeldii CGO (Thomas, 1904)	14	Acrocentric	Centromere	No	5	Neusser et al. 2001
	15	Acrocentric	Centromere	No	*9/22	
	16	Acrocentric	Centromere	No	*15/3	
	17	Acrocentric	Centromere	No	*13/17	
	21	Acrocentric	Centromere	No	20	
	22	Acrocentric (only in 1 homologous)	Centromere	No	*3/21	
Saguinus Oedipus SOE (Linnaeus, 1758)	20	Acrocentric	q arm	No	1	Neusser et al. 2001
	21	Acrocentric	q arm	No	1	
	22	Acrocentric	q arm	Yes	10	
Saimiri sciureus SSC (Linnaeus, 1758)	6	Submetacentric	Centromere	Yes	20/3	Stanyon et al. 2000
Ateles paniscus paniscus APA (Linnaeus, 1758)	8	Submetacentric	Centromere/q arm	Yes	19/*20	de Oliveira et al. 2005
Alouatta caraya ACA (Humboldt, 1812)	17	Acrocentric	q arm	Yes	8	de Oliveira et al. 2002
	23	Acrocentric	q arm	Yes	1	
Catarrhini						
Chlorocebus aethiops CAE (Linnaeus, 1758)	19	Subtelomeric	Centromere/q arm	Yes	22	Finelli et al. 1999
Colobus guereza CGU (Rüppell, 1835)	16	Submetacentric	Centromere/q arm	Yes	22/21	Bigoni et al. 1997
Erythrocebus patas EPA (Schreber, 1774)	26	Submetacentric	Centromere	No	22	Stanyon et al. 2005
Hylobates lar HLA (Linnaeus, 1771)	12	Submetacentric	q arm	Yes	2*/*3	Jauch et al. 1992
Symphalangus syndactylus SSY (Raffles, 1821)	21	Acrocentric	Centromere	No	3	Muller et al. 2003
	Y	Acrocentric	Centromere	No	Y	
Scandentia						
Tupaia minor TMI (Günther, 1876)	25	Acrocentric	Centromere	No	3	Dumas et al. 2012
	26	Acrocentric	Centromere	No	9	
	28	Acrocentric	Centromere	Yes	12*/*22	

Table 2. List of Primates - Scandentia species analyzed with the mapping data from rDNA probes and the respective references.

Species	rDNA mapping references
Catarrhini	
Colobus polykomos	Henderson et al. 1977
Gorilla gorilla	Henderson et al. 1976; Hirai et al. 1999
Hylobates agilis	Hirai et al. 1999
Hylobates lar	Warburton et al. 1975
Hylobates × *Nomascus hybrid*	Hirai et al. 2007
Macaca fuscata fuscata	Hirai et al. 1998
Macaca mulatta	Henderson 1974a
Pan paniscus	Henderson et al. 1976; Hirai et al. 1999
Pan troglodytes	Henderson 1974b; Hirai et al. 1999; Guillén et al. 2004
Pongo pygmaeus albei	Henderson et al. 1979
Papio cynocephalus	Henderson et al. 1977
Papio hamadryas	Henderson et al. 1977
Symphalangus syndactylus	Henderson et al. 1976; Hirai et al. 1999
Platyrrhini	
Ateles geoffroyi	Henderson et al. 1977
Pithecia pithecia	Henderson et al. 1977
Saguinus nigricollis	Henderson et al. 1977
Strepsirrhini	
Lemur fulvis	Henderson et al. 1977
Nycticebus bengalensis	Baicharoen et al. 2016

Results

FISH signals were located in different positions on primarily small particular chromosomes of taxa studied. The variation was observed between karyotypes regarding both the number and morphology of chromosomes bearing the signal as the rDNA site number per karyotype.

From one to five rDNA autosome markers were located at the tip of acrocentrics in 5 species: *Lemur catta* (pairs 21, 25) (Fig. 1A), *Otolemur garnettii* Ogilby, 1838, (pair 19) (Fig. 1B), *Callithrix jacchus* Linnaeus, 1758, (pairs 15, 17, 19) (Fig. 2A), *Callimico goeldii* Thomas, 1904, (pairs 14-17, 21 and, not frequent, 22 – single homolog) (Fig. 1G) and *Symphalangus syndactylus* (pair 21 and the Y-chromosome) (Fig. 1H).

In 7 species, pericentromeric position was recorded for a biarmed pair: *Saimiri sciureus* Linnaeus, 1758 (submetacentrics pair 6) (Fig. 1E), *Ateles paniscus paniscus* Linnaeus, 1758, (pair 8) (Fig. 1C), *Hylobates lar* (pair 12) (Fig. 1I), *Colobus guereza* Rüppell, 1835, (pair 16) (Fig. 2B), *Saguinus Oedipus* Linnaeus, 1758, (pair 20) (Fig. 1F), *Erythrocebus patas* Schreber, 1775, (pair 26) (Fig. 1M), or subtelocentric chromosomes 19 of *Chlorocebus aethiops* Linnaeus, 1758,(subtelocentric chromosomes 19) (Fig. 1L). Besides, in *Saguinus oedipus* the location on acrocentrics 21 and 22 was identified in a visible secondary constriction (Fig.1F).

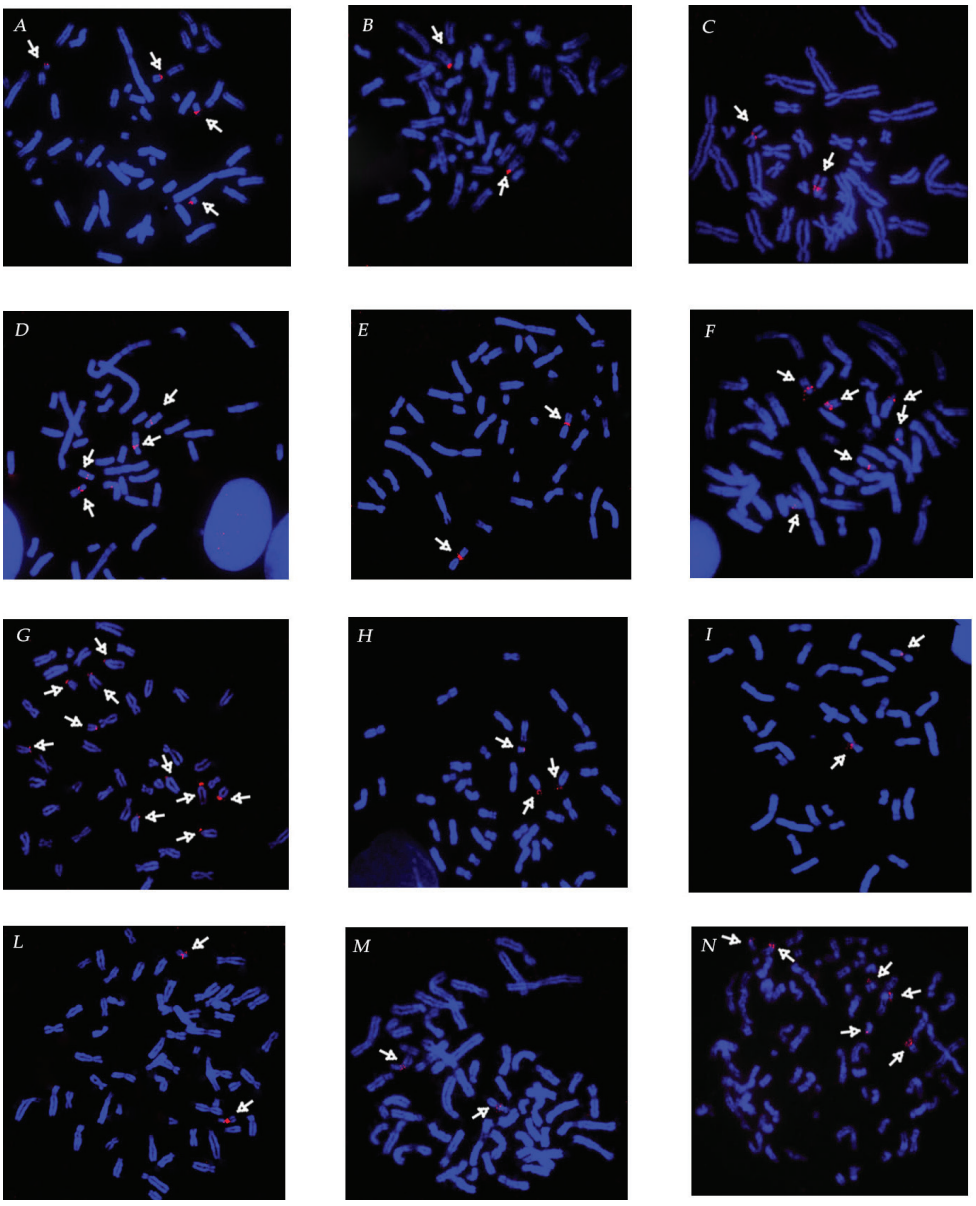

Figure 1. rDNA loci mapping (red signal highlighted by white arrows) on metaphases of: **A** *Lemur catta* **B** *Otolemur garnetti* **C** *Ateles paniscus paniscus* **D** *Alouatta caraya* **E** *Saimiri sciureus* **F** *Saguinus oedipus* **G** *Callimico goeldii* **H** *Symphalangus syndactilus* **I** *Hylobates lar* **L** *Chlorocebus aethiops* **M** *Erythrocebus patas* **N** *Tupaia minor*.

In *Alouatta caraya* Humboldt, 1812, signals were positioned on medium-small acrocentrics with a visible secondary constriction (pairs 17, 23) (Fig. 1D). Similarly, three small acrocentrics of *Tupaia minor* were marked (pairs 25, 26, 28) (Fig. 1N).

The results are reported also in Figure 3 and summarized in Table 1. Homology between marked chromosomes is below discussed.

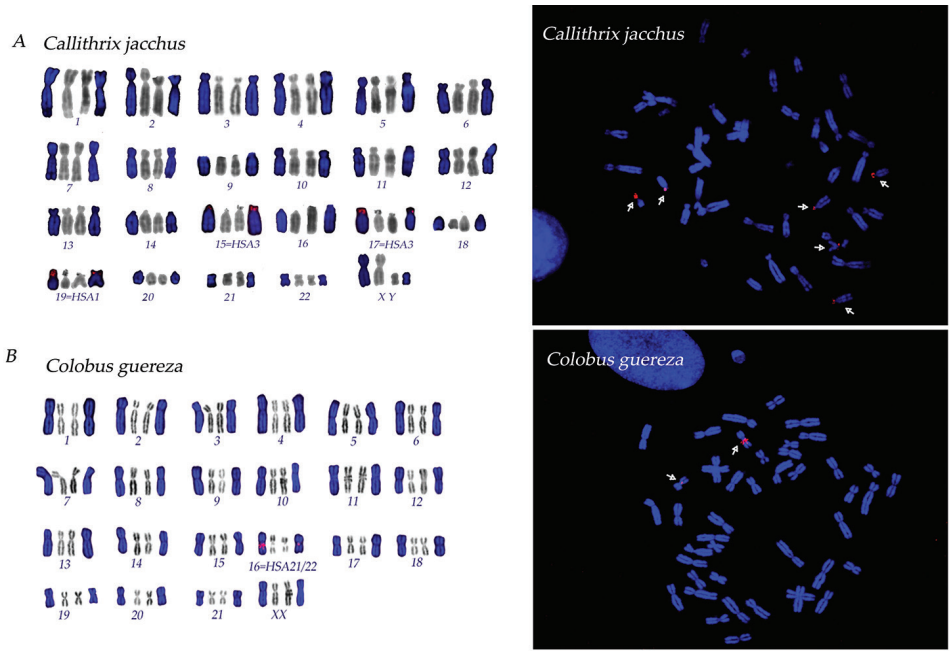

Figure 2. DAPI stained chromosomes (blue) with rDNA loci signal (red) are illustrated, together with DAPI inverted (grey) chromosomes arranged in karyotypes of **A** *Callithrix jacchus* **B** *Colobus guereza*. Corresponding metaphases (with red signals highlighted by white arrows) are shown on the left.

Discussion

rDNA mapping has been previously performed in a number of Primate species (Table 2), but in pioneering studies, the cross-species homology of chromosomes with rDNA could not be reliably identified due to limitations of G-banding and the lack of advanced molecular cytogenetic methods, such as chromosome painting. For example, the topology of rDNA loci was previously studied in *Hylobates lar, Lemur catta* and *Symphalangus syndactylus* (Warburton et al. 1975, Henderson et al. 1977, Hirai et al. 1999), but at that time, it was not always possible to identify the hybridized chromosomes nor their homology with human chromosomes. In our study, we were able to identify, in all studied species, the homology and synteny of each chromosome bearing rDNA loci to human karyotype, through DAPI inverted banding.

The data concerning the distribution of rDNA loci on the chromosomes of the analyzed species are discussed in an evolutionary perspective and illustrated in a graphical reconstruction (Fig. 4) based on chromosome characters such as is visualized in the tree; we report for each species the diploid number, rDNA-bearing chromosomes and the homology to human syntenies.

The comparative analysis of ours and other data demonstrated that rDNA loci are often localized in the chromosomes homologous to HSA synteny 3 and 22 in many Primates and in *Tupaia* as well (Fig. 3). Indeed, among Primates, we found the rDNA loci on HSA synteny 3 on Platyrrhini species *S. sciureus*, *C. jacchus*, *C.*

Figure 3. Chromosome pairs bearing rDNA probe signals for each species analyzed and corresponding human syntenies (HSA): chromosomes are in DAPI inverted banding; rDNA probe signals in red.

goeldii and in gibbons *H. lar* and *S. syndactilus*. In addition, data from literature on the Prosimian *Nycticebus bengalensis* Lacepede, 1800, (Baicharoen et al. 2016) show that rDNA loci exist on human synteny 3. Furthermore, we identified rDNA loci on HSA synteny 22 in the Prosimian representative *L. catta*. Similar topology of rDNA loci was presented previously in *N. bengalensis* (Baicharoen et al. 2016). Among Platyrrhini, even if the probe localized at the centromere of *C. goeldii* chromosome 15, close to human synteny 9, this last synteny is associated to human synteny 22, thus leading us to propose the hypothesis that an inversion could have relocated it after the fusion of the two involved syntenies. In all Cercopithecoidea studied (*C. aethiops, C. guereza* and *E. patas*), the rDNA loci were localized on human synteny 22; in *C. guereza* where it is between syntenies 22 and 21 presumably it conserved its position after the fusion of the first chromosome bearing the rDNA with the second one; other data from literature indicate that rDNA localized on human synteny 22 also in Hominoidea species such as *Pan paniscus* (Schwarz, 1929), *P. troglodytes*, *Gorilla gorilla* (Geoffroy, 1852) and in *H. sapiens* (Linnaeus, 1758) (Hirai et al. 1999, Tantravahi et al. 1976, Guillén et al. 2004). These results led us to suppose that rDNA on synteny 3 and 22 represents the ancestral status; presumably rDNA on synteny 3 has been lost in prosimians (LLC, OGR), Cercopithecoidea (CAE, CGU, EPA) and in many Platyrrhini, while the rDNA on synteny 22 has been lost in gibbons (HLA, SSY).

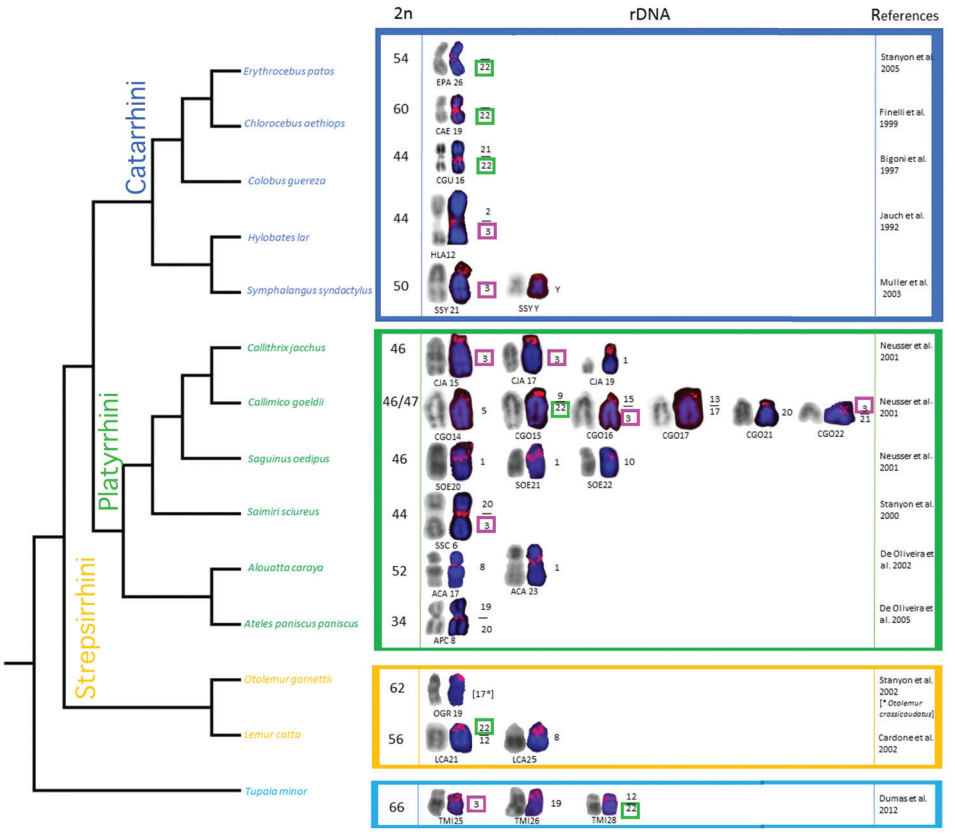

Figure 4. Primate molecular phylogenetic relationships as modified after Perelman et al. (2011). The tree was reconstructed in MESQUITE in consideration of the diploid number (2n), the DAPI stained chromosome (blue) with the rDNA probe signals localization (red) and the inverted DAPI (grey) for each species. In each chromosome pair, only a single chromosome is shown. Homologies to human chromosomes are indicated on the right side of chromosomes and are inferred through the analysis of the references listed in the last column. Ancestral localization of rDNA loci is underlined in color: green for human synteny 22, pink for human synteny 3.

Other multiple rDNA signals that we detected on different chromosomes, could be apomorphies with species specific locations such as, for example, the one found on chromosomes homologous to human synteny 17 in *O. garnettii*. Consistent with previous findings in *N. bengalensis* our data well correspond to species specific rDNA locations (Baicharoen et al. 2016). Furthermore, other rDNA loci could represent synapomorphisms in closely related species, such as the ones on HSA synteny 1 in *S. oedipus*, *A. caraya* and *C. jacchus* (Platyrrhini), as well as on HSA synteny 13/14 previously shown in Hominoidea (*Pan troglodytes*, *P. paniscus*, *H. sapiens*) (Tantravahi et al. 1976, Henderson et al. 1976, Hirai et al. 1999).

Despite the facts that have documented a conserved pattern in the topology of rDNA loci in many species (e.g. extensive homology to HSA synteny 3 and 22), we also showed the presence of multiple rDNA loci on distinct chromosomes (Fig. 4). Therefore, we assume that different mechanisms are responsible for their dispersion in genome, in agree-

ment with previous hypotheses (Hirai et al. 1999, Britton-Davidian et al. 2012). We conclude that intra- and interchromosomal rearrangements are probably not the single explanation of the rDNA pattern in Primates. Ectopic recombination might be responsible for the gain and loss of rDNA loci, resulting in the dispersal or loss of rDNA tandem repeats during meiosis, more prone to occur at the terminal tip of acrocentric chromosomes. For example, among the studied Primates, we found multiple topologies with up to five pairs of acrocentric chromosomes carrying the rDNA loci in *C. goeldii* (Platyrrhini). Actually, the similarity of five to eight pairs has been previously reported in literature for human (Henderson et al. 1972, Tantravahi et al. 1976), chimpanze and gorilla (Hirai et al. 1999).

In an alternative view, we cannot exclude the case that short tandem repeats of rDNA loci may exist on multiple chromosomes, beyond the detection efficiency of FISH, which were inherited by the ancestors of the extant Primates, and were subsequently amplified independently in different species during the evolution of their karyotypes, resulting in the extensive variability observed in this study. Concluding, our results indicate that rDNA distribution is due to different mechanisms; we found species with conserved signals on syntenic chromosomes, while in others, signal was detected in distinct chromosomes. There are reasons to pay more attention to the study of rDNA loci in Primates chromosomes as marks of the complex evolutionary relationships.

Acknowledgement

Thanks to the "Fondazione Intesa San Paolo Onlus" which has supported by funding the project "Evoluzione genomica in Primates" (2016-NAZ-0012, CUP: B72F16000130005) to F.D.

References

Baicharoen S, Hirai Y, Srikulnath K, Kongprom U, Hirai H (2016) Hypervariability of Nucleolus Organizer Regions in Bengal Slow Lorises, *Nycticebus bengalensis* (Primates, Lorisidae). Cytogenetic and Genome Research 149(4): 267–273. https://doi.org/10.1159/000449145

Bedard MT, Ma NSF, Jones TC (1978) Chromosome Banding Patterns and Nucleolar Organizing Regions in Three Species of Callithricidae. Journal of Medical Primatology 7: 82–97. https://doi.org/10.1159/000459791

Bigoni F, Stanyon R, Koehler U, Morescalchi AM, Wienberg J (1997) Mapping homology between human and black and white colobine monkey chromosomes by fluorescent in situ hybridization. American Journal of Primatology 42(4): 289–298. https://doi.org/10.1002/(SICI)1098-2345(1997)42:4<289::AID-AJP4>3.0.CO;2-T

Britton-Davidian J, Cazaux B, Catalan J (2012) Chromosomal dynamics of nucleolar organizer regions (NORs) in the house mouse: micro-evolutionary insights. Heredity (Edinb) 108(1): 68–74. https://doi.org/10.1038/hdy.2011.105

Bulatova N, Pavlova S (2016) A possible cytogenetic analogy to genomic «speciation islands» as revealed by chromosome study of a natural hybrid vole. Tsitologiya 58(5): 412–415.

Cardone MF, Ventura M, Tempesta S, Rocchi M, Archidiacono N (2002) Analysis of chromosome conservation in *Lemur catta* studied by chromosome paints and BAC/PAC probes. Chromosoma 111(5): 348–56. https://doi.org/10.1007/s00412-002-0215-3

Cazaux B, Catalan J, Veyrunes F, Douzery EJ, Britton-Davidian J (2011) Are ribosomal DNA clusters rearrangement hotspots?: a case study in the genus Mus (Rodentia, Muridae). BMC Evolutionary Biology 11(1): 124. https://doi.org/10.1186/1471-2148-11-124

Cioffi MB, Martins C, Bertollo LA (2010) Chromosome spreading of associated transposable elements and ribosomal DNA in the fish *Erythrinus erythrinus*. Implications for genome change and karyoevolution in fish. BMC Evolutionary Biology 10(1): 271. https://doi.org/10.1186/1471-2148-10-271

Da Silva Calixto M, de Andrade IS, Cabral-de-Mello DC, Santos N, Martins C, Loreto V, de Souza MJ (2014) Patterns of rDNA and telomeric sequences diversification: Contribution to repetitive DNA organization in Phyllostomidae bats. Genetica 142(1): 49–58. https://doi.org/10.1007/s10709-013-9753-2

De Oliveira EHC, Neusser M, Figueiredo WB, Nagamachi C, Pieczarka JC, Sbalqueiro IJ, Pieczarka JC, Sbalqueiro IJ, Wienberg J, Müller S (2002) The phylogeny of howler monkeys (*Alouatta*, Platyrrhini): Reconstruction by multicolor cross-species chromosome painting. Chromosome Research 10(8): 669–683. https://doi.org/10.1023/A:1021520529952

De Oliveira EHC, Neusser M, Pieczarka JC, Nagamachi C, Sbalqueiro IJ, Müller S (2005) Phylogenetic inferences of Atelinae (Platyrrhini) based on multi-directional chromosome painting in Brachyteles arachnoides, *Ateles paniscus paniscus* and *Ateles b. marginatus*. Cytogenetic and Genome Research 108(1–3): 183–190. https://doi.org/10.1159/000080814

Degrandi TM, Pita S, Panzera Y, De Oliveira EHC, Marques JRF, Figueiró MR, Marques LC, Vinadé Lucia, Gunski RJ, del Valle Garnero A (2014) Karyotypic evolution of ribosomal sites in buffalo subspecies and their crossbreed. Genetics and Molecular Biology 37(2): 375–380. https://doi.org/10.1590/S1415-47572014000300009

Dumas F, Houck ML, Bigoni F, Perelman P, Romanenko SA, Stanyon R (2012) Chromosome painting of the pygmy tree shrew shows that no derived cytogenetic traits link primates and scandentia. Cytogenetic and Genome Research 136(3): 175–179. https://doi.org/10.1159/000336976

Dumas F, Cuttaia H, Sineo L (2016) Chromosomal distribution of interstitial telomeric sequences in nine neotropical primates (Platyrrhini): possible implications in evolution and phylogeny. Journal of Zoological Systematics and Evolutionary Research (3): 226–236. https://doi.org/10.1111/jzs.12131

Averbeck KT, Eickbush TH (2005) Monitoring the mode and tempo of concerted evolution in the Drosophila melanogaster rDNA locus. Genetics 171: 1837–1846. https://doi.org/10.1534/genetics.105.047670

Eickbush TH, Eickbush DG (2007) Finely orchestrated movements: Evolution of the ribosomal RNA genes. Genetics 175(2): 477–485. https://doi.org/10.1534/genetics.107.071399

Endow SA (1982) Polytenization of the ribosomal genes on the X and Y chromosomes of *Drosophila melanogaster*. Genetics 100: 375–385.

Finelli P, Stanyon R, Plesker R, Ferguson-Smith MA, O'Brien PCM, Wienberg J (1999) Reciprocal chromosome painting shows that the great difference in diploid number between human and African green monkey is mostly due to non- Robertsonian fissions. Mammalian Genome 10(7): 713–718. https://doi.org/10.1007/s003359901077

Gornung E, Bezerra AMR, Castiglia R (2011) Comparative chromosome mapping of the rRNA genes and telomeric repeats in three Italian pine voles of the *Microtus savii s.l.* complex (Rodentia, Cricetidae). Comparative Cytogenetics 5(3): 247–257. https://doi.org/10.3897/compcytogen.v5i3.1429

Guillén AKZ, Hirai Y, Tanoue T, Hirai H (2004) Transcriptional repression mechanisms of nucleolus organizer regions (NORs) in humans and chimpanzees. Chromosome Research 12: 225–237. https://doi.org/10.1023/B:CHRO.0000021911.43225.eb

Henderson AS, Atwood KC, Warburton D (1976) Chromosomal Distribution of rDNA in *Pan paniscus*, *Gorilla gorilla beringei*, and *Symphalangus syndactylus*: Comparison to Related Primates. Chromosoma 59: 147–155. https://doi.org/10.1007/BF00328483

Henderson AS, Warburton D, Megraw-Ripley S, Atwood KC (1977) The chromosomal location of rDNA in selected lower primates. Cytogenetics and Cell Genetics 19: 281–302. https://doi.org/10.1159/000130821

Henderson AS, Warburton D, Megraw-Ripley S, Atwood KC (1979) The chromosomal location of rDNA in the Sumatran orangutan, *Pongo pygmaeus albei*. Cytogenetics and Cell Genetics 23: 213–216. https://doi.org/10.1159/000131328

Henderson AS, Warburton D, Atwood KC (1974) Localization of rDNA in the Chimpanzee (Pan troglodytes) chromosome complement. Chromosome 46: 135–441. https://doi.org/10.1007/BF00331631

Henderson AS, Warburton D, Atwood KC (1972) Location of ribosomal DNA in the human chromosome complement. Proceedings of the National Academy of Sciences 69: 3394–3398. https://doi.org/10.1073/pnas.69.11.3394

Hirai H, Yamamoto MT, Taylor RW, Imai H (1996) Genomic dispersion of 28S rDNA during karyotypic evolution on the ant genus Myrmecia (Formicidae). Chromosoma 105: 190–196. https://doi.org/10.1007/BF02509500

Hirai H, Hasegawa Y, Kawamoto Y, Tokita E (1998) Tandem duplication of nucleolus organizer region (NOR) in the Japanese macaque *Macaca fuscata fuscata*. Chromosome Research 6: 191–197. https://doi.org/10.1023/A:1009207600920

Hirai H, Taguchi T, Godwin AK (1999) Genomic differentiation of 18S ribosomal DNA and β-satellite DNA in the hominoid and its evolutionary aspects. Chromosome Research 7: 531–540. https://doi.org/10.1023/A:1009237412155

Hirai H, Hirai Y, Domae H, Kirihara Y (2007) A most distant intergeneric hybrid offspring (Larcon) of lesser apes, *Nomascus leucogenys* and *Hylobates lar*. Human Genetics 122: 477–483. https://doi.org/10.1007/s00439-007-0425-0

Jauch A, Wienberg J, Stanyon R, Arnold N, Tofanelli S, Ishida T, Cremer T (1992) Reconstruction of genomic rearrangements in great apes and gibbons by chromosome painting. Proceedings of the National Academy of Sciences 89(18): 8611–8615. https://doi.org/10.1073/pnas.89.18.8611

Lin J, Chen G, Gu L, Shen Y, Zheng M, Zheng W, Jiang C (2014) Phylogenetic affinity of tree shrews to Glires is attributed to fast evolution rate. Molecular Phylogenetics and Evolution 71(1): 193–200. https://doi.org/10.1016/j.ympev.2013.12.001

Maddison WP, Maddison DR (2011) Mesquite: a modular system for evolutionary analysis. Version 2.75. http://mesquiteproject.org

Masters JC, Stanyon R, Romagno D (1987) Standardized karyotypes for the greater Galagos, *Galago crassicaudatus* E. Geoffroy, 1812 and *G. garnettii* (Ogilby, 1838) (Primates: Prosimii). Genetica 75(2): 123–129. https://doi.org/10.1007/BF00055256

Mazzoleni S, Schillaci O, Sineo L, Dumas F (2017) Distribution of Interstitial Telomeric Sequences in Primates and the Pygmy Tree Shrew (Scandentia). Cytogenetic and Genome Research 151: 141–150. https://doi.org/10.1159/000467634

Müller S, Hollatz M, Wienberg J (2003) Chromosomal phylogeny and evolution of gibbons (Hylobatidae). Human Genetics 113(6): 493–501. https://doi.org/10.1007/s00439-003-0997-2

Nagamachi CY, Pieczarka JC, de Sousa Barros RM (1992) karyotypic comparison cebuella, *Callithrix jacchus* and *C. emilia* (Callitrichidae, Primates) and its taxomic implication. Genetica 85: 249–257. https://doi.org/10.1007/BF00132277

Neusser M, Stanyon R, Bigoni F, Wienberg J, Müller S (2001) Molecular cytotaxonomy of New World monkeys (Platyrrhini) - comparative analysis of five species by multi-color chromosome painting gives evidence for a classification of *Callimico goeldii* within the family of Callitrichidae. Cytogenetics and Cell Genetics 94(3–4): 206–215. https://doi.org/10.1159/000048818

Nguyen TT, Aniskin VM, Gerbault-Seureau M, Planton H, Renard JP, Nguyen BX, Volobouev VT (2008) Phylogenetic position of the saola *(Pseudoryx nghetinhensis*) inferred from cytogenetic analysis of eleven species of Bovidae. Cytogenetic and Genome Research 122(1): 41–54. https://doi.org/10.1159/000151315

Perelman P, Johnson WE, Roos C, Seuánez HN, Horvath JE, Moreira MAM, Kessing B, Pontius J, Roelke M, Rumpler Y, Schneider MPC, Silva A, O'Brien SJ, Slattery JP (2011) A molecular phylogeny of living primates. PLoS Genetics 7: 1–17. https://doi.org/10.1371/journal.pgen.1001342

Robicheau BM, Susko E, Harrigan AM, Snyder M (2017) Ribosomal RNA Genes Contribute to the Formation of Pseudogenes and Junk DNA in the Human Genome. Genome biology and evolution 9(2): 380–397. https://doi.org/10.1093/gbe/evw307

Rovatsos M, Johnson Pokorná M, Altmanová M, Kratochvíl L (2015a) Female heterogamety in Madagascar chameleons (Squamata: Chamaeleonidae: Furcifer): differentiation of sex and neo-sex chromosomes. Scientific Reports 5: 13196. https://doi.org/10.1038/srep13196

Rovatsos M, Johnson Pokorná M, Kratochvíl L (2015b) Differentiation of Sex Chromosomes and Karyotype Characterisation in the Dragonsnake *Xenodermus javanicus* (Squamata: Xenodermatidae). Cytogenetic and Genome Research 147(1): 48–54. https://doi.org/10.1159/000441646

Rovatsos M, Johnson Pokorná M, Altmanová M, Kratochvíl L (2016) Mixed-Up Sex Chromosomes: Identification of Sex Chromosomes in the X1X1X2X2/X1X2Y System of the Legless Lizards of the Genus *Lialis* (Squamata: Gekkota: Pygopodidae). Cytogenetic and Genome Research 149(4): 282–289. https://doi.org/10.1159/000450734

Schempp W, Zeitler S, Rietschel W (1998) Chromosomal localization of rDNA in the gorilla. Cytogenetics and Cell Genetics 80: 185–187. https://doi.org/10.1159/000014977

Sember A, Bohlen J, Slechtová V, Altmanová M, Symonová R, Ráb P (2015) Karyotype differentiation in 19 species of river loach fishes Nemacheilidae, Teleostei): extensive variability associated with rDNA and heterochromatin distribution and its phylogenetic and ecological interpretation. BMC Evolutionary Biology 15(1): 251. https://doi.org/10.1186/s12862-015-0532-9

Sineo L, Dumas F, Vitturi, R, Picone B, Privitera O, Stanyon R (2007) Williams-Beuren mapping in *Callithrix argentata, Callicebus cupreus* and *Alouatta caraya* indicates different patterns of chromosomal rearrangements in neotropical primates. Journal of Zoological Systematics and Evolutionary Research 45(4): 366–371. https://doi.org/10.1111/j.1439-0469.2007.00408.x

Small MF, Stanyon R, Smith DG, Sineo L (1985) High resolution chromosomes of rhesus macaques (*Macaca mulatta*). American Journal of Primatology 9: 63–67. https://doi.org/10.1002/ajp.1350090107

Srikulnath K, Matsubara K, Uno Y, Thongpan A, Suputtitada S, Apisitwanich S, Nishida C (2009) Karyological characterization of the butterfly lizard (*leiolepis reevesii rubritaeniata*, agamidae, squamata) by molecular cytogenetic approach. Cytogenetic and Genome Research 125(3): 213–223. https://doi.org/10.1159/000230005

Srikulnath K, Uno Y, Matsubara K, Thongpan A, Suputtitada S, Apisitwanich S, Matsuda Y (2011) Chromosomal localization of the 18S-28S and 5S rRNA genes and (TTAGGG) *n* sequences of butterfly lizards (*Leiolepis belliana belliana* and *Leiolepis boehmei*, Agamidae, Squamata). Genetics and Molecular Biology 34(4): 583–586. https://doi.org/10.1590/S1415-47572011005000042

Stanyon R, Bruening R, Stone G, Shearin A, Bigoni F (2005) Reciprocal painting between humans, de Brazza's and patas monkeys reveals a major bifurcation in the Cercopithecini phylogenetic tree. Cytogenetic and Genome Research 108(1–3): 175–182. https://doi.org/10.1159/000080813

Stanyon R, Consigliere S, Muller S, Morescalchi A, Neusser M, Wienberg J (2000) Fluorescence in situ hybridization (FISH) maps chromosomal homologies between the dusky titi and squirrel monkey. American Journal of Primatology 50(2): 95–107. https://doi.org/10.1002/(SICI)1098-2345(200002)50:2<95::AID-AJP1>3.0.CO;2-8

Stanyon R, Koehler U, Consigliere S (2002) Chromosome painting reveals that galagos have highly derived karyotypes. American Journal of Physical Anthropology 117: 319–326. https://doi.org/10.1002/ajpa.10047

Stults DM, Killen MW, Pierce HH, Pierce AJ (2008) Genomic architecture and inheritance of human ribosomal RNA gene clusters. Genome Research 18: 13–18. https://doi.org/10.1101/gr.6858507

Tantravahi R, Miller DA, Dev VG, Miller OJ (1976) Detection of nucleolus organizer regions in chromosomes of human, chimpanzee, gorilla, orangutan and gibbon. Chromosoma 56: 15–27. https://doi.org/10.1007/BF00293725

Tanomtong A, Khunsook S, Supanuam P, Kaewsri S (2009) A novel polymorphism of nuclear organizer regions (NORs) and complex inversion chromosome 8 of white-hended gibbons (Hylobates lar, Linnaeus, 1771) in Thailand. Cytologia 74(4): 379–384. https://doi.org/10.1508/cytologia.74.379

Warburton D, Henderson AS, Atwood KC (1975) Localization of rDNA and giemsa-banded chromosome complement of white-handed gibbon, *Hylobates lar*. Chromosoma 51(1): 35–40. https://doi.org/10.1007/BF00285805

Zhou X, Sun F, Xu S, Yang G, Li M (2015) The position of tree shrews in the mammalian tree: Comparing multi-gene analyses with phylogenomic results leaves monophyly of Euarchonta doubtful. Integrative Zoology 10(2): 186–198. https://doi.org/10.1111/1749-4877.12116

CompCytogen 12(1): 41–59 (2018)
doi: 10.3897/CompCytogen.v12i1.21744
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Molecular technique reveals high variability of 18S rDNA distribution in harvestmen (Opiliones, Phalangiidae) from South Africa

František Šťáhlavský[1], Vera Opatova[2], Pavel Just[1], Leon N. Lotz[3], Charles R. Haddad[4]

1 *Department of Zoology, Faculty of Science, Charles University, Viničná 7, CZ-12844 Praha, Czech Republic* **2** *Department of Biological Sciences and Auburn University Museum of Natural History, Auburn University, Auburn, AL 36849, USA* **3** *Department of Arachnology, National Museum, P.O. Box 266, Bloemfontein 9300, South Africa* **4** *Department of Zoology and Entomology, University of the Free State, P.O. Box 339, Bloemfontein 9300, South Africa*

Corresponding author: *František Šťáhlavský* (stahlf@natur.cuni.cz)

Academic editor: *M. Schneider* | Received 18 October 2017 | Accepted 27 December 2017 | Published 13 February 2018

http://zoobank.org/BD37B31A-62D0-48B6-B7AD-FCFF7050FD08

Citation: Šťáhlavský F, Opatova V, Just P, Lotz LN, Haddad CR (2018) Molecular technique reveals high variability of 18S rDNA distribution in harvestmen (Opiliones, Phalangiidae) from South Africa. Comparative Cytogenetics 12(1): 41–59. https://doi.org/10.3897/CompCytogen.v12i1.21744

Abstract

The knowledge of cytogenetics in the harvestmen family Phalangiidae has been based on taxa from the Northern Hemisphere. We performed cytogenetic analysis on *Guruia africana* (Karsch, 1878) (2n=24) and four species of the genus *Rhampsinitus* Simon, 1879 (2n=24, 26, 34) from South Africa. Fluorescence *in situ* hybridization with an 18S rDNA probe was used to analyze the number and the distribution of this cluster in the family Phalangiidae for the first time. The results support the cytogenetic characteristics typical for the majority of harvestmen taxa, i.e. the predominance of small biarmed chromosomes and the absence of morphologically well-differentiated sex chromosomes as an ancestral state. We identified the number of 18S rDNA sites ranging from two in *R. qachasneki* Kauri, 1962 to seven in one population of *R. leighi* Pocock, 1903. Moreover, we found differences in the number and localization of 18S rDNA sites in *R. leighi* between populations from two localities and between sexes of *R. capensis* (Loman, 1898). The heterozygous states of the 18S rDNA sites in these species may indicate the presence of XX/XY and ZZ/ZW sex chromosomes, and the possible existence of these systems in harvestmen is discussed. The variability of the 18S rDNA sites indicates intensive chromosomal changes during the differentiation of the karyotypes, which is in contrast to the usual uniformity in chromosomal morphology known from harvestmen so far.

Keywords
Karyotype, meiosis, sex chromosomes, FISH, 18S rDNA

Introduction

The harvestmen (Opiliones) represent one of the oldest terrestrial arachnid lineages (Dunlop et al. 2004). They are traditionally divided into four suborders (Cyphophthalmi, Eupnoi, Dyspnoi and Laniatores) (e.g. Pinto-da-Rocha et al. 2007), altogether comprising more than 6500 described species (Kury 2017). Harvestmen are present on all continents (except Antarctica) (Giribet and Kury 2007) and have a wide range of ecological functions within the ecosystem (e.g. predators, detritivores, scavengers). Typically, harvestmen have limited dispersal capabilities and show tendencies to local endemicity (Giribet and Kury 2007). Despite the species richness and ancient geographical isolation of many groups, harvestmen possess similar cytogenetic characteristics within each suborder.

So far, the chromosomes of 90 species of harvestmen have been examined, which ranks them as the third best cytogenetically explored arachnid order (Tsurusaki et al. 2017). All groups usually have biarmed monocentric chromosomes (Tsurusaki 2007). The highest numbers of chromosomes are known from Laniatores (2n=25–109, median 81) (Schneider et al. 2008) and Cyphopthalmi (2n=24–52, median 30) (Svojanovská et al. 2016). Both Dyspnoi (2n=10–28, median 16) and Eupnoi (2n=10–36, median 22) typically possess a lower number of chromosomes (Tsurusaki et al. 2017). In some species, intraspecific variability in chromosome numbers has been reported. Given the low vagility of harvestmen, the variability could be caused by a fixation of local karyotype changes in isolated populations. Geographical variation among populations is particularly known from sabaconids (Dypnoi) and sclerosomatids (Eupnoi) (Tsurusaki 2007), where narrow hybrid zones exist between the populations with different chromosome races (e.g. Tsurusaki et al. 1991, Gorlov and Tsurusaki 2000a). However, the intraspecific variability in the chromosome number may also be attributed to the presence of B chromosomes, i.e. the supernumerary chromosomes that remain univalent during meiosis and are usually heterochromatic and non-coding. The number of B chromosomes can vary even within one individual (White 1973). In opilionids, the presence of B chromosomes has been documented in the sclerosomatid harvestman *Psathyropus tenuipes* L. Koch, 1878 (Eupnoi), where some individuals possessed up to 18 B chromosomes (Tsurusaki 1993). Finally, the intraspecific variability could also be related to the presence of the heteromorphic XX/XY or ZZ/ZW sex chromosome systems. The XX/XY system evolved independently in both Dyspnoi and Eupnoi (Tsurusaki 2007), while the ZZ/ZW system is documented uniquely in *Mitopus morio* (Fabricius, 1779) (Eupnoi: Phalangiidae) from Rishiri island in Japan (Tsurusaki and Cokendolpher 1990).

The majority of the available chromosome data on harvestmen originates from the suborder Eupnoi (58 species examined) (Tsurusaki et al. 2017). However, all the Eupnoi taxa examined so far, with exception of *Holmbergiana weyenberghii* (Holmberg,

1876) (Sclerosomatidae) from Argentina (Rodríguez Gil and Mola 2010), occur in the Northern Hemisphere. Despite our relatively good knowledge of the cytogenetics of this group, it is likely that the variability within Eupnoi could still be underestimated, due to the obvious geographical bias of the available data. The suborder Cyphophthalmi could serve as an example of a similar situation; it was traditionally perceived as cytogenetically uniform, but a surprising amount of diversity was revealed when analyses encompassed material from a broad geographical range (Svojanovská et al. 2016).

The suborder Eupnoi is divided into six families comprising 1822 species (Pinto-da-Rocha et al. 2007, Kury 2013). The family Phalangiidae (394 described species), with the centre of diversity in the Northern Hemisphere (Giribet and Kury 2007), represents the second most diverse group of Eupnoi (Kury 2017). The presence of Eupnoi in South Africa is traditionally explained by the group's active dispersion from the Mediterranean region (Staręga 1984). Presently, only one species, *Guruia africana* (Karsch, 1878), and 30 species of the genus *Rhampsinitus* Simon, 1879 belonging to the family Phalangiidae are known from South Africa (Lotz 2009). In this study, we analyse the karyotypes of South African representatives of the family Phalangiidae and present the first data on the cytogenetics of this family of harvestmen from the Afrotropical Region. Moreover, we also use fluorescence in situ hybridization (FISH) to identify the number and position of the 18S rRNA genes, which is the first implementation of this method in the family Phalangiidae. The marker for 18S rRNA is frequently used to reveal concealed karyotype differences in groups with similar chromosomes (e.g. Cabral de Mello et al. 2011, Mattos et al. 2014, Sember et al. 2015) and to help inferring specific chromosomal changes along the course of the karyotype evolution (e.g. Nguyen et al. 2010, Grzywacz et al. 2011, Panzera et al. 2012).

Material and methods

The material examined in this study is deposited in the National Museum, Bloemfontein, South Africa (NMBA). We analyzed five species of harvestmen belonging to the family Phalangiidae from different localities in South Africa:

Guruia africana (Karsch, 1878): KwaZulu-Natal: Ndumo Game Reserve (26.8749°S, 32.2109°E), 4 males (NMBAO00900–NMBAO00903).

Rhampsinitus capensis (Loman, 1898): Eastern Cape: Hogsback (32.5888°S, 26.9352°E), 2 males, 2 females (NMBAO01016–NMBAO01019).

Rhampsinitus discolor (Karsch, 1878): Eastern Cape: Port St. Johns (31.5977°S, 29.5346°E), 2 males (NMBAO01023, NMBAO01024).

Rhampsinitus leighi Pocock, 1903: Eastern Cape: Silaka Nature Reserve (31.6529°S, 29.4919°E), 1 male (NMBAO01020); KwaZulu-Natal: Vernon Crookes Nature Reserve (30.27489°S, 30.6092°E), 2 males (NMBAO01021, NMBAO01022).

Rhampsinitus qachasneki Kauri, 1962: KwaZulu-Natal: Royal Natal National Park (28.7101°S, 28.9336°E), 1 male, 1 female (NMBAO01025, NMBAO01026).

The specimens were individually hand collected and kept alive until the gonad dissection. Chromosome preparation follows the "plate spreading" method (Traut 1976) widely used in arachnids (e.g. Šťáhlavský and Král 2004). After the dissection, the gonad tissue was hypotonized in 0.075 M KCl for 20 min, subsequently fixed in methanol: acetic acid (3:1) solution for 20 min, and finally dissociated in a drop of 60% acetic acid directly on the surface of the microscope slide. After the dissociation, the slide with the suspension was moved onto a histological plate (45 °C) to allow the evaporation of the liquid component of the suspension. The chromosomes were stained in a 5% Giemsa solution in Sörensen phosphate buffer for 20 min. Chromosomes were photographed using an ORCA-AG monochromatic camera (Hamamatsu) in an Olympus IX81 microscope. The karyotypes were analyzed from photographs using the LEVAN plugin (Sacamato and Zacaro 2009) for IMAGEJ 1.47 program (http://imagej.nih.gov/ij/). We used the classification of chromosomes of Levan et al. (1964) and measured the relative length of the chromosomes for the diploid set (Suppl. material 1).

FISH detection of 18S rDNA

The probe for 18S rDNA was prepared from *Euscorpius sicanus* (Koch, 1837). The whole genomic DNA was extracted using Genomic DNA Minikit - Tissue (Geneaid), following the manufacturer's guidelines. The 18S rDNA fragment (GenBank accession number MG761815) were amplified with the following primer combination 18S-Gal forward: 5'-CGAGCGCTTTTATTAGACCA-3' and 18S-Gal reverse: 5'-GGTTCACCTACGGAAACCTT-3' (Fuková et al. 2005), and 50 ng DNA template. The PCR protocol was as follows: 95°C for 3 min, 30 cycles at 94°C for 30 s, 55 °C for 30 s and 72 °C for 2 min, final extension at 72 °C for 3 min. PCR products were purified with GenElute PCR Clean-Up Kit (Sigma-Aldrich). The probe was labelled using PCR with biotin-14-dUTP (Roche) using Nick Translation Kit (Abbott Molecular) following the manufacturer's guidelines.

The FISH protocol was performed following Forman et al. (2013). Briefly, chromosome preparations were treated with RNase A (200 µg/ml in 2× SSC) for 60 min and then washed twice in 2× SSC for 5 min. Chromosomes were denaturized at 68 °C for 3 min 30 s in 70 % formamide in 2× SSC. The probe mixture, comprising 20 ng of the probe and 2.5 µl of salmon sperm DNA (Sigma-Aldrich) for each slide, was applied. The slides were left to hybridize overnight in a dark chamber at 37 °C. The following day, the preparations were treated with Cy3-conjugated streptavidin, followed by application of biotinylated antistreptavidin and another dose of Cy3-conjugated streptavidin. Chromosome preparations were counterstained with DAPI (4',6-diamidino-2-phenylindole), contained in FluoroshieldTM (Sigma-Aldrich) and observed in an Olympus IX81 microscope equipped with an ORCA-AG monochromatic charge-coupled device camera (Hamamatsu). The images were pseudocolored (red for Cy3 and blue for DAPI) and superimposed with Cell^R software (Olympus Soft Imaging Solutions GmbH).

Results

Guruia africana (Karsch, 1878)

The diploid number of chromosomes in the four males analysed was 24 (Fig. 1a). The karyotype of this species comprised five pairs of metacentric (pairs No. 1, 3, 5, 6, 9), seven pairs of submetacentric and one pair of subtelocentric (pair No. 12) chromosomes (Suppl. material 1). The chromosomes gradually decreased in length from 6.49 % to 2.74 % of the diploid set (Suppl. material 1). In this species, we observed visible modification of condensation of the chromosomes, such as positive heteropycnosis during mitosis. In the chromosomes, there were visible positive heteropycnotic blocks at the position of the centromere of mitotic metaphases (pairs No. 2, 5, 7, 10) (Fig. 1a); however, in some chromosomes, the heteropycnotic parts expanded to a large proportion of the arms (pairs No. 1, 3, 4, 8, 11), or even across the whole length of the chromosome (pairs No. 6, 9). Large positive heteropycnotic blocks were visible also during prophase I of meiosis, mainly during zygotene and pachytene (Fig. 1b). During the diffuse stage and the diplotene the heteropycnosis was only moderate (Fig. 1c). Later, during diakinesis (Fig. 1d), metaphase I and metaphase II (Fig. 1e), the whole chromosomes seemed to be isopycnotic (Fig. 1d, e). We did not clearly detect visible heteromorphic bivalents during diakinesis and metaphase I (Fig. 1d).

Two pairs of 18S rDNA clusters were detected by FISH in this species. The 18S rDNA probe signals were localized interstitially at approximately one third of the short arm of chromosome pair No. 1 and approximately at one third of the long arm of chromosome pair No. 3 (Fig. 1a). The signals on chromosome pair No. 3 were more intensive than on the longest chromosomes. During pachytene we identified the position of the 18S rDNA clusters on the positive heteropycnotic areas of the chromosomes, and the clusters were usually associated in this phase (Fig. 1f). Also during the diffuse stage and the diplotene the chromosomes bearing the NORs were close to each other (Fig. 1g). The condensation of the chromosomes caused the visualization of the 18S rDNA in the terminal part of the chromosomes during diakinesis and metaphase II (Fig. 1h, i).

Rhampsinitus capensis (Loman, 1898)

The diploid number of chromosomes in the two males and two females analysed was 34 (Fig. 2a, b). The karyotype of males comprised two pairs of metacentric (pairs No. 4, 16), three pairs of submetacentric (pairs No. 2, 5, 6, 14), eight pairs of subtelocentric, and four pairs of acrocentric (pairs No. 8, 11, 12, 15) chromosomes (Suppl. material 1). The first pair of chromosomes was slightly longer (5.49 % of the diploid set) than the following chromosome pair (4.50 %), and the rest of the chromosomes

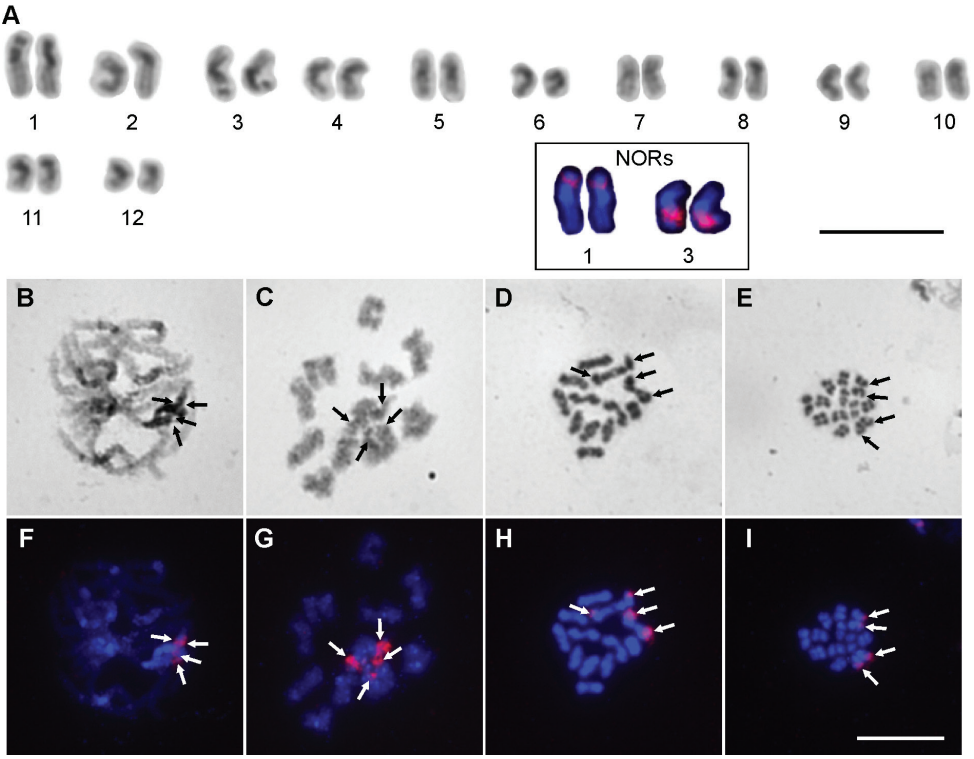

Figure 1. Chromosomes of *Guruia africana* (2n=24) males after Giemsa staining (**A–E**) and after FISH with 18S rDNA (**A** partly, **F–I**). **A** karyotype based on mitotic metaphase **B** pachytene **C** diplotene **D** diakinesis **E** one sister cell of metaphase II **F–I** are the same cells as **B–E** after FISH with 18S rDNA. White arrows indicate the position of 18S rDNA and black arrows indicate the same position after Giemsa staining. Bar = 10 μm.

gradually decreased in length to 1.49 % of the diploid set (Suppl. material 1). In some chromosomes, positive heteropycnotic blocks were visible at the position of the centromere during mitotic metaphase in both sexes (Figs 2a, b), and also during pachytene in females (Fig. 2d) (we did not observe meiosis in males). During this phase we only observed one pair with the positive heteropycnotic areas expanded to the larger proportion of the arms (Fig. 2d). In pachytene of females we detected one heteromorphic bivalent (Fig. 2d). Three signals of 18S rDNA clusters were detected by FISH in males of this species (Fig. 2a). The 18S rDNA probe signals were localized in the terminal position of the short arms of chromosome pair No. 14 and at one chromosome of pair No. 16. In females, we detected five signals of 18S rDNA clusters. During the mitotic metaphase they seemed to be in a terminal position (Figs 2b, c); however, during the pachytene it was clear that one pair was in a subterminal position (Fig. 2e). Interestingly, one cluster of 18S rDNA was very large (Fig. 2c) and covered nearly the whole arm of the large metacentric chromosome from the heteromorphic bivalent (Fig. 2e).

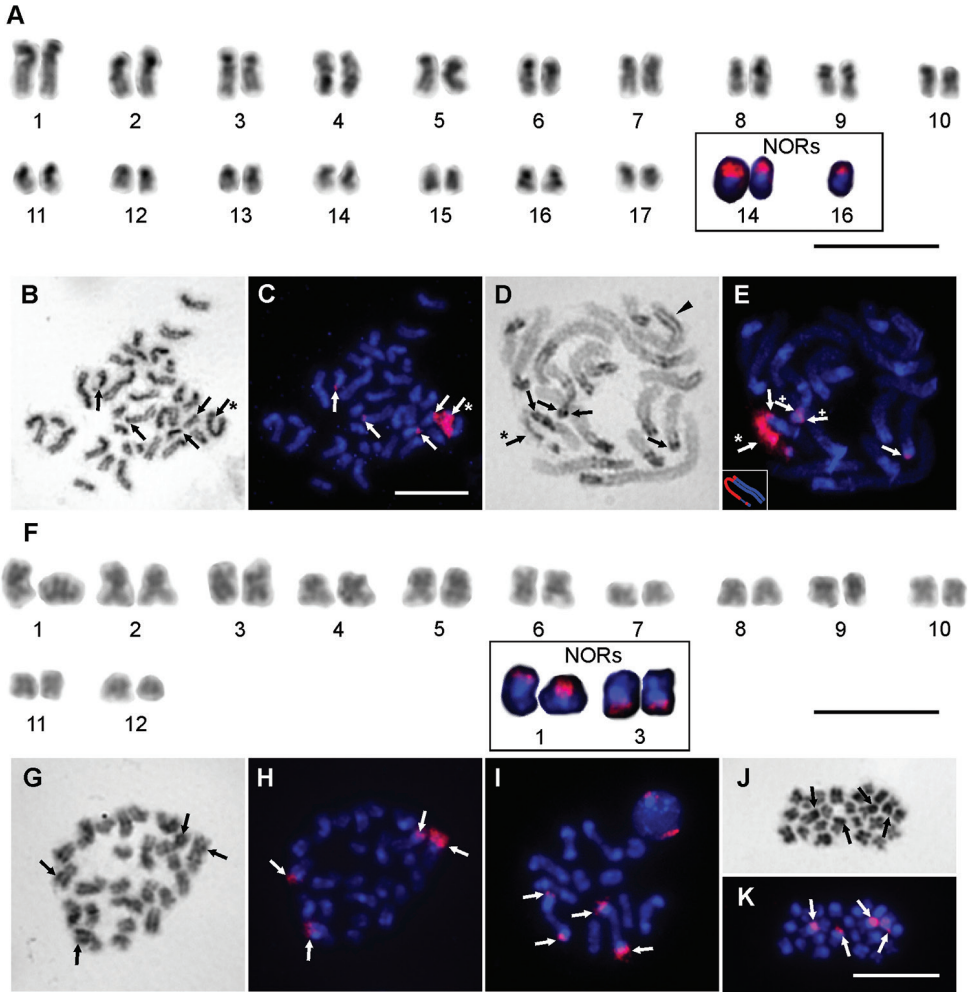

Figure 2. Chromosomes of *Rhampsinitus capensis* (2n=34) (**A-E**) and *Rhampsinitus discolor* (**F–K**) (2n=24) after Giemsa staining (**A, B, D, F, G, J**) and after FISH with 18S rDNA (**A** and **F** partly, **C, E, H, I, K**). **A** male karyotype based on mitotic metaphase **B, C** female mitotic metaphase **D, E** female pachytene, bottom left inset shows a reconstruction of the heteromorphic bivalent; crosses indicate the subterminal position of 18S rDNA; arrowhead indicates bivalent with the expanded positive heteropycnotic area **F** male karyotype based on two sister cells of metaphase II **G, H** male mitotic metaphase **I** diakinesis **J, K** two sister cells of metaphase II. White arrows indicate the position of 18S rDNA and black arrows indicate the position after Giemsa staining. Asterisks indicate large signal of heteromorphic bivalent. Bar = 10 μm.

Rhampsinitus discolor (Karsch, 1878)

The diploid number of chromosomes in the two males analysed was 24 (Fig. 2f). The karyotype comprised eight pairs of metacentric, two pairs of submetacentric (pairs No. 6, 9), and two pairs of subtelocentric (pairs No. 8, 12) chromosomes (Suppl. material 1).

The chromosomes gradually decreased in length from 5.96 % to 2.69 % of the diploid set (Suppl. material 1). In this species, we observed modification in spiralization, visible as positive heteropycnosis of large parts of the arms of almost all chromosomes during mitotic prophase. These large blocks of positive heteropycnotic regions were visible only in a few chromosomes later during mitotic metaphase (Fig. 2g). Large positive heteropycnotic blocks were visible also during meiosis, from leptotene. These positive heteropycnotic parts usually associated during pachytene, and they were still clearly visible during diplotene. Some chromosomes still showed the positive heteropycnosis during diakinesis and metaphase II (Fig. 2j). During meiosis, we did not detect clearly visible heteromorphic bivalents (Fig. 2i). Two pairs of 18S rDNA clusters were detected by FISH in this species (Fig. 2h, i, k). The 18S rDNA probe signals were localized at the terminal parts of the first and third pairs of chromosomes (Fig. 2f).

Rhampsinitus leighi Pocock, 1903

We identified two cytotypes from two different localities in this species. The diploid numbers of chromosomes in both of them were 26 (Fig. 3a, b), but the morphology of the chromosomes and the number and position of 18S rDNA clusters differed.

The karyotype of cytotype I from Vernon Crookes comprised six pairs of metacentric, five pairs of submetacentric (pairs No. 2, 4, 8, 9, 13), and one pair of subtelocentric (pair No. 5) chromosomes. Moreover, the karyotype of cytotype I included one heteromorphic bivalent (pair No. 11) composed of one metacentric and one submetacentric chromosome. The chromosomes of the first two pairs were distinctly longer (6.18 % and 5.67 % of the diploid set) than the next chromosomes, which gradually decrease in length from 4.29 % to 2.42 % of the diploid set (Suppl. material 1). In some chromosomes, positive heteropycnotic blocks were visible at the position of the centromere in mitotic metaphases, and in some pairs heteropycnotic parts expanded to a large proportion of the arms (Fig. 3a). Large heteropycnotic blocks were also visible during the early meiotic phases such as zygotene, pachytene (Fig. 3c) and diplotene (Fig. 3e). Later, during metaphase I and metaphase II the whole chromosome seemed to be isopycnotic. We only found bivalents with one chiasma in both males from Vernon Crookes during the diplotene (Fig. 3e, Suppl. material 2). Altogether seven signals of 18S rDNA clusters were detected by FISH in cytotype I (Fig. 3f). All of them were localized in the terminal position of the chromosomes. Interestingly, we identified a heterozygous state in two pairs of chromosomes (Fig. 3a). We identified a small signal in the terminal position of one chromosome of pair No. 5 (Fig. 3a), which is also visible during pachytene (Fig. 3d) and diplotene (Fig. 3f). In pair No. 11 we identified an expanded part of one chromosome that covered 18S rDNA clusters, which makes the bivalent heteromorphic (compare Fig. 3c, d, e, f).

The karyotype of cytotype II from Silaka (Fig. 3b) comprised seven pairs of metacentric and five pairs of submetacentric (pairs No. 3, 7, 9, 10, 12) chromosomes. Similar to cytotype I, cytotype II also included one heteromorphic bivalent (pair

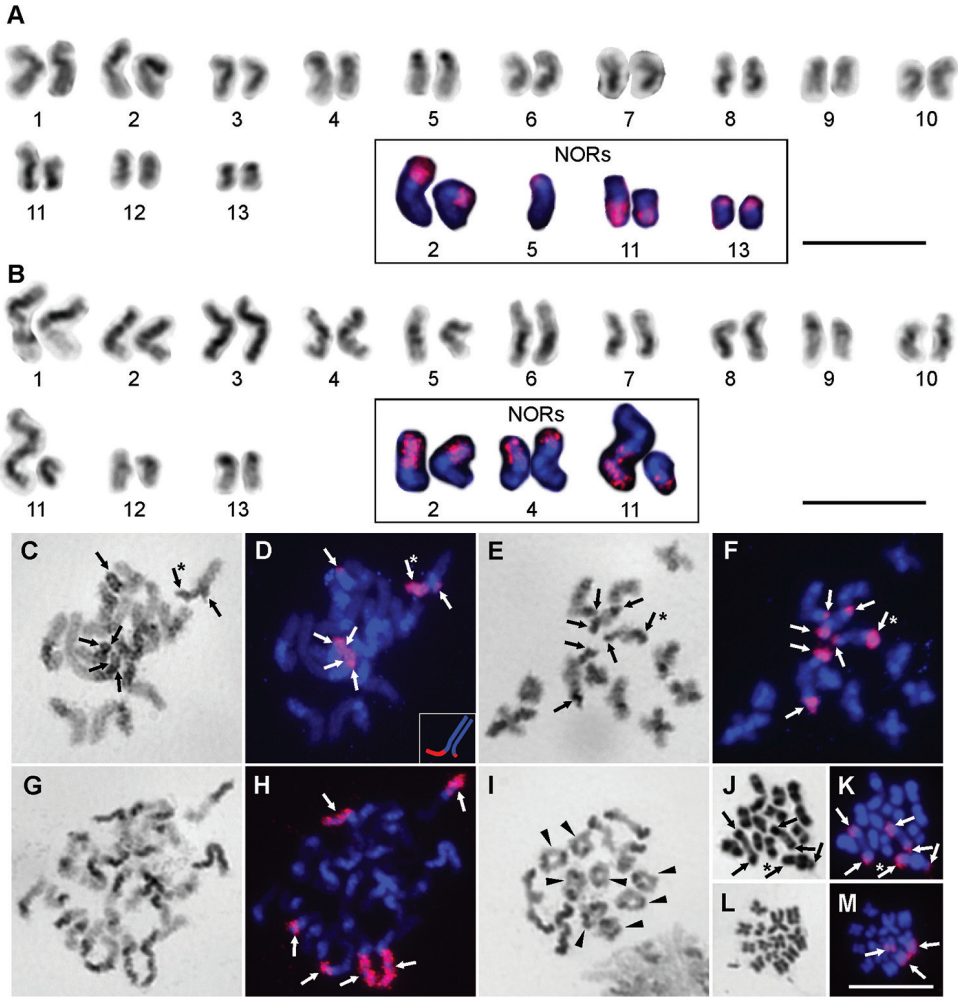

Figure 3. Chromosomes of *Rhampsinitus leighi* (2n=26) from Vernon Crookes (**A, C–F**) and from Silaka (**B, G–M**) after Giemsa staining (**A–C, E, G, I, J, L**) and after FISH with 18S rDNA (**A** and **B** partly, **D, F, H, K, M**). **A, B** karyotypes of males based on mitotic metaphase **C, D** pachytene with heterozygous bivalent (asterisk), bottom right inset shows a reconstruction of the heteromorphic bivalent **E, F** diplotene with heterozygous bivalent (asterisk) **G, H** mitotic prophase **I** diplotene, arrowheads indicate bivalents with two chiasmata. **J, K** diakinesis **L, M** one sister cell of metaphase II. White arrows indicate the position of 18S rDNA and black arrows indicate the same position after Giemsa staining. Asterisks indicate heteromorphic bivalent with large signal of rDNA. Bar = 10 μm.

No. 11) composed of one metacentric and one submetacentric chromosome (Suppl. material 1). The chromosomes of the first three pairs were slightly longer (6.04 %, 5.66 %, and 5.10 % of the diploid set) and the following chromosomes gradually decreased in length from 4.42 % to 2.21 % of the diploid set (Suppl. material 1). Also in cytotype II, positive heteropycnotic blocks were visible at the position of the

centromere of the chromosomes during mitotic prometaphase (Fig. 3g) and metaphase, and in some pairs the heteropycnotic parts expanded to a large portion of the arms (Fig. 3b). Large heteropycnotic blocks were also visible during the early meiotic phases until diplotene (Fig. 3i). Later, during diakinesis, metaphase I and metaphase II, the whole chromosome seemed to be isopycnotic (Figs 3j, l). We found from four to eight bivalents with two chiasmata in the male from Silaka during diplotene (Fig. 3i, Suppl. material 2). Six signals of 18S rDNA clusters were detected by FISH in cytotype II (Figs 3b, h, k). All of them were localized in the terminal position of the chromosomes (Figs. 3b, h, k, m). We detected a heterozygous state in one pair of chromosomes (Fig. 3b). In pair No. 11 we identified an expanded part of one chromosome that covered 18S rDNA clusters, which makes the bivalent heteromorphic (compare Fig. 3j, k).

Rhampsinitus qachasneki Kauri, 1962

The diploid number of chromosomes in this species was 24 (Fig. 4a, b). The karyotype of the male comprised six pairs of metacentric, four pairs of submetacentric (pair No. 2, 6, 9, 11), and two pairs of subtelocentric (pair No. 8, 12) chromosomes (Suppl. material 1). The chromosomes gradually decreased in length from 5.74 % to 2.87 % of the diploid set (Suppl. material 1). In some chromosomes, positive heteropycnotic blocks were visible at the position of the centromere of mitotic metaphases, but these blocks did not expand across the whole arms of the chromosomes. Large heteropycnotic blocks were also visible during the early phases of meiosis such as zygotene, pachytene and diplotene; however, later during the metaphase I and metaphase II

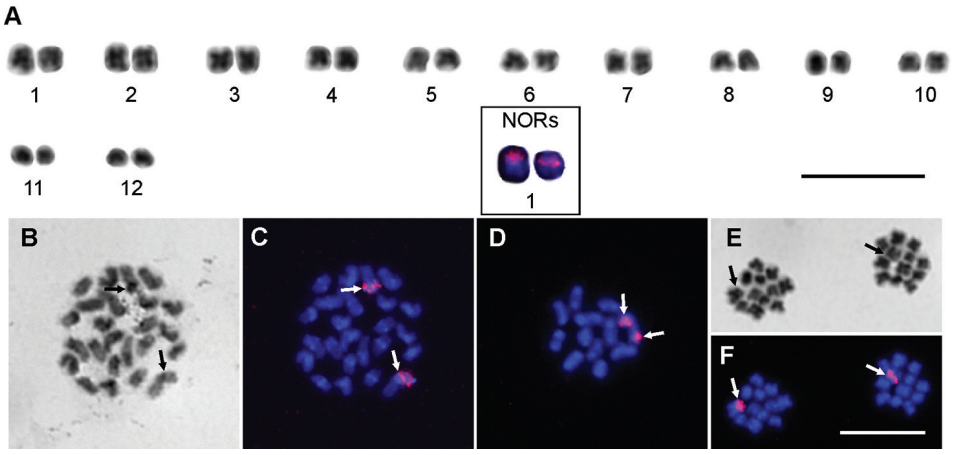

Figure 4. Chromosomes of *Rhampsinitus qachasneki* (2n=24) males after Giemsa staining (**A, B, E**) and after FISH with 18S rDNA (**A** partly, **C, D, F**). **A** male karyotype based on two sister cells of metaphase II **B, C** mitotic metaphase **D** diakinesis **E, F** two sister cells of metaphase II. White arrows indicate the position of 18S rDNA and black arrows indicate the same position after Giemsa staining. Bar = 10 μm.

the entire chromosomes seemed to be isopycnotic (Fig. 4e). One pair of 18S rDNA clusters was detected by FISH in the terminal part of one pair of chromosomes in this species (pair No. 1) (Fig. 4a, c, d, f).

Discussion

Phalangiid harvestmen have a centre of diversity in the Northern Hemisphere (Giribet and Kury 2007), and their presence in South Africa is traditionally explained by dispersal from the Mediterranean region (Staręga 1984). In this study, we focus on the genera *Guruia* and *Rhampsinitus* that are endemic to the Afrotropical biogeographical region (Staręga 1984), in order to provide further information about the cytogenetics of the family Phalangiidae from previously understudied parts of the World.

The results of the cytogenetic analyses of *Guruia africana* (2n=24) and four species of the genus *Rhampsinitus* (2n=24, 26, 34) show that the African taxa share the basic karyotype characteristics with the Northern Hemisphere phalangiids analysed so far (13 species). However, detailed information about the karyotypes is only available in three species, whereas the records for the remaining species only comprise chromosome numbers (see Tsurusaki et al. 2017). In Phalangiidae the chromosome number ranges from 2n=16 in *Oligolophus tridens* (C.L. Koch, 1836) (Tsurusaki 2007) to 2n=36 in *Rilaena triangularis* (Herbst, 1799) (Sokolow 1930), with the karyotypes predominated by biarmed chromosomes. The most frequent number of chromosomes in this group is 2n=24 (8 species) and 2n=32 (5 species) (Tsurusaki et al. 2017, present study). The hypothesised ancestral number of chromosomes in the order Opiliones is around 30 (Svojanovská et al. 2016). The low numbers of the chromosomes in Eupnoi may be explained in two different ways. The first hypothesis assumes independent reductions of the proposed ancestral number of chromosomes shared by the whole order in each family of Phalangioidea. The family Phalangiidae still harbours some species with an ancestral number close to 30 (see Tsurusaki et al. 2017). Therefore the 2n=34, found in *Rhampsinitus capensis* (the highest number of chromosomes), would represent a karyotype derived by centric fissions, since one-armed chromosomes (subtelocentrics and acrocentrics) are still common in this species' karyotype. The rest of *Rhampsinitus* species (2n=24 and 26) and *Guruia africana* (2n=24) would thus possess karyotypes derived by chromosome rearrangements, like fusions and inversions. This hypothesis would imply an independent reduction of the diploid number in the families Protolophidae (2n=18–22) and Sclerosomatidae (2n=10–24) (see Tsurusaki et al. 2017). The diploid number 2n=30 is also known from the family Caddidae (Tsurusaki and Cokendolpher 1990), a sister group to the superfamily Phalangioidea (Giribet et al. 2010), which would further indicate that the diploid number close to 30 probably represents the ancestral state in Eupnoi.

Alternatively, the second hypothesis presumes the ancestral number of chromosomes of the whole suborder Eupnoi being between 20 and 24 (the most frequent number in this suborder; see Tsurusaki et al. 2017). In this case, all higher numbers of

chromosomes would represent a derived state driven by chromosomal fissions. However, the direction of karyotype evolution in Eupnoi can only be resolved by interpreting the cytogenetic data within a wider phylogenetic framework. Unfortunately, a robust phylogenetic scheme for Eupnoi is not currently available.

The data concerning the number and position of the 18S rDNA clusters using the FISH technique in arachnids are still limited (see Svojanovská et al. 2016), and most of them come from scorpions (e.g. Mattos et al. 2014, Sadílek et al. 2015). The results suggest that the position of 18S rDNA clusters can be highly conserved among different genera/species of scorpions, e.g. in a terminal position of the genera *Rhopalurus* Thorell, 1876 and *Tityus* C. L. Koch, 1836 (e.g. Mattos et al. 2014) from South America, or in an interstitial position in *Androctonus* Ehrenberg, 1828 from Africa and Asia (Sadílek et al. 2015).

Contrastingly, the variability of the number of 18S rDNA sites may be high in some arachnids (e.g. Forman et al. 2013). However, the interpretation of the differences in the number of 18S rDNA clusters requires a precise knowledge of both the ancestral number of these clusters and the phylogeny of the group, otherwise the course of the karyotype evolution cannot be specified (e.g. Svojanovská et al. 2016), as in case of our results. There is no information available directly concerning the 18S rDNA sites of phalangiid harvestmen in the literature that would allow a comparison to our results. The only record available for the entire suborder Eupnoi belongs to the sclerosomatid harvestman *Psathyropus tenuipes* (L. Koch, 1878), which possesses one NOR (a transcription unit that encodes 28S, 5.8S and 18S rRNAs) in the diploid set (Gorlov and Tsurusaki 2000b). However, the NOR was visualised by silver staining, which only detects the NORs that are active during the preceding interphase (Miller et al. 1976), and thus the real number may be underestimated (Forman et al. 2013). Generally, it is hypothesized that one pair of NORs, as well as 18S rDNA sites, represents the ancestral state in the class Arachnida (Forman et al. 2013). We detected one pair of 18S rDNA clusters only in one species included in the analyses (*Rhampsinitus qachasneki*), while the number of clusters varied between 2 in *R. qachasneki* and 7 in *R. leighi*. The high variability in the number, position and even size of rDNA clusters between and also within the analysed species suggests intensive chromosomal changes and rapid evolutionary dynamics of 18S rDNA clusters on chromosomes of South African phalangiids. *Rhampsinitus* has a terminal position of 18S rDNA clusters, similarly to basal harvestmen of the suborder Cyphophthalmi (Svojanovská et al. 2016), and other arachnid groups such as spiders (e.g. Král et al. 2013), amblypygids (Paula-Neto et al. 2013), and South American scorpions (e.g. Mattos et al. 2014). The proximity to the telomeric region facilitates the efficiency of ectopic recombination (Goldman and Lichten 1996) and the preferential replacement of rDNA genes into a new subtelomeric position (Nguyen et al. 2010), as detected in all *Rhampsinitus* species. The effect of ectopic recombination may also be increased due to the association of positive heteropycnotic areas of different chromosomes during the meiotic prophase (e.g. Figs 1b, 3c). However, *Guruia africana* has 18S rDNA clusters placed in an interstitial position of one arm of biarmed chromosomes, which may be a consequence of another chromo-

some rearrangements, transposable element insertions or ectopic recombination (see Cabrero and Camacho 2008). Some 18S rDNA clusters in *Rhampsinitus* species were longer (see Fig. 3a, b), likely due to the presence of a higher number of copies of the 18S rDNA elements. This observation can be also explained by the duplication of the chromosome part including this cluster, or alternatively as a consequence of an insertion of transposable elements, which potentially accelerates the genomic reorganization after a speciation event (e.g. Symonová et al. 2013).

We also detected intraspecific variability and a heterozygous state in *R. capensis* and *R. leighi* that may correspond to population polymorphism. The low dispersal capability, typical for most harvestmen (Giribet and Kury 2007), can lead to a genetic differentiation among the populations on a relatively small geographical scale in many arachnid groups (e.g. Kotrbová et al. 2016, Opatova et al. 2016). The intraspecific variability in the number of 18S rDNA clusters between the populations from the two localities of *R. leighi* could thus be explained as an independent accumulation of the chromosomal changes at each locality, as is assumed for the wolf spider *Wadicosa fidelis* (O. Pickard-Cambridge, 1872) (Forman et al. 2013). However, the harvestmen are also an understudied group, so the differences in 18S rDNA clusters and karyotypes between both populations could indicate a possible existence of cryptic species that are common in this order (e.g. Murienne et al. 2010). Given this scenario, the presence of a heterozygous state could be a result of crossbreeding.

Interestingly, we also identified heteromorphic bivalents in both populations of *R. leighi* caused by distinctly enlarged 18S rDNA clusters on one chromosome. Similarly, the heteromorphic bivalents were also detected in *R. capensis*, but the enlarged rDNA sites were only identified in females. The heterozygous states in the size of 18S rDNA clusters could indicate the existence of XY sex chromosomes in *R. leighi* and ZW sex chromosomes in *R. capensis*. The XY sex chromosome system is known in the harvestmen *Sabacon makinoi* Suzuki, 1949 (Dyspnoi, Sabaconidae) (Tsurusaki 1989) and from 13 species of Sclerosomatidae (Eupnoi) (see Tsurusaki et al. 2017). In other arachnid orders the XY system is common in ticks (Oliver 1977), but it was also identified in few species from different spider families (Král et al. 2006, Řezáč et al. 2006), and some species of the pseudoscorpion family Neobisiidae (Troiano 1990, 1997, Šťáhlavský and Zaragoza 2008). The ZW system is completely exceptional in arachnids (Tsurusaki 2007). It is described only from *Mitopus morio* and it is hypothesized in *Oliogloplus aspersus* (Karsch, 1881) (both Phalangiidae) (Tsurusaki and Cokendolpher 1990). However, the ZW system in *M. morio* was described from a low quality single standard stained mitotic metaphase of one female (see Tsurusaki and Cokendolpher 1990). In this study, heteromorphic bivalents were detected in both females of *R. capensis* (Fig. 2b, d); however, we did not obtain any information about meiosis and chromosome pairing in males. The number and position of 18S rDNA clusters in mitotic metaphases of the analysed males does not indicate the presence of homomorphic chromosomes that would correspond to the heteromorphic state in females. Similarly, the evidence of a XY system in *R. leighi* is unclear, since we have no information concerning the organization of 18S rDNA clusters in females.

The size variation of rDNA clusters on homologous chromosomes is frequently considered a result of unequal crossing-over or gene duplication that does not represent sex-specific differences (e.g. Foresti et al. 1981). However, when the sex locus is located close to the rDNA, the recombination in this region can be limited and thus accelerate the evolution of the sex chromosome(s) (Reed and Phillips 1997). For that reason, we cannot rule out that the heteromorphic bivalents in *R. leighi* and *R. capensis* may represent the initial stages of sex differentiation from the ancestral nondifferentiated state typical for harvestmen (Tsurusaki 2007). Our analysis indicates that the differences in morphology and size of the chromosomes, considered in some harvestmen to be the sex chromosomes (e.g. Tsurusaki and Cokendolpher 1990), may in fact represent the variability in rDNA clusters induced by ectopic recombination, unequal crossing-over or gene duplication mentioned above. It would be necessary to analyse the species with heteromorphic sex chromosomes in order to know whether this heteromorphism is also associated with the rDNA. These findings bring a new light to our understanding of the presence of heteromorphic bivalents in harvestmen, and they highlight the necessity for the implementation of molecular cytogenetic techniques such as CGH in order to assess the presence of sex chromosomes in this group.

Acknowledgment

We are grateful to J.A. Neethling for technical support during the field work and to J. Štundlová for technical assistance during probe preparation. The project was supported by the Czech Republic Ministry of Education, Youth and Sports grant SVV 260 434 / 2017 and by grants from the National Research Foundation of South Africa in the Knowledge Interchange and Collaboration programme (No.'s 97495 and 105318) and Competitive Funding for Rated Researchers programme (No. 95569) to the last author. The specimens were collected under the collecting permits OP 4072/2016 from Ezemvelo KZN Wildlife (KwaZulu-Natal Province) and RA-0201 from the Eastern Cape Parks and Tourism Agency (Eastern Cape Province). Microscopy was performed in the Laboratory of Confocal and Fluorescence Microscopy, co-financed by the European Regional Development Fund and the state budget of the Czech Republic. Project no. CZ.1.05/4.1.00/16.0347 and CZ.2.16/3.1.00/21515.

References

Cabral de Mello DC, Moura RC, Martins C (2011) Cytogenetic mapping of rRNAs and histone H3 genes in 14 species of *Dichotomius* (Coleoptera, Scarabaeidae, Scarabaeinae) beetles. Cytogenetic and Genome Research 134: 127–135. https://doi.org/10.1159/000326803

Cabrero J, Camacho JPM (2008) Location and expression of ribosomal RNA genes in grasshoppers: abundance of silent and cryptic loci. Chromosome Research 16: 595–607. https://doi.org/10.1007/s10577-008-1214-x

Dunlop JA, Anderson LI, Kerp H, Hass H (2004) A harvestman (Arachnida, Opiliones) from the Early Devonian Rhynie cherts, Aberdeenshire, Scotland. Transactions of the Royal Society of Edinburgh: Earth Sciences. 94: 341–354. https://doi.org/10.1017/S0263593300000730

Foresti F, Almeida Toledo LF, Toledo SA (1981) Polymorphic nature of nucleolus organizer regions in fishes. Cytogenetics and Cell Genetics 31: 137–144. https://doi.org/10.1159/000131639

Forman M, Nguyen P, Hula V, Král J (2013) Sex chromosome pairing and extensive NOR polymorphism in *Wadicosa fidelis* (Araneae: Lycosidae). Cytogenetic and Genome Research 141: 43–49. https://doi.org/10.1159/000351041

Fuková I, Nguyen P, Marec F (2005) Codling moth cytogenetics: karyotype, chromosomal location of rDNA, and molecular differentiation of sex chromosomes. Genome 48: 1083–1092. https://doi.org/10.1139/G05-063

Giribet G, Kury AB (2007) Phylogeny and Biogeography. In: Pinto-da-Rocha R, Machado G, Giribet G (Eds) Harvestmen: The Biology of Opiliones. Harvard University Press, Cambridge, MA, USA, 62–87.

Giribet G, Vogt L, González AP, Sharma P, Kury AB (2010) A multilocus approach to harvestman (Arachnida: Opiliones) phylogeny with emphasis on biogeography and the systematics of Laniatores. Cladistics 26: 408–437. https://doi.org/10.1111/j.1096-0031.2009.00296.x

Goldman AS, Lichten M (1996) The efficiency of meiotic recombination between dispersed sequences in *Saccharomyces cerevisiae* depends upon their chromosomal location. Genetics 144: 43–55. http://www.genetics.org/content/144/1/43.short

Gorlov IP, Tsurusaki N (2000a) Staggered clines in a hybrid zone between two chromosome races of the harvestman *Gagrellopsis nodulifera* (Arachnida: Opiliones). Evolution 54: 176–190. https://doi.org/10.1111/j.0014-3820.2000.tb00018.x

Gorlov IP, Tsurusaki N (2000b) Morphology and meiotic/mitotic behavior of B chromosomes in a Japanese harvestman, *Metagagrella tenuipes* (Arachnida: Opiliones): No evidence for B accumulation mechanisms. Zoological Science 17: 349–355. https://doi.org/10.2108/jsz.17.349

Grzywacz B, Maryanska-Nadachowska A, Chobanov DP, Karamysheva T, Warchalowska-Sliwa E (2011) Comparative analysis of the location of rDNA in the Palaearctic bushcricket genus *Isophya* (Orthoptera: Tettigoniidae: Phaneropterinae). European Journal of Entomology 108: 509–517. https://doi.org/10.14411/eje.2011.066

Kotrbová J, Opatova V, Gardini G, Šťáhlavský F (2016) Karyotype diversity of pseudoscorpions of the genus *Chthonius* (Pseudoscorpiones, Chthoniidae) in the Alps. Comparative Cytogenetics 10: 325–345. https://doi.org/10.3897/CompCytogen.v10i3.8906

Král J, Kořínková T, Krkavcová L, Musilová J, Forman M, Herrera IMA, Haddad CR, Vítková M, Henriques S, Vargas JGP, Hedin M (2013) Evolution of karyotype, sex chromosomes, and meiosis in mygalomorph spiders (Araneae: Mygalomorphae). Biological Journal of the Linnean Society 109: 377–408. https://doi.org/10.1111/bij.12056

Král J, Musilová J, Šťáhlavský F, Řezáč M, Akan Z, Edwards RL, Coyle FA, Almerje CR (2006) Evolution of the karyotype and sex chromosome systems in basal clades of araneomorph spiders. Chromosome Research 14: 859–880. https://doi.org/10.1007/s10577-006-1095-9

Kury AB (2013) Order Opiliones Sundevall, 1833. In: Zhang Z-Q (Ed.) Animal Biodiversity: An Outline of Higher-level Classification and Survey of Taxonomic Richness (Addenda 2013). Zootaxa 3703: 27–33. https://doi.org/10.11646/zootaxa.3703.1.7

Kury AB (2017) Classification of Opiliones. Museu Nacional/UFRJ website. Online at: http://www.museunacional.ufrj.br/mndi/Aracnologia/opiliones.html

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position of chromosomes. Hereditas 52: 201–220. https://doi.org/10.1111/j.1601-5223.1964.tb01953.x

Lotz LN (2009) Harvestmen (Arachnida: Opiliones) in southern Africa - an annotated catalogue with notes on distribution. Navorsinge van die Nasionale Museum, Bloemfontein 25: 1–45.

Mattos VF, Carvalho LS, Cella DM, Schneider MC (2014) Location of 45S ribosomal genes in mitotic and meiotic chromosomes of buthid scorpions. Zoological Science 31: 603–607. https://doi.org/10.2108/zs140005

Miller DA, Dew V, Tantravahi R, Miller O (1976) Suppression of human nucleolar organizer activity in mouse human somatic hybrid cells. Experimental Cell Research 101: 235–243. https://doi.org/10.1016/0014-4827(76)90373-6

Murienne J, Karaman I, Giribet G (2010) Explosive evolution of an ancient group of Cyphophthalmi (Arachnida: Opiliones) in the Balkan Peninsula. Journal of Biogeography 37: 90–102. https://doi.org/10.1111/j.1365-2699.2009.02180.x

Nguyen P, Sahara K, Yoshido A, Marec F (2010) Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). Genetica 138: 343–354. https://doi.org/10.1007/s10709-009-9424-5

Oliver JH (1977) Cytogenetics of mites and ticks. Annual Reviews of Entomology 22: 407–429. https://doi.org/10.1146/annurev.en.22.010177.002203

Opatova V, Bond JE, Arnedo MA (2016) Uncovering the role of the Western Mediterranean tectonics in shaping the diversity and distribution of the trap-door spider genus *Ummidia* (Araneae, Ctenizidae). Journal of Biogeography 43: 1955–1966. https://doi.org/10.1111/jbi.12838

Panzera Y, Pita S, Ferreiro MJ, Ferrandis I, Lages C, Pérez R, Silva AE, Guerra M, Panzera F (2012) High dynamics of rDNA cluster location in kissing bug holocentric chromosomes (Triatominae, Heteroptera). Cytogenetic and Genome Research 138: 56–67. https://doi.org/10.1159/000341888

Paula-Neto E, Araujo D, Carvalho LS, Cella DM, Schneider MC (2013) Chromosomal characteristics of a Brazilian whip spider (Amblypygi) and evolutionary relationships with other arachnid orders. Genetics and Molecular Research 12: 3726–3734. https://doi.org/10.4238/2013.September.19.3

Pinto-da-Rocha R, Giribet G (2007) Taxonomy. In: Pinto-da-Rocha R, Machado G, Giribet G (Eds) Harvestmen: The Biology of Opiliones. Harvard University Press, Cambridge, MA, USA, 88–246.

Reed KM, Phillips RB (1997) Polymorphism of the nucleolus organizer region (NOR) on the putative sex chromosomes of Arctic char (*Salvelinus alpinus*) is not sex related. Chromosome Research 5: 221–227. https://doi.org/10.1023/A:1018411417816

Rodríguez-Gil SG, Mola LM (2010) Chromosome complement and meiosis of *Holmbergiana weyenberghii* (Opiliones: Sclerosomatidae: Gagrellinae) from Argentina. Revista de la Sociedad Entomológica Argentina 69: 164–170. https://www.biotaxa.org/RSEA/article/view/28588

Řezáč M, Král J, Musilová J, Pekár S (2006) Unusual karyotype diversity in the European spiders of the genus *Atypus* (Araneae: Atypidae). Hereditas 143: 123–129. https://doi.org/10.1111/j.2006.0018-0661.01949.x

Sadílek D, Nguyen P, Koç H, Kovařík F, Yağmur EA, Šťáhlavský F (2015) Molecular cytogenetics of *Androctonus* scorpions: an oasis of calm in the turbulent karyotype evolution of the diverse family Buthidae. Biological Journal of the Linnean Society 115: 69–75. https://doi.org/10.1159/000445863

Sakamoto Y, Zacaro AA (2009) LEVAN, an ImageJ plugin for morphological cytogenetic analysis of mitotic and meiotic chromosomes. Initial version. http://rsbweb.nih.gov/ij/

Schneider MC, Zacaro AA, Pinto-da-Rocha R, Candido DM, Cella DM (2008) Conventional and ultrastructural analyses of the chromosomes of *Discocyrtus pectinifemur* (Opiliones, Laniatores, Gonyleptidae). Journal of Zoological Systematics and Evolutionary Research 47: 203–207. https://doi.org/10.1111/j.1439-0469.2008.00490.x.

Sember A, Bohlen J, Šlechtová V, Altmanová M, Symonová R, Ráb P (2015) Karyotype differentiation in 19 species of river loach fishes (Nemacheilidae, Teleostei): extensive variability associated with rDNA and heterochromatin distribution and its phylogenetic and ecological interpretation. BMC Evolutionary Biology 15: 251. https://doi.org/10.1186/s12862-015-0532-9

Sokolow I (1930) Untersuchungen über die Spermatogenese bei den Arachniden. IV. Über die spermatogenese der Phalangiden (Opiliones). Zeitschrift für Zellforschung und Mikroskopische Anatomie 10: 164–194. https://doi.org/10.1007/BF02450640

Staręga W (1984) Revision der Phalangiidae (Opiliones), III. Die afrikanischen Gattungen der Phalangiinae, nebst Katalog aller afrikanischen Arten der Familie. Annales Zoologici (Polska Akademia Nauk) 38: 1–79. http://rcin.org.pl/Content/58500/WA058_10835_P255-T38_Annal-Zool-Nr-1.pdf

Svojanovská H, Nguyen P, Hiřman M, Tuf IH, Wahab RA, Haddad CR, Šťáhlavský F (2016) Karyotype evolution in harvestmen of the suborder Cyphophthalmi (Opiliones). Cytogenetic and Genome Research 148: 227–236. https://doi.org/10.1159/000445863

Symonová R, Majtánová Z, Sember A, Staaks GBO, Bohlen J, Freyhof J, Rábová M, Ráb P (2013) Genome differentiation in a species pair of coregonine fishes: an extremely rapid speciation driven by stress-activated retrotransposons mediating extensive ribosomal DNA multiplications. BMC Evolutionary Biology 13: 42. https://doi.org/10.1186/1471-2148-13-42

Šťáhlavský F, Král J (2004) Karyotype analysis and achiasmatic meiosis in pseudoscorpions of the family Chthoniidae (Arachnida: Pseudoscorpiones). Hereditas 140: 49–60. https://doi.org/10.1111/j.1601–5223.2004.01783.x

Traut W (1976) Pachytene mapping in the female silkworm *Bombyx mori* L. (Lepidoptera). Chromosoma 58: 275–284. https://doi.org/10.1007/BF00292094

Troiano G (1990) Karyotype and male meiosis of four species of *Roncus* L. Koch, 1873 (Pseudoscorpionida, Neobisiidae). Bolletino di Zoologia 57: 1–9. https://doi.org/10.1080/11250009009355666

Troiano G (1997) Further studies on karyology of the pseudoscorpions of the gen. *Roncus*: the karyotype of *Roncus gestroi* and *Roncus belluatii*. Caryologia 50: 271–279. https://doi.org/10.1080/00087114.1997.10797401

Tsurusaki N (1989) Geographic variation of chromosomes in *Sabacon makinoi* Suzuki (Arachnida, Opiliones, Sabaconidae). Bulletin of the Biogeographical Society of Japan 44: 111–116.

Tsurusaki N (1993) Geographic variation of the number of B-chromosomes in *Metagagrella tenuipes* (Opiliones, Phalangiidae, Gagrellinae). Memoirs of the Queensland Museum 33: 659–665.

Tsurusaki N (2007) Cytogenetics. In: Pinto-da-Rocha R, Machado G, Giribet G (Eds) Harvestmen: The Biology of Opiliones. Harvard University Press, Cambridge, MA, USA, 266–279.

Tsurusaki N, Cokendolpher J (1990) Chromosomes of sixteen species of harvestmen (Arachnida, Opiliones, Caddidae and Phalangididae). Journal of Arachnology 18: 151–166. http://www.jstor.org/stable/3705833

Tsurusaki N, Murakami M, Shimokawa K (1991) Geographic variation of chromosomes in the Japanese harvestman, *Gagrellopsis nodulifera* with special reference to hybrid zone in western Honshu. Zoological Science 8: 265–275.

Tsurusaki N, Svojanovská H, Schöenhofer A, Šťáhlavský F (2017) The harvestmen cytogenetic database. Version 4.0. Online at: http://www.arthropodacytogenetics.bio.br/harvestmen-database/index.html

White MJD (1973) Animal cytology and evolution, 3rd edn., Cambridge University Press.

Zaragoza JA, Šťáhlavský F (2008) A new *Roncus* species (Pseudoscorpiones: Neobisiidae) from Montseny Natural Park (Catalonia, Spain), with remarks on karyology. Zootaxa 1693: 27–40.

Supplementary material 1

Table S1

Authors: František Šťáhlavský, Vera Opatova, Pavel Just, Leon N. Lotz, Charles R. Haddad

Data type: Microsoft Excel Worksheet (.xls).

Explanation note: Measurements of the relative chromosome length (% RCL) and arm ratio of South African harvestmen from family Phalangiidae (± standard deviation). Numbers of measured metaphases are given in brackets following the species names.

Link: https://doi.org/10.3897/CompCytogen.12.21744.suppl2

Supplementary material 2

Table S2

Authors: František Šťáhlavský, Vera Opatova, Pavel Just, Leon N. Lotz, Charles R. Haddad

Data type: Microsoft Excel Worksheet (.xls).

Explanation note: Numbers of nuclei with different numbers of chiasmata in diplotene of *Rhampsinitus leighi* (2n = 26) from Vernon Crookes (cytotype I) and Silaka (cytotype II).

Link: https://doi.org/10.3897/CompCytogen.12.21744.suppl3

CompCytogen 12(1): 61–82 (2018)
doi: 10.3897/CompCytogen.v12i1.21554
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Variation in chromosome number and breeding systems: implications for diversification in *Pachycereus pringlei* (Cactaceae)

Carina Gutiérrez-Flores[1], José L. León-de la Luz[2], Francisco J. García-De León[3], J. Hugo Cota-Sánchez[1]

1 *Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon, SK, S7N5E2, Canada* **2** *HICB Herbarium, Centro de Investigaciones Biológicas del Noroeste, Instituto Politécnico Nacional 195, Playa Palo de Santa Rita Sur, C.P. 23096, La Paz, B.C.S., México* **3** *Laboratorio de Genética para la Conservación, Centro de Investigaciones Biológicas del Noroeste, Instituto Politécnico Nacional 195, Playa Palo de Santa Rita Sur, C.P. 23096, La Paz, B.C.S., México*

Corresponding author: *J. Hugo Cota-Sánchez* (hugo.cota@usask.ca)

Academic editor: *I. Kirov* | Received 10 October 2017 | Accepted 30 January 2018 | Published 14 February 2018

http://zoobank.org/E86A06ED-10E7-4F4B-B409-F3365A41A56F

Citation: Gutiérrez-Flores C, León-de la Luz JL, García-De León FJ, Cota-Sánchez JH (2018) Variation in chromosome number and breeding systems: implications for diversification in *Pachycereus pringlei* (Cactaceae). Comparative Cytogenetics 12(1): 61–82. https://doi.org/10.3897/CompCytogen.v12i1.21554

Abstract

Polyploidy, the possession of more than two sets of chromosomes, is a major biological process affecting plant evolution and diversification. In the Cactaceae, genome doubling has also been associated with reproductive isolation, changes in breeding systems, colonization ability, and speciation. *Pachycereus pringlei* (S. Watson, 1885) Britton & Rose, 1909, is a columnar cactus that has long drawn the attention of ecologists, geneticists, and systematists due to its wide distribution range and remarkable assortment of breeding systems in the Mexican Sonoran Desert and the Baja California Peninsula (BCP). However, several important evolutionary questions, such as the distribution of chromosome numbers and whether the diploid condition is dominant over a potential polyploid condition driving the evolution and diversity in floral morphology and breeding systems in this cactus, are still unclear. In this study, we determined chromosome numbers in 11 localities encompassing virtually the entire geographic range of distribution of *P. pringlei*. Our data revealed the first diploid (2n = 22) count in this species restricted to the hermaphroditic populations of Catalana (ICA) and Cerralvo (ICE) Islands, whereas the tetraploid (2n = 44)

condition is consistently distributed throughout the BCP and mainland Sonora populations distinguished by a non-hermaphroditic breeding system. These results validate a wider distribution of polyploid relative to diploid individuals and a shift in breeding systems coupled with polyploidisation. Considering that the diploid base number and hermaphroditism are the proposed ancestral conditions in Cactaceae, we suggest that ICE and ICA populations represent the relicts of a southern diploid ancestor from which both polyploidy and unisexuality evolved in mainland BCP, facilitating the northward expansion of this species. This cytogeographic distribution in conjunction with differences in floral attributes suggests the distinction of the diploid populations as a new taxonomic entity. We suggest that chromosome doubling in conjunction with allopatric distribution, differences in neutral genetic variation, floral traits, and breeding systems has driven the reproductive isolation, evolution, and diversification of this columnar cactus.

Keywords
Diploid, Cactaceae, cryptic speciation, cytogeography, karyotype, *Pachycereus pringlei*, polyploidy

Introduction

Polyploidy and hybridisation are major biological events in plant evolution and speciation (Grant 1981, Wendel and Doyle 2005), often leading to complex patterns of genetic diversity, reproductive isolation, and discrepancy in breeding systems (De Wet 1971, Tate et al. 2005, Marques et al. 2014). Therefore, studies focusing on changes in chromosome numbers are instrumental to identify reproductive variability and distribution of different cytotypes at the intra- and inter-population levels and to make inferences about the origins of polyploids.

The Cactaceae, a family with approximately 1,430 species (Hunt et al. 2006), exhibits an extensive habitat radiation and reproductive versatility linked to striking specialized floral morphology (Cota-Sánchez and Croutch 2008, Almeida et al. 2013) and variation in chromosome numbers (Cota 1991, Baker et al. 2009a). As stated by Stebbins (1950, p. 369), "polyploidy ... is one of the quickest biological process producing totally different and more vigorous and well-adapted genotypes." In the same way, polyploidy, along with variation in breeding systems, has been considered an important factor directing the evolutionary history and disparity of the Cactaceae, often resulting in the formation of new species (Baker and Pinkava 1987, 1999, Baker 2002, 2006). Remarkably, approximately 28% (154 out of 551 species) of cacti cytologically investigated have increased genome dosage, primarily in subfamily Opuntioideae (Rebman and Pinkava 2001). Genome doubling prompts the evolution of some sexual systems in the Cactaceae, i.e., gynodioecy and trioecy (Rebman and Pinkava 2001), and additional chromosome sets have been correlated with physiology and differences in morphological and geographic distribution. For example, polyploidy in cacti allows the adaptation to freezing temperatures (Cota-Sánchez 2002), taxonomic diversification (Majure et al. 2012), the colonization of higher latitudes (Cota and Philbrick 1994), wider geographical range (Barthlott and Taylor 1995, Cota-Sánchez and Bomfim-Patrício 2010), and acts as a predictor of responses to environment and evolution (Segura et al. 2007). However, for many taxa with wide ecological and geographic distribution the role and extent of polyploidy is still unknown because different ploidy

levels come to light only after a cytological survey has been made across populations in an extensive geographic area.

Surveys of chromosome variation, both numerical and structural, have been successfully applied in systematic studies of the Cactaceae (Pinkava et al. 1977, Pinkava and Parfitt 1982, Mazzola et al. 1988). Chromosomal structural rearrangements in the cactus family vary from translocations in *Opuntia leptocaulis* de Candolle, 1828 (Pinkava et al. 1985) and inversions in *O. curvospina* Griffiths, 1916 (Pinkava et al. 1973) to cryptic structural changes in *Pyrrhocactus* (A. Berger, 1929) Backeberg et F.M. Knuth, 1935 (Las Peñas et al. 2008) and stable nuclear content of DNA in species of *Mammillaria* Haworth, 1812 (Christian et al. 2006). Similarly, analyses of meiotic chromosome behavior and polyploidy have been effective in addressing taxonomic problems in several genera and the hybrid status of *Opuntia* × *spinosibacca* M.S. Anthony, 1956 (Pinkava and Parfitt 1988). The natural history of cacti has also involved karyotypic studies to clarify species boundaries, the correlation of geographic range with ploidy levels and morphology, and phylogenetic relationships (Beard 1937, Grant and Grant 1979, Parfitt 1987, Cid and Palomino 1996, Das 1999, Baker and Johnson 2000, Baker and Cloud-Hughes 2014, Stock et al. 2014, Wellard 2016). Yet, there are still numerous cacti for which cytological information remains unknown.

The columnar cactus *Pachycereus pringlei* (S. Watson, 1885) Britton & Rose, 1909, has been an excellent model plant for ecological and evolutionary studies because this species has an extensive distribution range in the Mexican portion of the Sonoran Desert (Turner et al. 1995, Drezner and Lazarus 2008). Unlike most cacti, this taxon exhibits variation in genders and breeding systems (Fleming et al. 1998, Medel-Narváez 2008, Gutiérrez-Flores et al. 2016, 2017). While the vegetative morphological variability in this species is seemingly conservative to the extent that the species can be easily recognized by these attributes, the existence of polymorphism in floral traits associated with breeding systems and geographic distribution of populations is highly diverse, suggesting reproductive isolation (Gutiérrez-Flores et al. 2017). Recent studies of neutral genetic variation (Gutiérrez-Flores 2015, Gutiérrez-Flores et al. 2016) identified five genetic populations of *P. pringlei* unexpectedly associated with different breeding systems, namely two hermaphrodite populations restricted to Catalana and Cerralvo Islands in the Gulf of California, a mainly dioecious assemblage in Cabo San Lucas (CBS) at the tip of the BCP, another trioecious cohort from CBS to northern BCP (~28°N), and a mostly gynodioecious population in northern BCP and the coast of Sonora in mainland Mexico.

The biogeographic distribution pattern of *P. pringlei* has been driven by long-standing climatic fluctuations associated with differential colonization abilities of genders, geographic variation of selfing and outcrossing rates, and the effect of biotic and abiotic factors (Gutiérrez-Flores et al. 2016, 2017). As a result, the spatial segregation of genders in *P. pringlei* has long been a magnet to ecologists, geneticists, and systematists (Fleming et al. 1994, 1998, Molina-Freaner et al. 2003, Gutiérrez-Flores et al. 2016, 2017). Even so, several important evolutionary questions, such

as the distribution of chromosome numbers and whether the diploid condition is dominant over a polyploid condition influencing the evolution and diversity of this cactus, remain unclear.

Chromosome counts and allozyme data have revealed that *Pachycereus pringlei* is tetraploid (2n = 44), but these reports are supported by scanty evidence from northern BCP at El Rosario (Pinkava et al. 1973) and another site in mainland Mexico in Bahía Kino (Murawski et al. 1994). Consequently, chromosome numbers in the vast area of distribution in the BCP remain unexplored. Moreover, to date there is no record indicating the existence of the family's base chromosome number (n = 11) in this species. Since the characterization of the geographic distribution and potential variability of ploidy levels is useful to gain new insights into the natural history of this long-lived cactus in connection with the distribution of reproductive systems and genetic variation, in this paper we present a survey of chromosome numbers in new and different populations of *P. pringlei* throughout the BCP and mainland Mexico. We combine cytological data with information about breeding systems and floral and genetic diversity to discuss their relationships and role in the diversification and evolution of this species. Explicitly, the goals of the study were 1) to expand knowledge about the geographic distribution and possible variation in chromosome numbers (diploid versus polyploid cytotypes) throughout the geographic range of *P. pringlei* and 2) to examine the correspondence of ploidy levels with genetic populations, floral attributes, and breeding systems. When appropriate, a discussion dealing with taxonomic implications of variation in chromosome number with respect to morphological traits is included.

Material and methods

The study species

Pachycereus pringlei, a cactus commonly known as Cardón, is circumscribed within the subfamily Cactoideae. The species dominates rocky slopes and alluvial plains in the deserts of the BCP, most islands of the Gulf of California, and coastal areas of mainland Sonora, Mexico (Turner et al. 1995, Gutiérrez-Flores et al. 2017). Old plants reach an average height of eight to nine m, have an impressive candelabra-like shape (Fig. 1A), and bear from a few to up to 30 large branches (Bravo-Hollis and Sánchez-Mejorada 1991). The flowering season is from late March to early June with a peak from late April to mid-May. The flowers are white to cream in color (Fig. 1B) with abundant nectar and pollen, open early in the evening, and are pollinated by the long-nosed bat *Leptonycteris yerbabuenae* Martínez & Villa-R., 1940 (Phyllostomidae: Glossophaginae (Fig. 1C); however, the blossoms persist open for several hours the next morning allowing visits from diurnal pollinators, such as birds and insects, mainly bees (Fleming et al. 1996, 1998). The large, fleshy fruits with red pulp (Fig. 1D) attract frugivorous animals, facilitating seed dispersal.

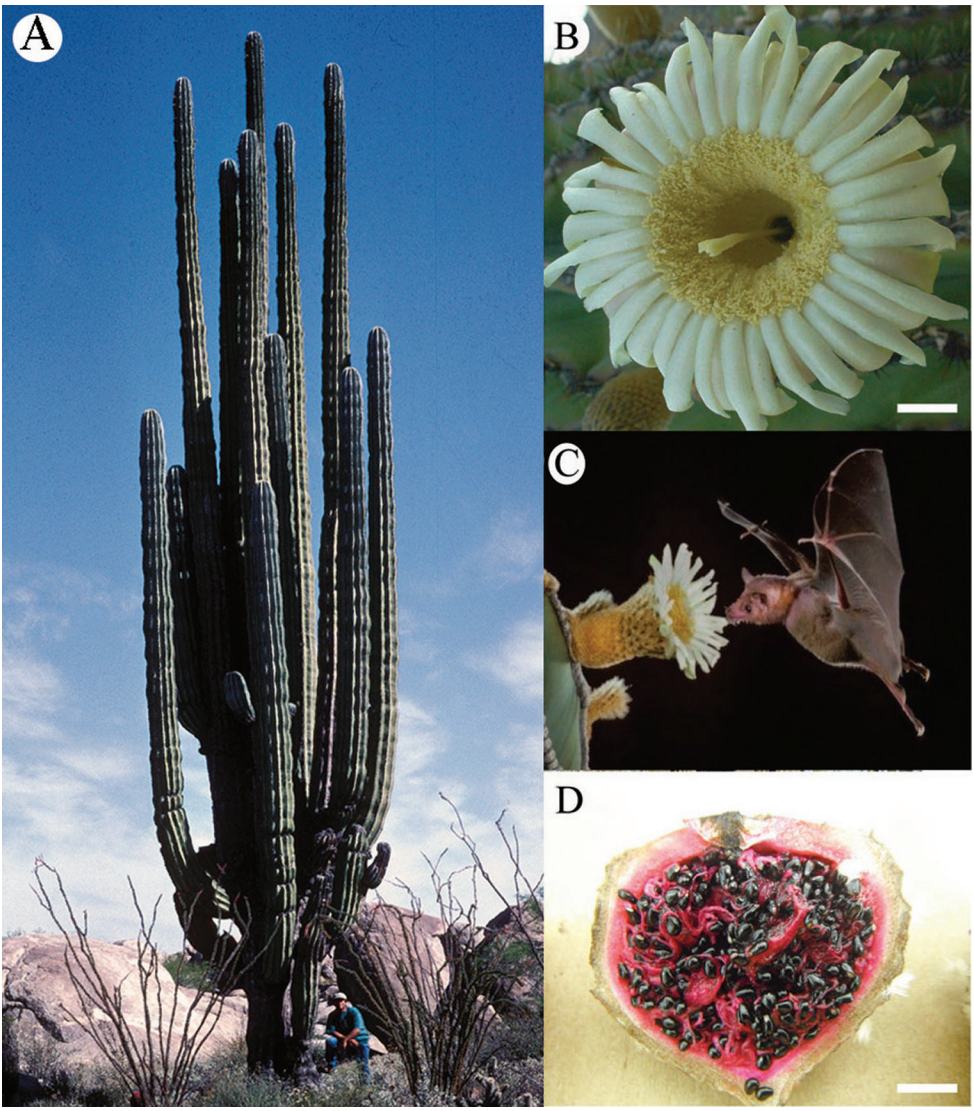

Figure 1. Typical vegetative and floral morphology of the emblematic cactus *Pachycereus pringlei*. **A** Mature individual with candelabra-like structure in the Cataviña region. **B** Archetypal funnel-form flower. **C** Main pollinator, the long-nosed bat *Leptonycteris yerbabuenae*. **D** Mature, fleshy fruit. Photo **A** by Jon Rebman; photo **C** by Merlin D. Tutle.

Inspection of chromosome numbers

The chromosome numbers inspected in this study were obtained from individual plants from natural populations across the wide distributional range of *P. pringlei* encompassing variation in ecology, latitude, and longitude as well as floral morphology, breeding systems, and levels of genetic diversity. Fruits with mature seeds were collected in the

field from three to 10 individuals in the following localities of the BCP: Bahía de Los Ángeles (BAN), Cabo San Lucas (CBS), Catalana Island (ICA), Cerralvo Island (ICE), El Comitán (COM), López Mateos (LMA), Loreto (LOR), San Felipe (SFE), Puente Querétaro (PQU), and Santa Rosalía (SRO). Also, one fruit collected in mainland Mexico from one locality, Álamos (ALA), Sonora, was scrutinised (Table 1). We also included one previous count by Pinkava et al. (1973) from El Rosario (ROS) and an allozymatic inference of the ploidy level by Murawski et al. (1994) from Bahía Kino (BKI) (Table 1) for a grand total of 13 locations. The distance among the different sample sites varies from 20 km between López Mateos (LMA) and Puente Querétaro (PQU) to 983.7 km between Cabo San Lucas (CBS) and San Felipe (SFE).

Mitotic chromosome numbers were determined using meristematic cells from fresh root tips following a modified protocol by Cota et al. (1996). Approximately 30 seeds per fruit were first rinsed with 10 % commercial bleach (NaClO) and then germinated in Petri dishes with moistened filter paper under controlled greenhouse conditions. The one-week-old root tips of ca. 1 cm in length were trimmed and immersed in Colchicine 0.2 % to arrest chromosomes at metaphase and kept at 4 °C for 2–4 h, then rinsed twice with distilled water and fixed in Carnoy's fluid (3:1 ethanol 95 % and glacial acetic acid v/v) overnight. Next, the roots were rinsed with distilled water, hydrolysed in 1N HCl at 60 °C, rinsed twice with distilled water, and stained with aceto-orcein at room temperature for 1 h. Semi-permanent slides were prepared by squashing root tips in Hoyer's medium and then examined in a Zeiss Axio Imager Z1 microscope (Carl Zeiss, Toronto, ON, Canada) at 40×, 60×, and 100× (immersion oil). Photographs were taken using an AxioCam MRm charge-coupled digital CCD camera and AxioVision 4.8 imaging software. Chromosome size was estimated using the measuring tool available in the AxioVision 4.8 imaging software to add the corresponding scale bar to the pictures. For consistency, meiotic chromosome counts in CBS were also performed in anthers from mature flower buds of one hermaphrodite and two male plants.

For meiotic figures and counts, anthers from floral buds at different developmental stages were fixed in Carnoy's solution, then stained with aceto-orcein at room temperature for 1 h, squashed, mounted in Hoyer's medium and microscopically examined as indicated above. A minimum of three cytological figures from different individuals were scrutinized in each population for confirmation of chromosome number. Finally, the geographic distribution of diploid and polyploid cytotypes from the localities examined was plotted on a base map obtained from the Comisión Nacional para el Conocimiento y Uso de la Biodiversidad (CONABIO, http://www.conabio.gob.mx) using the ArcGIS 10.4 software (ESRI).

Idiogram construction

Idiograms were reconstructed based on microscopic observation of mitotic figures. Chromosome homology for diploid and tetraploid cytotypes follows Cota and Wallace (1995), which is based on similarities in morphology, length, and centromere position, the primary physical features used because no satellites or secondary constrictions were detected.

Table 1. Sample sites of the columnar cactus *Pachycereus pringlei* for which chromosome numbers were investigated, including counts by Pinkava et al. 1973 (†) and Murawski et al. 1994 (*). All diploid counts represent new reports for this species. Breeding systems, genetic diversity and genetic populations according to Gutiérrez-Flores et al. (2016). ND = not determined.

Locality	Code	Latitude	Longitude	Chromosome number	Ploidy level	Breeding system	Genetic diversity	Genetic population
Cabo San Lucas	CBS	22.9438	-109.9905	2n = 44	Tetraploid	Mainly dioecious	0.38	CBS
Catalana Island	ICA	25.6768	-110.8087	2n = 22	Diploid	Herma-phroditic	0.40	ICA
Cerralvo Island	ICE	24.1868	-109.8775	2n = 22	Diploid	Herma-phroditic	0.26	ICE
El Comitán	COM	24.1332	-110.4317	2n = 44	Tetraploid	Mainly trioecious	0.45	South
López Mateos	LMA	25.2726	-111.8942	2n = 44	Tetraploid		0.45	
Loreto	LOR	25.8918	-111.4698	2n = 44	Tetraploid		ND	
Puente Querétaro	PQU	25.3508	-111.6094	2n = 44	Tetraploid		0.45	
Santa Rosalía	SRO	27.2408	-112.3615	2n = 44	Tetraploid	Mainly gynodio-ecious	ND	North
Bahía de Los Ángeles	BAN	28.9164	-113.5541	2n = 44	Tetraploid		0.35	
El Rosario	ROS	30.0861	-115.6795	n = 22	Tetraploid†		ND	
San Felipe	SFE	30.3716	-114.8537	2n = 44	Tetraploid		0.35	
Álamos	ALA	26.8978	-109.4578	2n = 44	Tetraploid		0.35	
Bahía Kino	BKI	28.5000	-111.8063	ND	Tetraploid*		ND	

Phenotypic variation in floral characters

To compare floral variability among diploid and polyploid populations of *P. pringlei*, 17 floral traits were selected and a one-way ANOVA of morphological attributes from flowers gathered for the five genetic populations *fide* Gutiérrez-Flores et al. (2017) was performed. Only data from bisexual flowers were used to avoid potential misinterpretations due to gender variation. Measurements of floral phenotypic traits and ANOVA tests are summarized in Table 2.

Results

Chromosome number and morphology

Chromosome counts performed in the populations of *P. pringlei* investigated revealed variation in ploidy level. Foremost, our survey unveiled the first diploid (2n = 2x = 22) count for this species and expanded the current cytological knowledge with additional tetraploid (2n = 4x = 44) cytotypes in different localities of the BCP. The diploid counts were consistently determined in all mitotic cells in plants from Cerralvo (ICE) and Catalana (ICA) Islands (Figs 2, 3; Table 1), which are composed of hermaphrodite populations. Double chromosome dosage was found in all the remaining populations

Table 2. Measurements of phenotypic floral traits, including sample size (mean ± SE) and statistical comparisons of bisexual flowers between diploid (ICE, ICA) and polyploid (CBS, SOUTH, NORTH) populations of *Pachycereus pringlei* in the Baja California Peninsula, Mexico. Lower case superscript letters indicate floral characters having statistically significant differences.

Floral trait	Diploid		Polyploid		
	ICE	ICA	CBS	SOUTH	NORTH
	n = 50	n = 35	n = 7	n = 20	n = 38
Corolla width (mm)	33.3 ± 0.7	36.2 ± 0.9	29.4 ± 1.2	33.1 ± 1.0	36.0 ± 0.7
Floral length (mm)	90.7 ± 1.2	86.8 ± 0.4	76.4 ± 2.8	85.1 ± 2.1	96.2 ± 1.5
Nectary length (mm)	12.9 ± 0.3	13.0 ± 0.3	9.2 ± 0.4	11.9 ± 0.4	13.0 ± 0.3
Nectary width (mm)	11.1 ± 0.2^{b}	10.7 ± 0.3^{b}	8.7 ± 0.5^{a}	9.3 ± 0.2^{a}	9.9 ± 0.3^{a}
No. of pollen grains (x10^{6})	2.8 ± 0.3^{c}	2.3 ± 0.2^{cb}	1.0 ± 0.2^{a}	1.3 ± 0.2^{a}	1.6 ± 0.2^{ab}
No. of stamens (in 0.5 cm^{2})	48.6 ± 1.0^{ab}	43.0 ± 1.8^{a}	49.0 ± 3.14^{ab}	53.4 ± 1.9^{b}	51.6 ± 2.0^{b}
No. of tepals	49.9 ± 0.6	46.7 ± 1.2	44.3 ± 1.6	47.9 ± 1.4	49.9 ± 0.8
P:O ratio (x10^{3})	2337 ± 255^{b}	2638 ± 307^{b}	1178 ± 289^{a}	911 ± 117^{a}	2166 ± 203^{a}
Stamen length (mm)	10.0 ± 0.2	10.2 ± 0.2	11.9 ± 0.7	10.0 ± 0.2	10.9 ± 0.2
Tepal length (mm)	23.0 ± 0.4	23.5 ± 0.4	19.0 ± 0.6	20.4 ± 0.6	23.0 ± 0.5
Tepal width (mm)	7.7 ± 0.3	8.1 ± 0.3	5.9 ± 0.3	8.0 ± 0.3	9.1 ± 0.2
Ovary length (mm)	14.0 ± 0.4	9.4 ± 0.4	8.3 ± 0.7	13.4 ± 0.7	15.5 ± 0.5
Ovary width (mm)	8.9 ± 0.3	7.9 ± 0.3	8.1 ± 0.5	8.7 ± 0.4	9.6 ± 0.3
Pistil length (mm)	44.6 ± 0.7	47.1 ± 0.9	43.8 ± 1.2	47.9 ± 1.4	51.0 ± 0.9
Stigma length (mm)	9.8 ± 0.3	8.8 ± 0.3	7.7 ± 0.5	8.9 ± 0.3	10.7 ± 0.4
No. of ovules (mm)	1550 ± 66^{b}	907 ± 40^{a}	849 ± 115^{a}	1505 ± 118^{b}	1614 ± 112^{b}
Stamen-stigma distance (mm)	0.5 ± 0.5^{a}	2.0 ± 0.6^{ab}	1.5 ± 1.1^{ab}	4.2 ± 1.0^{bc}	5.4 ± 0.6^{c}

investigated from both the BCP and mainland Mexico (Figs 2, 3; Table 1). All counts performed in pollen grains from flower buds were diploid (n = x = 22), and no abnormal figures or disruptive cell divisions were noted. These observations supported tetraploidy in two male individuals and one hermaphrodite plant from CBS.

The overall morphology of mitotic chromosomes for diploid and tetraploid cytotypes of this columnar cactus is, in general, symmetric and uniform in shape, i.e., chromosomes are mostly metacentric (M) with a few submetacentric (SM) and relatively small in size (measuring in average 2μm in length) (Figs 2, 4). No visible physical structural differences or secondary constrictions (satellites) were detected in any of the diploid and polyploid populations investigated. The karyotype in diploid and tetraploid populations is also symmetric (Fig. 4) and the variation mainly involves numerical changes with insignificant differences in chromosome shape and proportions.

Phenotypic variation in floral characters

Morphological comparisons of bisexual flowers among populations revealed a few significant statistical differences that can be associated with variation in ploidy level. For instance, diploid individuals from ICE and ICA have wider nectaries (11.1 ± 0.2 and 10.7

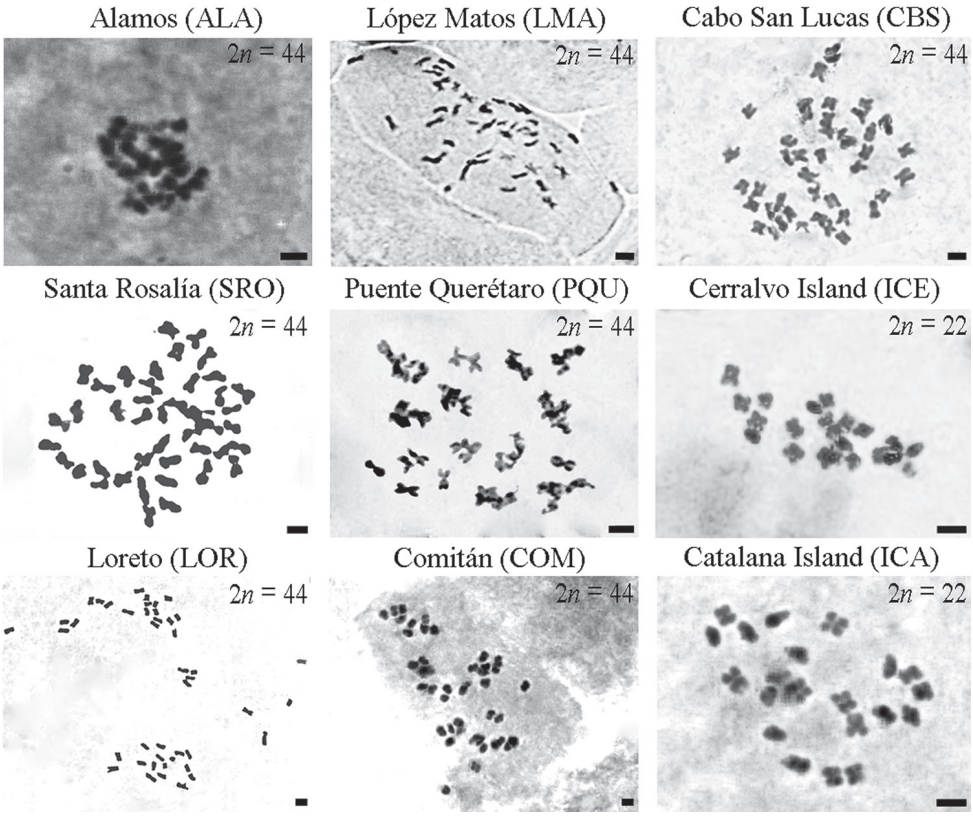

Figure 2. Representative metaphase chromosomes of *Pachycereus pringlei*. Cerralvo Island (ICE) and Catalana Island (ICA) are the sole localities for diploid cytotypes. Remaining pictures in plate are typical chromosomes in different tetraploid populations. Scale Bar: 2 μm.

± 0.3 mm, respectively) and larger P:O ratios (2,337 ± 255 and 2,638 ± 307, respectively). The diploid cytotypes also tend to have larger amount of pollen grains (2.8 x10^6 and 2.3 x10^6, respectively), fewer number of stamens (48.6 ± 1.0, and 43.0 ± 1.8, respectively), fewer number of ovules (1,550 ± 66 and 907 ± 40, respectively), and closer proximity between stamen and stigmas (0.5 ± 0.5 and 2.0 ± 0.6 mm, respectively). See also Table 2.

Discussion

Geographic and range expansion of chromosome numbers in *P. pringlei*

Chromosome number variation, especially polyploidy, is one of the major biological processes that has affected angiosperm evolution (Stebbins 1971), leading to different or new evolutionary lines promoting new genome combinations in organisms (Wendel and Doyle 2005), including the Cactaceae (Cota and Philbrick 1994). The signifi-

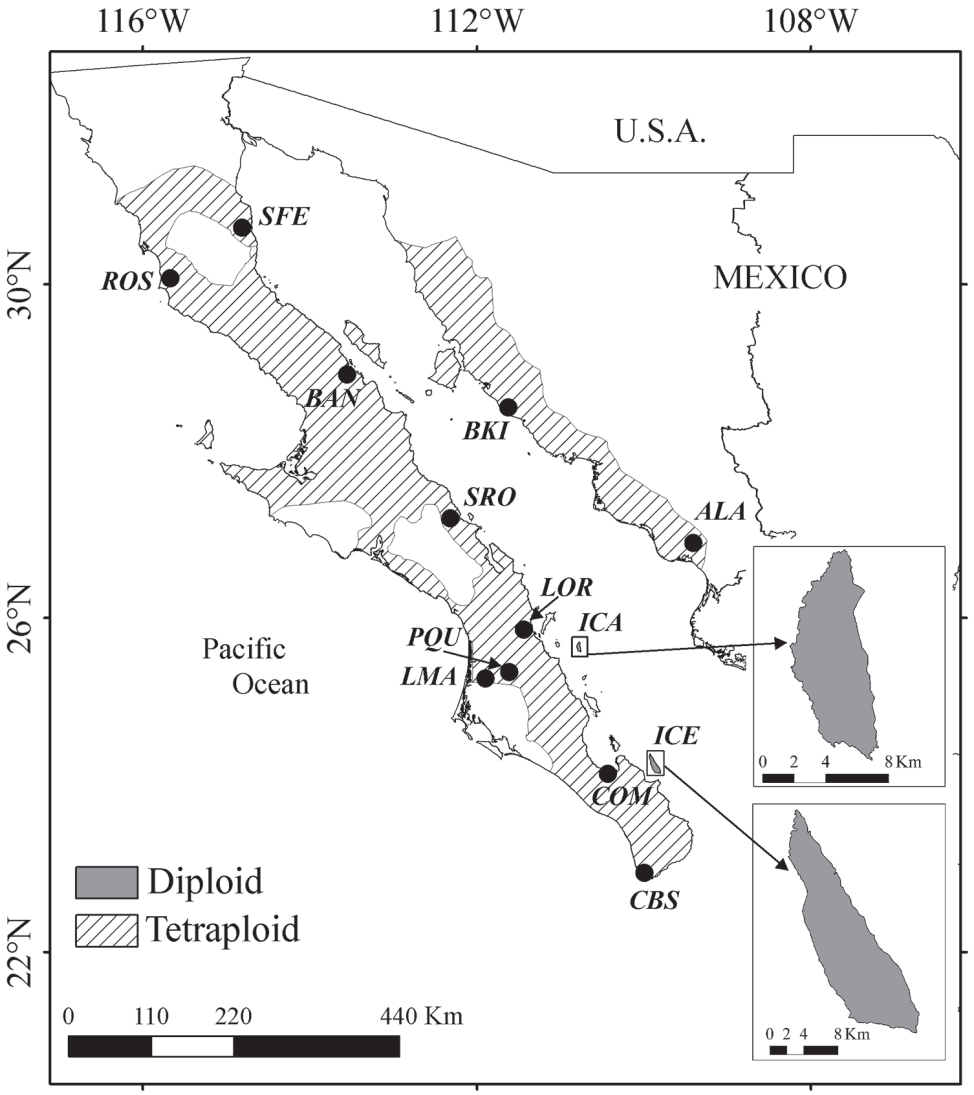

Figure 3. Distribution of diploid and tetraploid individuals in populations (black dots) of *Pachycereus pringlei*. Dark gray areas in ICA and ICE indicate distribution of diploid (2n = 22) cytotypes. Diagonal shaded area indicates the predicted coverage of tetraploid (2n = 44) cytotypes. See Table 1 for full names of the abbreviated localities indicated in the map.

cance of polyploidy in the cactus family is more evident in the subfamily Opuntioideae, in which polyploid taxa, primarily in the genus *Opuntia* Miller, 1754 (Pinkava and McLeod 1971, Pinkava et al. 1973, 1998, Baker and Pinkava 1987, Baker et al. 2009b, Majure et al. 2012), are common. In subfamily Cactoideae, polyploidy is more sporadic despite the large number of species circumscribed in this group, probably due to the relatively recent origin and the unexplored cytological aspects of this lineage.

Different levels of polyploidy have been reported in various clades of the Cactoideae including terrestrial, e.g., *Blossfeldia liliputana* Wendermann, 1937 (Ross 1981), *Echinocereus* Engelmann, 1848 (Cota 1991, Cota and Wallace 1995), *Mammillaria* spp. (Ross 1981), *Weberbauerocereus weberbaueri* (K. Schumann ex Vaupel, 1913) Backeberg, 1958 (Sahley 1996), and epiphytic taxa, e.g., *Hylocereus* (A. Berger, 1905) Britton & Rose, 1909 and *Selenicereus* (A. Berger, 1905) Britton & Rose, 1909 (Lichtenzveig et al. 2000), and *Rhipsalis baccifera* (Solander, 1771) Stearn, 1939 (Cota-Sánchez and Bomfim-Patrício 2010). Additionally, autotetraploidy (four copies of a single genome due to doubling of an ancestral chromosome complement) has been documented in the columnar cactus *W. weberbaueri* (Sahley 1996) and in *P. pringlei* (Murawski et al. 1994).

This study has unveiled the first report of diploid (2n = 2x = 22) cytotypes in *P. pringlei* and expands the distributional range of the tetraploid (2n = 4x = 44) condition known for this species. Geographically, our survey also revealed that the base diploid number is maintained exclusively in the two deep-water islands (ICA and ICE) of the Gulf of California characterised by the prevalence of hermaphrodite individuals. In turn, double chromosome dosage (tetraploidy) is consistent throughout the three BCP populations (CBS, North and South) (Fig. 3) and is associated with the presence of unisexual plants and a dioecious, gynodioecious or trioecious breeding system (Table 1). We predict that the tetraploid condition extends to other continental islands, such as Espíritu Santo, San José, Monserrat, San Lorenzo, and Tiburón, which are areas with unisexual plants (Gutiérrez-Flores 2015). Conceivably, an increment in chromosome number has enabled *P. pringlei* to colonize wide areas of the BCP. The same premise has been proposed in other cacti, e.g., *Echinocereus*, in which polyploid taxa occupy wider territories and ecological sorting, from medium to high latitudes and elevations relative to the overall distribution of the genus and diploid relatives (Cota and Philbrick 1994, Cota-Sánchez 2008). Also, comparable biogeographic patterns exist for other angiosperm taxa with different cytotypes, e.g., *Chamerion* (Rafinesque, 1818) Rafinesque ex Holub, 1972 (Husband and Schemske 1998), *Chrysolaena* H. Robinson, 1988 (Do Pico and Dematteis 2014), and members of the Asparagaceae (Azizi et al. 2016).

Among polyploids, tetraploidy is the most successful condition (De Wet 1980). In fact, tetraploidy is the most frequent form in the Cactaceae (Pinkava et al. 1985), and the radiation success of *Echinocereus* from central Mexico to the southwest of the US has been attributed to the prevalence of tetraploids throughout the distribution range (Cota 1991, Cota and Philbrick 1994) and the *Humifusa* clade of *Opuntia* s.s. (Majure et al. 2012). Similarly, the relatively fast radiation of the South American epiphytic cactus *Rhipsalis baccifera* into areas of the New and Old Worlds has been accompanied by successive cycles of polyploidy (di-, tetra- and hexaploid), in conjunction with life history traits, such as facultative selfing, asexual reproduction, and vivipary (Cota-Sánchez and Bomfím Patrício 2010). Accordingly, the incidence of polyploidy can be associated with increasing the colonizing ability and diversification of species into new environments due to relaxed selection in the additional genome copies (Stebbins 1971, Adams and Wendel 2005, Cota-Sánchez 2008). Thus, it is not surprising that the dominance and success of polyploid (tetraploid) cytotypes in *P. pringlei* is reflected in their extensive distribution and ability to

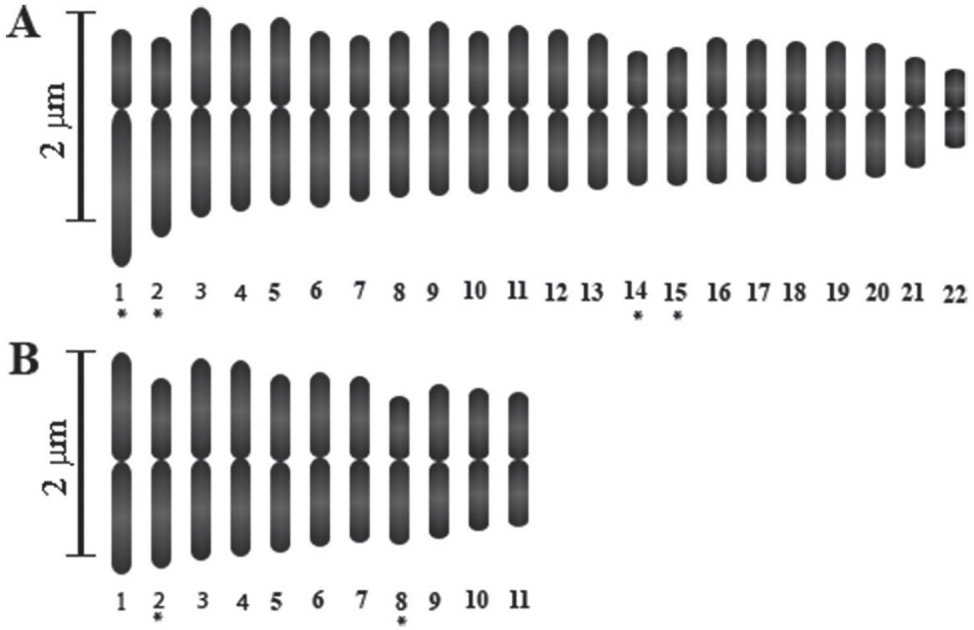

Figure 4. Idiograms of tetraploid (**A**) and diploid (**B**) cytotypes of the columnar cactus *Pachycereus pringlei*. Asterisk (*) denotes submetacentric chromosomes.

colonize a widespread range of habitats and ecological conditions in relation to the diploid state, which is restricted to ICA and ICE. Recent studies, e.g., Levy and Feldman (2004), Adams and Wendel (2005), have shown that the paramount ability of polyploids to adapt to a gamut of novel factors is based on a wide spectrum of molecular and physiological adjustments conferred by the amalgamation of two or more genomes.

During past geological events, the southern BCP, including ICE and ICA Islands, exhibited suitable niche conditions that served as refugia for populations of *P. pringlei* during the Last Glacial Maximum (LGM), as evidenced by Ecological Niche Modelling (Gutiérrez-Flores et al. 2016). Consequently, we suggest that ICE and ICA could be the relicts of a southern BCP diploid ancestor of *P. pringlei* from which both the polyploid and unisexual conditions gradually evolved in concert in mainland BCP. This, in turn, facilitated the northward range expansion during the Holocene, resulting in the modern dispersal of this neopolyploid complex with concomitant colonisation of suitable habitats available after the glacial retreat, leading to the present-day distribution of this cactus throughout the BCP and mainland Sonora. The same rationale has been used to explain the colonisation of large geographic areas of the polyploid *Opuntia humifusa* (Rafinesque, 1820) Rafinesque, 1830 s.l. and *O. macrorhiza* Engelmann, 1850 s.l. based on LGM events (Majure et al. 2012). Similarly, rapid high polyploidisation caused by recurrent population fragmentation and expansion during the Pleistocene has taken place in other angiosperms, including *Cerastium* Linnaeus, 1753, *Draba* Linnaeus, 1753, *Parnassia* Linnaeus, 1753, *Saxifraga* Linnaeus, 1753, and *Vaccinium* Linnaeus, 1753 (Ab-

bott and Brochmann 2003, Brochmann et al. 2004), and other plants. Nonetheless, it is unclear whether the extent of polyploid populations of *P. pringlei* in the BCP is a consequence of higher diversification rates following the ubiquitous genome duplication.

Chromosome morphology

In terms of morphology, the chromosomes of *P. pringlei* follow the overall homogeneous pattern reported for the sister taxon *P. weberi* (J.M. Coulter, 1896) Backeberg, 1960 (Gama-López 1994) and, in general, for the cactus family (Cota and Wallace 1995, Las Peñas et al. 2008 and references therein). Because of the similarity and minute size of chromosomes among cytotypes, no major structural differences were detected. That is, the chromosomes are consistently small and mostly metacentric with no evident secondary satellites. Therefore, except for the numerical difference, the homogeneous chromosome morphology in terms of arm length and shape makes the characterization between diploid and tetraploid cytotypes difficult. In addition to uniformity, chromosome size between diploid and tetraploid individuals is also insignificant, and structural changes, if any, remain cryptic. Similarly, in *Opuntia*, morphological differentiation is equivocal and has been a commonly reported event among diploid and polyploid cytotypes (Majure et al. 2012). Thus, structural chromosomal arrangements, either cryptic or physical, should not be ruled out in this columnar cactus because speciation without detectable chromosomal changes or divergence has been documented in other plants, e.g., *Platanus* Linnaeus, 1753 (Swanson et al. 1981) and *Stephanomeria* Nuttall, 1841 and *Clarkia* Pursh, 1814 (Crawford 1985).

Stebbins' (1971) hypothesis on the frequency of chromosome types in plants is useful to explain the existence of symmetric idiograms in diploid and tetraploid cytotypes of *P. pringlei*. In plants, metacentric chromosomes are fairly common, e.g., Araceae (Turco et al. 2014), Arecaceae (Oliveira et al. 2016) and Asparagaceae (Chen et al. 2017), and originate by the fusion of two telocentric chromosomes with relatively little effect in gene sequence (Stebbins 1971). In the Cactaceae, symmetric karyotypes are also ordinary, e.g., *Echinocereus* (Cota and Wallace 1995), *Nyctocereus* (A. Berger, 1905) Britton & Rose, 1909 (Palomino et al. 1988), *Setiechinopsis* Backeberg, 1950 (Las Peñas et al. 2011), *Opuntia* Ser. *Armata* (Las Peñas et al. 2017), and other species exhibiting low degree of variability in karyotype morphology. Hence, the morphological homogeneity of chromosomes in *P. pringlei* is not surprising.

Chromosome number and diversification of breeding systems

Foremost, it is worth noting that the correspondence of the base chromosome number (x = 11) with a hermaphroditic mating system in most cacti, including members of the basal subfamily (Pereskioideae) and species closely related to *P. pringlei*, such as *P. weberi* (Gama-López, 1994) and *P. pecten-aboriginum* (Engelmann ex S. Watson, 1886) Brit-

ton & Rose, 1909 (Pinkava et al. 1977), indicates that both the evolution of unisexuality and polyploidy are derived conditions in the Cactaceae, an idea put forward by Gutiérrez-Flores et al. (2017). In fact, there is an apparent transition between breeding system and ploidy level. The change from hermaphroditism to trioecy is coupled with an increase in chromosome number in *P. pringlei* and is consistent with polyploidisation events reported for other cacti with non-hermaphroditic sexual systems (Valiente-Banuet et al. 1997, Fleming et al. 1998, Strittmatter 2002, Díaz and Cocucci 2003, Strittmatter et al. 2006, 2008). Our results imply that in *P. pringlei* the hermaphrodite system is diploid and restricted to ICA and ICE, whereas the tetraploid condition is essentially associated with unisexual, dioecious, gynodioecious and trioecious breeding systems (Table 1). Also, the high level of genetic divergence reported for the ICE and ICA populations (Gutiérrez-Flores et al. 2016) support the general idea that difference in ploidy level is an important factor for reproductive isolation, as proposed by Baker and Pinkava (1987, 1999), Cota and Philbrick (1994), and Baker (2002, 2006). However, intra- and inter-population experimental crosses between different cytotypes are needed to determine the degree of compatibility and reproductive potential.

Taxonomic implications in relation to morphology and ploidy level

Diploid and tetraploid plant populations may or may not be ecologically differentiated (Cota 1991). According to De Wet (1980), in the absence of chromosome information, close morphological resemblance may imply genetic continuity, a characteristic of conspecific populations. However, when a difference in ploidy level is known to exist, the issue is that barriers to gene exchange characteristic of distinct species are generally found between diploid and polyploid populations, which poses a taxonomic problem, i.e., whether or not to recognize these entities as two different species on the basis of reproductive isolation due to differences in chromosome number.

Unveiling polyploid individuals from diploid ancestors leads to the discovery of new cytotypes and potential taxonomic issues because different populations are frequently associated with an assortment of floral and/or vegetative phenotypes (Brickford et al. 2007). In this regard, the phenotypic variation reported in vegetative (Medel-Narváez et al. 2006) and reproductive (Gutiérrez-Flores et al. 2017) traits in populations of *P. pringlei* has been interpreted as a physiological response to a gradual change in environmental conditions and sex-specific selection acting at different magnitudes on sexual characters of floral morphs and populations (Gutiérrez-Flores et al. 2017). However, considering floral attributes (Table 1) and data reported by Gutiérrez-Flores et al. (2017), it is clear that the flowers from the diploid populations in ICA and ICE have, in general, wider nectaries, shorter stamen-stigma distance, larger amounts of pollen grains, larger P:O ratios, and fewer number of stamens and ovules compared to flowers of the polyploid counterparts. These morphological differences in conjunction with discrepancy in chromosome number, and geographic isolation are key elements suggesting the description of a new subspecies. Although some may argue

that these few, somewhat obscure, floral traits may not warrant the recognition of a new taxonomic entity even with the evidence of a difference in ploidy, we argue that these floral features play a role in pollinator selection and breeding systems, reinforcing reproductive isolation between polyploids in mainland BCP and Sonora and their diploid progenitors in populations from the ICA and ICE islets, further suggesting a distinct taxonomic unit. Thus, rather than one species with different cytotypes, which can hinder insights into evolution, speciation, and conservation, we propose two genetically divergent subspecies with distinct ploidy levels, geographic ranges, breeding systems, and floral morphological differences. In concert, all these factors may also be largely responsible for the genetic divergence and putative reproductive isolation between diploid and tetraploid *P. pringlei* populations. This idea is also substantiated by several cytological studies providing compelling evidence to effectively distinguish diploid from polyploid species of cacti by correlating morphology with geography. For instance, the existing geographic variation of diploid and tetraploid phenotypically similar cytotypes in *Echinocereus* spp. (Cota and Philbrick 1994), the morphological disparities, sometimes cryptic, among diploid and polyploid cytotypes of the *Humifusa* clade of *Opuntia* s.s. (Majure et al. 2012), the dimorphic hexaploid (2n = 66) *Echinocereus yavapaiensis* M.A. Baker, 2006 (Baker 2006), as well as in other higher plants (Bickford et al. 2007, Rani et al. 2015, Molgo et al. 2017) and animals (Vacher et al. 2017) have been used as evidence to delimit new taxa.

Evidently, genomic changes potentially produce new gene complexes, facilitating rapid evolution of individuals and their new attributes (Soltis and Soltis 1999). However, although some functional traits are important in explaining species success, genome flexibility, and versatility in reproductive systems, morphological evaluations encompassing a large, wide-ranging number of individuals are needed to deal with a formal taxonomic description and the implications arising from differences in ploidy and patterns of geographic variation and inconsistency in morphological features throughout the populations of *P. pringlei*. At present, we can only say that variations in morphology, genetic diversity, and ploidy level suggest reproductive isolation and support the recognition of a new taxonomic entity.

Concluding remarks

Merging chromosome number information, genetic data, breeding systems, and floral morphology has provided new insights to better understand the evolutionary history and reproductive success of this iconic cactus in northwestern Mexico. *P. pringlei* has diploid and tetraploid populations with distinctive distribution. Although tetraploids have not been named as distinct species due to the tradition of including multiple cytotypes derived from diploid relatives as a single species and the practicality of adhering to the general morphological species concept (Soltis et al. 2007), our results allude to the possibility of describing a new subspecies in *P. pringlei*. The diploid condition is endemic to Catalana and Cerralvo Islands, whereas polyploid populations characterise the populations from

mainland BCP and Sonora. This cytogeographic distribution suggests the distinction of the diploid populations as a new taxonomic entity, which is likely the ancestral condition of the broadly distributed tetraploid throughout the BCP. Succinctly, we suggest that chromosome doubling in conjunction with allopatric distribution, differences in neutral genetic variation, floral traits, and breeding systems has driven the reproductive isolation, evolution, and diversification of this columnar cactus. These functional attributes render this species an ideal candidate to conduct ecological genetic investigations to further explore the selective forces acting on plants and their life history traits.

Acknowledgements

The authors thank the anonymous reviewers for providing feedback to improve the manuscript. This research was funded by the National Geographic Society, Grant No. 7382-02, to JHCS, and the Consejo Nacional de Ciencia y Tecnología (CONACYT), Grant No. CB-2008-01-106925, to FJGDL. CONACYT (Grant No. 165852) and the Emerging Leaders of the Americas Program, Government of Canada, subsidized scholarships for CGF. We are thankful to R. Domínguez-Cadena, A. Medel-Narváez, and A. Lozano-Garza for field assistance, to D. Litwiller and the Cota-Sánchez Plant Systematics Lab personnel for critical discussions and editorial comments in earlier drafts of the manuscript, and to Dr. J. Rebman and Merlyn D. Tutle for providing photographs.

References

Abbott RJ, Brochmann C (2003) History and evolution of the arctic flora: in the footsteps of Eric Hultén. Molecular Ecology 12: 299–313. https://doi.org/10.1046/j.1365-294X.2003.01731.x

Adams JK, Wendel JF (2005) Polyploidy and genome evolution in plants. Current Opinion in Plant Biology 8: 135–141. https://doi.org/10.1016/j.pbi.2005.01.001

Almeida OJG, Cota-Sánchez JH, Paoli AAS (2013) The systematic significance of floral morphology, nectaries, and sugar nectar concentration in epiphytic cacti of tribes Hylocereeae and Rhipsalideae (Cactaceae). Perspectives in Plant Ecology, Evolution and Systematics 15: 255–268. https://doi.org/10.1016/j.ppees.2013.08.001

Azizi N, Amirouche R, Amirouche N (2016) Karyological investigations and new chromosome number reports in *Bellevalia* Lapeyrouse, 1808 and *Muscari* Miller, 1758 (Asparagaceae) from Algeria. Comparative Cytogenetics 10: 171–187. https://doi.org/10.3897/CompCytogen.v10i1.6445

Baker MA (2002) Chromosome numbers and their significance in some Opuntioideae and Cactoideae (Cactaceae) of mainland Ecuador and Peru. Haseltonia 9: 69–77.

Baker MA (2006) A new florally dimorphic hexaploid, *Echinocereus yavapaiensis* sp. nov. (section *Triglochidiatus*, Cactaceae) from central Arizona. Plant Systematics and Evolution 258: 63–83. http://www.jstor.org/stable/23655485

Baker MA, Cloud-Hughes MA (2014) *Cylindropuntia chuckwallensis* (Cactaceae), a new species from Riverside and Imperial Counties, California. Madroño 61: 231–43. https://doi.org/10.3120/0024-9637-61.2.231

Baker MA, Johnson RA (2000) Morphometric analysis of *Escobaria sneedii* var. *sneedii*, *E. sneedii* var. *leei* and *E. guadalupensis* (Cactaceae). Systematic Botany 25: 577–587. https://doi.org/10.2307/2666722

Baker MA, Pinkava DJ (1987) A cytological and morphometric analysis of a triploid apomict, *Opuntia* x *kelvinensis* (subgenus *Cylindropuntia*, Cactaceae). Brittonia 39: 387–401. https://doi.org/10.2307/2807141

Baker MA, Pinkava DJ (1999) A new Arizona hybrid cholla, *Opuntia* x *campii* (Cactaceae). Cactus and Succulent Journal (Los Angeles) 71: 320–322.

Baker MA, Rebman JP, Parfitt BD, Pinkava DJ, Christy C, Salywon A, Puente-Martínez R, Zimmerman A, Cota JH (2009a) Chromosome numbers of miscellaneous angiosperm taxa. Journal of the Botanical Research Institute of Texas 3: 279–283. http://www.jstor.org/stable/41972167

Baker MA, Rebman JP, Parfitt BD, Pinkava DJ, Zimmerman AD (2009b) Chromosome numbers in some cacti of western North America-VIII. Haseltonia 15: 117–134. https://doi.org/10.2985/026.015.0112

Barthlott W, Taylor NP (1995) Notes towards a monograph of Rhipsalideae (Cactaceae). Bradleya 13: 43–79. https://doi.org/10.25223/brad.n13.1995.a7

Beard EC (1937) Some chromosome complements in the Cactaceae and a study of meiosis in *Echinocereus papillosus*. Botanical Gazette 99: 1–21. https://doi.org/10.1086/334687

Bickford D, Lohman DJ, Sodhi NS, Ng PK, Meier R, Winker K, Ingram KK, Das I (2007) Cryptic species as a window on diversity and conservation. Trends in Ecology and Evolution 22: 148–155. https://doi.org/10.1016/j.tree.2006.11.004

Bravo-Hollis H, Sánchez-Mejorada HS (1991) Las cactáceas de México. Vol. 1, Universidad Nacional Autónoma de México, Mexico City.

Brochmann C, Brysting AK, Alsos IG, Borgen L, Grundt HH, Scheen A-C, Elven R (2004) Polyploidy in arctic plants. Biological Journal of the Linnean Society 82: 521–536. https://doi.org/10.1111/j.1095-8312.2004.00337.x

Chen JS, Gao Q, Zhou H, Huang YS, Ogisu M, Cao M (2017) Distribution, karyomorphology, and morphology of *Aspidistra subrotata* (Asparagaceae) at different ploidy levels in limestone areas of Asia. Comparative Cytogenetics. 11: 1–13. https://doi.org/10.3897/CompCytogen.v11i1.9803

Christian DA, Palomino G, García A, Mendez I (2006) Nuclear genome size and karyotype analysis in *Mammillaria* species (Cactaceae). Caryologia 59: 177–186. http://dx.doi.org/10.1080/00087114.2006.10797914

Cid R, Palomino G (1996) Cytotypes and meiotic behavior in Mexican populations of *Myrtillocactus geometrizans* var. *geometrizans* (Cactaceae). Caryologia 61: 343–348. http://doi.org/10.1508/cytologia.61.343

Cota JH (1991) Karyotype evolution in the genus *Echinocereus* (Cactaceae). M.Sc. Thesis, Claremont, CA, USA: The Claremont Graduate School, 89 pp.

Cota JH, Philbrick CT (1994) Chromosome number variation and polyploidy in the genus *Echinocereus* (Cactaceae). American Journal of Botany 81: 1054–1062. http://www.jstor.org/stable/2445300

Cota JH, Wallace RS (1995) Karyotypic studies in the genus *Echinocereus* and its taxonomic implications. Caryologia 48: 105–122. http://dx.doi.org/10.1080/00087114.1995.10797321

Cota JH, Rebman JP, Wallace RS (1996) Chromosome numbers in *Ferocactus* (Cactaceae: Cactoideae). Cytologia 61: 431–437. http://doi.org/10.1508/cytologia.61.431

Cota-Sánchez JH (2002) Taxonomy, distribution, rarity status and uses of Canadian cacti. Haseltonia 9: 17–25.

Cota-Sánchez JH (2008) Evolución de cactáceas en la región del Golfo de California, México. In: Flores CLM (Ed.) Estudios de las islas del Golfo de California, Universidad Autónoma de Sinaloa, Consejo Nacional de Ciencia y Tecnología, Gobierno del Estado de Sinaloa, Culiacán, Mexico, 67–79.

Cota-Sánchez JH, Bomfim-Patrício MC (2010) Seed morphology, polyploidy and the evolutionary history of the epiphytic cactus *Rhipsalis baccifera* (Cactaceae). Polibotánica 29: 107–129.

Cota-Sánchez JH, Croutch DS (2008) Notes on the floral biology of *Praecereus euchlorus* subsp. *euchlorus* (Cactaceae). Schumannia 5: 98–103.

Crawford DJ (1985) Electrophoretic data and plant speciation. Systematic Botany 10: 405–416. https://doi.org/10.2307/2419134

Das AB, Mohanty S, Marrs RH, Das P (1999) Somatic chromosome number and karyotype diversity in fifteen species of *Mammillaria* of the family Cactaceae. Cytobios 97: 141–151.

De Wet JMJ (1971) Polyploidy and evolution in plants. Taxon 20: 29–35. https://doi.org/10.2307/1218531

De Wet JMJ (1980) Origin of polyploids. In: Lewis WH (Ed.) Polyploidy: biological relevance. Plenum Press, New York, NY, USA, 3–15. https://doi.org/10.1007/978-1-4613-3069-1_1

Díaz L, Cocucci AA (2003) Functional gynodioecy in *Opuntia quimilo* (Cactaceae), a tree cactus pollinated by bees and hummingbirds. Plant Biology 5: 531–539. https://doi.org/10.1055/s-2003-44783

Do Pico GM, Dematteis M (2014) Cytotaxonomy of two species of genus *Chrysolaena* H. Robinson, 1988 (Vernonieae, Asteraceae) from Northeast Paraguay. Comparative Cytogenetics 8: 125–137. https://doi.org/10.3897/compcytogen.v8i2.7209

Drezner TD, Lazarus BL (2008) The population dynamics of columnar and other cacti: a review. Geography Compass 2: 1–29. https://doi.org/10.1111/j.1749-8198.2007.00083.x

Fleming TH, Maurice S, Buchmann SL, Tuttle MD (1994) Reproductive biology and relative male and female fitness in a trioecious cactus, *Pachycereus pringlei* (Cactaceae). American Journal of Botany 81: 858–867. http://www.jstor.org/stable/2445767

Fleming TH, Maurice S, Hamrick JL (1998) Geographic variation in the breeding system and the evolutionary stability of trioecy in *Pachycereus pringlei* (Cactaceae). Evolutionary Ecology 12: 279–289. https://doi.org/10.1023/A:1006548132606

Fleming TH, Tuttle MD, Horner MA (1996) Pollination biology and the relative importance of nocturnal and diurnal pollinators in three species of Sonoran Desert columnar cacti. Southwestern Naturalist 41: 257–269. http://www.jstor.org/stable/30055122

Gama-López S (1994) Análisis morfólogico y cariológico de *Pachycereus weberi* (J. Coulter) Backeb. y *Pachycereus* aff. *weberi* (J. Coulter) Backeb. (Cactaceae). Ph.D. dissertation. México City, México: Universidad Nacional Autónoma de México, 147 pp.

Grant V (1981) Plant speciation. Columbia University Press, New York, NY, 563 pp.

Grant V, Grant KA (1979) Systematics of the *Opuntia phaeacantha* group in Texas. Botanical Gazette 140: 199–207. https://doi.org/10.1086/337076

Gutiérrez-Flores C (2015) Filogeografía y estructura genética poblacional del cardón *Pachycereus pringlei* en el noroeste de México. Ph.D. dissertation. La Paz, Baja California Sur, Mexico: Centro de Investigaciones Biológicas del Noroeste, S.C., 174 pp.

Gutiérrez-Flores C, Cota-Sánchez JH, León-de La Luz JL, García-De León FJ (2017) Disparity in floral traits and breeding systems in the iconic columnar cactus *Pachycereus pringlei* (Cactaceae). Flora 235: 18–28. https://doi.org/10.1016/j. ora.2017.08.007

Gutiérrez-Flores C, García-De León FJ, León-de La Luz JL, Cota-Sánchez JH (2016) Microsatellite genetic diversity and mating systems in the columnar cactus *Pachycereus pringlei* (Cactaceae). Perspectives in Plant Ecology, Evolution and Systematics 22: 1–10. https://doi.org/10.1016/j.ppees.2016.06.003

Hunt DR, Taylor NP, Charles G (2006) The new cactus lexicon. DH Books, Milborne Port, UK, 869 pp.

Husband BC, Schemske DW (1998) Cytotype distribution at a diploid–tetraploid contact zone in *Chamerion* (*Epilobium*) *angustifolium* (Onagraceae). American Journal of Botany 85: 1688–1694. http://www.jstor.org/stable/2446502

Las Peñas ML, Bernardello G, Kiesling R (2008) Karyotypes and fluorescent chromosome banding in *Pyrrhocactus* (Cactaceae). Plant Systematics and Evolution 272: 211–222. https://doi.org/10.1007/s00606-007-0611-5

Las Peñas ML, Kiesling R, Bernardello G (2011) Karyotype, heterochromatin, and physical mapping of 5S and 18-5.8-26S rDNA genes in *Setiechinopsis* (Cactaceae), an Argentine endemic genus. Haseltonia 16: 83–90. https://doi.org/10.2985/1070-0048-16.1.83

Las Peñas ML, Oakley L, Moreno NC, Bernardello G (2017) Taxonomic and cytogenetic studies in *Opuntia* ser. Armatae (Cactaceae). Botany 95: 101–120. https://doi.org/10.1139/cjb-2016-0048

Levy AA, Feldman M (2004) Genetic and epigenetic reprogramming of the wheat genome upon allopolyploidization. Biological Journal of the Linnean Society 82: 607–613. https://doi.org/10.1111/j.1095-8312.2004.00346.x

Lichtenzveig J, Abbo S, Nerd A, Tel-Zur N, Mizrahi Y (2000) Cytology and mating systems in the climbing cacti *Hylocereus* and *Selenicereus*. American Journal of Botany 87: 1058–1065. http://www.jstor.org/stable/2657005

Majure LC, Judd WS, Soltis PS, Soltis DE (2012) Cytogeography of the *Humifusa* clade of *Opuntia* s.s. Mill. 1754 (Cactaceae, Opuntioideae, Opuntieae): correlations with pleistocene refugia and morphological traits in a polyploid complex. Comparative Cytogenetics 6: 53–77. https://doi.org/10.3897/CompCytogen.v6i1.2523

Marques I, Draper D, Riofrío L, Naranjo C (2014) Multiple hybridization events, polyploidy and low postmating isolation entangle the evolution of neotropical species of *Epidendrum* (Orchidaceae). BMC Evolutionary Biology 14: 20. https://doi.org/10.1186/1471-2148-14-20.

Mazzola P, Romano S, Fici P (1988) Contributo alla conoscenza del genere *Opuntia* Miller. Dati cariologici e distributivi delle specie spontaneizzate e coltivate in Sicilia. Naturalista Siciliano IV 12: 159–168.

Medel-Narváez A (2008) Estudio ecológico y genético del cardón (*Pachycereus pringlei*) en el desierto Sonorense. Ph.D. dissertation. La Paz, Baja California Sur, México: Centro de Investigaciones Biológicas del Noroeste, 114 pp.

Medel-Narváez A, León-de la Luz JL, Freaner-Martínez F, Molina-Freaner F (2006) Patterns of abundance and population structure of *Pachycereus pringlei* (Cactaceae), a columnar cactus of the Sonoran Desert. Plant Ecology 187: 1–14. https://doi.org/10.1007/s11258-006-9128-1

Molgo IE, Soltis DE, Soltis PS (2017) Cytogeography of *Callisia* section *Cuthbertia* (Commelinaceae). Comparative Cytogenetics 11: 553–557. https://doi.org/10.3897/CompCytogen.v11i3.11984.

Molina-Freaner F, Cervantes-Salas M, Morales-Romero D, Buchmann S, Fleming TH (2003) Does the pollinator abundance hypothesis explain geographic variation in the breeding system of *Pachycereus pringlei*? International Journal of Plant Sciences 164: 383–393. https://doi.org/10.1086/368394

Murawski DA, Fleming TH, Ritland K, Hamrick JL (1994) Mating system of *Pachycereus pringlei*: an autotetraploid cactus. Heredity 72: 86–94. https://doi.org/10.1038/hdy.1994.11.

Oliveira LC, de Oliveira MD, Davide LC, Torres GA (2016) Karyotype and genome size in *Euterpe* Mart.(Arecaceae) species. Comparative Cytogenetics 10: 17–25. https://doi.org/10.3897/CompCytogen.v10i1.5522

Palomino H, Zuleta S, Scheinvar L (1988) Estudios citogenéticos de dos especies y una variedad de *Nyctocereus* (Cactaceae). Boletín de la Sociedad Botánica de México 48: 75–80.

Parfitt BD (1987) *Echinocereus nicholii* (L. Benson) Cactaceae. Phytologia 63: 157–158.

Pinkava DJ, McLeod MG (1971) Chromosome numbers in some cacti of western North America. Brittonia 23: 171–176. https://doi.org/10.2307/2805433

Pinkava DJ, Parfitt BD (1982) Chromosome numbers in some cacti of western North America. IV. Bulletin of the Torrey Botanical Club 109: 121–128. https://doi.org/10.2307/2996251

Pinkava DJ, Parfitt BD (1988) Nomenclatural changes in the Chihuahuan Desert *Opuntia* (Cactaceae). SIDA Contributions to Botany (USA) 13: 125–130. http://www.jstor.org/stable/41966769

Pinkava DJ, Baker MA, Parfitt BD, Mohlenbrock MW, Worthington RD (1985) Chromosome numbers in some cacti of western North America-V. Systematic Botany 10: 471–483. https://doi.org/10.2307/2419140

Pinkava DJ, McGill LA, Reeves T (1977) Chromosome numbers in some cacti of western North America-III. Bulletin of the Torrey Botanical Club 104: 105–110. https://doi.org/10.2307/2484355

Pinkava DJ, McLeod MG, McGill LA, Brown RC (1973) Chromosome numbers in some cacti of western North America-II. Brittonia 25: 2–9. https://doi.org/10.2307/2805484

Pinkava DJ, Rebman JP, Baker MA (1998) Chromosome numbers in some cacti of western North America-VII. Haseltonia 6: 32–41.

Rani S, Sharma TR, Kapila R, Chahota RK (2015) Identification of new cytotypes of *Valeriana jatamansi* Jones, 1970 (Valerianaceae) from North-Western Himalayan region of India. Comparative Cytogenetics 9: 499–512. https://doi.org/10.3897/CompCytogen.v9i4.8875

Rebman JP, Pinkava DJ (2001) *Opuntia* cacti of North America: an overview. Florida Entomologist 84: 474–483. https://doi.org/10.2307/3496374

Ross R (1981) Chromosome counts, cytology, and reproduction in the Cactaceae. American Journal of Botany 68: 463–470. http://www.jstor.org/stable/2443022

Sahley CT (1996) Bat and hummingbird pollination of an autotetraploid columnar cactus, *Weberbauerocereus weberbaueri* (Cactaceae). American Journal of Botany 83: 1329–1336. http://www.jstor.org/stable/2446118

Segura S, Scheinvar L, Olalde G, Leblanc O, Filardo S, Muratalla A, Gallegos C, Flores C (2007) Genome sizes and ploidy levels in Mexican cactus pear species *Opuntia* (Tourn.) Mill. series *Streptacanthae* Britton et Rose, *Leucotrichae* DC., *Heliabravoanae* Scheinvar and *Robustae* Britton et Rose. Genetic Resources and Crop Evolution 54: 1033–1041. https://doi.org/10.1007/s10722-006-9196-z

Soltis DE, Soltis PS (1999) Polyploidy: recurrent formation and genome evolution. Trends in Ecology and Evolution 14: 348–52. https://doi.org/10.1016/S0169-5347(99)01638-9

Soltis DE, Soltis PS, Schemske DW, Hancock JF, Thompson JN, Husband BC, Judd WS (2007) Autopolyploidy in angiosperms: have we grossly underestimated the number of species? Taxon 56: 13–30. http://www.jstor.org/stable/25065732

Stebbins GL (1950) Variation and evolution in plants. Columbia University Press, New York, NY, 643 pp.

Stebbins GL (1971) Chromosomal evolution in higher plants. Addison Wesley, London, 216 pp.

Stock AD, Hussey N, Beckstrom MD (2014) A new species of *Opuntia* (Cactaceae) from Mojave Co, Arizona. Cactus and Succulent Journal (Los Angeles) 86: 79–83. https://doi.org/10.2985/015.086.0201

Strittmatter LI (2002) Subdioecy in *Consolea spinosissima* (Cactaceae): breeding system and embryological studies. American Journal of Botany 89: 1373–1387. http://www.jstor.org/stable/4124055

Strittmatter LI, Hickey RJ, Negrón-Ortiz V (2008) Heterochrony and its role in sex determination of cryptically dioecious *Consolea* (Cactaceae) staminate flowers. Botanical Journal of the Linnean Society 156: 305–326. https://doi.org/10.1111/j.1095-8339.2007.00754.x

Strittmatter LI, Negrón-Ortiz V, Hickey RJ (2006) Comparative microsporangium development in male-fertile and male-sterile flowers of *Consolea* (Cactaceae): when and how does pollen abortion occur. Grana 45: 81–100. http://dx.doi.org/10.1080/00173130500537101

Swanson CP, Merz T, Young WJ (1981) Cytogenetics. The chromosome in division, inheritance and evolution. Prentice Hall, NY, USA, 577 pp.

Tate JA, Soltis DE, Soltis PS (2005) Polyploidy in plants. In: Gregory TR (Ed) The evolution of the genome. Elsevier Academic Press, Burlington, MA, USA, 371–426. https://doi.org/10.1016/B978-012301463-4/50009-7

Turco A, Medagli P, Albano A, D'Emerico S (2014) Karyomorphometry on three polyploid species of *Arum* L. (Araceae, Aroideae). Comparative Cytogenetics 8: 71–80. https://doi.org/10.3897/compcytogen.v8i1.6444

Turner RM, Bowers JE, Burgess TL (1995) Sonoran Desert plants: an ecological atlas. University of Arizona Press, Tucson, AZ, USA, 504 pp.

Vacher JP, Kok PJ, Rodrigues MT, Lima JD, Lorenzini A, Martinez Q, Fallet M, Courtois EA, Blanc M, Gaucher P, Dewynter M (2017) Cryptic diversity in Amazonian frogs: integrative taxonomy of the genus *Anomaloglossus* (Amphibia: Anura: Aromobatidae) reveals a unique case of diversification within the Guiana Shield. Molecular Phylogenetics and Evolution 112: 158–173. https://doi.org/10.1016/j.ympev.2017.04.017

Valiente-Banuet A, Rojas-Martínez A, Del Coro Arizmendi M, Dávila P (1997) Pollination biology of two columnar cacti (*Neobuxbaumia mezcalaensis* and *Neobuxbaumia macrocephala*) in the Tehuacán Valley, central Mexico. American Journal of Botany 84: 452–455. http://www.jstor.org/stable/2446020

Wellard B (2016) A new ancestral diploid species of *Echinocereus* (Cactaceae) endemic to southwestern Utah and northwestern Arizona (USA). Journal of the Botanical Research Institute of Texas 10: 345–359.

Wendel J, Doyle J (2005) Polyploidy and evolution in plants. In: Henry RJ (Ed.) Plant diversity and evolution: genotypic and phenotypic variation in higher plants. CABI, Wallingford, UK, 97–117.

CompCytogen 12(1): 83–95 (2018)
doi: 10.3897/CompCytogen.v12i1.14995
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Genomic *in situ* hybridization in interspecific hybrids of scallops (Bivalvia, Pectinidae) and localization of the satellite DNA *Cf303*, and the vertebrate telomeric sequences (TTAGGG)n on chromosomes of scallop *Chlamys farreri* (Jones & Preston, 1904)

Liping Hu[1,2,*], Liming Jiang[1,*], Ke Bi[3], Huan Liao[1], Zujing Yang[1], Xiaoting Huang[1], Zhenmin Bao[1,4]

1 *Ministry of Education Key Laboratory of Marine Genetics and Breeding, College of Marine Life Sciences, Ocean University of China, 5 Yushan Road, Qingdao 266003, China* **2** *Yantai Fisheries Research Institute, Yantai 264003, China* **3** *Museum of Vertebrate Zoology, University of California, Berkeley, California 94720, USA* **4** *Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China*

Corresponding author: *Xiaoting Huang* (xthuang@ouc.edu.cn)

Academic editor: *V. Stunžėnas* | Received 14 July 2017 | Accepted 27 November 2017 | Published 13 March 2018

http://zoobank.org/086A7DEC-6CDA-43C1-8BD5-34754BC289D8

Citation: Hu L, Jiang L, Bi K, Liao H, Yang Z, Huang X, Bao Z (2018) Genomic *in situ* hybridization in interspecific hybrids of scallops (Bivalvia, Pectinidae) and localization of the satellite DNA *Cf303*, and the vertebrate telomeric sequences (TTAGGG)n on chromosomes of scallop *Chlamys farreri* (Jones & Preston, 1904). Comparative Cytogenetics 12(1): 83–95. https://doi.org/10.3897/CompCytogen.v12i1.14995

Abstract

Mitotic chromosome preparations of the interspecific hybrids *Chlamys farreri* (Jones & Preston, 1904) × *Patinopecten yessoensis* (Jay, 1857), *C. farreri* × *Argopecten irradinas* (Lamarck, 1819) and *C. farreri* × *Mimachlamys nobilis* (Reeve, 1852) were used to compare two different scallop genomes in a single slide. Although genomic *in situ* hybridization (GISH) using genomic DNA from each scallop species as probe painted mitotic chromosomes of the interspecific hybrids, the painting results were not uniform; instead it showed species-specific distribution patterns of fluorescent signals among the chromosomes. The most prominent GISH-bands were mainly located at centromeric or telomeric regions of scallop chromosomes. In order to illustrate the sequence constitution of the GISH-bands, the satellite *Cf303* sequences of *C. farreri* and the vertebrate telomeric $(TTAGGG)_n$ sequences were used to map mitotic chromosomes

* These authors contributed equally to this work.

of *C. farreri* by fluorescence *in situ* hybridization (FISH). The results indicated that the GISH-banding pattern presented by the chromosomes of *C. farreri* is mainly due to the distribution of the satellite *Cf303* DNA, therefore suggesting that the GISH-banding patterns found in the other three scallops could also be the result of the chromosomal distribution of other species-specific satellite DNAs.

Keywords
GISH banding, FISH, *Cf303*, telomere, scallop

Introduction

Chromosomal bandings were used to identify chromosomes and provided insight into the substructure and organization of whole chromosomes (Rønne 1990). Some chromosome banding techniques have been applied to bivalves, e.g. C-banding was used for investigating the distribution and composition of heterochromatin (Pasantes et al. 1996, Insua et al. 1998, Pauls and Affonso 2000, Huang et al. 2007b, García-Souto et al. 2016), silver staining was used for localizing the nucleolus organizer regions (Ag-NORs) (Martínez-Expósito et al. 1997, Pauls and Affonso 2000, Boroń et al. 2004), fluorescence counterstaining with chromomycin A_3 or DAPI/PI used to identify the GC-rich or AT-rich heterochromatic regions (Martínez et al. 2002, Huang et al. 2007b, Zhang et al. 2007c, Pérez-García et al. 2010a, García-Souto et al. 2015, 2016), and fluorescent *in situ* hybridization (FISH) were used to localize 28S rDNA, 5S rDNA, and histone H3 gene to study karyotypic evolution on a variety of bivalves (Insua et al. 1998, 2006, Wang and Guo 2004, López-Piñón et al. 2005, Huang et al. 2006, 2007a, Odierna et al. 2006, Zhang et al. 2007b, Hu et al. 2011, Li et al. 2016, Yang et al. 2016, García-Souto et al. 2015, 2016). In addition, FISH was proved to be a valuable tool for mapping vertebrate telomere sequence $(TTAGGG)_n$ on chromosomes of some bivalves (Wang and Guo 2001, Huang et al. 2007a, 2007b, García-Souto et al. 2016).

Genomic *in situ* hybridization (GISH) has been successfully used for discrimination of genome constitutions in hybrids and allopolyploids (Schwarzacher et al. 1989, 1992; Heslop-Harrison and Schwarzacher 1996; Hae-Woonet et al. 2008; Huang et al. 2011; Hu et al. 2013) and estimating phylogenetic relationships (Markova et al. 2007, Zhou et al. 2008). GISH-banding was occasionally observed on chromosomes with conventional GISH, which revealed chromosomal distribution of the randomly repeated sequences (Zhou et al. 2010). Patterns of GISH-banding coincident with those of Giemsa C-banding were first reported in the genus *Alstroemeria* (Linnaeus, 1762) using standard GISH with blocking DNA (Kuipers et al. 1997). Belyayev et al. (2001) constructed a GISH-banding karyotype of *Aegilops speltoides* (Tausch, 1837) and investigated the evolutionary dynamics of repetitive sequences in *Aegilops* (Linnaeus, 1753). Zhou et al. (2008) developed a GISH-banding protocol, built a universal reference karyotype of the *Secale cereale* (Linnaeus, 1753) chromosome 1R to 7R, and discriminate the repetitive sequence polymorphism in species or subspecies

of *Secale*. In Pectinidae, GISH was used to discriminate parental genomes in hybrids and some GISH-bandings were observed which implied the different distribution of repetitive sequences (Huang et al. 2011, Hu et al. 2013, Huang et al. 2015). Recently, the genome of Yesso scallop, *Patinopecten yessoensis* (Jay, 1857) was sequenced and assembled, providing a thorough overview of the repetitive sequences constitution in Pectinidae (Wang et al. 2017). In addition, by paired-end sequencing of 2016 fosmid clones, a total of 2500 tandem repeats of *Chlamys farreri* (Jones & Preston, 1904), including 313 satellites, 1816 minisatellites and 371 microsatellites, were described (Zhang et al. 2008). However, the distributions of repetitive DNA sequences in chromosomes of different species of the family Pectinidae are still uncertain.

Repetitive DNA refers to DNA sequences that occur in multiple copies and makes up the major proportion of the nuclear DNA in most eukaryotic genomes. Changes in repetitive DNA likely contribute to the karyotypical features and variations, as well as genome sizes (Flavell 1986, San Miguel and Bennetzen 1998). Repetitive DNAs usually evolve faster than coding regions, and their sequence divergence may reflect evolutionary distances between species (Belyayev and Raskina 1998). Satellite DNAs, as the tandem arrays of repeated units, are paramount among repetitive sequences and can be located in centromeric, intercalary and/or subtelomeric chromosomal regions (Plohl et al. 2008, Plohl 2010, García-Souto et al. 2017), which are chiefly heterochromatic regions of chromosomes (Brutlag 1980).

In the present study, GISH was carried out on chromosomal slides of interspecific hybrids *C. farreri* × *P. yessoensis*, *C. farreri* × *Argopecten irradians* (Lamarck, 1819) and *C. farreri* × *Mimachlamys nobilis* (Reeve, 1852). Chromosomal distributions of the randomly repeated DNA sequences were revealed by GISH-banding in the four scallop species (*C. farreri*, *P. yessoensis*, *A. irradians* and *M. nobilis*). In order to verify the sequences constitution of GISH-banding, FISH with the satellite DNA *Cf303* and vertebrate telomere sequence $(TTAGGG)_n$ probes were performed to compare the GISH-banding of *C. farreri*. Our results provided the first application of GISH-banding in Pectinidae, and first physical mapping of the satellite DNA *Cf303* and vertebrate telomere sequence $(TTAGGG)_n$ in *C. farreri*, aiding to understanding chromosome distribution and composition of the repetitive DNA sequences in the studied scallops.

Material and methods

Specimens and chromosome preparations

The sexually mature scallops, *C. farreri*, *P. yessoensis*, *A. irradians* and *M. nobilis*, were obtained from hatcheries in Shandong Province, China. The interspecific hybrids *C. farreri* × *P. yessoensis*, *C. farreri* × *A. irradians* and *C. farreri* × *M. nobilis* were carried out in the laboratory. Eggs and sperm were collected from sexually mature scallops. Eggs were fertilized by adding sperm suspension. After fertilization, excessive sperm was removed by rinsing with seawater on a 20 μm screen (Wang and Wang

2008). The progeny individuals were sampled at the trochophore stage. Chromosome preparations were performed following the method of Zhang et al. (2007a). Briefly, following a treatment with colchicine (0.01 %, 1.5 h) and KCl (0.075 M, 20 min), trochophores were fixed three times (15 min each) in fresh ethanol/ glacial acetic acid solution (3:1 v/v). The fixed larvae were dissociated in 50 % acetic acid to obtain a cell suspension and that was then dropped onto hot-wet glass slides. The chromosome preparations were air-dried and stored at -20 °C until use.

Probe preparation

Telomeric $(TTAGGG)_7$ probes were synthesized and 5'-end labelled with biotin-16-dUTP (Invitrogen). Plasmids were isolated from a fosmid clone containing the satellite DNA *Cf303* by standard laboratory methods (Sambrook et al. 1989). Genomic DNA was extracted from adductor muscle tissue using a standard phenolchloroform procedure (Sambrook et al. 1989). Then they were both labeled with biotin-16-dUTP by nick translation (Nick translation kit, Roche) following manufacturer's instructions. The lengths of the DNA fragments were estimated by 2 % agarose gels and 100–600 bp were suitable as the probes for next GISH analysis.

GISH and FISH

GISH and FISH were performed according to the methods of Huang et al. (2011) and Zhang et al. (2007a). Detection of biotin-labeled probes was carried out with fluorescein avidin DCS (Vector). Chromosomes were then counterstained with PI (Vector). Slides were examined with a Nikon Eclipse-600 epifluorescence microscope equipped with a CCD camera, and the signals were collected using appropriate filter sets for FITC and PI. The digital images were analyzed with a Lucia-FISH Image System Software. At least 10 complete metaphase chromosome spreads were examined for each sample.

Results

Using labeled total genomic DNA from *C. farreri* as probe, the hybridization signal (yellow-green) covered only chromosomes of *C. farreri*, but not *P. yessoensis*, *A. irradians* and *M. nobilis*, on the mitotic chromosomes of *C. farreri* hybrids with these scallop species (Fig. 1A1, B1, C1). Moreover, the signals on chromosomes of *C. farreri* were not uniform. The brighter fluorescent signals (GISH-bandings) clustered in the centromeric and/or peri-centromeric regions of 11–12 subtelocentric chromosomes, and the telomeric and/or peri-telomeric regions of the long arms of 13–14 submetacentric or subtelocentric chromosomes of *C. farreri*. In addition, the signal in telomeric regions was apparently stronger than that in the centromeric regions.

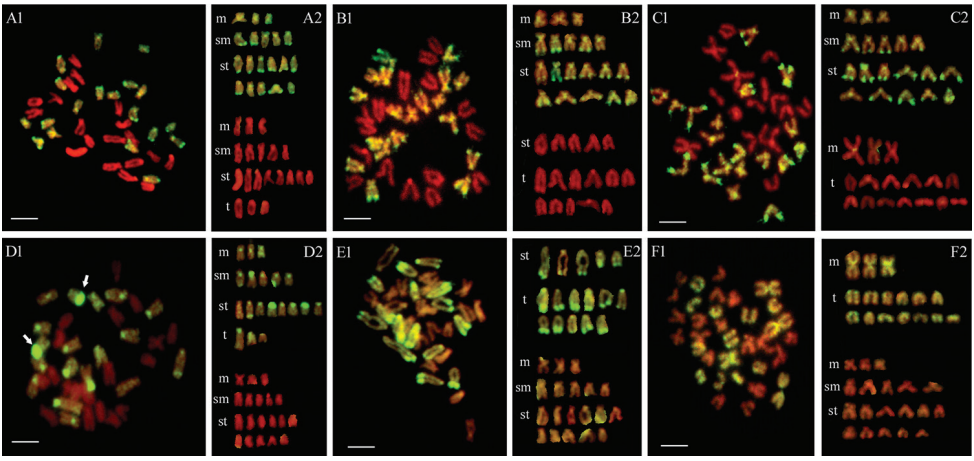

Figure 1. Metaphase chromosomes and karyotypes of scallop hybrids. **A1, A2, D1, D2** *C. farreri* × *P. yessoensis* **B1, B2, E1, E2** *C. farreri* × *A. irradians* **C1, C2, F1, F2** *C. farreri* × *M. nobilis* **A1, A2, B1, B2, C1, C2** the chromosomes originating from *C. farreri* were painted in yellow-green using the labeled genomic DNA probes from *C. farreri* **D1, D2** - the chromosomes originating from *P. yessoensis* were painted in yellow-green using the labeled genomic DNA probes from *P. yessoensis*. Nucleolus organizer regions (NORs) in *P. yessoensis* were marked with arrows in (**D1**). **E1, E2** the chromosomes originating from *A. irradians* were painted in yellow-green using the labeled genomic DNA probes from *A. irradians*. **F1, F2** the chromosomes originating from *M. nobilis* were painted in yellow-green using the labeled genomic DNA probes from *M. nobilis*. Scale bars: 5μm.

Similarly, the hybridization signals only covered chromosomes of one parent whose genomic DNA was labeled as probes: the metaphase of *C. farreri* × *P. yessoensis* – by genomic DNA from *P. yessoensis* (Fig. 1D1), the metaphase of *C. farreri* × *A. irradians* – by genomic DNA from *A. irradians* (Fig. 1E1) and the metaphase of *C. farreri* × *M. nobilis* – by genomic DNA from *M. nobilis* (Fig. 1F1). Interestingly, the GISH-bandings of different scallops were significantly different. The GISH-bandings of *P. yessoensis* (Fig. 1D1, D2) were mainly distributed on centromeric regions of almost all chromosomes, the nucleolus organizer regions (NORs) where the signals were particularly strong in the peri-telomeric regions of the short arms of two subtelocentric chromosomes (arrows in Fig. 1D1), and peri-telomeric region of two subtelocentric chromosomes. The chromosomes of *A. irradians* were clearly labeled by the brighter signals in telomeric and/or peri-telomeric regions of the long arms of all chromosomes, in the short arms of two subtelocentric or telocentric chromosomes, and in interstitial regions of one chromosome (Fig. 1E1, E2). On the chromosomes of *M. nobilis*, the signals were dispersed along all them and some specific signals were located on centromeric regions (Fig. 1F1, F2).

Considering that the GISH-bandings were mainly discovered in the telomeric and/or peri-telomeric regions of some chromosomes in *C. farreri*, the vertebrate telomeric sequence $(TTAGGG)_n$, as well as satellite DNA *Cf303* were used as probes to compare the signal distribution. Telomeric repeats were hybridized to the ends of all *C. farreri*

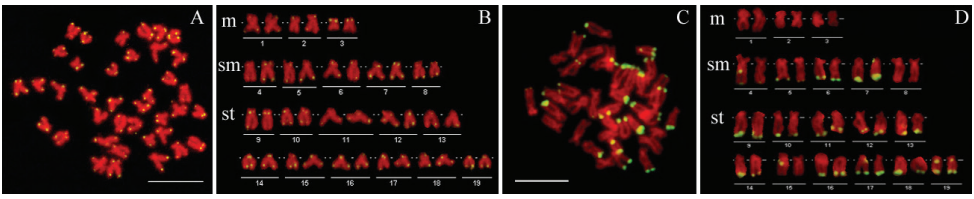

Figure 2. Metaphase chromosomes and karyotype of *C. farreri*. FISH mapping with vertebrate telomeric sequence $(TTAGGG)_7$ (**A, B**) and satellite DNA *Cf303* (**C, D**). Scale bars: 5μm.

chromosomes, the signal intensity was weak and varied among different chromosomes, and no interstitial hybridization signal was observed (Fig. 2A, B). Satellite DNA *Cf303*, detected by FISH, were located on the centromeric region of a pair of subtelocentric chromosomes, and the telomeric regions of the long arms of 13–14 pairs of submetacentric or subtelocentric chromosomes in *C. farreri*, as shown in Fig. 2C, D.

Discussion

Constitutive heterochromatin has been defined as a structurally distinct kind of chromatin comprising noncoding, largely repetitive DNA, which is permanently not transcribed (John 1988). The constitutive heterochromatic regions were usually detectable by C-banding (Sumner 1990, Sharma and Raina 2005). All C-bands correspond to heterochromatin but some heterochromatin was not stained by C-banding methods (Sumner 1990). In bivalves, the C-banding techniques were carried out in many species, such as mussels (Vitturi et al. 2000, Boroń et al. 2004), scallops (Insua et al. 1998, Pauls and Affonso 2000, Huang et al. 2007b) and oysters (Leitão et al. 2001, Pereira et al. 2011). Moreover, the C-banding patterns obtained in some species were consist with the chromomycin A3 positive bands or 4'6'-diamidino-2-phenylindole (DAPI) / propidium iodide (PI) banding patterns, revealing the GC-rich or AT-rich heterochromatic regions on chromosomes of bivalves (Boroń et al. 2004, Huang et al. 2007b, Zhang et al. 2007c, Petrović et al. 2009). However, C-bandings were not stable and still couldn't be obtained successfully in many bivalve species (Xu et al. 2011).

Using GISH, Kuipers et al. (1997) found that GISH-banding patterns coincided with Giemsa C-banding patterns in the genus *Alstroemeria*. The consistent results between GISH-banding patterns and Giemsa C-banding patterns had been attributed to specific repetitive sequences, such as a tandem repeat from *Allium fistulosum* (Linnaeus, 1753), which was found to occur in major heterochromatic blocks (Irifune et al. 1995). FISH results of several highly repetitive sequences showed a significantly correspondence with the C-banding pattern in comparable studies of *S. montanum* (Gussone, 1825) (Cuadrado and Jouve 1995). The researches mentioned above were mainly focused on plant. However, the GISH-bandings in animals were rarely reported. In the present study, we observed significant GISH-bandings on the chromosomes of *C. farreri*, *P. yessoensis* and *A. irradians* after GISH. The results suggested the distribution of repetitive DNA

(heterochromatin regions) were multifarious in scallops. In addition, the GISH-banding results of *P. yessoensis* and *A. irradians* corresponded roughly to their C-band-like patterns revealed by PI staining (Zhang et al. 2007c). Because there wasn't reported about the herterochromatic region in chromosomes of *C. farreri*, we speculated that the GISH-bandings may reveal heterochromatic regions in *C. farreri*.

Highly repeated DNA exists within each eukaryotic genome. Satellite DNAs, as the tandem arrays of repeated units, are chiefly localized at heterochromatic regions of chromosomes (Brutlag 1980). The vertebrate telomeric repeat has been located at chromosome ends in some bivalves (Wang and Guo 2004, Huang et al. 2007a, García-Souto et al. 2016). To verify the constitution of GISH-banding, vertebrate telomeric sequences $(TTAGGG)_n$ and satellite DNA *Cf303* were selected as probes to locate on chromosomes of *C. farreri* by FISH.

Vertebrate-type telomeric sequences $(TTAGGG)_n$ located at terminal regions of each chromosome of *C. farreri* in our study. The signal intensities were weak and varied among different chromosomes; no interstitial hybridization signal was observed. This is the first report about the chromosomal distribution of telomeric sequences in *C. farreri.* The locations of these sequences were coincident with the results reported in *P. yessoensis* and *A. irradians* (Huang et al. 2007a, 2007b). In other bivalves, mostly terminal signals for these sequences were located in mussels (Mytilidae) (Martínez-Expósito et al. 1997, Plohl et al. 2002, Pérez-García et al. 2010a, 2010b, 2011), oysters (Ostreidae) (Guo and Allen 1997, Wang et al. 2001), wedge-shells (Donax) (Plohl et al. 2002, Petrović et al. 2009) and trough shells (Mactridae) (García-Souto et al. 2016, 2017). Indicia of intercalary signals were only reported in species of genus Mytilus (Martínez-Expósito et al. 1997, Plohl et al. 2002) and genus Brachidontes (Pérez-García et al. 2010b), probably as a result of the interspersion of telomeric sequences and the subterminal major rDNA. The telomeric signal intensity varied among different chromosomes in *C. farreri*, which suggested the length of telomeric repeat sequences were different among different chromosomes.

Satellite DNAs are highly repetitive DNA sequences that can be located in pericentromeric (Plohl et al. 1998), telomeric regions (Petrović et al. 2009) or intercalary regions (García-Souto et al. 2017). In bivalves, a highly repetitive satellite sequence *Cg*170 was located on the centromeric regions of 7 chromosomes in the *Crassostrea gigas* (Thunberg, 1793) (Wang et al. 2001). Our FISH results showed that the satellite DNA *Cf303* was located in centromeric region of one or two subtelocentric chromosome and the telomeric regions of the long arms of most submetacentric or subtelocentric chromosomes in *C. farreri*. Satellite DNAs could act as informative cytogenetic markers for the identification of chromosomal abnormalities, pairs of homologous chromosomes and specific regions of chromosomes, such as the α satellites of human (Gusella et al. 1982, Looijenga et al. 1990), CL1 and CL25 satellite repeats of *Raphanus sativus* (Linnaeus, 1753) (He et al. 2015), and the *Cg*170 satellites of *C. gigas* (Clabby et al. 1996). In this study, the satellite DNA *Cf303* could be developed as specific probe for identification of chromosomes in *C. farreri*.

In contrast to the location of telomere sequence in scallops, the satellite DNA *Cf303* existed only on the chromosomes of *C. farreri*, but not on the chromosomes of closely related species *P. yessoensis*, *A. irradians* and *M. nobilis*, which suggested that the satellite DNA *Cf303* was species-specific. After comparing the signal distribution and intensity of GISH-banding, vertebrate telomeric sequences and satellite DNA *Cf303*, we found the GISH-banding pattern in *C. farreri* was not consistent with the result of telomeric repeats. Interestingly, the GISH-bands overlapped the FISH signals obtained with satellite DNA *Cf303*. Generally, satellite DNAs are chiefly localized at heterochromatic regions of chromosomes (Brutlag 1980). Our results suggested that satellite DNA *Cf303* may represent the dominating component of heterochromatic regions in the chromosomes of *C. farreri*, as shown by GISH-banding.

Zhou et al. (2008) believed GISH-banding has displayed rapidly evolving repetitive sequences in the study of repetitive sequences polymorphism in *S.cereale*. In view of this, we speculated that the GISH-banding patterns in *C. farreri* were ascribed to rapidly evolving repetitive sequences, especially satellite DNA *Cf303*. In addition, the GISH-banding patterns of *P. yessoensis*, *A. irradians* and *M. nobilis* were completely different with that of *C. farreri*, which indicated that the repetitive sequences in the GISH-banding regions were distinctly species-specific in different scallop species. These species-specific satellite DNA and GISH-banding patterns would represent a useful tool in the scallop taxonomy for closely related species studies. Results of this study would further contribute to a better understanding the characteristics of genomic structure and to assess the evolutionary relationships within Pectinidae.

Acknowledgement

This research was funded by the National Natural Science Foundation of China (41676132), the Fundamental Research Funds for the Central Universities (201762016, 201562018), the Earmarked Fund for Modern Agro-industry Technology Research System (CARS-49), the key Research and Development Program of Shandong Province (2016ZDJQ0208), and the Seed Improvement Project of Shandong Province.

References

Belyayev A, Raskina O (1998) Heterochromatin discrimination in *Aegilops speltoides* by simultaneous genomic *in situ* hybridization. Chromosome Research 6: 559–565. https://doi.org/10.1023/A:1009292726034

Belyayev A, Raskina O, Nevo E (2001) Evolutionary dynamics and chromosomal distribution of repetitive sequences on chromosomes of *Aegilops speltoides* revealed by genomic *in situ* hybridization. Heredity 86(6): 738–742. https://doi.org/10.1046/j.1365-2540.2001.t01-1-00891.x

Boroń A, Woźnicki P, Skuza L, Zieliński R (2004) Cytogenetic characterization of the zebra mussel *Dreissena polymorpha* (Pallas) from Miedwie Lake, Poland. Folia Biologica (Kraków) 52: 33–38.

Brutlag DL (1980) Molecular arrangement and evolution of heterochromatic DNA. Annual Review of Genetics 14: 121–144. https://doi.org/10.1146/annurev.ge.14.120180.001005

Clabby C, Goswami U, Flavin F, Wilkins NP, Houghton JA, Powell R (1996) Cloning, characterization and chromosomal location of a satellite DNA from the Pacific oyster, *Crassostrea gigas*. Gene 168: 205–209. https://doi.org/10.1016/0378-1119(95)00749-0

Cuadrado A, Jouve N (1995) Fluorescent *in situ* hybridization and C-banding analyses of highly repetitive DNA sequences in the heterochromatin of rye (*Secale montanum* Guss.) and wheat incorporating *S. montanum* chromosome segments. Genome 38(4): 795–802. https://doi.org/10.1139/g95-101

Flavell RB (1986) Repetitive DNA and chromosome evolution in plants. Philosophical Transactions of the Royal Society B–Biological Sciences 312: 227–242. https://doi.org/10.1098/rstb.1986.0004

García-Souto D, Pérez-García C, Morán P, Pasantes JJ (2015) Divergent evolutionary behavior of H3 histone gene and rDNA clusters in venerid clams. Molecular Cytogenetics 8(1): 40. https://doi.org/10.1186/s13039-015-0150-7

García-Souto D, Pérez-García C, Kendall J, Pasantes JJ (2016) Molecular cytogenetics in trough shells (Mactridae, Bivalvia): Divergent GC-rich heterochromatin content. Genes 7(8): 47. https://doi.org/10.3390/genes7080047

García-Souto D, Mravinac B, Šatović E, Plohl M, Morán P, Pasantes JJ (2017) Methylation profile of a satellite DNA constituting the intercalary G+C-rich heterochromatin of the cut trough shell *Spisula subtruncata* (Bivalvia, Mactridae). Scientific Reports 7(1): 6930. https://doi.org/10.1038/s41598-017-07231-7

Guo X, Allen SK (1997) Fluorescence *in situ* hybridization of vertebrate telomere sequence to chromosome ends of the Pacific oyster, *Crassostrea gigas* Thunberg. Journal of Shellfish Research. 16: 87–89.

Gusella JF, Jones C, Kao F, Housman D, Puck TT (1982) Genetic fine-structure mapping in human chromosome 11 by the use of repetitive DNA sequences. Proceedings of the National Academy of Sciences of the United States of America 79(24): 7804–7808. https://doi.org/10.1073/pnas.79.24.7804

Hae-Woon C, Jeong-Soon K, Sang-Hoon L, Jae-Wook B (2008) Physical mapping by FISH and GISH of rDNA loci and discrimination of genomes A and B in *Scilla scilloides* complex distributed in Korea. Journal of Plant Biology 51(6): 408–412. https://doi.org/10.1007/BF03036061

He Q, Cai Z, Hu T, Liu H, Bao C, Mao W, Jin W (2015) Repetitive sequence analysis and karyotyping reveals centromere-associated DNA sequences in radish (*Raphanus sativus* L.). BMC Plant Biology 15: 105. https://doi.org/10.1186/s12870-015-0480-y

Heslop-Harrison JS, Schwarzacher T (1996) Genomic Southern and in situ hybridization for plant genome analysis. In: Jauhar PP, Methods of Genome Analysis in Plants. Boca Raton: CRC Press 163–179 pp.

Hu L, Shang W, Sun Y, Wang S, Ren X, Huang X, Bao Z (2011) Comparative cytogenetics analysis of *Chlamys farreri*, *Patinopecten yessoensis*, and *Argopecten irradians* with C_0t-1 DNA by fluorescence *in situ* hybridization. Evidence-Based Complementary and Alternative Medicine. 2011: 785831. https://doi.org/10.1155/2011/785831

Hu L, Huang X, Mao J, Wang C, Bao Z (2013) Genomic characterization of interspecific hybrids between the scallops *Argopecten purpuratus* and *A. irradians irradians*. PLoS ONE 8(4): e62432. https://doi.org/10.1371/journal.pone.0062432

Huang X, Bao Z, Bi K, Hu J, Zhang C, Zhang Q, Hu X (2006) Chromosomal localization of the major ribosomal RNA genes in scallop *Chlamys farreri*. Acta Oceanologica Sinica 25: 108–115.

Huang X, Hu X, Hu J, Zhang L, Wang S, Lu W, Bao Z (2007a) Mapping of ribosomal DNA and $(TTAGGG)_n$ telomeric sequence by FISH in the bivalve *Patinopecten yessoensis* (Jay, 1857). Journal of Molluscan Studies 73(4): 393–398. https://doi.org/10.1093/mollus/eym036

Huang X, Hu J, Hu X, Zhang C, Zhang L, Wang S, Lu W, Bao Z (2007b) Cytogenetic characterization of the bay scallop, *Argopecten irradians irradians*, by multiple staining techniques and fluorescence *in situ* hybridization. Genes & Genetic Systems 82: 257–263. https://doi.org/10.1266/ggs.82.257

Huang X, Bi K, Hu L, Sun Y, Lu W, Bao Z (2011) Fertilization and cytogenetic examination of interspecific reciprocal hybridization between the scallops, *Chlamys farreri* and *Mimachlamys nobilis*. PLoS ONE 6(11): e27235. https://doi.org/10.1371/journal.pone.0027235

Huang X, Bi K, Lu W, Wang S, Zhang L, Bao Z (2015) Genomic in situ hybridization identifies parental chromosomes in hybrid scallop (Bivalvia, Pectinoida, Pectinidae) between female *Chlamys farreri* and male *Argopecten irradians irradians*. Comparative Cytogenetics 9(2): 189–200. https://doi.org/10.3897/CompCytogen.v9i2.8943

Insua A, López-Piñón MJ, Méndez J (1998) Characterization of *Aequipecten opercularis* (Bivalvia: Pectinidae) by different staining techniques and fluorescent *in situ* hybridisation. Genes & Genetic Systems 73(4): 193–200. https://doi.org/10.1266/ggs.73.193

Insua A, López-Piñón MJ, Freire R, Méndez J (2006) Karyotype and chromosomal location of 18S–28S and 5S ribosomal DNA in the scallops *Pecten maximus* and *Mimachlamys varia* (Bivalvia: Pectinidae). Genetica 126(3): 291–301. https://doi.org/10.1007/s10709-005-7408-7

Irifune K, Hirai K, Zheng J, Tanaka R (1995) Nucleotide sequence of a highly repeated DNA sequence and its chromosomal localization in *Allium fistulosum*. Theoretical and Applied Genetics 90: 312–316. https://doi.org/10.1007/BF00221970

John B (1988) The biology of heterchromatin. Heterochromatin: Molecular and structural aspects, ed. By R. S. Verma, Cambridge University Press, Cambridge, 1–47.

Kuipers AGJ, van Os DPM, de Jong JH, Ramanna MS (1997). Molecular cytogenetics of *Alstroemeria*: identification of parental genomes in interspecific hybrids and characterization of repetitive DNA families in constitutive heterochromatin. Chromosome Research 5: 31–39. https://doi.org/10.1023/A:1018489318300

Leitão A, Chaves R, Santos S, Boudry P, Guedespinto H (2001) C-banding in the oysters *Crassostrea gigas* and *Ostrea edulis*. 14th International Chromosome Conference: 2001, Würzburg, Germany. https://doi.org/10.1023/A:1016718306415

Li X, Yang Z, Liao H, Zhang Z, Huang X, Bao Z (2016) Chromosomal mapping of tandem repeats in the Yesso scallop, *Patinopecten yessoensis* (Jay, 1857), utilizing fluorescence *in situ*

hybridization. Comparative Cytogenetics 10: 157–169. https://doi.org/10.3897/CompCytogen.v10i1.7391

Looijenga LHJ, Smit VTHBM, Wessels JW, Mollevanger P, Oosterhuis JW, Cornelisse CJ, Devilee P (1990) Localization and polymorphism of a chromosome 12-specific a satellite DNA sequence. Cytogenetic and Genome Research 53: 216–218. https://doi.org/10.1159/000132934

López-Piñón MJ, Insua A, Méndez J (2005) Chromosome analysis and mapping of ribosomal genes by one- and two color fluorescent *in situ* hybridization in *Hinnites distortus* (Bivalvia: Pectinidae). Journal of Heredity 96: 52–58. https://doi.org/10.1093/jhered/esi001

Markova M, Michu E, Vyskot B, Janousek B, Zluvova J (2007) An interspecific hybrid as a tool to study phylogenetic relationships in plants using the GISH technique. Chromosome Research 15: 1051–1059. https://doi.org/10.1007/s10577-007-1180-8

Martínez A, Mariñas L, González-Tizón A, Méndez J (2002). Cytogenetic characterization of *Donax trunculus* (Bivalvia: Donacidae) by means of karyotyping, fluorochrome banding and fluorescent in situ hybridization. Journal of Molluscan Studies 68: 393–396. https://doi.org/10.1093/mollus/68.4.393

Martínez-Expósito MJ, Méndez J, Pasantes JJ (1997) Analysis of NORs and NOR-associated heterochromatin in the mussel *Mytilus galloprovincialis* Lmk. Chromosome Research 5(4): 268–273.

Odierna G, Aprea G, Barucca M, Canapa A, Capriglione T, Olmo E (2006) Karyotype of the Antarctic scallop *Adamussuim colbecki*, with some comments on the karyological evolution of pectinids. Genetica 127: 341–349. https://doi.org/10.1007/s10709-005-5366-8

Pasantes JJ, Martínez-Expósito MJ, Méndez J (1996) C-band polymorphism in the chromosomes of the mussel *Mytilus galloprovincialis* Lmk. Caryologia 49: 233–245. https://doi.org/10.1080/00087114.1996.10797368

Pauls E, Affonso PR (2000) The karyotype of *Nodipecten nodosus* (Bivalvia: Pectinidae). Hydrobiologia 420: 99–102. https://doi.org/10.1007/978-94-017-2184-4_11

Pereira JC, Chaves R, Batista FM, Guedespinto H, LeltãoA (2011) Cytogenetic characterization of the dwarf oyster *Ostrea stentina* (Mollusca: Bivalvia) and comparative karyological analysis within Ostreinae. Journal of Shellfish Research 30(2): 211–216. https://doi.org/10.2983/035.030.0203

Pérez-García C, Cambeiro JM, Morán P, Pasantes JJ (2010a) Chromosomal mapping of rDNAs, core histone genes and telomeric sequences in *Perumytilus purpuratus* (Bivalvia: Mytilidae). Journal of Experimental Marine Biology and Ecology 395: 199–205. https://doi.org/10.1016/j.jembe.2010.09.004

Pérez-García C, Guerra-Varela J, Morán P, Pasantes JJ (2010b). Chromosomal mapping of rRNA genes, core histone genes and telomeric sequences in *Brachidontes puniceus* and *Brachidontes rodriguezi* (Bivalvia: Mytilidae). BMC Genetics 11: 109. https://doi.org/10.1186/1471-2156-11-109

Pérez-García C, Morán P, Pasantes JJ (2011) Cytogenetic characterization of the invasive mussel species *Xenostrobus securis* Lmk. (Bivalvia: Mytilidae). Genome 54: 771–778. https://doi.org/10.1139/G11-040

Petrović V, Pérez-García C, Pasantes JJ, Satović E, Prats E, Plohl M (2009). A GC-rich satellite DNA and karyology of the bivalve mollusk *Donax trunculus*: a dominance of GC-

rich heterochromatin. Cytogenetic and Genome Research 124(1): 63–71. https://doi.org/10.1159/000200089

Plohl M, Mestrovic N, Bruvo B, Ugarkovic D (1998) Similarity of structural features and evolution of satellite DNAs from *Palorus subdepressus* (Coleoptera) and related species. Journal of Molecular Evolution 46: 234–239. https://doi.org/10.1007/PL00006298

Plohl M, Prats E, Martínez-Lage A, González-Tizón A, Méndez J, Cornudella L (2002) Telomeric localization of the vertebrate-type hexamer repeat, $(TTAGGG)_n$ in the wedge shell clam *Donax trunculus* and other marine invertebrate genomes. The Journal of Biological Chemistry 277: 19839–19846. https://doi.org/10.1074/jbc.M201032200

Plohl M, Luchetti A, Meštrović N, Mantovani B (2008) Satellite DNAs between selfshness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero) chromatin. Gene 409: 72–82. https://doi.org/10.1016/j.gene.2007.11.013

Plohl M (2010) Those mysterious sequences of satellite DNAs. Periodicum Biologorum 112: 403–410.

Rønne M (1990) Chromosome preparation and high resolution banding (review). Journal of Dairy Science 4(6): 337–365.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NewYork, 26–32.

San Miguel P, Bennetzen JL (1998) Evidence that a recent increase in maize genome size was caused by the massive amplification of intergene retrotransposons. Annals of Botany 82: 37–44. https://doi.org/10.1006/anbo.1998.0746

Schwarzacher T, Anamthawat-Jonsson K, Harrison GE, Islam AKMR, Jia JZ, King IP, Leitch AR, Miller TE, Reader SM, Rogers WJ, Shi M, Heslop-Harrison JS (1992) Genomic *in situ* hybridization to identify alien chromosomes and chromosome segments in wheat. Theoretical and Applied Genetics 84: 778–786. https://doi.org/10.1007/BF00227384

Schwarzacher T, Leitch AR, Bennet MD, Heslop-Harrison JS (1989) In situ localization of parental genomes in a wide hybrid. Annals of Botany 64: 315–324. https://doi.org/10.1093/oxfordjournals.aob.a087847

Sharma S, Raina SN (2005) Organization and evolution of highly repeated satellite DNA sequences in plant chromosomes. Cytogenetic and Genome Research 109: 15–26. https://doi.org/10.1159/000082377

Sumner AT (1990) Chromosome banding. Academic Division of Unwin Hyman, London, 434 pp.

Vitturi R, Gianguzza P, Colomba MS, Riggio S (2000) Cytogenetic characterization of *Brachidontes pharaonis* (Fisher P, 1870): karyotype, banding and fluorescent *in situ* hybridization (FISH) (Mollusca: Bivalvia: Mytilidae). Ophelia 52: 213–220. https://doi.org/10.1080/00785236.1999.10409430

Wang R, Wang Z (2008) Science of marine shellfish culture. Qingdao: China Ocean University Press 61–72.

Wang S, Zhang J, Jiao W, Li J, Xun X, Sun Y, Guo X, Huan P, Dong B, Zhang L, Hu X, Sun X, Wang J, et al. (2017) Scallop genome provides insights into evolution ofbilaterian karyotype and development. Nature Ecology and Evolution 1: 0120. https://doi.org/10.1038/s41559-017-0120

Wang Y, Guo X (2001) Chromosomal mapping of the vertebrate telomeric sequences $(TTAGGG)_n$ in four bivalve molluscs by fluorescence *in situ* hybridization. Journal of Shellfish Research 20(3): 1187–1190.

Wang Y, Guo X (2004) Chromosomal rearrangement in Pectinidae revealed by rRNA loci and implications for bivalve evolution. Biological Bulletin 207: 247–256. https://doi.org/10.2307/1543213

Wang Y, Xu Z, Guo X (2001) A centromeric satellite sequence in the pacific oyster (*Crassostrea gigas* Thunberg) identified by fluorescence *in situ* hybridization. Marine Biotechnology 3: 486–492. https://doi.org/10.1007/s10126-001-0063-3

Xu J, Bao Z, Ren X, Wang S, Hu L, Huang X (2011) DAPI banding and PI banding of Zhikong scallop (*Chlamys farreri*). Periodical of Ocean University of China 41(4): 77–80. [In Chinese with English abstract]

Yang Z, Li X, Liao H, Hu L, Zhang Z, Zhao B, Huang X, Bao Z (2016) Physical mapping of immune-related genes in Yesso scallop (*Patinopecten yessoensis*) using fluorescent *in situ* hybridization. Comparative Cytogenetics 10(4): 529–541. https://doi.org/10.3897/CompCytogen.v10i4.10047

Zhang L, Bao Z, Wang S, Hu X, Hu J (2007a) FISH mapping and identification of Zhikong scallop (*Chlamys farreri*) chromosomes. Marine Biotechnology 10: 151–157. https://doi.org/10.1007/s10126-007-9045-x

Zhang L, Bao Z, Wang S, Hu X, Hu J (2007b) Chromosome rearrangements in Pectinidae (Bivalvia: Pteriomorphia) implied based on chromosomal localization of histone H3 gene in four scallops. Genetica 130: 193–198. https://doi.org/10.1007/s10709-006-9006-8

Zhang L, Bao Z, Wang J, Wang S, Huang X, Hu X, Hu J (2007c) Cytogenetic analysis in two scallops (Bivalvia: Pectinidae) by PRINS and PI banding. Acta Oceanologica Sinica 26(5): 153–158.

Zhang L, Chen C, Cheng J, Wang S, Hu X, Hu J, Bao Z (2008) Initial analysis of tandemly repetitive sequences in the genome of Zhikong scallop (*Chlamys farreri* Jones et Preston). DNA Sequence 19(3): 195–205. https://doi.org/10.1080/10425170701462316

Zhou J, Yang Z, Li G, Liu C, Ren Z (2008). Discrimination of repetitive sequences polymorphism in *Secale cereale* by genomic *in situ* hybridization-banding. Journal of Integrative Plant Biology 50(4): 452–456. https://doi.org/10.1111/j.1744-7909.2008.00644.x

Zhou J, Yang Z, Li G, Liu C, Tang Z, Zhang Y, Ren Z (2010) Diversified chromosomal distribution of tandemly repeated sequences revealed evolutionary trends in *Secale* (Poaceae). Plant Systematics and Evolution 287: 49–56. https://doi.org/10.1007/s00606-010-0288-z

CompCytogen 12(1): 97–110 (2018)
doi: 10.3897/CompCytogen.v12i1.22344
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Multidirectional chromosome painting in *Synallaxis frontalis* (Passeriformes, Furnariidae) reveals high chromosomal reorganization, involving fissions and inversions

Rafael Kretschmer[1], Vanusa Lilian Camargo de Lima[2], Marcelo Santos de Souza[2], Alice Lemos Costa[3], Patricia C. M. O'Brien[4], Malcolm A. Ferguson-Smith[4], Edivaldo Herculano Corrêa de Oliveira[5,6], Ricardo José Gunski[2], Analía Del Valle Garnero[2]

1 *Programa de Pós-graduação em Genética e Biologia Molecular, PPGBM, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, RS, Brazil* **2** *Programa de Pós-graduação em Ciências Biológicas, PPCGCB, Universidade Federal do Pampa, São Gabriel, Rio Grande do Sul, Brazil* **3** *Graduação em Ciências Biológicas, Universidade Federal do Pampa, São Gabriel, Rio Grande do Sul, Brazil* **4** *Cambridge Resource Centre for Comparative Genomics, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK* **5** *Faculdade de Ciências Naturais, Instituto de Ciências Exatas e Naturais, Universidade Federal do Pará, Belém, Pará, Brazil* **6** *Laboratório de Cultura de Tecidos e Citogenética, SAMAM, Instituto Evandro Chagas, Ananindeua, Brazil*

Corresponding author: *Rafael Kretschmer* (rafa.kretschmer@gmail.com)

Academic editor: *A. Barabanov* | Received 17 November 2017 | Accepted 15 February 2018 | Published 14 March 2018

http://zoobank.org/C379B8F3-45B9-455E-9009-AC6E06A2B667

Citation: Kretschmer R, de Lima VLC, de Souza MS, Costa AL, O'Brien PCM, Ferguson-Smith MA, de Oliveira EHC, Gunski RJ, Garnero ADV (2018) Multidirectional chromosome painting in *Synallaxis frontalis* (Passeriformes, Furnariidae) reveals high chromosomal reorganization, involving fissions and inversions. Comparative Cytogenetics 12(1): 97–110. https://doi.org/10.3897/CompCytogen.v12i1.22344

Abstract

In this work we performed comparative chromosome painting using probes from *Gallus gallus* (GGA) Linnaeus, 1758 and *Leucopternis albicollis* (LAL) Latham, 1790 in *Synallaxis frontalis* Pelzeln, 1859 (Passeriformes, Furnariidae), an exclusively Neotropical species, in order to analyze whether the complex pattern of intrachromosomal rearrangements (paracentric and pericentric inversions) proposed for Oscines and Suboscines is shared with more basal species. *S. frontalis* has 82 chromosomes, similar to most Avian species, with a large number of microchromosomes and a few pairs of macrochromosomes. We found polymorphisms in pairs 1 and 3, where homologues were submetacentric and acrocentric. Hybridization

of GGA probes showed syntenies in the majority of ancestral macrochromosomes, except for GGA1 and GGA2, which hybridized to more than one pair of chromosomes each. LAL probes confirmed the occurrence of intrachromosomal rearrangements in the chromosomes corresponding to GGA1q, as previously proposed for species from the order Passeriformes. In addition, LAL probes suggest that pericentric inversions or centromere repositioning were responsible for variations in the morphology of the heteromorphic pairs 1 and 3. Altogether, the analysis of our data on chromosome painting and the data published in other Passeriformes highlights chromosomal changes that have occurred during the evolution of Passeriformes.

Keywords

Avian cytogenetics, chromosome painting, macrochromosome syntenies, chromosome fission, intrachromosomal rearrangements

Introduction

Passeriformes (passerines) are the largest and most diverse order of birds, with approximately 5,700 species, representing almost 60 % of all living birds (Ericson et al. 2014). The order is divided into two suborders: Oscines (songbirds), which comprise 776 genera and approximately 80 % of all species of Passeriformes, and Suboscines (vocal non-learners), with 284 genera (Selvatti et al. 2015). Among the Suboscines, the family Furnariidae (ovenbirds and woodcreepers) is outstanding for its exceptional diversification and ecological adaptation (Chesser et al. 2004, Moyle et al. 2009). Among its three subfamilies, Furnariinae is the richest in number of species (Irestedt et al. 2002, Remsen et al. 2016).

Among birds, Passeriformes have the highest number of species analyzed by classical cytogenetics (Santos and Gunski 2006). Most species show diploid numbers (2n) ranging between 76–80 chromosomes, although there are exceptions, such as *Platyrinchus mystaceus* Vieillot, 1818 a Suboscine species belonging to the Platyrinchidae family, which has 60 chromosomes (Gunski et al. 2000, Santos and Gunski 2006, Correia et al. 2009). Among the Furnariidae, only two species have been described cytogenetically - *Sittasomus griseicapillus* Vieillot, 1818 and *Lepidocolaptes angustirostris* Vieillot, 1818, both with 2n=82 (Barbosa et al. 2013).

Besides information on diploid number and chromosome morphology, classical cytogenetic analyses have detected examples of chromosomal polymorphisms in some species of Passeriformes, such as *Saltator similis* d'Orbigny and Lafresnaye, 1837 (dos Santos et al. 2015) and *Zonotrichia albicollis* Gmelin, 1789 (Thomas et al. 2008). The polymorphism found in the latter species was associated with plumage and behavioral variations (Thomas et al. 2008), corroborating the fact that chromosomal alterations may have important effects on genome function, aside from being important phylogenetic markers.

Fourteen species of the suborder Oscines have been analyzed by chromosome painting (Guttenbach et al. 2003, Derjusheva et al. 2004, Itoh and Arnold 2005, Nanda et al. 2011, Kretschmer et al. 2014, dos Santos et al. 2015, 2017), and only four species of Suboscines (Kretschmer et al. 2015, Rodrigues et al. In press). Eight of these species were compared using only whole chromosome probes of *Gallus gallus* Linnaeus, 1758

(GGA) (Guttenbach et al. 2003, Derjusheva et al. 2004, Itoh and Arnold 2005, Nanda et al. 2011). The results have shown mostly the same syntenic groups found in the putative avian ancestral karyotype (PAK) proposed by Griffin et al. (2007), with the exception of PAK1 (GGA1), which is split into two chromosome pairs, representing a synapomorphy shared by all the species of Passeriformes analyzed so far. However, the other ten species were analyzed with whole chromosome probes from two different species – GGA and *Leucopternis albicollis* Latham, 1790 (LAL), an Accipitridae with 2n=66, in which syntenic groups corresponding to PAK pairs 1, 2, 3 and 5 each correspond to 2–5 different pairs (de Oliveira et al. 2010, Kretschmer et al. 2014, 2015, dos Santos et al. 2015, 2017, Rodrigues et al. In press). This approach revealed additional rearrangements in which chromosome pairs corresponding to PAK1p and PAK1q were reshuffled through a series of paracentric and pericentric inversions in both Oscines and Suboscines species, suggesting that these rearrangements had occurred early in the history of Passeriformes, before their split into two suborders (Kretschmer et al. 2014, 2015, dos Santos et al. 2015, 2017). The occurrence of such intrachromosomal rearrangements has been confirmed by data from genome sequencing in Passeriformes (Frankl-Vilches et al. 2015).

Species belonging to the genus *Synallaxis* Vieillot, 1818 (Subfamily Furnariinae) show higher diversification when compared to other Furnariidae, probably due to the shift in their nesting habits and an expansion of their habitats to open areas (Irested et al. 2009), but only a few species of this family have been karyotyped. Hence, the aim of this study was to analyze the karyotype of *Synallaxis frontalis* Pelzeln, 1859, a species belonging to family Furnariidae, by chromosome painting using GGA and LAL probes, in order to verify if this complex pattern of intrachromosomal rearrangements is also present in more basal species for Passeriformes.

Material and methods

Samples and chromosome preparations

The experiments followed protocols approved by the ethics committee (CEUA-Universidade Federal do Pampa, no. 026/2012, SISBIO 33860-3 and 44173-1). Seven specimens of *Synallaxis frontalis* (SFR), four males and three females, were caught in São Gabriel, Rio Grande do Sul State, Brazil, within the natural area of Universidade Federal do Pampa. Skin biopsies were used for fibroblast cultures according to Sasaki et al. (1968) and chromosomes were obtained by standard protocols using colcemid and fixation with Carnoy fixative.

Classical cytogenetics

Diploid number and chromosome morphology were determined by the analysis of at least 20 metaphases per individual, conventionally stained with Giemsa. C-banding

(Ledesma et al. 2002) was used to analyze the distribution of blocks of constitutive heterochromatin. Chromosomes were ordered following size and centromere position, according to Ladjali-Mohammedi et al. (1999).

Fluorescent *in situ* hybridization

Fluorescent *in situ* hybridization (FISH) experiments were performed with whole chromosome probes from two different species – *Gallus gallus* (pairs GGA1-GGA10) and *Leucopternis albicollis* (LAL), pairs homologous to GGA 1 (LAL 3, 6, 7, 15 and 18), GGA 2 (LAL 2, 4 and 20), GGA 3 (LAL 9, 13, 17 and 26), GGA 4 (LAL 1 and 16), GGA 5 (LAL 5) and GGA 6 (LAL 3) (de Oliveira et al. 2010). Both sets of probes were obtained by flow cytometry at the Cambridge Resource Centre for Comparative Genomics (Cambridge, United Kingdom), and labeled by DOP-PCR, using biotinilated nucleotides. Hybridization, stringency washes and detection were performed according to de Oliveira et al. (2010). Slides were analyzed using a fluorescence microscope (Zeiss Imager Z2) and images were captured using the software Axiovision 4.8 (Zeiss, Germany).

Results

Classical cytogenetics

We found a karyotype of 2n=82 in *Synallaxis frontalis*, with 11 pairs of macrochromosomes, including the Z and the W chromosomes, and 30 pairs of microchromosomes (Figure 1A–B). In some individuals, pairs 1 and 3 showed heteromorphism of the length of their short arms (Figure 1). Hence, pair 1 was represented by a submetacentric and an acrocentric element in one male and one female, while in the other five individuals both homologues of this pair were acrocentric. Additionally, chromosomes of pair 3 were heteromorphic (acrocentric and submetacentric) in two males and one female, while in the other four individuals this pair was acrocentric. In two individuals, pairs 1 and 3 were both acrocentric. Pair 2 and 4–7 were acrocentric, while pair 8 was metacentric and the Z chromosome was submetacentric in all individuals. The W chromosome was metacentric (Fig. 1A–D).

C-banding showed that blocks of constitutive heterochromatin were located in the centromeric region of the autosomes and Z chromosome, while the W chromosome was almost completely heterochromatic (Figure 1B, D).

GGA whole chromosome probes showed that most syntenic groups found in the putative avian ancestral karyotype (PAK) were conserved in SFR, except for GGA 1 and GGA 2, which were fissioned into two pairs each - SFR1/SFR5 and SFR3/SFR7, respectively (Fig. 2). LAL probes confirmed that both fissions were centric (Fig. 3). The results also suggested that pericentric inversions or centromere repositioning were

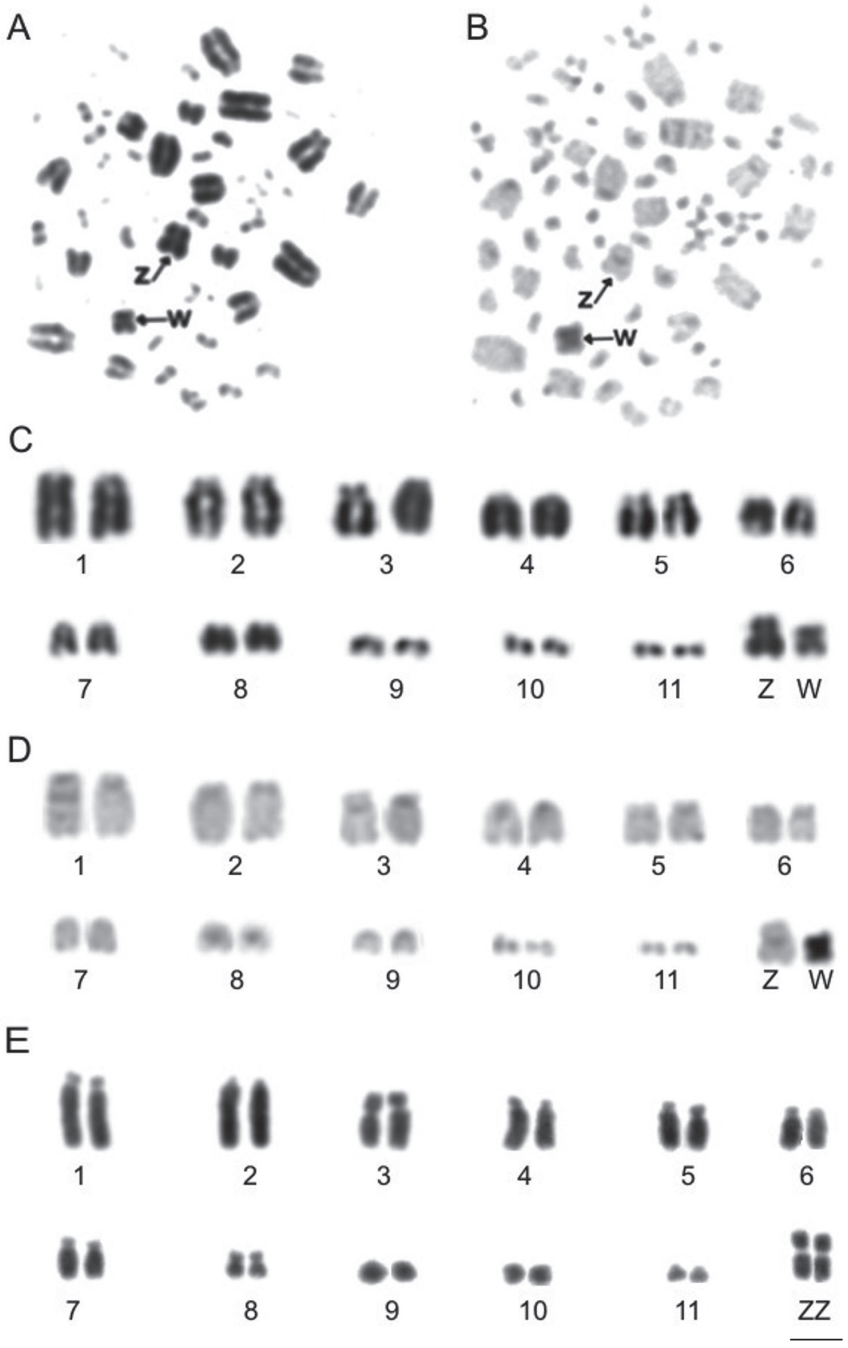

Figure 1. Metaphases and partial karyotype of female *Synallaxis frontalis* with heteromorphism in pair 1: Giemsa (**A, C**), C-banding (**B, D**). Partial karyotype of male *S. frontalis* with heteromorphism in pair 3 (**E**). Arrows indicate the Z and W chromosomes. Scale bar: 5 μm.

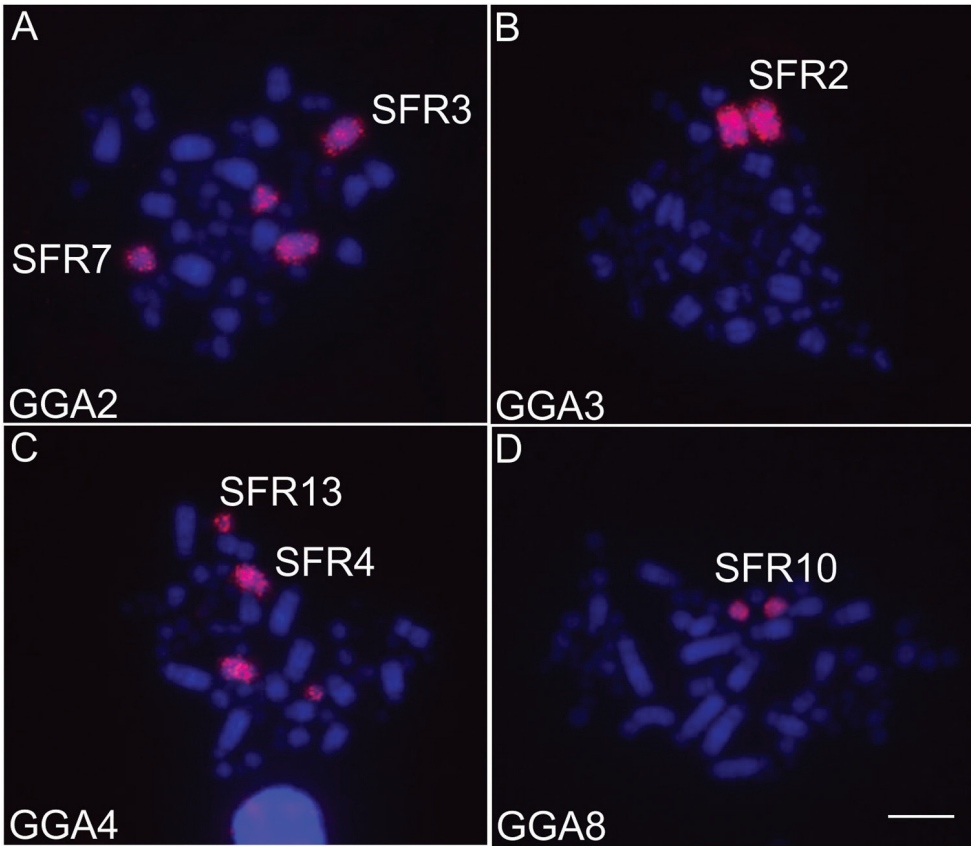

Figure 2. Representative FISH experiments using *Gallus gallus* GGA (**A–D**) probes on metaphase chromosomes of *Synallaxis frontalis* (SFR). Chromosomes were counterstained with DAPI (blue), and probes detected with Cy3 (red). Probes used are indicated in the lower left corner of the images. Scale bar: 5 μm.

responsible for the heteromorphism observed in pairs SFR1 and SFR3 (figs 5, 6). Moreover, the complex pattern of intrachromosomal rearrangements involving paracentric and pericentric inversions previously described in other Passeriformes in the chromosome corresponding to GGA1q were also detected by LAL probes. The homology map comparing SFR, GGA and LAL chromosomes is shown in Figure 4A.

Discussion

The genome of *S. frontalis* shows a chromosomal organization typical for Class Aves and order Passeriformes (Gunski et al. 2000, Santos and Gunski 2006, Kretschmer et al. 2014), with 2n=82. The two species of the family Furnariidae described cytogenetically so far, *Sittasomus griseicapillus* and *Lepidocolaptes angustirostris*, also have the same diploid number found in *S. frontalis*, but with variations in the morphologies of some

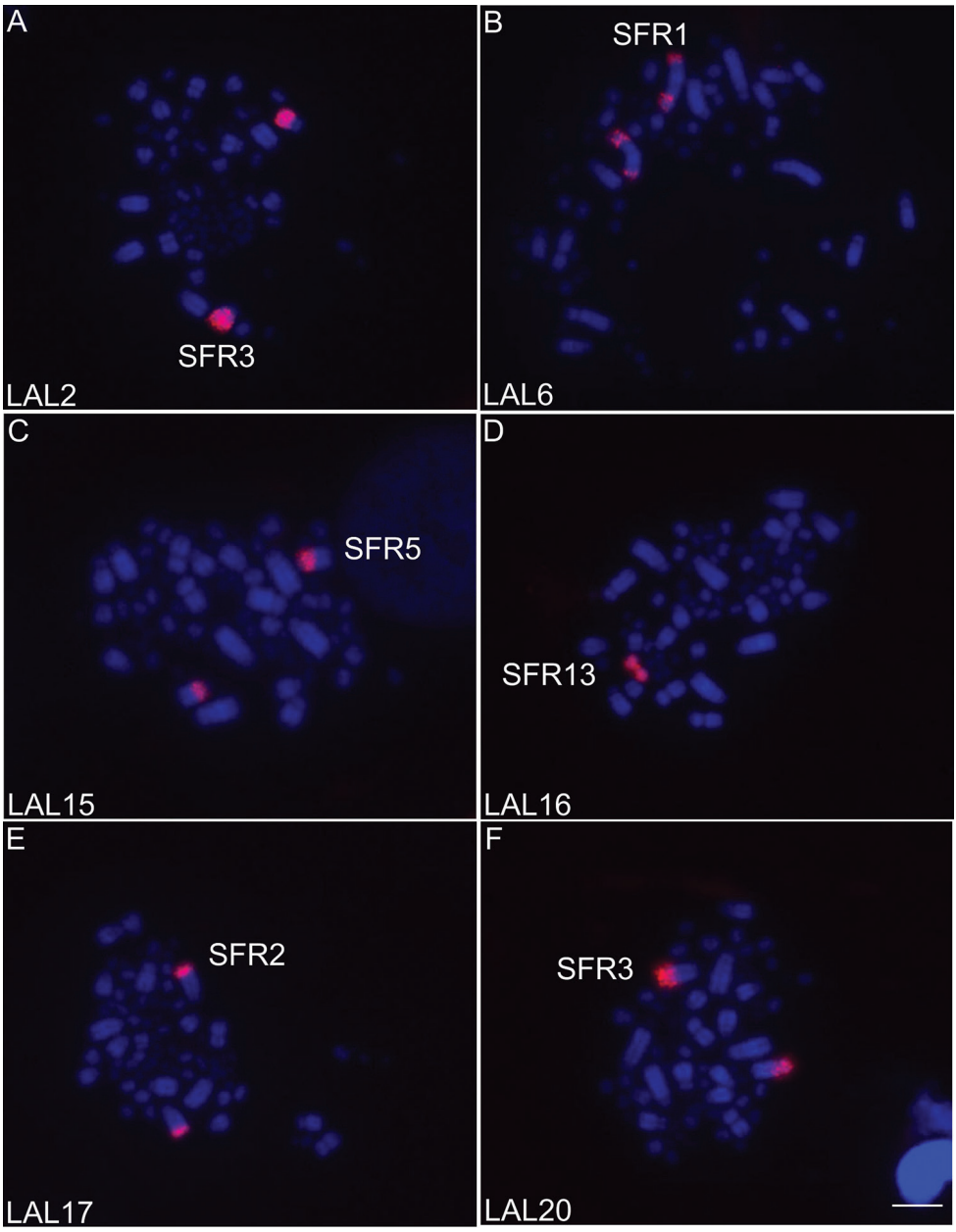

Figure 3. Representative FISH experiments using *Leucopternis albicollis* LAL (**A–F**) probes on metaphase chromosomes of *Synallaxis frontalis* (SFR). Chromosomes were counterstained with DAPI (blue), and probes detected with Cy3 (red). Probes used are indicated in the lower left corner of the images. Scale bar: 5 μm.

macrochromosomes (Barbosa et al. 2013). The Z chromosome of *S. frontalis* is submetacentric, unlike the acrocentric morphology found in *S. griseicapillus*, and *L. angustirostris*. The variation in morphology of this chromosome is common, even in species

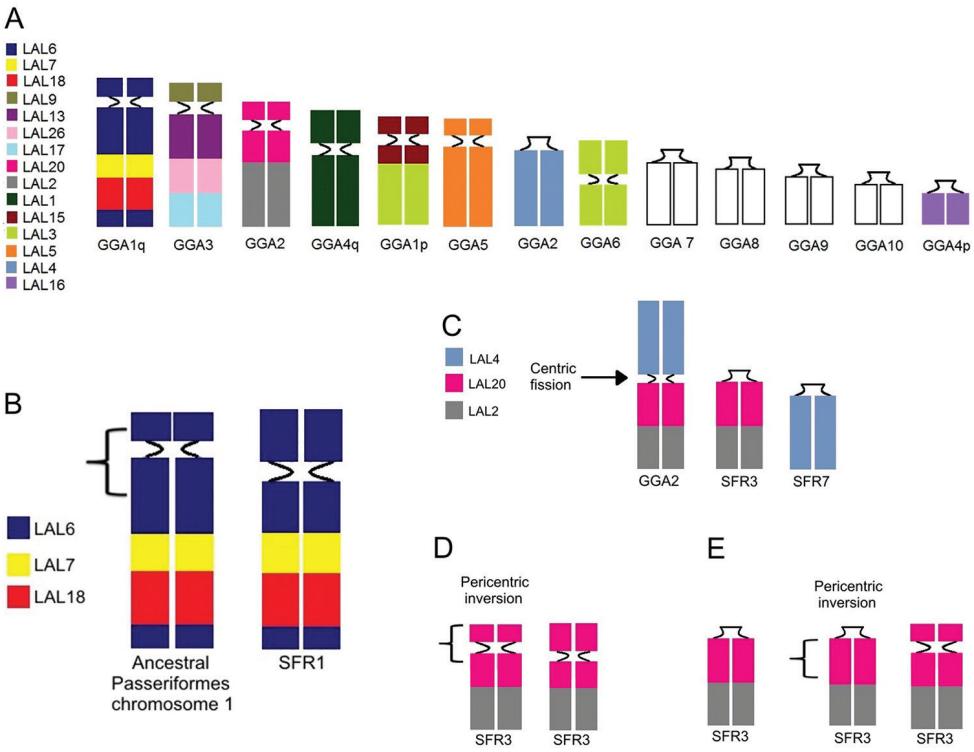

Figure 4. Homology map (13 first autosomal pairs) comparing the syntenic groups of *Synallaxis frontalis* to *Gallus gallus* (bottom) and *Leucopternis albicollis* (colors) (**A**). Schematic diagram showing the hypothetical pericentric inversion responsible for the heteromorphism observed in pair 1 from two individuals of *Synallaxis frontalis* (SFR1) (**B**). Hypothetical rearrangements observed in *Synallaxis frontalis* (SFR) PAK 2 (GGA2) that would have given rise to SFR3 and SFR7 (C–E). First, a centric fission in the ancestral synteny homologous to GGA2, created two distinct chromosome pairs, homologous to GGA2p (SFR7) and GGA2q (SFR3) (**C**). A pericentric inversion in SFR3 changed its morphology to acrocentric (**D**). A second pericentric inversion gave rise to the heteromorphic element in pair 3, which corresponds to a submetacentric chromosome (**E**).

of the same family, as observed in family Tyrannidae (Gunski et al. 2000, Kretschmer et al. 2015), probably due to the presence of repetitive sequences in this chromosome (Nanda et al. 2002). Similarly to these two species, the W chromosome is metacentric. Concerning heteromorphic pairs 1 and 3, it is interesting to notice that heteromorphisms in autosomal chromosomes are not exclusive to *S. frontalis*, since they have been reported in species of the Emberizidae family (genera *Zonotrichia* Swainson, 1832 and *Junco* Wagler, 1831), as well as in the Z chromosome of *Saltator similis* (Thraupidae) (Shields 1973, de Lucca 1985, Thomas et al. 2008, dos Santos et al. 2015). Additionally, the C-banding pattern in *S. frontalis* is similar to most bird species, with blocks of constitutive heterochromatin in the centromeric region of chromosomes and in most of the W chromosome (Kretschmer et al. 2014, dos Santos et al. 2015).

Most of the ancestral macrochromosomes are conserved as whole chromosomes in *S. frontalis*, as shown by the hybridizations of *G. gallus* macrochromosomes. Only the first two pairs (GGA1 and GGA2) are not conserved, due to the occurrence of fissions, and correspond to SFR1 and SFR5, SFR3 and SFR7 pairs, respectively. The fission of the ancestral chromosome 1 has been found in all species of the order Passeriformes studied to date (19 species, including *S. frontalis*) (Guttenbach et al. 2003, Derjusheva et al. 2004, Itoh and Arnold 2005, Nanda et al. 2011, Kretschmer et al. 2014, 2015, dos Santos et al. 2015, 2017, Rodrigues et al. In press). Probably this characteristic is shared by all Passeriformes, since it was found in species of both suborders, Oscines (14 species) and Suboscines (5 species, including *S. frontalis*). The presence of this characteristic in the genome of *S. frontalis* is important for the confirmation of this hypothesis, since only four species of the Suboscines suborder had been studied by chromosome painting, and now we can verify that this characteristic is shared between two species in different families, Tyrannidae and Furnariidae (Kretschmer et al. 2015, Rodrigues et al. In press). In addition, the Furnariidae family is more basal than the family Tyrannidae (Selvatti et al. 2015).

Unlike the fission of the GGA1 chromosome, the fission of the GGA2 chromosome has been described previously in only one species of the order Passeriformes, *Satrapa icterophrys* Vieillot, 1818 (Rodrigues et al. In press). This rearrangement is probably shared with two other species of the Furnariidae family described by Barbosa et al. (2013), because in these species the two first autosomes pairs are similar in size, a fact also observed in *S. frontalis*. Two species of the Formicariidae family (Furnariidae sister group) also present the first two pairs with similar size, so the fission of the GGA2 chromosome may be a characteristic shared by the species of Parvordem Furnariida (Ledesma et al. 2002, Selvatti et al. 2015). This similarity was not observed in other Passeriformes analyzed by chromosome painting until now, suggesting the possibility of fission of chromosome 2 in other species of Furnariidae and Formicariidae (Guttenbach et al. 2003, Derjusheva et al. 2004, Itoh and Arnold 2005, Nanda et al. 2011, Kretschmer et al. 2014, 2015, dos Santos et al. 2015, 2017). It is necessary to confirm this hypothesis by chromosome painting in different families of Parvordem Furnariida. In addition, as the fission of chromosome GGA2 has been observed in only one Tyrannidae species up to this moment, this rearrangement corresponds probably to a convergent character in *Satrapa icterophrys* and in Furnariidae species.

Hybridizations with LAL probes was not enough to identify the mechanism responsible for the heteromorphisms observed in the first and third chromosomes pairs in some SFR individuals. Both heteromorphisms may have originated either by pericentric inversions or centromere repositioning. Pericentric and paracentric inversions have been reported in several species of Passeriformes (Warren et al. 2010, Volker et al. 2010, Skinner and Griffin 2012, Kretschmer et al. 2014, 2015, dos Santos et al 2015, 2017). However, an alternative explanation is the centromere repositioning that was also reported in Galliformes species (Kasai et al. 2003, Zlotina et al., 2012). We have assumed that the mechanism was a pericentric inversion, since several *in silico* and chromosome painting studies have demonstrated a high frequency of inversions in bird species, especially Pas-

seriformes (Warren et al. 2010, Volker et al. 2010, Skinner and Griffin 2012, Kretschmer et al. 2014, 2015). Thus, an extra inversion may had occurred in the region corresponding to LAL6 (GGA1q) (Fig. 4B) in one of the homologous chromosomes in individuals with heteromorphisms in the first pair, in addition to the three inversions common to all Passeriformes. Similarly, in individuals with heteromorphisms in the third pair, there was an inversion in the segment corresponding to LAL20 in one homologue (Fig. 4C–D).

In addition to the *in silico* analysis demonstrating several intrachromosomal rearrangements, chromosome painting studies with *L. albicollis* probes have also identified some of these rearrangements, especially in the GGA1q chromosome in Passeriformes (Kretschmer et al. 2014, 2015, dos Santos et al. 2015, 2017). Here, we have identified inversions already proposed for the Passeriformes (GGA1p and q) and hypothetical inversions responsible for the heteromorphisms in the first and third pairs. However, the rearrangements of the chromosome that corresponds to GGA1q detected by chromosome painting with LAL probes is more complex than we imagined initially. In 2014, Kretschmer and colleagues first described three inversions on chromosome two (GGA1q) in two species of the genus *Turdus* Linnaeus, 1758 (Oscines). In 2015, the same three inversions were detected in *Elaenia spectabilis* Pelzeln, 1868 (Suboscines) (Kretschmer et al. 2015). After the publication of the *E. spectabilis* observation, dos Santos et al. (2015) and dos Santos et al. (2017) also detected the reorganization of chromosome 2 (GGA1q) in two species of the genus *Saltator* Vieillot, 1816 (Oscines), *Taeniopygia guttata* Reichenbach, 1862 and in *Serinus canaria* Linnaeus, 1758, but this rearrangement was slightly different from that in *Turdus* and *E. spectabilis*. The main difference is that the block corresponding to LAL 18 is conserved integrally in the four species described by dos Santos (et al. 2015, 2017), whereas in *Turdus* and *E. spectabilis* this region is separated into two blocks. The most likely explanation would be the occurrence of independent rearrangements in Oscines and Suboscines, since a block of LAL 18 appears in *Saltator*, *Taeniopygia guttata*, *Serinus canaria* and *Synallaxis* while two blocks appear in *Turdus* (Oscines) and *Elaenia* (Suboscines). However, we still cannot determine which of these characters was present in the last common ancestor of Passeriformes. Perhaps it was the pattern observed in *S. frontalis*, since it is the most basal species of the order Passeriformes studied to date, but other species of the Furnariidae family must be analyzed to confirm or reject the hypothesis of independent rearrangement. However, the current scenario leads us to assume that the ancestral genome of the Passeriformes had a complex reorganization of the chromosomes corresponding to GGA1q, although it is necessary to determine which of the two situations was the first to occur – the one observed in *S. frontalis*, *Saltator*, *Taeniopygia guttata* and *Serinus canaria* or the one found in *Turdus* and *Elaenia*.

Future studies on this species could use other probes such as BACs clones (Damas et al. 2017) to test if the heteromorphisms described here were originated by pericentric inversions or centromere repositioning. Besides that, it would be interesting to carry out similar work to the present study in the sympatric sister species *S. spixi*, since Hooper and Price (2017) proposed that chromosomal inversion differences correlate with range overlap in passerine birds. The conventional analysis with Giemsa in other individuals of *S. frontalis* would also be useful in order to verify if these heteromor-

phisms are fixed in the population sampled and in other populations. In addition, it would be interesting to analyze the possible effects of these heteromorphisms on the phenotype of carriers, since in *Zonotrichia albicollis* it has been proposed that the heteromorphisms caused changes in behavior and plumage (Thomas et al. 2008).

Acknowledgements

We are grateful to members of the Research Group Diversidade Genética Animal of the Universidade Federal do Pampa, SISBIO for the license to sampling of specimens studied in this paper and for the Pró-Reitoria de Pesquisa (PROPESQ-UNIPAMPA) for the financial contribution through notices of Support to Research Groups. We are also grateful to members of the Laboratory of Tissue Culture and Cytogenetics of the Evandro Chagas Institute (Ananindeua, PA, Brazil) for technical and financial support. Kretschmer R and de Lima VLC received student fellowships from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

Barbosa MO, da Silva RR, Correia VCS, dos Santos LP, Garnero ADV, Gunski RJ (2013) Nucleolar organizer regions in *Sittasomus griseicapillus* and *Lepidocolaptes angustirostris* (Aves, Dendrocolaptidae): Evidence of a chromosome inversion. Genetics and Molecular Biology 36(1): 70–73. https://doi.org/10.1590/S1415-47572013000100010

Chesser RT (2004) Molecular systematics of New World suboscine birds. Molecular Phylogenetics and Evolution 32(1): 11–24. https://doi.org/10.1016/j.ympev.2003.11.015

Correia VCS, Garnero ADV, dos Santos LP, Rodrigues RR, Barbosa MO, Gunski RJ (2009) Alta similaridade cariotípica na família Emberizidae (Aves: Passeriformes). Journal of Biosciences 25(2): 99–111.

Damas J, O'Connor R, Farré M, Lenis VPE, Martell HJ, Mandawala A, Fowler KE, Jospeh S, Swain M, Griffin DK, Larkin DM (2017) Upgrading short-read animal genome assemblies to chromosome level using comparative genomics and a universal probe set. Genome Research 27(5): 875–884. https://doi.org/10.1101/gr.213660.116

de Lucca EJ, Rocha GT (1985) Chromosomal polymorphism in *Zonotrichia capensis* (Passeriformes, Aves). Brazilian Journal of Genetics 8: 71–78.

Derjusheva S, Kurganova A, Haberman F, Gaginskaia E (2004) High chromosome conservation detected by comparative chromosome painting in chicken, pigeon and passerine birds. Chromosome Research 12(7): 715–723. https://doi.org/10.1023/B:CHRO.0000045779.50641.00

de Oliveira EHC, Tagliarini MM, Rissino JD, Pieczarka JC, Nagamachi CY, O'Brien PCM, Ferguson-Smith MA (2010) Reciprocal chromosome painting between white hawk (*Leucopternis albicollis*) and chicken reveals extensive fusions and fissions during karyotype evolution of Accipitridae (Aves, Falconiformes). Chromosome Research 18(3): 349–355. https://doi.org/10.1007/s10577-010-9117-z

dos Santos MS, Kretschmer R, Silva FAO, Ledesma MA, O'Brien PCM, Ferguson-Smith MA, Garnero ADV, de Oliveira EHC, Gunski RJ (2015) Intrachromosomal rearrangements in two representatives of the genus *Saltator* (Thraupidae, Passeriformes) and the occurrence of heteromorphic Z chromosomes. Genetica 143(5): 535–543. https://doi.org/10.1007/s10709-015-9851-4

dos Santos MS, Kretschmer R, Frankl-Vilches C, Bakker A, Gahr M, O'Brien PCM, Ferguson-Smith MA, de Oliveira EHC (2017) Comparative Cytogenetics between Two Important Songbird, Models: The Zebra Finch and the Canary. PLoS ONE 12(1): e0170997. https://doi.org/10.1371/journal.pone.0170997

Ericson PGP, Klopfstein S, Irestedt M, Nguyen JMT, Nylander JAA (2014) Dating the diversification of the major lineages of Passeriformes (Aves). BMC Evolutionary Biology 14: 8. https://doi.org/10.1186/1471-2148-14-8

Frankl-Vilches C, Kuhl H, Werber M, Klages S, Kerick M, Bakker A, de Oliveira EHC, Reusch C, Capuano F, Vowinckel J, Leitner S, Ralser M, Timmermann B, Gahr M (2015) Using the canary genome to decipher the evolution of hormone-sensitive gene regulation in seasonal singing birds. Genome Biology 16: 19. https://doi.org/10.1186/s13059-014-0578-9

Griffin DK, Robertson LBW, Tempest HG, Skinner BM (2007) The evolution of the avian genome as revealed by comparative molecular cytogenetic. Cytogenetic and Genome Research 117(1–4): 64–77. https://doi.org/10.1159/000103166

Gunski RJ, Cabanne GS, Ledesma MA, Garnero, ADV (2000) Análisis cariotípico de siete especies de tiránidos (Tyrannidae). El Hornero 15(2): 103–109.

Guttenbach M, Nanda I, Feichtinger W, Masabanda JS, Griffin DK, Schmid M (2003) Comparative chromosome painting of chicken autosomal paints 1–9 in nine different bird species. Cytogenetic and Genome Research 103(1–2): 173–184. https://doi.org/10.1159/000076309

Hooper DM, Price TD (2017) Chromosomal inversion differences correlate with range overlap in passerine birds. Nature Ecology & Evolution 1: 1526–1534. https://doi.org/10.1038/s41559-017-0284-6

Itoh Y, Arnold AP (2005) Chromosomal polymorphism and comparative painting analysis in the zebra finch. Chromosome Research 13(1): 47–56. https://doi.org/10.1007/s10577-005-6602-x

Irestedt M, Fjeldså J, Dalén L, Ericson PGP (2009) Convergent evolution, habitat shifts and variable diversification rates in the ovenbird-woodcreeper family (Furnariidae). BMC Evolutionary Biology 9: 268. https://doi.org/10.1186/1471-2148-9-268.

Irestedt M, Fjeldså J, Johansson US, Ericson PGP (2002) Systematic relationships and biogeography of the tracheophone suboscines (Aves: Passeriformes). Molecular Phylogenetics and Evolution 23(3): 499–512. https://doi.org/10.1016/S1055-7903(02)00034-9

Kasai F, Garcia C, Arruga MV, Ferguson-Smith MA (2003) Chromosome homology between chicken (*Gallus gallus domesticus*) and the red-legged partridge (*Alectoris rufa*): evidence of the occurrence of a neocentromere during evolution. Cytogenetic Genome Research 102(1–4): 326–330. https://doi.org/10.1159/000075770

Kretschmer R, Gunski RJ, Garnero ADV, Furo IdO, O'Brien PCM, Ferguson-Smith MA, de Oliveira EHC (2014) Molecular cytogenetic characterization of multiple intrachromosomal rearrangements in two representatives of the genus *Turdus* (Turdidae, Passeriformes). PLoS ONE 9: e103338. https://doi.org/10.1371/journal.pone.0103338

Kretschmer R, de Oliveira EHC, dos Santos MS, Furo IDO, O'Brien PCM, Ferguson-Smith MA, Garnero ADV, Gunski RJ (2015) Chromosome mapping of the large elaenia (*Elaenia spectabilis*): evidence for a cytogenetic signature for passeriform birds? Biological Journal of the Linnean Society 115(2): 391–398. https://doi.org/10.1111/bij.12504

Ladjali-Mohammedi K, Bitgood JJ, Tixier-Boichard M, Ponce de Leon FA (1999) International System for Standardized Avian Karyotypes (ISSAK): standardized banded karyotypes of the domestic fowl (*Gallus domesticus*). Cytogenetics and Cell Genetics 86: 271–276. https://doi.org/10.1159/000015318

Ledesma MA, Garnero ADV, Gunski RJ (2002) Análise do cariótipo de duas espécies da família Formicariidae (Aves, Passeriformes). Ararajuba 10(1):15–19.

Moyle RG, Chesser RT, Brumfield RT, Tello JG, Marchese DJ, Cracraft J (2009) Phylogeny and phylogenetic classification of the antbirds, ovenbirds, woodcreepers, and allies (Aves: Passeriformes: Furnariides). Cladistics 25(4):386–405. https://doi.org/10.1111/j.1096-0031.2009.00259.x

Nanda I, Schrama D, Feichtinger W, Haaf T, Schartl M, Schmid M (2002) Distribution of telomeric (TTAGGG)n sequences in avian chromosomes. Chromosoma 111(4): 215–227. https://doi.org/10.1007/s00412-002-0206-4

Nanda I, Benisch P, Fetting D, Haaf T, Schmid M (2011) Synteny conservation of chicken macrochromosomes 1–10 in different Avian lineages revealed by cross-species chromosome painting. Cytogenetic and Genome Research 132(3): 165–181. https://doi.org/10.1159/000322358

Remsen JVJr, Areta JI, Cadena CD, Jaramillo A, Nores M, Pacheco JF, Pérez-Emán J, Robbins MB, Stiles FG, Stotz DF, Zimmer KJ (2016) A classification of the bird species of South America. American Ornithologists' Union. http://www.museum.lsu.edu/~Remsen/SACCBaseline.html [Accessed June 2016]

Rodrigues BS, Kretschmer R, Gunski RJ, Garnero ADV, O'Brian PCM, Ferguson-Smith MA, de Oliveira EHC (In press) Chromosome painting in tyrant flycatchers confirms a set of inversions shared by Oscines and Suboscines (Aves, Passeriformes). Cytogenetic and Genome Research. https://doi.org/10.1159/000486975

Santos LP, Gunski RJ (2006) Revisão de dados citogenéticos sobre a avifauna brasileira. Revista Brasileira de Ornitologia 14(1): 35–45.

Sasaki M, Ikeuchi T, Makino SA (1968) Feather pulp culture for avian chromosomes with notes on the chromosomes of the peafowl and the ostrich. Experientia 24(12):1923–1929.

Selvatti AP, Gonzaga LP, Russo CAM (2015) A Paleogene origin for crown passerines and the diversification of the Oscines in the New World. Molecular Phylogenetics and Evolution 88: 1–15. https://doi.org/10.1016/j.ympev.2015.03.018

Shields GF (1973) Chromosomal polymorphism common to several species of *Junco* (Aves). Canadian Journal of Genetics and Cytology 15(3): 461–471.

Skinner BM, Griffin DK (2012) Intrachromosomal rearrangements in avian genome evolution: evidence for regions prone to breakpoints. Heredity 108(1): 37–41. https://doi.org/10.1038/hdy.2011.99

Thomas JW, Cáceres M, Lowman JJ, Morehouse CB, Short ME, Baldwin EL, Maney DL, Martin CL (2008) The chromosomal polymorphism linked to variation in social behavior in the white-throated sparrow (*Zonotrichia albicollis*) is a complex rearrangement and suppressor of recombination. Genetics 179(3): 1455–1468. https://doi.org/10.1534/genetics.108.088229

Volker M, Backstrom N, Skinner BM, Langley EJ, Bunzey SK, Ellegren H, Griffin DK (2010) Copy number variation, chromosome rearrangement, and their association with recombination during avian evolution. Genome Research 20(4): 503–511. https://doi.org/10.1101/gr.103663.109

Zlotina A, Galkina S, Krasikova A, Crooijmans RPMA, Groenen MAM, Gaginskaya E, Deryusheva S (2012) Centromere positions in chicken and Japanese quail chromosomes: de novo centromere formation versus pericentric inversions. Chromosome Research 20(8): 1017–1032. https://doi.org/10.1007/s10577-012-9319-7

Warren WC, Clayton DF, Ellegren H, Arnold AP, Hillier LW, Künstner A, Searle S, White S, Vilella AJ et al (2010) The genome of a songbird. Nature 464(7289): 757–762. https://doi.org/10.1038/nature08819

CompCytogen 12(1): 111–140 (2018)
doi: 10.3897/CompCytogen.v12i1.20334
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

The genome structure of *Arachis hypogaea* (Linnaeus, 1753) and an induced *Arachis* allotetraploid revealed by molecular cytogenetics

Eliza F. de M. B. do Nascimento[1,2], Bruna V. dos Santos[2], Lara O. C. Marques[2,3], Patricia M. Guimarães[2], Ana C. M. Brasileiro[2], Soraya C. M. Leal-Bertioli[4], David J. Bertioli[4], Ana C. G. Araujo[2]

1 *University of Brasilia, Institute of Biological Sciences, Campus Darcy Ribeiro, CEP 70.910-900, Brasília, DF, Brazil* **2** *Embrapa Genetic Resources and Biotechnology, PqEB W5 Norte Final, CP 02372, CEP 70.770-917, Brasília, DF, Brazil* **3** *Catholic University of Brasilia, Campus I, CEP 71966-700, Brasília, DF, Brazil* **4** *Center for Applied Genetic Technologies, University of Georgia, 111 Riverbend Road, 30602-6810, Athens, Georgia, USA*

Corresponding author: *Ana Claudia Guerra Araujo* (ana-claudia.guerra@embrapa.br)

Academic editor: *N. Golub* | Received 15 August 2017 | Accepted 23 January 2018 | Published 14 March 2018

http://zoobank.org/02035703-1636-4F8E-A5CF-54DE8C526FCC

Citation: do Nascimento EFMB, dos Santos BV, Marques LOC, Guimarães PM, Brasileiro ACM, Leal-Bertioli SCM, Bertioli DJ, Araujo ACG (2018) The genome structure of *Arachis hypogaea* (Linnaeus, 1753) and an induced *Arachis* allotetraploid revealed by molecular cytogenetics. Comparative Cytogenetics 12(1): 111–140. https://doi.org/10.3897/CompCytogen.v12i1.20334

Abstract

Peanut, *Arachis hypogaea* (Linnaeus, 1753) is an allotetraploid cultivated plant with two subgenomes derived from the hybridization between two diploid wild species, *A. duranensis* (Krapovickas & W. C. Gregory, 1994) and *A. ipaensis* (Krapovickas & W. C. Gregory, 1994), followed by spontaneous chromosomal duplication. To understand genome changes following polyploidy, the chromosomes of *A. hypogaea*, IpaDur1, an induced allotetraploid (*A. ipaensis* × *A. duranensis*)^{4x} and the diploid progenitor species were cytogenetically compared. The karyotypes of the allotetraploids share the number and general morphology of chromosomes; DAPI$^+$ bands pattern and number of 5S rDNA loci. However, one 5S rDNA locus presents a heteromorphic FISH signal in both allotetraploids, relative to corresponding progenitor. Whilst for *A. hypogaea* the number of 45S rDNA loci was equivalent to the sum of those present in the diploid species, in IpaDur1, two loci have not been detected. Overall distribution of repetitive DNA sequences was similar in both allotetraploids, although *A. hypogaea* had additional CMA_3^+ bands and few slight differences in the LTR-retrotransposons distribution compared to IpaDur1. GISH showed that the chromosomes of both allotetraploids had preferential hybridization to their corresponding diploid genomes. Nevertheless, at

least one pair of IpaDur1 chromosomes had a clear mosaic hybridization pattern indicating recombination between the subgenomes, clear evidence that the genome of IpaDur1 shows some instability comparing to the genome of *A. hypogaea* that shows no mosaic of subgenomes, although both allotetraploids derive from the same progenitor species. For some reasons, the chromosome structure of *A. hypogaea* is inherently more stable, or, it has been at least, partially stabilized through genetic changes and selection.

Keywords
Chromosomes, DNA content, FISH, GISH, heterochromatic bands, LTR-retrotransposons, peanut, rDNA

Introduction

The genus *Arachis* (Linnaeus, 1753) is native to South America, with *Arachis* as the largest botanical section. Most species in this section are diploids (2n = 2x = 20), but there are a few aneuploids and two tetraploids: *A. hypogaea* (Linnaeus, 1753), the cultivated peanut (groundnut) and *A. monticola* (Krapovickas & Rigoni, 1958) (2n = 4x = 40) (Krapovickas and Gregory 1994, Valls and Simpson 2005). *A. hypogaea* has its origin estimated between 3,500 and 9,400 years ago (Bonavia 1982, Simpson et al. 2001, Bertioli et al. 2016), from one or few events of hybridization between two wild diploid species, followed by spontaneous polyploidization (Singh 1986, Kochert et al. 1996, Grabiele et al. 2012).

Whereas the chromosomes of *A. hypogaea* are of mostly similar size and metacentric, cytogenetic analysis can distinguish two different genome components: the A subgenome comprising ten pairs of chromosomes, with the centromeres strongly stained by DAPI, including the small pair termed 'A' (Husted 1936) and the B subgenome, with another ten pairs of chromosomes that have no, or just weak DAPI$^+$ bands (Seijo et al. 2004, 2007, Robledo and Seijo 2010). Fluorescence in situ hybridization (FISH) and many different lines of evidence show that the distribution of rDNA loci and heterochromatic DNA inww *A. hypogaea* are almost equivalent to the sum of those of the progenitor diploid species: *A. duranensis* (Krapovickas & W. C. Gregory, 1994), which has A genome chromosome, and *A. ipaensis* (Krapovickas et W. C. Gregory, 1994), which has B genome chromosomes (Grabiele et al. 2012, Robledo et al. 2009, Robledo and Seijo 2010). The only exception to this is that in both diploid species, the 45S rDNA hybridization signals bear the thread-like constriction of the pair of chromosomes SAT that strongly suggests transcriptional activity (Fernández and Krapovickas 1994). However, in *A. hypogaea*, the secondary constrictions observed on the B subgenome chromosomes have been silenced (Seijo et al. 2004), a common event in polyploids called nucleolar dominance (Navashin 1934, Preuss and Pikaard 2007).

Dhillon et al. (1980), using renaturation kinetics, estimated that 64 % of the *A. hypogaea* genome was composed of repetitive sequences. Genomic in situ hybridization (GISH) on chromosomes of *A. hypogaea*, with labeled whole genomic DNA from *A. duranensis* and *A. ipaensis* hybridized concomitantly showed that whilst the probes hybridize indistinctly to some genomic regions, the chromosomes of A and B genome

components (A and B subgenomes) are easily distinguishable (Seijo et al. 2007). Since the hybridization kinetics favors repetitive DNA sequences, this indicates that whereas the *A. hypogaea* A and B subgenomes share common repetitive DNA sequences with both diploid progenitors, in other aspects, the repetitive sequences are quite distinct between the subgenomes (Raina and Mukai 1999, Seijo et al. 2007).

Cytogenetic analysis mainly reveals the faster evolving repetitive DNA sequences; therefore, it tends to emphasize the differences between the subgenomes in allopolyploids. On the other hand, observations using genetic mapping and genes in *Arachis* tended to detect the similarities between the subgenomes: high collinearity between A and B subgenomes has been shown by comparing genetic linkage maps and sequencing of homeologous regions (Burow et al. 2001, Shirasawa et al. 2013, Bertioli et al. 2013, Bertioli et al. 2016). In addition, sequencing has shown very high DNA identity between A and B genes: around 97 % (Ramos et al. 2006, Nielen et al. 2012, Moretzsohn et al. 2013). The distinct fractions are thought to have evolved independently, following the evolutionary divergence of the progenitor species, which is estimated to have occurred 2–3 million years ago (Nielen et al. 2010, Bertioli et al. 2013, Moretzsohn et al. 2013, Samoluk et al. 2015a, Bertioli et al. 2016).

An important step in the understanding the genetics of many crops has been obtained by whole genome sequencing. However, for *A. hypogaea*, the very high similarity of the subgenomes makes the characterization of its genome, at the whole genome level, very challenging, although various lines of evidence suggested that the progenitor genomes had undergone relatively few changes since polyploidization (Fávero et al. 2015, Foncèka et al. 2012, Shirasawa et al. 2012, Bertioli et al. 2016, Chen et al. 2016). Phenotypic and genetic observations of progeny derived from crosses between *A. hypogaea* and the induced allotetraploid [(*A. ipaensis* K30076 × *A. duranensis* V14167)^{4x}] (Fávero et al. 2006), here called IpaDur1, strongly supported the close relationship between the diploid genomes and corresponding *A. hypogaea* subgenomes (Foncèka et al. 2012, Shirasawa et al. 2012).

The availability of the genome sequences of two representatives of *A. hypogaea* diploid progenitor species, *A. duranensis* V14167 and *A. ipaensis* K30076, (Bertioli et al. 2016) made possible to analyze their assembled chromosomal pseudomolecules. Homeologous chromosomes were given corresponding numbers based on previous genetic linkage maps, which most unfortunately, do not have correspondence with cytogenetic chromosome assignments (Bertioli et al. 2016). Comparisons of the diploid genome sequences with those of *A. hypogaea* confirmed the high sequence identity between the diploid genomes and their corresponding tetraploid components (Bertioli et al. 2016, Chen et al. 2016). However, as may have been expected for closely related highly collinear homeologous chromosomes, some recombination between the subgenomes of *A. hypogaea* was detected. Small terminal chromosome regions have changed from the expected genome formula of AABB, to AAAA, and others had changed to BBBB. These events were similar to, but smaller than, the recombination between subgenomes previously detected using genetic markers in this same induced allotetraploid IpaDur1 (Leal-Bertioli et al. 2015). There were also, in *A. hypogaea*, distinct signs of

migration of B subgenome alleles to A subgenome, especially in collinear homeologs (Bertioli et al. 2016).

In addition to genetic recombination between *A. hypogaea* subgenomes, other genomic changes are likely to have occurred following what McClintock (1984) termed as "genomic shock" of polyploid formation (Adams and Wendel 2005). Such changes may be caused by transposable element activation and re-organization of repetitive DNA sequences. While the overall patterns of GISH and evidences of the abundance of retrotransposons (Nielen et al. 2010, 2012, Samoluk et al. 2015a) indicate that, there has not been a mass movement of transposons between the genomes, or large-scale re-organization of repetitive DNA sequences, further investigations using IpaDur1 could disclose modifications that are frequently found in new hybrids (Comai 2000, Kashkush et al. 2003, Shcherban 2013, Kim 2017).

Interestingly, the recent cytogenetic observations of Zhang et al. (2016) showed that whilst the subgenome B chromosomes of *A. hypogaea* were very similar compared to its *A. ipaensis* counterpart, there were differences between the A subgenome and *A. duranensis* chromosomes. The authors suggested the participation of distinct *A. duranensis* accessions in the origin of *A. hypogaea*. However, this is not consistent with DNA marker data, which strongly implies a single origin (Kochert et al. 1991, 1996, Grabiele et al. 2012, Moretzsohn et al. 2013). Instability in the A subgenome chromosomes since polyploidy is an alternative explanation for that.

With the aim of understanding genome changes that have occurred after the polyploidization in *A. hypogaea*, a detailed comparative cytogenetic study of *A. hypogaea*, IpaDur1and progenitor diploid species is here presented. It was expected that the recently synthesized allotetraploid would undergo similar changes to those in *A. hypogaea* in the first years following polyploidization. Here is shown that IpaDur1 shows some alterations also observed in *A. hypogaea*, such as possible A genome nucleolar dominance, genome deletions and transposons activity. However, further alterations in IpaDur1, such as the smaller number of 45S rDNA loci and evident large-scale recombination between subgenomes in at least one chromosome pair of IpaDur1 were here evidenced. Current data contributes directly to the understanding of immediate effects of allotetraploidization in *Arachis* and to the overall understanding of *Arachis* genomes.

Material and methods

Plant material

Seeds from the wild diploid species (2n = 20) *A. duranensis*, accession V14167 and *A. ipaensis*, accession K30076; the allotetraploids (2n = 40) *A. hypogaea* subsp. *fastigiata* var. *fastigiata* 'IAC Tatu-ST' (AABB) and the induced allotetraploid IpaDur1 (*A. ipaensis* K30076 × *A. duranensis* V14167)^{4x} (Fávero et al. 2006) (BBAA) were obtained from the Embrapa Genetic Resources and Biotechnology Active Germplasm Bank (genotypes summarized in Table 1), and growing plants were maintained in open plan greenhouse.

Genome sizes

Genome sizes were estimated using the CyFlow Space system (Sysmex Partec GmbH, Görlitz, Germany), with leaf cells labeled with propidium iodide, as described by Galbraith et al. (1983). Leaflets of the third leaf, from three weeks old plants were removed from five different individuals, for each genotype. Samples were distributed as three technical replicates, for each genotype. Data was analyzed using built-in FORMAX 2.7 software, using *Solanum lycopersicum* (Linnaeus, 1753) and *Glycine max* (Linnaeus, 1753) Merril, 1917 genomes as size standards, according to Doležel et al. (1992).

Metaphase spreads

Meristem cells from root tips were isolated to obtain metaphase chromosome spreads. Root tips were collected from at least five different plants, of each genotype, then fixed in ethanol: glacial acetic acid (3:1v/v) solution for 60 min at 4 °C and finally digested with 2 % cellulase and 20 % pectinase (Maluszynska and Heslop-Harrison 1993, Schwarzacher and Heslop-Harrison 2000). Each root tip was squashed in a drop of 60 % acetic acid on a histological slide, under a cover glass. The cover glass was then removed using liquid N_2 and the slide, air-dried. Slides containing chromosomes with high quality were selected using phase contrast mode in the AxiosKop microscope (Zeiss, Oberkochen, Germany).

DAPI staining

Slides containing metaphase spreads were stained with DAPI (4', 6-diamino-2-phenylindole; 2 μg/ml) to determine the presence of heterochromatic bands (AT-rich regions). The chromosomes were analyzed using the epifluorescent Zeiss AxioPhot photomicroscope (Zeiss, Oberkochen, Germany), with the corresponding DAPI fluorescent filter. Images were captured using the Zeiss AxioCam MRc digital camera (Carl Zeiss Light Microscopy, Göttingen, Germany) and Axiovision Rel. 4.8 software (https://www.zeiss.com/microscopy/int/products/microscope-software/axiovision.html). Images were acquired and further analyzed using the Adobe Photoshop CS software, applying only functions, except cropping, that affect the whole image equally.

***CMA_3* banding**

For CMA_3 banding, the nuclear dye chromomycin A3 (CMA_3, Sigma Aldrich) was used following Schweizer and Ambros (1994). Aged slides (72 h) were treated with

Table 1. DNA content and size, CMA_3^+ bands and distribution of the in situ hybridization signals (GISH and FISH) on chromosomes of the four *Arachis* genotypes.

	Genotypes	*A. duranensis*	*A. ipaensis*	IpaDur1	*A. hypogaea*
	Karyotype formula	9 m + 1 sm	9 m + 1 sm	18 m + 2 sm	18 m + 2 sm
	DNA content (2C) (pg)	2.62	3.34	5.92	5.70
	Size (1C) (Gb	1.28	1.63	2.89	2.79
	CV (%)	2.67	4.14	2.36	3.25
	CMA_3^+	Proximal regions on cyt-A10*	Proximal region on cyt-B10*	Proximal region on cyt-A10* and cyt-B10	Proximal regions on cyt-A10*, cyt-B10 and another three pairs
GISH (genomic probes)	**IpaDur1**	–	–	On all chromosomes, for both subgenomes. Few signals on centromeres of A subgenome chromosomes and terminal regions. Cyt-B10 entirely covered by signals	On all chromosomes, for both subgenomes. Seldom signals on cyt-A9. Few signals on centromeres of A subgenome chromosomes and terminal regions. Cyt-B10 entirely covered by signals
	A. hypogaea	–	–	On all chromosomes, for both subgenomes. Seldom signals on cyt-A9. Few signals on centromeres of A subgenome chromosomes and terminal regions. Cyt-B10 with alternated pattern	On all chromosomes, of both subgenomes. Seldom signals on cyt-A9. Few signals on centromeres of A subgenome chromosomes and terminal regions. Cyt-B10 entirely covered by signals
	A. duranensis* and *A. ipaensis	–	–	Higher affinity to chromosomes of each corresponding subgenome. Hybridized poorly on cyt-A9, centromeres of A chromosomes and terminal regions of all chromosomes. Cyt-B10 with mosaic pattern	Higher affinity to chromosomes of each corresponding subgenome. Hybridized poorly on cyt-A9, centromeres of A chromosomes and terminal regions of all chromosomes. Cyt-B10 with higher affinity to *A. ipaensis* probe
rDNA FISH	**5S**	Proximal region on cyt-A3	Proximal region on cyt-B3	Interstitial region on cyt-A3 and proximal region on cyt-B3	Interstitial region on cyt-A3 and proximal region on cyt-B3
	45S	Proximal region on cyt-A2 and A10*	Proximal region on cyt-B3 and B10* and on terminal region on cyt-B7	Proximal region on cyt-A2; A10* and B10	Proximal regions on cyt-A2; A10*; B3 and B10 and in terminal regions on cyt-B7
LTR-RT FISH	**RE128-84**	Dispersed on arms and proximal regions of all chromosomes. Seldom detected on centromeric and terminal regions	Dispersed on the arms and proximal regions of most chromosomes. Lacking on two pairs. Seldom detected on centromeric and terminal regions	Dispersed on the arms and proximal regions of most chromosomes. Lacking on one pair of chromosome of the subgenome A Seldom detected on centromeric and terminal regions	Dispersed on arms and proximal regions of most chromosomes. Lacking on cyt-A9 and cyt-A10. Seldom detected on centromeric and terminal regions

	Genotypes	*A. duranensis*	*A. ipaensis*	IpaDur1	*A. hypogaea*
LTR-RT FISH	**Pipoka**	Dispersed on arms and proximal regions of most chromosomes. Poorly on cyt-A9 and cyt-A10. Seldom detected on centromeric and terminal regions	Dispersed on the arms and proximal regions of most chromosomes. Seldom detected on centromeric and terminal regions	Dispersed on the arms and proximal regions of most chromosomes. Lacking on cyt-A9, cyt-A10 and on two pairs of A subgenome. Seldom detected on centromeric and terminal regions	Dispersed on the arms and proximal regions of few chromosomes. Lacked on cyt-A9, cyt-A10. Seldom detected on centromeric and terminal regions
	Athena	Dispersed on arms and proximal regions of most chromosomes. Seldom on centromeric and terminal regions	Dispersed on the arms and proximal regions of most chromosomes Lacking on terminal regions of all chromosomes	Dispersed on the arms and proximal regions of most chromosomes on B subgenome. Lacking on cyt-A9 and cyt-A10. Seldom detected on centromeric and terminal regions	Dispersed on the arms and proximal regions of most chromosomes, Lacking on cyt-A9 and cyt-A10. Seldom detected on centromeric and terminal regions

CV: coefficient of variance; m: metacentric; sm: submetacentric; *: NOR (Nucleolar Organizing Region); –: not analysed.

CMA_3 and the slides mounted with glycerol / McIlvaine buffer, added to $MgCl_2$. Slides were observed in the Zeiss AxioPhot photomicroscope, with the CMA_3 corresponding fluorescent filter. Capture and treatment of the images were performed as described above.

GISH

Genomic DNA from all four genotypes was isolated according to the CTAB protocol (Ferreira and Grattapaglia 1998) in order to obtain the probes for GISH. Four young leaflets, collected from five different plants, for each genotype were assembled to form three DNA pools, for each genotype. Purified DNA (1µg) was then labeled with, either digoxigenin-11-dUTP (Roche Diagnostics Deutschland GmbH) or Cy3-dUTP (Roche Diagnostics Deutschland GmbH) by Nick Translation (Roche Diagnostics Deutschland GmbH). Incorporation of digoxigenin labeled nucleotides and the estimate concentration of the probes were determined by dot blot, followed by immunocytochemical detection. Metaphase spreads were pre-treated with RNase A and pepsin prior to fixation with 4 % paraformaldehyde and then with the hybridization solution, as described by Schwarzacher and Heslop-Harrison (2000).

GISH was performed according to Schwarzacher and Heslop-Harrison (2000). To obtain the *A. hypogaea* probe, approximately 50 ng/µl/slide of the genomic DNA of *A. hypogaea* was used. Similar amount of IpaDur1 genomic DNA was used to prepare the other probe. Hybridizations were carried out for 16 h at 37 °C, followed by 73 % stringent washes.

For single GISH, metaphase spreads of IpaDur1were hybridized with the *A. hypogaea* probe. After analysis and images acquisition, the *A. hypogaea* probe and DAPI stain were removed (Heslop-Harrison et al. 1992), and the same slides were re-hybridized with the IpaDur1 probe and DAPI. On the same way, the *A. hypogaea* chromosomes were hybridized with the IpaDur1 probe and then, with its own probe. No blocking DNA (unlabeled DNA) was used. The hybridization sites were detected using the antibody anti-digoxigenin conjugated to fluorescein (Fab fragments from sheep; Roche Diagnostics Deutschland GmbH) or by the direct observation of the Cy3 fluorescence. Chromosomes were counterstained with DAPI after the hybridization detection step in the case of digoxigenin labeled probe or after stringent washes whenever the probe was labeled with Cy3. Images were captured using corresponding fluorescent filters for DAPI, FITC and Cy3 and the analyses conducted as described before.

For double GISH, approximately 50 ng/µl/slide of each diploid labeled DNA was used concomitantly. Slides were hybridized as above described, with no blocking DNA. Detection of hybridization sites, DAPI counterstaining, analysis and images acquisition were conducted as described above.

5S and 45S rDNA chromosome mapping

The ribosomal sequences (rDNA) coding for 5S and 45S (18S-5.8S-25S) of *Lotus japonicus* (Regel) K. Larsen, 1955 (Pedrosa et al. 2002) and *Arabidopsis thaliana* (Linnaeus, 1753) Heynhold, 1842 (Wanzenböck et al. 1997), respectively were used to obtain the rDNA probes for FISH. DNA was labeled with either digoxigenin-11-dUTP or Cy3-dUTP by Nick Translation (Roche Diagnostics Deutschland GmbH).

LTR retrotransposons chromosome mapping

The LTR retrotransposon families, RE128-84 (Genbank KF729744.1; KF729735.1; KC608796.1; KC608788.1), representing the Ty1-copia group; Pipoka (Genbank KF729742.1 and KC608774.1) from Ty3-gypsy and Athena (Genbank KC608817.1), a non-autonomous transposon (which lacks the reverse transcriptase coding sequence) were chosen as the representatives of the most abundant LTR-retrotransposon families, and amongst the most and least frequent LTR-retrotransposons in *A. duranensis* and *A. ipaensis* genomes. DNA corresponding to the sequence coding for the reverse transcriptase enzyme of RE128-84 (Revtrans-RE) and Pipoka (Revtrans-PIP) were used to obtain the probes for FISH. Since Athena family comprises non-autonomous elements, there is no DNA sequence coding for the reverse transcriptase enzyme. Therefore, a non-genic, internal conserved DNA sequence, specific to the Athena family (Conserved-Ath) was used to obtain Athena probe. DNAs were PCR-amplified and the size of the amplicons confirmed in 1 % (w/v) agarose gel. DNAs were then purified and sequenced. Each DNA was labeled with either digoxigenin-11-dUTP or Cy3-dUTP by Nick Translation (Roche Diagnostics

Deutschland GmbH). Primers, sizes of the amplicons and the sequences are listed in Table 2. Hybridization conditions, detection of the hybridization sites, DAPI counterstaining, analysis and images acquisition were conducted as described above.

In silico coverage and mapping of the LTR-retrotransposons on the diploid genomes

The conserved DNA sequences specific for each LTR-retrotransposon family (Table 2) were used as the query to assess the estimate coverage of each LTR-retrotransposon in *A. duranensis* and *A. ipaensis* diploid genomes, using the REPEATMASKER (www.repeatmasker.org), with default parameters, except with the parameters -nolow and -norna to not mask low-complexity sequences and rDNA. The estimate coverage included all members of each LTR-retrotransposon family, thus including complete sequences, reminiscent fragments, nested sequences and solo LTRs. Output files were processed using a custom Perl script, and regions masked by more than one sequence in the repeat library were recognized and counted only once.

These conserved DNA sequences from each LTR-retrotransposon family were used as queries to assess their distribution in the chromosomal pseudomolecules, of both diploid species, using the PeanutBase BLAT tool (http://www.peanutbase.org). The match score was set to ≥ 80 %. Data was manually curated to remove sequences with different size than the expected one (Table 2); misalignments, overlapping of similar sequences and tandem organized sequences, here considered as a single hit. After trimming, the number of hits for each LTR-retrotransposon was determined for each chromosomal pseudomolecule, designated Aradu.A01 or Araip.B01, for example, according to nomenclature previously used (Bertioli et al. 2016, http://www.peanutbase.org). To avoid confusion of cytogenetic and pseudomolecule numbering, which might not fully correspond, here in this manuscript, the cytogenetic numbering will be prefix with "cyt- xxx" (for example, cyt-A1, for chromosome 1 of the subgenome A and cyt-B1 for chromosome 1 of the subgenome B), for both allotetraploids and diploids.

Results

Genome sizes

The DNA content estimated by flow cytometry revealed that IpaDur1 had a value very close to the sum of those of *A. duranensis* and *A. ipaensis*, however, slightly different from that of *A. hypogaea* (Table 1). Therefore, the estimate size of IpaDur1 genome is 2.89 Gb. The DNA content of the accession V14167 of *A. duranensis* was herein determined for the first time and its value was very close to those previously determined for other accessions of this species (Temsch and Greilhuber 2001, Samoluk et al. 2015a, b). On the other hand, the herein estimate value for *A. ipaensis* (3.34 pg) was slight higher than previous data (3.19 pg; Samoluk et al. 2015a, b).

Organization of chromosomes

IpaDur1 harbored 40 chromosomes, with similar morphology to those chromosomes of *A. hypogaea* and their progenitors, *A. ipaensis* and *A. duranensis*, being mostly metacentric (36 m + 4 sm), with the two submetacentric pairs of chromosomes designated as cyt-A10 and cyt-B10, both SAT chromosomes (Table 1; Fig. 1A). IpaDur1 A subgenome chromosomes, as well as those of *A. hypogaea* and *A. duranensis* had evident $DAPI^+$ bands, situated at centromeric regions (Fig. 1A, B, C). $DAPI^+$ bands on B subgenome chromosomes of both allotetraploids, as well as those on the chromosomes of *A. ipaensis* were not detected (Fig. 1D). Proximally located CMA_3^+ bands (DNA regions rich in C-G) on cyt-A10 and cyt-B10 were observed in the four genotypes analyzed (Fig. 2A, B, C, D). However, *A. hypogaea* had another three pairs of chromosomes with CMA_3^+ bands (Fig. 2B). The karyotype formulae and CMA_3^+ banding patterns are compiled in Table 1.

GISH1

GISH with the allotetraploid genomic probes

Genomic in situ hybridization used either *A. hypogaea* or IpaDur1 labeled genomic DNA as the probe (single GISH). Hybridization with IpaDur1 or *A. hypogaea* probes indicated a similar and overall affinity of both probes to all chromosomes of IpaDur1, except for the signals on cyt-A9 (equivalent to Aradu.A08; Bertioli et al. 2016), which were observed only after hybridization with the IpaDur1 probe (Fig. 3A, B), but not after hybridization with the *A. hypogaea* probe. Additionally, hybridization on IpaDur1 cyt-B10 chromosomes (Fig. 3A, inset) with the IpaDur1 probe generated signals evenly distributed, all along the chromosomes, whilst signals after the hybridization with *A. hypogaea* probe had an alternated pattern, with dark and lighter bands (Fig. 3B, C, insets), indicating different affinity of this probe to different regions of these chromosomes. On the other hand, the cyt-B10 of *A. hypogaea* had signals evenly spread along the chromosomes, independently of the probe used. Furthermore, cyt-A9 of *A. hypogaea* showed weak signals, independently of the probe used (Fig. 3D, E, F), whilst in IpaDur1, the signals were evident.

GISH with the diploid genomic probes

Simultaneous hybridization with *A. duranensis* and *A. ipaensis* genomic probes (double GISH) confirmed that each diploid probe hybridized preferentially with the chromosomes of its corresponding subgenome, for both IpaDur1 and *A. hypogaea*. IpaDur1 showed evident hybridization on all chromosomes, as single or overlapping signals (one or both probes hybridizing to the same region of the chromosome, respectively),

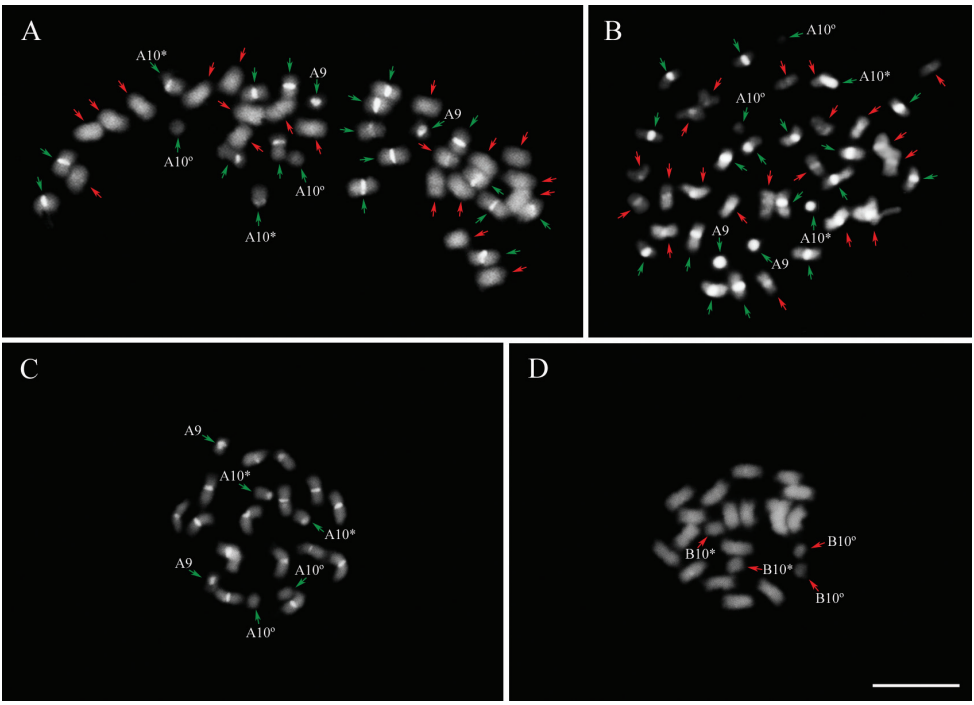

Figure 1. Metaphase chromosome spreads from root tips after DAPI staining (bright white) of **A** IpaDur1 **B** *A. hypogaea* **C** *A. duranensis* and **D** *A. ipaensis.* Chromosomes of the A subgenome (green arrows) and B subgenome (red arrows). Cyt-A9 (A9). Whenever the secondary constriction on cyt-A10 and cyt-B10 is extended, forming the thread-like constriction; the short arm and the proximal segment of the long arm are indicated by an asterisk (*) and the separated satellite is marked by a degree sign (°). Bar = 5μm.

except for cyt-A9; centromeres of A subgenome chromosomes and terminal chromosomal regions, which hybridized poorly (Fig. 4A, B, C, D).

Strikingly, a distinct intercalated mosaic-banding pattern was also observed on the pair of chromosomes cyt-B10: bands with higher affinity to *A. duranensis* genomic probe (Fig. 4A, inset) and bands with higher affinity to *A. ipaensis* probe (Fig. 4B, inset). This pattern on IpaDur1 cyt-B10 is similar to that observed after single GISH using the *A. hypogaea* probe (intercalated dark and light bands) (Fig. 3B, C). These lighter bands correspond to the subgenome A of IpaDur1, as showed after double GISH, which showed stronger signals with *A. duranensis* probe, whilst the dark bands correspond to the B subgenome, as indicated after hybridization with *A. ipaensis* probe. Together, results suggest that the A subgenome component in the cyt-B10 of IpaDur1 might had changed after polyploidization, or that it is derived from a different accession of *A. duranensis*. At least another chromosome pair of IpaDur1 chromosomes also appears to show weaker affinity to the *A. duranensis* probe in one part, and stronger affinity to *A. ipaensis* probe, in another.

A. hypogaea chromosomes showed patterns similar to those observed in IpaDur1 after double GISH, except cyt-B10 that showed uniform hybridization signals along

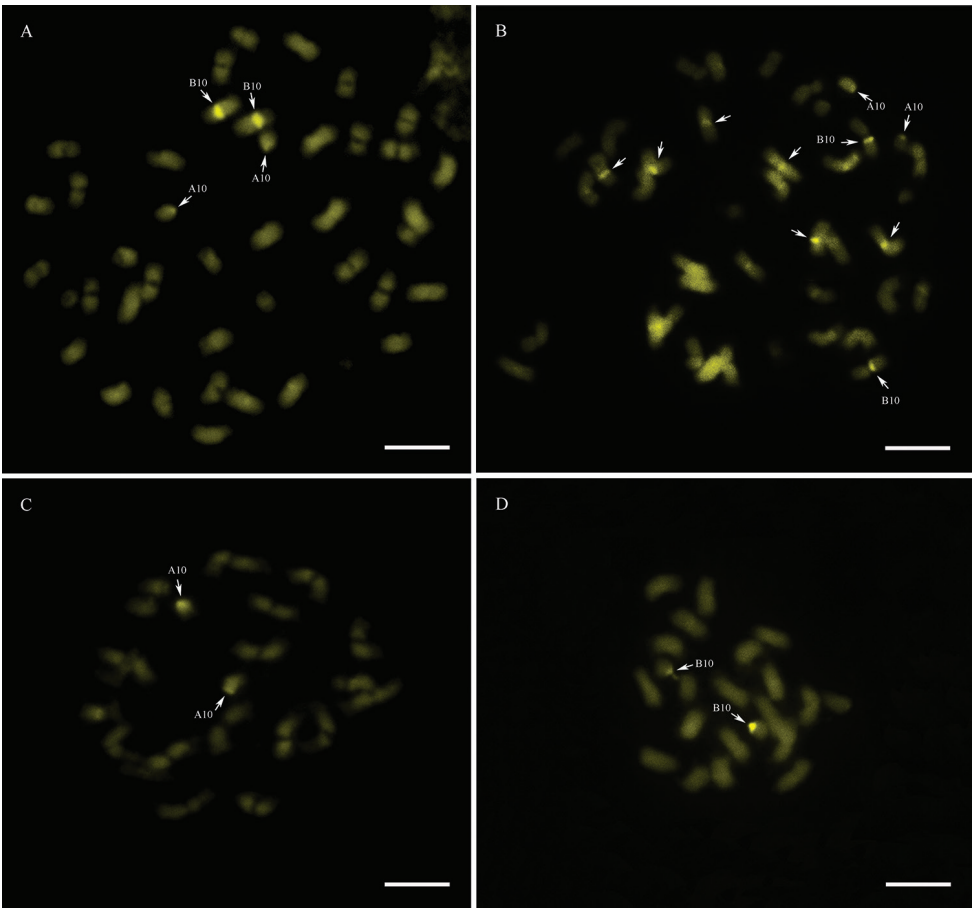

Figure 2. Chromosomes with CMA_3^+ bands (arrows) on their proximal regions. **A** IpaDur1 **B** *A. hypogaea* **C** *A. duranensis* **D** *A. ipaensis*. Cyt-A10 (A10) and cyt-B10 (B10). Bar = 5μm.

the chromosomes (Fig. 4D, E, F). Both allotetraploids had few signals on centromeres of the A subgenome chromosomes and terminal regions of all chromosomes, after both (single and double) GISH. These results are compiled in Table 1.

5S and 45S rDNA chromosome mapping

The number of 5S rDNA loci was an additive character for both IpaDur1 and *A. hypogaea*: one locus on the cyt-A3, originating from the corresponding chromosome in *A. duranensis*, and another locus on cyt-B3, from the corresponding chromosome in *A. ipaensis* (Fig. 5A, B). Observations of cyt-A3 in both IpaDur1 and *A. hypogaea* indicated that the 5S signals extended from the proximal into the interstitial chromosomal regions (Fig. 5A, B), whereas in the corresponding chromosomes of *A. duranensis*, the

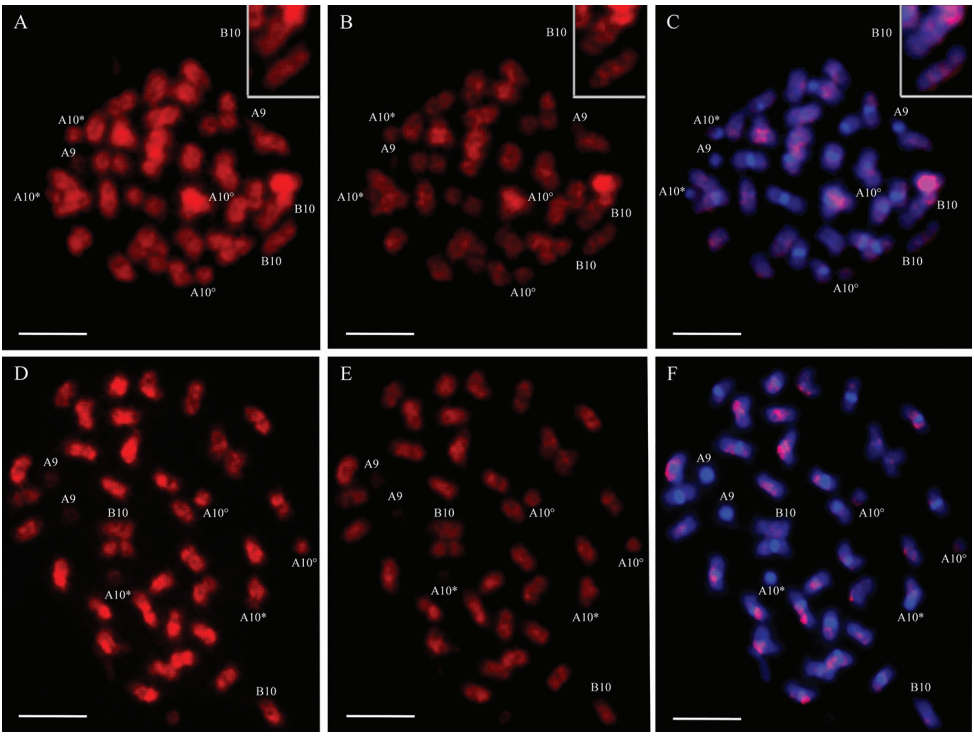

Figure 3. Single GISH on IpaDur1 (**A, B, C**) and *A. hypogaea* (**D, E, F**) chromosomes, followed by DAPI counterstaining (blue **C, F**). Hybridization with the genomic probe of IpaDur1 **A, E** *A. hypogaea* probe **B, D** and **C** overlapping of DAPI and *A. hypogaea* probe on IpaDur1 chromosomes **F** overlapping of DAPI and IpaDur1 probe on *A. hypogaea*. Cyt-A9 (A9), CytB-10 (B10). Insets of cyt-B10 of IpaDur1 (**A, B, C**) showing alternate dark and light bands. When the secondary constriction on cyt-A10 is extended, forming the thread-like constriction, the short arm and the proximal segment of the long arm are indicated by an asterisk (*) and the separated satellite is marked by a degree sign (°). Bar = 5μm.

signals were restricted to the proximal region (Fig. 6A). Further analysis on meiotic chromosomes is needed to confirm the possible increase of these loci in allotetraploids. The 5S rDNA signals on cyt-B3 had a similar pattern in both allotetraploids and *A. ipaensis* (Fig. 6B).

Considering the FISH with the 45S rDNA probe, there were only three loci in IpaDur1 and five *A. hypogaea*, thus being an addictive character only for the latter. In IpaDur1 (Fig. 5A), the signals were proximally located on cyt-A2 and cytB-10, while on cyt-A10, signal was near the secondary constriction of the SAT region, forming a thread-like constriction, characteristic of NORs (Nucleolar Organizing Regions), as observed on the corresponding chromosome of *A. duranensis* (Fig. 6C). The *A. hypogaea* 45S rDNA loci (Fig. 5B) were proximally located on cyt-A2, cyt-B10 and cyt-B3; terminally positioned on cyt-B7 and, on cyt-A10, they were situated near the secondary constriction of the SAT region, as observed in the corresponding chromosome of the progenitor diploid species, *A. duranensis* (Figs. 5B; 6C, D). No differences

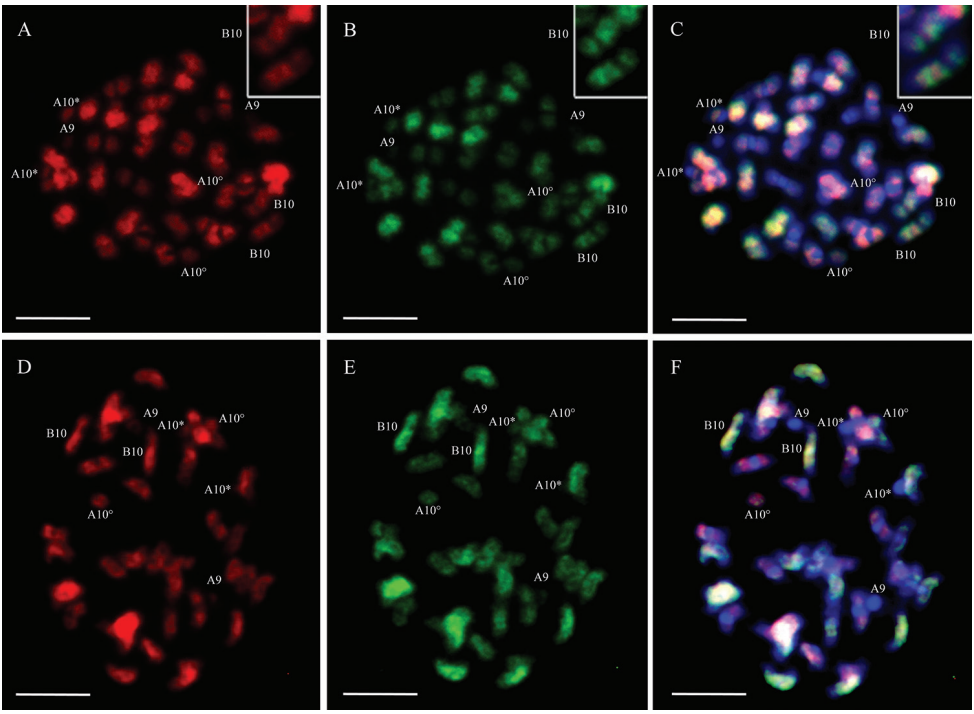

Figure 4. Double GISH on IpaDur1 (**A, B, C**) and *A. hypogaea* (**D, E, F**) chromosomes, followed by DAPI counterstaining (blue **C, F**). Hybridization with the genomic probe of *A. duranensis* (red **A, D**) and *A. ipaensis* (green **B, E**). Overlapping of DAPI and both diploid probes on **C** IpaDur1 and on **F** *A. hypogaea*. Cyt-A9 (A9), cyt-B10 (B10). Insets of IpaDur1 cyt-B10 (**A, B, C**), showing a colored mosaic. When the secondary constriction on cyt-A10 is extended, forming the thread-like constriction, the short arm and the proximal segment of the long arm are indicated by an asterisk (*) and the separated satellite is marked by a degree sign (°). Bar = 5μm.

were detected in the signals produced by either 5S and 45S rDNA dig-dUTP or Cy3-dUTP labeled probes. Interestingly to note that the co-localization of 5S and 45S rDNA loci on cyt-B3 was detected only in *A. hypogaea*, but not in IpaDur1 (Fig. 5A, B, inset). In contrast, the 45S rDNA loci co-localized with CMA_3^+ bands on cyt-A10 and cyt-B10, for both allotetraploids, as well as on the corresponding chromosomes, for both diploid species. FISH results are summarized in Table 1.

LTR-retrotransposon chromosome mapping

RE128-84

In all genotypes, the RE128-84 signals were preferentially dispersed on proximal regions and along the arms of the chromosomes, and seldom detected on centromeric

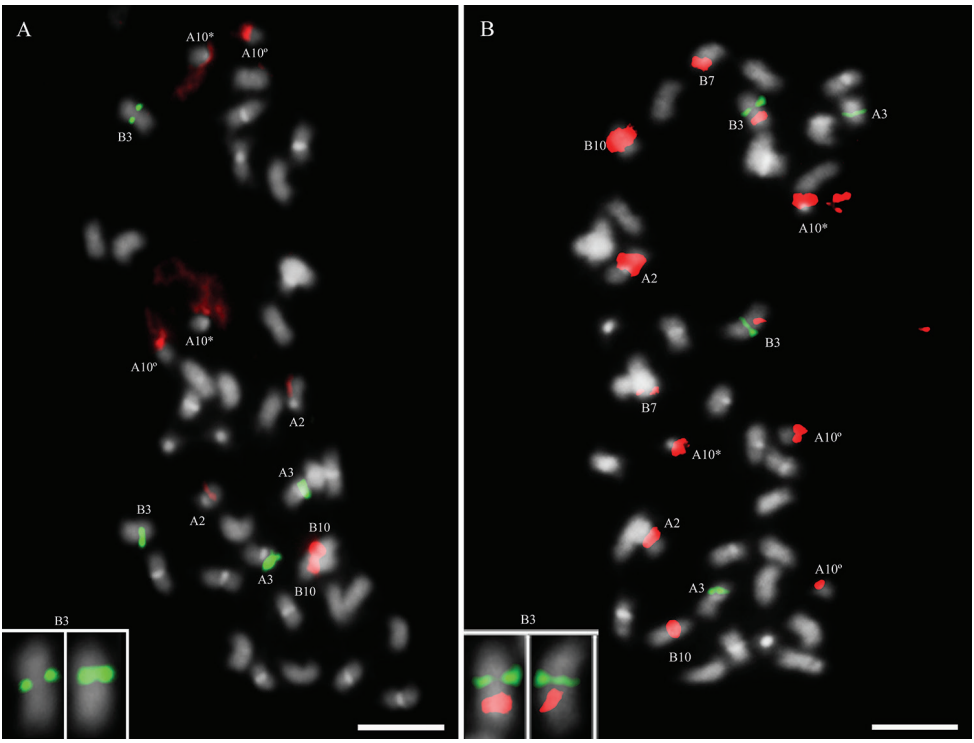

Figure 5. A IpaDur1 and **B** *A. hypogaea* chromosomes hybridized with the 5S rDNA probe (green) and 45S (red), followed by DAPI counterstaining (bright white). Cyt-A2 (A2), cyt-A3 (A3), cyt-B3 (B3), cyt-B7 (B7) and cyt-B10 (B10). *A. hypogaea* cyt-B3 with the co-localization of 5S and 45S rDNA signals. When the secondary constriction on cyt-A10 is extended, forming the thread-like constriction, the short arm and the proximal segment of the long arm are indicated by an asterisk (*) and the separated satellite is marked by a degree sign (°). Bar = 5μm.

and terminal regions. For both allotetraploids (Fig. 7A, B), the majority of the chromosomes had signals, except on cyt-A9 and cyt-A10 of *A. hypogaea* and on another pair of A subgenome chromosomes of IpaDur1. Signals lacked also on two pairs of chromosomes in *A. ipaensis*, whilst *A. duranensis* showed overall more evident signals than those in the other diploid species (Fig. 7C, D). However, chromosomes of the subgenome B of both allotetraploids generally had more signals than on *A. ipaensis* chromosomes, although no quantitative analysis could be performed.

Pipoka

As for RE128-84, Pipoka signals observed were spread along the chromosomes, except on centromeric and terminal regions. The majority of the IpaDur1 chromosomes showed signals (Fig. 7E), while *A. hypogaea* had only few signals (Fig. 7F). *A. duranensis* showed comparable signals than those in *A. ipaensis*, with signals on all chromosomes

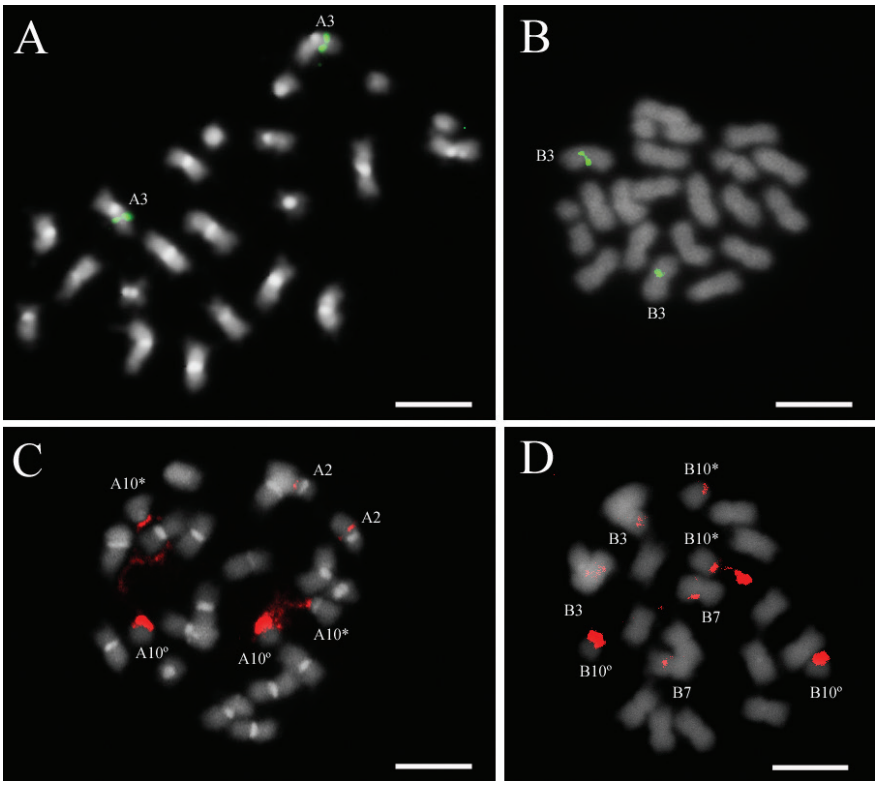

Figure 6. Mitotic metaphase chromosome hybridized with the 5S rDNA probe (green **A, B**) and 45S rDNA probe (red **C, D**), followed by DAPI counterstaining (bright white). **A** *A. duranensis* (2n = 2x = 20) showing signals on cyt-A3 (A3) **B** *A. ipaensis* (2n = 2x = 20) showing signals on cyt-B3 (B3) **C** *A. duranensis* with signals on cyt-A2 (A2) and cyt-A10 (A10) **D** *A. ipaensis* showing signals on cyt-B3, B7 and B10. When the secondary constriction on chromosome 10 is extended, forming the thread-like constriction, the short arm and the proximal segment of the long arm are indicated by an asterisk (*) and separated satellite is marked by a degree sign (°). Bar = 5μm.

of both diploid species (Fig. 7G, H). This probe did not hybridize to cyt-A9 or cyt-A10 of both allotetraploids and just poorly hybridized on these same chromosomes of *A. duranensis*. The hybridization patterns on the chromosomes of IpaDur1 suggested being closer to those detected in both diploid species than those observed for *A. hypogaea*.

Athena

In a similar way, chromosomes of all genotypes had *Athena* dispersed signals that lacked on centromeric and terminal regions. The abundance of signals in IpaDur1 seemed to be lower than in *A. hypogaea* (Fig. 7I, J), whilst *A. duranensis* apparently showed more signals than *A. ipaensis* (Fig. 7K, L). The signals in IpaDur1 were mostly on the B subgenome chromosomes, whilst in *A. hypogaea*, the signals were present on

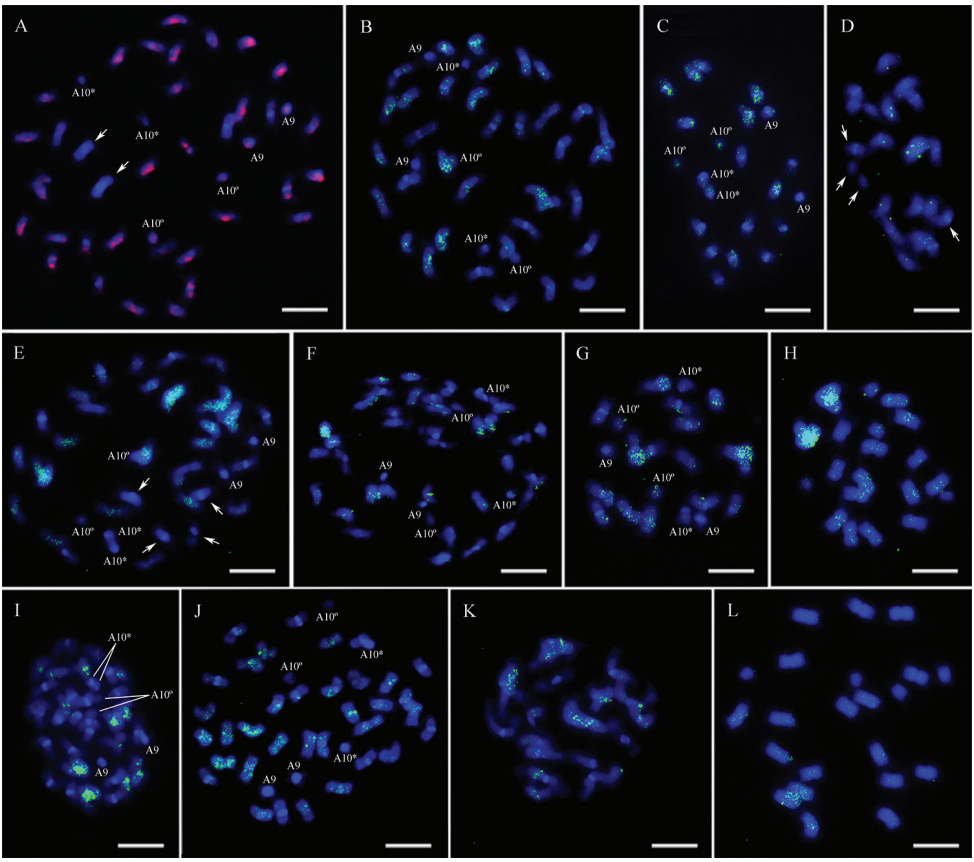

Figure 7. IpaDur1 (**A, E, I**), *A. hypogaea* (**B, F, J**), *A. duranensis* (**C, G, K**) and *A. ipaensis* (**D, H, L**) chromosomes hybridized with the LTR-retrotransposon probes RE-128-84 (**A, B, C, D**), Pipoka (**E, F, G, H**) and Athena (**I, J, K, L**), followed by DAPI counterstaining (blue). Cyt-A9 (A9). Chromosomes lacking signals (arrow). When the secondary constriction on cyt-A10 is extended, forming the thread-like constriction, the short arm and the proximal segment of the long arm are indicated by an asterisk (*) and the separated satellite is marked by a degree sign (°). Bar = 5μm.

chromosomes of both subgenomes (Fig. 7I, J). Athena signals lacked on cyt-A9 and cyt-A10 of IpaDur1, *A. hypogaea* and *A. duranensis* (not shown). The hybridization patterns on IpaDur1 chromosomes suggested being closer to the sum of those in the diploid species, while in *A. hypogaea*, signals seemed to be more abundant. Results of the LTR- retrotransposons FISH are compiled in Table 1.

LTR-retrotransposons coverage and mapping on pseudomolecules

The coverage of the LTR-retrotransposons indicated that these elements covered for RE128-84 family, around 1.20 % and 1.17 % of the *A. duranensis* and *A. ipaensis* chro-

Table 2. Characteristics of the LTR-retrotransposon families, RE128-84; Pipoka and Athena. Conserved DNA sequence used as probes; transposition autonomy character; superfamily; primers for amplification; sequences, sizes and names of the amplified DNA.

RT-LTR	Superfamily	Primers	Name and fragment size (bp)	DNA conserved sequences
Athena non--autonomous	-	Athena-FWD CCATCATAATTATCATAGTTGTGG Athena-REV CTCCAAACCAAGAGGGTGATAAC	Conserved-Ath 618	TTATGGAAAGGAAGGGATCCCATAACTCATCCCAAGTCAAGGTTTCATTACGTTTTAAACCACTTTTTCATCAATTTTGAGTCTTACTTGTTTATATTAGATACATAGTTCTTTTATTCCTTCATTAGTTTATTAATTACAATTTTGCCTTGTTCTTTTATCTCTTTATTGTTTACTTCAAACATTGAAAACCCTTTTGATCTTCACAACCAATTTTATGCACTTGTTGTCACTAGTTCCTAGGGAGAACAAATACTCTCGGTATATATATTTGCTTTGAATTGTGACAATCTTTAGAGTAATAATTTGACTATTGGCCAATTGTTGGTTCGAAGCTATACTTGCAACGAAGATCTATTTGGAGAAAATTCCAACCTACAATTTGGTCTTTGTCAAATTTTGGCGCCGTTGCCGGGGAGCTAATGTCATGAGTGCTATATTTTGGTTGTTGTAAATATGTCCATAGTATGAATAGATACTTTTTGGTTGCTTGTTTATTTTTGTTGGTAATTAGGATTTTGTTTATTTTGTTAATTGATGTCTTTAGTTGTTATTTTCAATTTTCTCTATGA
RE128-84 autonomous	Ty1-*Copia*	RE128-84-FWD CCACTAGATCCTCAAGCAAG RE128-84-REV AGAAGGCACTAAGCCTTTC	Revtrans--RE 558	AGCAAGAAGCAAGTAGAACCGAGCAATGTTGCCTTCTTGTCCCAATTGGAGCCTCTCAATGTGAAACAAGATCTTGAAGACCCCTCATGGGTTAAAGCCATGGAAAAAGAGCTGGCACAATTTGAAAAGAATGAGGTGTGGACACTTGTACCAAATCCAAATGATAAGAAGGTAACCGGTACAAGGTGGATTTTTAAAAATAAATTGGTTGAGGATGGTAGTGTTGTTCGTAACAAGGCTAGATTAATGGCCCAAGGTTACGATCAAGAAGAAGGAATTGATTTTGATGAGTCATTTTCCCCGGTAGCTAGAATGGAAGCAATTAGGTTGCTTCTTGCCTATGCTGCCCACAAGGGTTTTCAAGATGTTCCAAATGGATGTCAGATGTGCATTCCTTAATGGTTTTATAGATAGGGAAGTATTTGTGACTCAACCCCTCGGTTTTGAAAGTAAAGAATTTCCAAACCATGTTTTTAAATTATCAAAGGCTCTTTATGGCCTTAGGCAAGCTCCAAGAGCTCGGTAT
Pipoka autonomous	Ty3-*Gypsy*	Pipoka-FWD CCACATTGCTTTAGAGGATC Pipoka-REV GCTTGTCAAAAGCCTCCATGC	Revtrans--Pip 535	AAGAAAAAACAACCTTTACATGCCCCTTTGGCACTTATGCCTACAAGCGTATGCCATTTGGCTTATGCAACGCACCGGTAACTTTCCAAAGGTGTATGATGAGCATATTTGCAGATCTTCAAGAGCATTGGATGGAGGTGTTCATGGACGATTTTAGTGTCTATGGGGACTCTTTTGATCTTTGCTTGGACAACCTTGCAAAAGTGTTGGAGAGGTGTACTAAAACAAATATTGTCTTAAATTTTGAGAAGTGTCATTTTATGGTTAGACAAGGTATTGTTTTAGGACACATTATCTCTAACGATGGTATTTCTATGGATCCAGCAAAGATAAATGTTATATCTAGTTTACCTTACCCCTCCTCCGAGAGGGAAGTCCGTGCGTTCCTTGGACATACAGGTTTTTACTGGTGATTTATTAAGGACTTTAGCAAGGTGGCATTACCTCTATCTTGATTGTTGCAAAAAGACGTTGAATTTGATCGAAGCAAAGAGT

Table 3. In silico coverage of the LTR-retrotransposons on the chromosomal pseudomolecules of *A. duranensis* and *A. ipaensis* (accordingly to www.peanutbase.org).

Frequency of LTR-retrotransposons (%)				
Pseudomolecule	**RE128-84**	**Pipoka**	**Athena**	**Total/ pseudomolecule**
Aradu.A01 (≅ 107 Mb)	1.14	3.42	0.96	5.51
Aradu.A02 (≅ 93 Mb)	1.28	2.62	0.52	4.42
Aradu.A03 (≅ 135 Mb)	1.12	2.76	0.74	4.62
Aradu.A04 (≅ 123 Mb)	1.35	2.90	0.58	4.83
Aradu.A05 (≅ 110 Mb)	1.17	2.49	0.59	4.25
Aradu.A06 (≅ 112 Mb)	1.10	2.99	0.66	4.75
Aradu.A07 (≅ 79 Mb)	1.38	2.37	0.64	4.38
Aradu.A08 (≅ 49 Mb)	1.67	0.85	0.25	2.76
Aradu.A09 (≅ 120 Mb)	1.08	3.24	0.73	5.06
Aradu.A10 (≅ 109 Mb)	1.09	3.20	0.75	5.05
Total in A genome (1.25 Gb)	1.20	2.81	0.77	4.68
Araip.B01 (≅ 137 Mb)	1.07	6.54	1.27	8.89
Araip.B02 (≅ 108 Mb)	1.30	5.22	1.09	7.61
Araip.B03 (≅ 135 Mb)	1.21	4.75	0.97	6.93
Araip.B04 (≅ 133 Mb)	1.26	6.03	1.05	8.34
Araip.B05 (≅ 149 Mb)	1.09	6.40	1.28	8.77
Araip.B06 (≅ 137 Mb)	1.03	5.76	1.09	7.89
Araip.B07 (≅ 126 Mb)	1.09	7.61	1.32	10.01
Araip.B08 (≅ 129 Mb)	1.35	6.08	1.25	8.67
Araip.B09 (≅ 147 Mb)	1.20	5.91	1.25	8.36
Araip.B10 (≅ 136 Mb)	1.10	6.49	1.25	8.84
Total in B genome (1.56 Gb)	1.17	6.09	1.19	8.44

Mb: megabase. Gb: gigabase.

mosomal pseudomolecules, respectively, for Pipoka, 2.81 % and 6.09 % and for Athena, 0.77 % and 1.19 %. These three families covered about 4.68 % and 8.44 % of *A. duranensis* and *A. ipaensis*, mostly due to the large abundance of Pipoka members (Table 3). The *A. duranensis* pseudomolecules with the lowest and highest frequencies were respectively, Aradu.A08 and Aradu.A01, while in *A. ipaensis*, Araip.B03 and Araip.B07.

Accordingly, the number of LTR-retrotransposon hits after the LTR-retrotransposons in silico mapping on the diploid pseudomolecules were higher in *A. ipaensis* than in *A. duranensis* (Fig. 8), with the pseudomolecules with the highest and lowest number of hits being in accordance with the results of the estimate coverage. RE128-84 hits were on all pseudomolecules, but more abundant on Aradu.A04 and Araip. B02. Hits were found along the arms, but not on centromeric regions. Pipoka hits were more abundant on Aradu.A09 (do not correspond to the cyt-A9) and Araip.B07; less abundant on Aradu.A07 and Araip.B01, and lacked on Aradu.A08. Hits were mostly concentrated on centromeric and proximal regions, for both diploids, and lacked on terminal regions of most of the pseudomolecules, except for Aradu.A09 and on some *A. ipaensis* pseudomolecules. Because of the low number of hits generated by Athena,

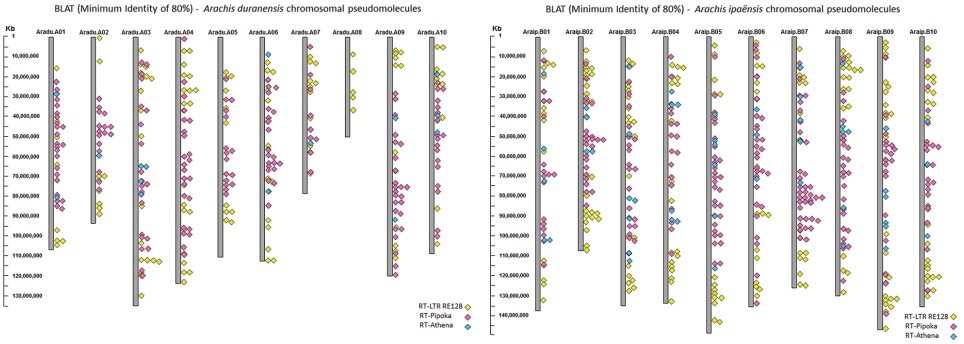

Figure 8. In silico mapping of the LTR-retrotransposon families, RE128-84, Pipoka and Athena on the chromosomal pseudomolecules of *A. duranensis* (left) and *A. ipaensis* (right).

no clear distribution pattern could be recognized, although the highest number of hits was on Aradu.A03 and Araip.B03; the lowest on Aradu.A02, Aradu.A07 and Araip. B10, whilst no hits were observed on Aradu.A04, Aradu.A05 and Aradu.A08 (Fig. 8).

The distribution of the LTR-retrotransposons, both in silico and in situ showed general similar patterns for the RE128-84 and Athena in *A. duranensis*; Pipoka in *A. ipaensis* and Athena, for both diploid genomes. The results shared by these two approaches enabled the inference of putative assignments by numbers for some of the IpaDur1 chromosomes, based on the abundance of hits on the numbered pseudomolecules (www.peanutbase.org). For example, *A. duranensis* chromosomal pseudomolecule Aradur.A04 had the largest number of RE128-84 hits; therefore, the chromosomes with more abundance of RE128-84 in situ hybridization signals in IpaDur1 could be putatively assigned as cyt-A4. In this same way, the pseudomolecule Araip.B02 was the one with the highest number of RE128-84 hits in *A. ipaensis*, thus the pair of chromosomes with more abundance of in situ signals would be called cyt-B2. Additionally, Araip.B07 had more Pipoka hits; therefore, the putative corresponding chromosome would be the cyt-B7. Aradur. A05 and Aradur.A08 pseudomolecules had no Athena hits, thus the corresponding chromosomes lacking in situ signals would be cyt-A5 and cyt-A9.

Discussion

Cultivated peanut (*A. hypogaea*) is an allotetraploid with an AABB type genome, originated from the diploid progenitor wild species *A. duranensis* (A genome; female progenitor) and *A. ipaensis* (B genome; male donor) (Kochert et al. 1991, Seijo et al. 2007, Moretzsohn et al. 2013). Earlier evidence from cytogenetics, genetic mapping and analysis of progeny derived-from crosses of *A. hypogaea* with an induced allotetraploid [(*A. ipaensis* K30076 × *A. duranensis* V14167)^{4x}] (Fávero et al. 2006) showed that their genomes had not undergone large-scale rearrangements since polyploidization (Fávero et al. 2015, Ramos et al. 2006, Seijo et al. 2007, Foncèka et al. 2009, Shirasawa et al. 2013).

However, considering the behavior of other polyploids in general, it seemed that some changes following polyploidy were extremely likely to have occurred. Accordingly, comparisons at the genome sequence level have shown some recombination between the subgenomes of *A. hypogaea* and evidence of the A subgenome erosion by gene conversion with the B subgenome (Bertioli et al. 2016). Additionally, although meiotic pairing in *A. hypogaea* is described as presenting the bivalents, with rare univalents, trivalents, and quadrivalent exceptions (Husted 1936), there is an indication of limited homeologous pairing between A and B subgenomes, as the recent genetic studies suggested that cultivated peanut may be better classified as a segmental allotetraploid with predominantly disomic, but partially tetrasomic genetics (Leal-Bertioli et al. 2015, Bertioli et al. 2016, Clevenger et al. 2017).

In this study, in order to investigate genome structure alterations, cytogenetics was used to make a detailed comparison of *A. hypogaea*, an induced allotetraploid IpaDur1 [(*A. ipaensis* K30076 × *A. duranensis* V14167)^{4x}] and their progenitor species, *A. duranensis* and *A. ipaensis*. The use of an induced allotetraploid is advantageous because this hybrid approximates an early *A. hypogaea*, and it was expected to undergo similar changes to those that peanut underwent in the early generations following polyploidy, although *A. duranensis* was the male progenitor in IpaDur1 and the female in *A. hypogaea*. Furthermore, comparisons are more accurate, because the exact diploid progenitors are known, and both have their reference genome sequences available.

Genome sizes

The sum of the estimated genome sizes of the diploid species, herein using the flow cytometry was very similar to the one estimated for IpaDur1, but somewhat larger (4 %) than the one estimated for *A. hypogaea* (Table 1). This difference is small, but might indicate a different *A. duranensis* accession(s), as the A subgenome donor to *A. hypogaea* (Zhang et al. 2016), and / or that deletions in *A. hypogaea* subgenome A had occurred following polyploidy. Either explanation is very plausible, because *A. duranensis* is known to vary significantly in genome size (Temsch and Greilhuber 2001) and genome deletions in polyploids are known to be common (Ma and Gustafson 2006, Bento et al. 2008, Eilam et al. 2008, Petit et al. 2010). Although the estimate value for the DNA content of *A. ipaensis* herein determined slightly differed from previous data (Singh et al. 1996, Samoluk et al. 2015a, b), this new value estimates the genome size of *A. ipaensis* as being 22 % larger than that of *A. duranensis*, very similar to the size difference between their chromosomal pseudomolecules (29 %, Bertioli et al. 2016).

Organization of chromosomes

Current analysis indicates that *A. hypogaea* and IpaDur1 share many similarities derived from the progenitor diploids, however variations relative to progenitors were also

cytogenetically revealed during this study. Chromosomes of IpaDur1 are morphologically similar to those of *A. hypogaea* (Seijo et al. 2004, Robledo et al. 2009, Robledo and Seijo 2010), including the cultivar Tatu that was here studied. Staining of AT-rich heterochromatin with DAPI showed that both allotetraploids had additive patterns (Fig. 1A, B), that is, the sum of the patterns in the progenitor diploid species was equal to the patterns in the allotetraploids. However, the CG-rich regions revealed by the CMA_3 did not have an additive pattern in *A. hypogaea*, since it that has bands on three extra pairs of chromosomes than the sum in the progenitor species (Fig. 2B). CMA_3^+ banding in *Arachis* species was described only for few species, which did not include species of the section *Arachis* (Cai et al. 1987, Pierozzi and Baroni 2014, Ortiz et al. 2017). It is difficult to interpret the significance of these differences currently, but the lack of CMA_3^+ bands could be a possible inaccessibility of the CMA_3 fluorophore due to immediate structural changes in chromatin organization.

Genome affinity by GISH

Double GISH using simultaneously both labeled genomic DNAs from the diploid species as probes and, the single GISH using separately each of the allotetraploid genomic labeled DNA as probe were used to study the overall affinities of the genomes, especially considering the known biases of hybridization kinetics related to DNA repetitive fractions. Our hybridizations generated patterns generally consistent with previous observations in *A. hypogaea* (Ramos et al. 2006, Seijo et al. 2007). Single GISH indicated that these allotetraploid genomes shared most of its contents, which correspond to that of the diploid progenitor genomes. Both IpaDur1and *A. hypogaea* showed scarcity of signals on cyt-A9, the small pair "A" (Fig. 3), indicating its low repetitive content and possible equivalence to Aradu.A08 (Bertioli et al. 2016). Double GISH confirmed the preferential hybridization of each diploid probe to its corresponding subgenome, thus allowing chromosomes of the A and B subgenome to be easily distinguished in IpaDur1 (Fig. 4), as it was previously recognized for the genome components of *A. hypogaea* (Raina and Mukai 1999, Seijo et al. 2007). Although the genome of IpaDur1, *A. hypogaea* and progenitor species share the majority of the DNA content, remarkable differences were detected in IpaDur1, such as the striking mosaic hybridization patterns observed on cyt-B10 (Fig. 4), suggesting that this pair of chromosomes might have undergone multiple recombination events between subgenomes. Relatively strong residual hybridization in its bands suggests partial, and not complete, subgenome replacement. At first sight, this different affinity to the genomes along cyt-B10 of IpaDur1 seems consistent with the recombination between Aradu.A04 and AraipB04 that has been reported in this induced allotetraploid (Leal-Bertioli et al. 2015). However, on closer inspection, it seems likely that this possible genome instability cytogenetically observed is in a different chromosome (cyt-B10 has a conspicuous constriction indicating the presence of a large 45S rDNA cluster, but Araip.B04 does not have any 45S rDNA sequences). Although A-B subgenomes recombinations

between distal euchromatic regions of homeologous chromosomes have been shown in *A. hypogaea* (Bertioli et al. 2016), it is believed that most likely, they were not detected here because of their relatively small size and poor hybridization in the repeat-poor distal portions of the chromosomes.

5S and 45S rDNA chromosome mapping

Hybridizations with ribosomal DNAs (rDNAs) probes were carried out since they generate strong signals, the positions of ribosomal loci are important landmarks for cytogenetic chromosome identification and it is known that their concerted evolution drives changes following polyploidy (Grabiele et al. 2012). The number of 5S rDNA loci herein determined for IpaDur1 showed to be additive, as it was here confirmed for *A. hypogaea* cultivar Tatu (Fig. 5), in accordance to previous reports including other *A. hypogaea* cultivars (Seijo et al. 2004, Robledo et al. 2009, Robledo and Seijo 2010). However, the heteromorphic signal on cyt-A3, for both allotetraploids, relative to the corresponding chromosome in the diploid *A. duranensis*, could indicate possible genome instability in the allopolyploids. Similar heteromorphic signal was also observed for other *A. hypogaea* accessions by Seijo et al. (2004). Sequence similarity searches on *A. ipaensis* chromosomal pseudomolecules identified a single location of 5S rDNA, on Araip.B06, thus allowing its correspondence to cyt-B3. Nonetheless, similarity searches of the sequences of *A. duranensis* detected multiple rDNA locations (data not shown), making ascertained further cytogenetic - pseudomolecule correspondences, still a challenge.

Generally, 45S rDNA loci inherited from both parents often remain structurally (not necessarily functionally) intact in first generation hybrids, and ancient allopolyploids usually display uniparental inheritance and / or structural rearrangements of parental 45S rDNA (Volkov et al. 2017). Our analysis indicated that the sum of the 45S rDNA loci in the diploid species is equivalent to the number detected in *A. hypogaea* cultivar Tatu (Fig. 5B), in accordance to previous reports for other cultivars (Seijo et al. 2004, Robledo et al. 2009, Robledo and Seijo 2010). Nonetheless, and notably, the number of 45S rDNA loci in IpaDur1 differed from the sum of the progenitor diploid species, since signals on cyt-B3 and cyt-B7 were not detected (Fig. 5A), thus constituting another hint of genome instability. Nucleolar dominance was the same in both allotetraploids: NORs were present on cyt-B10 of *A. ipaensis* (Seijo et al. 2004), but might not be active on cyt-B10 of *A. hypogaea* (only on cytA-10; Seijo et al. 2004) or IpaDur1. Such alterations could be consequences of different mechanisms of heritance of these sequences, yet to be clarified in further studies.

Chromosome cyt-A10 of IpaDur1 (Fig. 5A) is the only pair comprising a potential active NOR (Nucleolus Organizer Region) in this genotype, since the thread-like constricted with 45S rDNA hybridization signals, typical of NORs are consistently present. In a similar way, our analysis of cyt-A10 in *A. hypogaea* (Fig. 5B), *A. duranensis* (Fig. 6C) and cyt-B10 in *A. ipaensis* (Fig. 6D) indicated similar patterns of 45S rDNA

signals, which are in accordance with the previous reports for these diploid species and *A. hypogaea* (Seijo et al. 2004, Robledo et al. 2009, Robledo and Seijo 2010). On the other hand, cyt-B10 for both allotetraploids did not show a distended rDNA 45S signal, suggesting that this locus might have been silenced, and hence suggesting a nucleolar dominance of cyt-A10. The possible cyt-B10NOR silencing in this newly synthetized allotetraploid indicates that this possible nucleolar dominance could be a rapid event after polyploidization, besides being independent of the maternal or paternal role played by *A. duranensis* during allotetraploidization. NORs / rDNA 45S loci losses, such as those described for the allopolyploids hybrids *Tragopogon mirus* (G.B. Ownbey, 1950) and *T. miscellus* (G.B. Ownbey, 1950) (Soltis et al. 2004); *A. thaliana* and the natural *A. suecica* (Pontes et al. 2004) and the induced *Triticum* (Linnaeus, 1753) /*Aegilops* (Linnaeus, 1753) (Guo and Han 2014) are usually attributed to rapid chromosomal rearrangements after polyploidization, although longer periods are usually necessary for a selective elimination of one parental NORs / 45S rDNA. Moreover, if there are some DNA regions in the chromosomes with gaps or constrictions that have tendency to break/gap, among possible consequences, there are changes in number, position and activity of 45S rDNA sites (reviewed by Rocha et al. 2017).

LRT-retrotransposons coverage and mapping

Differences in the repetitive content created, for example, by the activation of transposons, following polyploidy could explain why the variation of the intensity of signals on *A. hypogaea* chromosomes hybridized to its own genomic probe and IpaDur1 probe. In this regard, distribution of three retroelements from different classes was further inspected, both in situ and in silico: the Ty1-copia transposon RE128-84, the Ty3-gypsy transposon Pipoka, and the non-autonomous Athena (Fig. 8 and Table 3). FISH using the selected LTR-retroelements probes produced dispersed signals, corresponding to larger or smaller clusters of the members of these retroelement families. Generally, although there are some indications of changes, signals in the allotetraploids were additive, mostly considering the RE128-84 (Fig. 7A, B, C, D). This indicates that there has not been large-scale activation of these retrotransposon families after allopolyploidization. Nevertheless, in silico analysis of the coverage of these LTR-retroelements on the diploid pseudomolecules did not show a complete association with their in situ distribution.

Overall, in this study, whilst there are some indications that genome changes have occurred after polyploidy in *A. hypogaea*, they are quite small: possible nucleolar dominance and genome deletions, and indications of transposon activity. Whilst recombination between subgenomes has been clearly shown by the sequence analysis in *A. hypogaea* (Bertioli et al. 2016), evidences of similar genome rearrangements could not be detected in this species using the cytogenetic tools applied in this study. This could be due to the limited power of detection of genomic hybridization (GISH) in the euchromatic chromosomal regions. In contrast, IpaDur1 has clearly undergone further alterations that

could be evidenced cytogenetically: lack of two 45S sites on B subgenome chromosomes, large-scale multiple recombination between subgenomes, in at least one chromosome pair, the cyt-B10. Yet, this pair of chromosome is probably different to the pair in which A-B rearrangements were genetically detected (Leal-Bertioli et al. 2015).

It seems that IpaDur1 has a more unstable genome, and had larger recombination between subgenomes than *A. hypogaea*. IpaDur1 might be undergoing, at least in part, a route of 'autotetraploidization' and genetic degradation, process that has been termed the "Polyploid Ratchet" (Gaeta and Pires 2010). Since A and B subgenomes of IpaDur1 and *A. hypogaea* are mostly similar, it may have been expected that they would have similar propensities to recombination between subgenomes and stability, when incorporated in an allotetraploid form. However, this does not seem to be the case, perhaps because a distinct *A. duranensis* accession was the A subgenome donor, inheritance has been stabilized to some degree though genetic changes and selection (Jenczewski and Alix 2004, Gaeta and Pires 2010) or due to the reversed male/female roles played by the diploid species. The exact extent and the basis of genetics present in *A. hypogaea*, the cultivated peanut, are questions still unanswered and will be pursued with further investigation.

Acknowledgements

The authors thank Drs. J. F. M. Valls, M. T. Pozzobon, M.C. Moretzsohn for seed supply, technical support, and useful discussions; Ana C. Gomes for laboratory support; Leandro Mesquita, for greenhouse assistance; the Plant Cytogenetics and Evolution Laboratory at the Federal University of Pernambuco (UFPE), PE, Brazil for FaCS use and rDNA stabs and Artur Fonseca for the flow cytometry analysis; A. Pedrosa from UFPE, PE, Brazil for the plasmids with the rDNA sequences.

References

Adams KL, Wendel JF (2005) Polyploidy and genome evolution in plants. Current Opinionin Plant Biology 8: 135–141. http://dx.doi.org/10.1016/j.pbi.2005.01.001

Bento M, Pereira HS, Rocheta M, Gustafson P, Viegas W, Silva M (2008) Polyploidization as a retraction force in plant genome evolution: Sequence rearrangements in *Triticale*. PLoS One 3: 1402. http://dx.doi.org/10.1371/journal.pone.0001402

Bertioli DJ, Cannon SB, Froenicke L, Huang G, Farmer AD, Cannon EKS, Liu X, Gao D, Clevenger J, Dash S, Ren L, Moretzsohn MC, Shirasawa K, Huang W, Vidigal B, Abernathy B, Chu Y, Niederhuth CE, Umale P, Araújo ACG, Kozik A, Kim KD, Burow MD, Varshney RK, Wang X, Zhang X, Barkley N, Guimarães PM, Isobe S, Guo B, Liao B, Stalker HT, Schmitz RJ, Scheffler BE, Leal-Bertioli SCM, Xun X, Jackson SA, Michelmore R, Ozias-Akins P (2016) The genome sequences of *Arachis duranensis* and *Arachis ipaënsis*, the diploid ancestors of cultivated peanut. Nature Genetics 48: 438–446.

Bertioli DJ, Vidigal B, Nielen S, Ratnaparkhe MB, Lee TH, Leal-Bertioli SCM, Kim M, Guimarães PM, Seijo G, Schwarzacher T, Paterson AH, Heslop-Harrison P, Araujo ACG (2013) The repetitive component of the A genome of peanut (*Arachis hypogaea*) and its role in remodelling intergenic sequence space since its evolutionary divergence from the B genome. Annals of Botany 112: 545–559. https://doi.org/10.1093/aob/mct128

Bonavia D (1982) Los Gavilanes: mar, desierto y oásis en la historia del hombre: precerámico peruano. Corporación Financiera de Desarrollo, Instituto Arqueologico Aleman.

Burow MD, Simpson CE, Starr JL, Paterson AH (2001) Transmission genetics of chromatin from a synthetic amphidiploid to cultivated peanut (*Arachis hypogaea* L.): Broadening the gene pool of a monophyletic polyploid species. Genetics 159: 823–837.

Cai Q, Lu S, Chinnappa CC (1987) Analysis of karyotypes and Giemsa C-banding patterns in eight species of *Arachis*. Genome 29: 187–194. https://doi.org/10.1139/g87-032

Chen X, Li H, Pandey MK, Yang Q, Wang X, Garg V, Li H, Chi X, Doddamani D, Hong Y, Upadhyaya H, Guo H, Khan AW, Zhu F, Zhang X, Pan L, Pierce GL, Zhou G, Krishnamohan KVS, Chen M, Zhong N, Agarwal G, Li S, Chitikineni A, Zhang GQ, Sharma S, Chen N, Liu H, Janila P, Li S, Wang M, Wang T, Sun J, Li X, Li C, Wang M, Yu L, Wen S, Singh S, Yang Z, Zhao J, Zhang C, Yu Y, Bi J, Zang X, Liu ZJ, Paterson AH, Wang S, Liang X, Varshney RK, Yu S (2016) Draft genome of the peanut A-genome progenitor (*Arachis duranensis*) provides insights into geocarpy, oil biosynthesis, and allergens. Proceedings of the National Academy of Sciences 113: 6785–6790. https://doi.org/10.1073/pnas.1600899113

Clevenger J, Chu Y, Guimaraes LA, Maia T, Bertioli D, Leal-Bertioli SCM, Pandey MK, Vaughn J, Abernathy B, Barkley NA, Hovav R, Burow M, Nayak SN, Chitikineni A, Isleib TG, Holbrook C, Jackson SJ, Varshney RK, Ozias-Akins P (2017) Genome-wide SNP genotyping resolves signatures of selection and tetrasomic recombination in peanut. Molecular Plant 10: 309–322. https://doi.org/10.1016/j.molp.2016.11.015

Comai L (2000) Genetic and epigenetic interactions in allopolyploid plants. Plant Molecular Biology 43: 387–399. https://doi.org/10.1023/A:1006480722854

Dhillon SS, Rake AV, Miksche JP (1980) Reassociation Kinetics and Cytophotometric Characterization of Peanut (*Arachis hypogaea* L.) DNA. Plant Physiology 65: 1121–1127.

Doležel J, Sgorbati S, Lucretti S (1992) Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. Physiologia Plantarum 85: 625–631. https://doi.org/10.1111/j.1399-3054.1992.tb04764.x

Eilam T, Anikster Y, Millet E, Manisterski J, Feldman M (2008) Nuclear DNA amount and genome downsizing in natural and synthetic allopolyploids of the genera *Aegilops* and *Triticum*. Genome 51: 616–627. https://doi:10.1139/G08-043

Fávero AP, Simpson CE, Valls JFM, Vello NA (2006) Study of the evolution of cultivated peanut through crossability studies among *Arachis ipaënsis*, *A. duranensis*, and *A. hypogaea*. Crop Science 46: 1546–1552. https://doi.org/10.2135/cropsci2005.09-0331

Fávero AP, Santos RF, Simpson CE, Valls JFM, Vello NA (2015) Successful crosses between fungal-resistant wild species of *Arachis* (Section *Arachis*) and *Arachis hypogaea*. Genetics and Molecular Biology 38: 353–365. http://dx.doi.org/10.1590/S1415-475738320140376

Fernández A, Krapovickas A (1994) Cromosomas y evolucion en *Arachis* (Leguminosae). Bonplandia 8: 187–220.

Ferreira ME, Grattapaglia D (1998) Introdução ao uso de marcadores moleculares em análise genética. 3 ed. Brasília: Embrapa Recursos Geneticos e Biotecnologia.

Foncèka D, Tossim H-A, Rivallan R, Vignes H, Faye I, Ndoye O, Moretzsohn MC, Bertioli DJ, Glaszmann JC, Courtois B, Rami JF (2012) Fostered and left behind alleles in peanut: interspecific QTL mapping reveals footprints of domestication and useful natural variation for breeding. BMC Plant Biology 12: 26. http://dx.doi.org/10.1186/1471-2229-12-26

Foncèka D, Hodo-Abalo T, Rivallan R, Faye I, Sall MN, Ndoye O, Fávero AP, Bertioli DJ, Glaszmann JC, Courtois B, Rami JF (2009) Genetic mapping of wild introgressions into cultivated peanut: a way toward enlarging the genetic basis of a recent allotetraploid. BMC Plant Biology 9: 1–103. https://doi.org/10.1186/1471-2229-9-103

Gaeta RT, Pires CJ (2010) Homoeologous recombination in allopolyploids: The polyploid ratchet. New Phytologist 186: 18–28. https://doi.org/10.1111/j.1469-8137.2009.03089.x

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220: 1049–1051. https://doi.org/10.1126/science.220.4601.1049

Grabiele M, Chalup L, Robledo G, Seijo G (2012) Genetic and geographic origin of domesticated peanut as evidenced by 5S rDNA and chloroplast DNA sequences. Plant Systematics and Evolution 298: 1151–1165. https://doi.org/10.1007/s00606-012-0627-3

Guo X, Han F (2014) Asymmetric epigenetic modification and elimination of rDNA sequences by polyploidization in wheat. The Plant Cell 26: 4311–4327. http://dx.doi.org/10.1105/tpc.114.129841

Heslop-Harrison JS, Harrison GE, Leitch IJ (1992) Reprobing of DNA: DNA in situ hybridization preparations. Trends in Genetics 8: 372–373. http://dx.doi.org/10.1016/0168-9525(92)90287-E

Husted L (1936) Cytological studies on the peanut, *Arachis*. II. Chromosome number, morphology and behavior, and their application to the problem ofthe origin of the cultivated forms. Cytologia 7: 396–423. https://doi.org/10.1508/cytologia.7.396

Jenczewski E, Alix K (2004) From Diploids to Allopolyploids: the Emergence of Efficient Pairing Control Genes in Plants. Critical Reviews in Plant Sciences 23: 21–45. http://dx.doi.org/10.1080/07352680490273239

Kashkush K, Feldman M, Levy AA (2003) Transcriptional activation of retrotransposons alters the expression of adjacent genes in wheat. Nature Genetics 33:102–106.

Kim NS (2017) The genomes and transposable elements in plants: are they friends or foes? Genes and Genomics 39: 359–370. https://link.springer.com/article/10.1007/s13258-017-0522-y

Kochert G, Halward T, Branch WD, Simpson CE (1991) RFLP variability in peanut (*Arachis hypogaea* L.) cultivars and wild species. Theoretical and Applied Genetics 81: 565–570. http://dx.doi.org/10.1007/BF00226719

Kochert G, Stalker HT, Gimenes M, Galgaro L, Lopes CR, Moore K (1996) RFLP and Cytogenetic Evidence on the Origin and Evolution of Allotetraploid Domesticated Peanut, *Arachis hypogaea* (Leguminosae). American Journal of Botany 83: 1282–1291. https://doi.org/10.1002/j.1537-2197.1996.tb13912.x

Krapovickas A, Gregory W (1994) Taxonomia del genero. Bomplandia 8: 1–186.

Leal-Bertioli S, Shirasawa K, Abernathy B, Moretzsohn M, Chavarro C, Clevenger J, Ozias-Akins P, Jackson S, Bertioli DJ (2015) Tetrasomic recombination is surprisingly frequent in allotetraploid *Arachis*. Genetics 199: 1093–1105. https://doi.org/10.1534/genetics.115.174607

Ma X-F, Gustafson JP (2006) Timing and rate of genome variation in Triticale following allopolyploidization. Genome 49: 950–958. https://doi.org/10.1139/g06-078

Maluszynska J, Heslop-Harrison JS (1993) Molecular cytogenetics of the genus *Arabidopsis*: in situ localization of rDNA sites, chromosome numbers and diversity in centromeric heterochromatin. Annals of Botany 71: 479–484. https://doi.org/10.1006/anbo.1993.1063

McClintock B (1984) The significance of responses of the genome to challenge. Science 256: 792–801. https://doi.org/10.1126/science.15739260

Moretzsohn MC, Gouvea EG, Inglis PW, Leal-Bertioli SCM, Valls JFM, Bertioli DJ (2013) A study of the relationships of cultivated peanut (*Arachis hypogaea*) and its most closely related wild species using intron sequences and microsatellite markers. Annals of Botany 111: 113–126. https://doi.org/10.1093/aob/mcs237

Moretzsohn MC, Barbosa AV, Alves-Freitas DM, Teixeira C, Leal-Bertioli SCM, Guimarães P, Pereira RW, Lopes CR, Cavallari MM, Valls JFM, Bertioli DJ, Gimenes MA (2009) A linkage map for the B-genome of *Arachis* (Fabaceae) and its synteny to the A-genome. BMC Plant Biology 9: 40. https://doi.org/10.1186/1471-2229-9-40

Navashin M (1934) Chromosome alterations caused by hybridization and their bearing upon certain general genetic problems. Cytologia 5: 169–203. http://doi.org/10.1508/cytologia.5.169

Nielen S, Vidigal BS, Leal-Bertioli SCM, Ratnaparkhe M, Paterson AH, Garsmeur O, D'Hont A, Guimarães PM, Bertioli DJ (2012) Matita, a new retroelement from peanut: Characterization and evolutionary context in the light of the *Arachis* A-B genome divergence. Molecular Genetics and Genomics 287: 2138. https://doi.org/10.1007/s00438-011-0656-6

Nielen S, Fonseca F, Leal-Bertioli SCM, Guimarães PM, Seijo G, Christopher T, Arrial R, Bertioli DJ (2010) FIDEL - a retrovirus-like retrotransposon and its distinct evolutionary histories in the A- and B-genome components of cultivated peanut. Chromosome Research 18: 227–246. https://doi.org/10.1007/s10577-009-9109-z

Ortiz AM, Robledo G, Seijo G, Valls JFM, Lavia GI (2017) Cytogenetic evidences on the evolutionary relationships between the tetraploids of the section *Rhizomatosae* and related diploid species (*Arachis*, Leguminosae). Journal of Plant Research 130: 791–807. https://doi.org/10.1007/s10265-017-0949-x

PeanutBase. http://peanutbase.org/blat [accessed 10 April 2017]

Pedrosa A, Sandal N, Stougaard J, Schweizer D, Bachmair A (2002) Chromosomal Map of the Model Legume *Lotus japonicus*. Genetics 161: 1661–1672.

Petit M, Guidat C, Daniel J, Denis E, Montoriol E, Bui QT, Lim KY, Kovarik A, Leitch AR, Grandbastien MA, Mhiri C (2010) Mobilization of retrotransposons in synthetic allotetraploid tobacco. New Phytologist 186: 135–147. http://doi.org/10.1111/J.1469-8137.2009.03140.X

Pierozzi NI, Baroni RM (2014) Karyotype analysis using C-and NOR-banding in Ramie [*Boehmeria nivea* (L.) Gaud.]. Cytologia 79: 261–268. http://doi.org/10.1508/cytologia.79.261

Pontes O, Neves N, Silva M, Lewis MS, Madlung A, Comai L, Viegas W, Pikaard CS (2004) Chromosomal locus rearrangements are a rapid response to formation of the allotetraploid *Arabidopsis suecica* genome. Proceedings of the National Academy of Sciences 101: 18240–18245. https://doi.org/10.1073/pnas.0407258102

Preuss S, Pikaard CS (2007) rRNA gene silencing and nucleolar dominance: insights into a chromosome-scale epigenetic on/off switch. Biochimica et Biophysica Acta - Gene Structure and Expression 1769: 383–392. https://doi.org/10.1016/j.bbaexp.2007.02.005

Raina SN, Mukai Y (1999) Genomic in situ hybridization in *Arachis* (Fabaceae) identifies the diploid wild progenitors of cultivated (*A. hypogaea*) and related wild (*A. monticola*) peanut species. Plant Systematics and Evolution 214: 251–262. https://doi.org/10.1007/BF00985743

Ramos ML, Fleming G, Chu Y, Akiyama Y, Gallo M, Ozias-Akins P (2006) Chromosomal and phylogenetic context for conglutin genes in *Arachis* based on genomic sequence. Molecular Genetics and Genomics 275: 578–592. https://doi.org/10.1007/s00438-006-0114-z

Robledo G, Lavia GI, Seijo G (2009) Species relations among wild *Arachis* species with the A genome as revealed by FISH mapping of rDNA loci and heterochromatin detection. Theoretical and Applied Genetics 118: 1295–1307. https://doi.org/10.1007/s00122-009-0981-x

Robledo G, Seijo G (2010) Species relationships among the wild B genome of *Arachis* species (section *Arachis*) based on FISH mapping of rDNA loci and heterochromatin detection: A new proposal for genome arrangement. Theoretical and Applied Genetics 121: 1033–1046. https://doi.org/10.1007/s00122-010-1369-7

Rocha LC, Jankowska M, Fuchs J, Mittelmann A, Techio VH, Houben A (2017) Decondensation of chromosomal 45S rDNA sites in *Lolium* and *Festuca* genotypes does not result in karyotype instability. Protoplasma 254: 285–292. https://doi.org/10.1007/s00709-016-0942-6

Samoluk SS, Robledo G, Podio M, Chalup L, Ortiz JPA, Pessino SC, Seijo G (2015a) First insight into divergence, representation and chromosome distribution of reverse transcriptase fragments from L1 retrotransposons in peanut and wild relative species. Genetica 143: 113–125. https://doi.org/10.1007/s10709-015-9820-y

Samoluk SS, Chalup L, Robledo G, Seijo G (2015b) Genome sizes in diploid and allopolyploid *Arachis* L. species (section *Arachis*). Genetic Resources and Crop Evolution 62: 747–763. https://doi.org/10.1007/s10722-014-0193-3

Schwarzacher T, Heslop-Harrison J (2000) Practical in situ hybridization. BIOS Scientific Publishers Ltd., Oxford.

Schweizer D, Ambros P (1994) Chromosome banding: stain combinations for specific regions. Methods in Molecular Biology 29: 97–112. https://doi.org/10.1385/0-89603-289-2:97

Seijo JG, Robledo G, Ortíz A, Lavia GI (2014) Classical and Molecular Cytogenetics in *Arachis*. Genetics, Genomics and Breeding of Peanuts 37.

Seijo G, Lavia GI, Fernández A, Krapovickas A, Ducasse DA, Bertioli DJ, Moscone EA (2007) Genomic relationships between the cultivated peanut (*Arachis hypogaea*, Leguminosae) and

its close relatives revealed by double GISH. American Journal of Botany 94: 1963–1971 https://doi.org/10.3732/ajb.94.12.1963

Seijo JG, Lavia GI, Fernández A, Krapovickas A, Ducasse D, Moscone EA (2004) Physical mapping of the 5S and 18S-25S rRNA genes by FISH as evidence that *Arachis duranensis* and *A. ipaënsis* are the wild diploid progenitors of *A. hypogaea* (Leguminosae). American Journal of Botany 91: 1294–1303. https://doi.org/10.3732/ajb.91.9.1294

Shcherban AB (2013) The reorganization of plant genomes during allopolyploidization. Russian Journal of Genetics: Applied Research 3: 444–450. https://doi.org/10.1134/S2079059713060087

Shirasawa K, Bertioli DJ, Varshney RK, Moretzsohn MC, Leal-Bertioli SCM, Thudi M, Pandey MK (2013) Integrated consensus map of cultivated peanut and wild relatives reveals structures of the a and b genomes of arachis and divergence of the legume genomes. DNA Research 20: 173–184. https://doi.org/10.1093/dnares/dss042

Shirasawa K, Hirakawa H, Tabata S, Hasegawa M, Kiyoshima H, Suzuki S, Sasamoto S (2012) Characterization of active miniature inverted-repeat transposable elements in the peanut genome. Theoretical and Applied Genetics 124: 1429–1438. https://doi.org/10.1007/s00122-012-1798-6

Simpson CE, Krapovickas A, Valls JFM (2001) History of *Arachis* Including Evidence of *A. hypogaea* L. Progenitors. Peanut Science 28: 78–80. https://doi.org/10.3146/i0095-3679-28-2-7

Singh AK (1986) Utilization of wild relatives in the genetic improvement of *Arachis hypogaea* L. Theoretical and Applied Genetics 72: 433–439. https://doi.org/10.1007/BF00289523

Soltis DE, Soltis PS, Pires JC, Kovarik A, Tate JA, Mavrodiev E (2004) Recent and recurrent polyploidy in *Tragopogon* (Asteraceae): cytogenetic, genomic and genetic comparisons. Biological Journal of the Linnean Society 82: 485–501. https://doi.org/10.1111/j.1095-8312.2004.00335.x

Temsch EM, Greilhuber J (2001) Genome size in *Arachis duranensis*: a critical study. Genome 44: 826–830. https://doi.org/10.1139/g01-081

Valls JFM, Simpson CE (2005) New Species of *Arachis* (Leguminosae) from Brazil, Paraguay an Bolivia. Bonplandia 14: 35–63.

Volkov RA, Panchuk II, Borisjuk NV, Hosiawa-Baranska M, Maluszynska J, Hemleben V (2017) Evolutional dynamics of 45S and 5S ribosomal DNA in ancient allohexaploid *Atropa belladonna*. BMC Plant Biology 17: 21. https://doi.org/10.1186/s12870-017-0978-6

Wanzenböck EM, Schöfer C, Schweizer D, Bachmair A (1997) Ribosomal transcription units integrated via T - DNA transformation associate with the nucleolus and do not require upstream repeat sequences for activity in *Arabidopsis thaliana*. The Plant Journal 11: 1007–1016. https://doi.org/10.1046/j.1365-313X.1997.11051007.x

Zhang L, Yang X, Tian L, Chen L, Yu W (2016) Identification of peanut (*Arachis hypogaea*) chromosomes using a fluorescence in situ hybridization system reveals multiple hybridization events during tetraploid peanut formation. New Phytologist 211: 1424–1439. https://doi.org/10.1111/nph.13999

CompCytogen 12(1): 141–144 (2018)
doi: 10.3897/CompCytogen.v12i1.24550
http://compcytogen.pensoft.net

IN MEMORIAM

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

In Memoriam: Professor Iya Kiknadze (1930–2017)

Paraskeva Michailova[1], Valentina Kuznetsova[2], Snejana Grozeva[1], Julia Ilkova[1], Ninel Petrova[2]

1 *Research Group Cytotaxonomy and Evolution, Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 1 Tsar Osvoboditel blvd, 1000 Sofia, Bulgaria* **2** *Department of Karysystematics, Zoological Institute, Russian Academy of Sciences, Universitetskaya emb. 1, 199034 St. Petersburg, Russia*

Corresponding author: *Paraskeva Michailova* (pmichailova@yahoo.com)

Received 19 February 2018 | Accepted 20 February 2018 | Published 23 March 2018

http://zoobank.org/642E5FD3-EE90-48CE-8F18-0850BB15B7E2

Citation: Michailova P, Kuznetsova V, Grozeva S, Ilkova J, Petrova N (2018) In Memoriam: Professor Iya Kiknadze (1930–2017). Comparative Cytogenetics 12(1): 141–144. https://doi.org/10.3897/CompCytogen.v12i1.24550

With very deep sympathies and very great regret we announce that our best friend, the dearest colleague and excellent researcher Professor Iya Kiknadze passed away in Novosibirsk on December 17, 2017. She was a scientist who made a great and very valuable contribution in the field of cytology – structure and function of the eukaryotic chromosome, cytogenetics and karyotype evolution of family Chironomidae.

Iya Kiknadze was the founder of a very important field in the Cell Biology in Russia – studies of the functional and structural organization of the salivary gland polytene chromosomes in Diptera, especially in the Chironomidae. In many of her studies she followed gene activity during ontogenesis (Kiknadze 1975, Kiknadze et al. 1989). One of the most important and impressive studies was dedicated to gene activity of the key structures of the polytene chromosomes: Balbiani Ring (BR) and Nucleolus Organizer Region (NOR). Her study on the transcriptional activity of these structures represents a great contribution to the genome research on the Chironomidae. Even more, the study on the functional activity of these structures during larval development of the model Chironomid species *Chironomus riparius* Mg. (Kiknadze 1978) provides valuable information about the processes of the environmental mutagenesis. The chromosome map of this species developed by Iya Kiknadze and her collaborators (Kiknadze et al. 1991) is successfully used in the field of genotoxicology for monitoring the environment and

Professor Iya Kiknadze (1930–2017).
Photo credit: AG Istomina.

for assessing the potential environmental impact (Michailova et al. 2012). Her own study on the chromosome alterations of Chironomids from Republic of Sakha (Yakutia) showed the sensitivity of their genomes to different stress agents in the environment. Her beneficial monograph "Functional organization of the chromosomes" (Kiknadze 1972) presented very well the gene activity and functional organization of the salivary gland chromosomes of the Chironomidae. Dealing with chironomid polytene chromosomes, Iya Kiknadze was the first who has proved that the evolutionarily conserved structure, NOR, is a transcriptionally active region of the interphase chromosomes. Most significant and marvelous studies of Iya Kiknadze on the functional organization of the genomes were done and published in collaboration with well-known specialists as Drs B. Daneholdt, M. Lezzi, J. Edstrom, and U. Grossbach. Together with other scientists, Drs T. Hankeln, E. Schmidt, and her collaborators Drs M. Filippova and K. Aimanova, Professor Iya Kiknadze discovered significant differences in the amount of centromeric heterochromatin between sibling species of the *Chironomus plumosus* group. By *in situ* hybridization they studied the localization and genomic organization of a Sau3AI restriction site (Sau elements) in 24 *Chironomus* Mg. species (Hankeln et al. 1994).

As discussed above, the presence of giant polytene chromosomes in chironomid larvae makes them prospective subjects for genetic, cytogenetic, biochemical and molecular studies. However, to realize the full potential of these subjects precise species identification is necessary. In many cases, a conventional morphological method does not help to identify the species in the larva stage, as the larvae have no distinct differences in external morphology in some genera, e.g. genus *Chironomus* which is in fact rich in sibling species. In all those cases for solving different taxonomic problems of these insects, together with external morphology the markers of the salivary gland chromosomes are being applied. Iya Kiknadze was the author or a co-author of many original chromosome maps of different Chironomid species (Kiknadze et al. 1991, 1996) where banding patterns of polytene chromosomes have been combined with detailed morphological analyses of larvae. These maps allow the scientists to solve many problems in taxonomy, cytogenetics, and chromosomal polymorphism of natural populations of the species, to trace the path of species divergence. Based on the chromo-

some maps, the rearrangements in the polytene chromosomes and different biomarkers are applied to analyze precisely the genome response to various stress agents in the environment. Of especial interest is her book "Karyotypes of Palearctic and Holarctic species of the genus *Chironomus*" (Kiknadze et al. 2016), where Iya Kiknadze, together with her closest colleagues Drs A. Istomina, V. Golygina and L. Gunderina, presented the cytogenetic characteristics of 63 species of genus *Chironomus*, collected from different geographical areas, including Russia (European part, Ural, West and East Siberia, Altai, Tuva, the Far East), Kazakhstan, West Europe (Germany, Belgium, the Netherlands), Bulgaria, USA, Canada, China, and Japan. The pictures of the polytene chromosomes of all studied species are brilliant and allow very easy and correct species identification as well as analyzing chromosomal polymorphisms, the chromosomal rearrangements involved in the species divergence. Here it is important to underline that phylogenetic analysis of these data allowed her to reconstruct the cytogenetic history of many Chironomid species (Kiknadze et al. 2008). The authors evaluated the role of chromosome aberrations in population divergence and speciation. They found that in each continent there are endemic species-specific sequences together with common sequences for different species and cytocomplexes.

Iya Kiknadze had a very fruitful collaboration not only with colleagues from different parts of Russia (besides some mentioned above, these are Drs S. Belyanina, N. Petrova etc.) but also with specialists from all over the world: Australia – Dr J. Martin, USA – Dr M. Butler, Europe – Germany – Dr W. Wülker, Bulgaria – Dr P. Michailova, Netherlands – Dr H. Vallenduuk etc. As a result, they described together many new chironomid species, presented a number of unique chromosome maps and suggested the main paths of karyotype evolution in the Chironomidae.

Iya Kiknadze was the teacher and supervisor of many entomologists, cytogeneticists, geneticists, karyosystematicists in Russia, especially in Novosibirsk. Her scientific ideas are followed by many scientists in Europe, Asia and America. As a famous scientist and specialist in different fields of Genetics she has been invited in the editorial boards of many journals as Tsitologiya, Ontogenesis, Eurasian Entomological Journal and Comparative Cytogenetics.

Prof. Iya Kiknadze was not only a remarkable scientist but she had a real talent for organization of different Symposia and Conferences. She initiated the first International Symposium on Organization and Expression of Tissue Specific Genes in Novosibirsk in 1982 and it became a regular every second year workshop on Chironomid Balbiani Ring, organized in different countries. An important dimension of her career was the participation in many international Symposia, workshops in the field of structure and function of the genome as well as in the field of Karyosystematics and Evolution of the Chironomidae. Always her presentations were followed with a great interest.

With the death of Professor Iya Kiknadze we lost a great specialist in genetics, cytogenetics and karyosystematics of the Chironomidae. We will miss our best friend and a great person. We will always remember Iya Kiknadze for her work, commitment and energy. She will always live in our hearts and memories.

References

Hankeln T, Filippova M, Kiknadze II, Aimanova K, Schmidt E (1994) Centromeric heterochromatin and satellite DNA in the *Chironomus plumosus* species group. Genome 37: 925–934. https://doi.org/10.1139/g94-132

Kiknadze II (1972) The functional organization of the chromosomes. Nauka, Leningrad, 212 pp. [In Russian]

Kiknadze II (1975) Subject of Development Biology. Problems of Development Biology, 95–127. [In Russian]

Kiknadze II (1978) Comparative characteristics of puffing pattern in salivary gland chromosomes of *Chironomus thummi* during larval development and metamorphosis. II. Puffing pattern in chromosomes I, II, III. Tsitologia 20: 514-521. [In Russian with English Summary].

Kiknadze II, Blinov A, Kolesnokov N (1989) Molecular and Cytological Organization of Chironomid Genome. In: Shumnii B (Ed.) Structure and functional organization of the Genome. Nauka, Novosibirsk, 4–58. [In Russian]

Kiknadze II, Shilova A, Kerkis I, Shobanov N, Zelenzov N, Grebenjuk F, Istomina A, Praslov B (1991) Karyotypes and Morphology of larvae in the tribe Chironomini. Atlas, Novosibirsk, 117 pp. [In Russian]

Kiknadze II, Istomina A, Gunderina L, Salova T, Aimanova K, Savvinov D (1996) Chironomid karyopools of Yakutian cryolitozone. In: Gruzdev A, Sergeev M (Eds). Novosibirsk, 166 pp. [In Russian]

Kiknadze II, Istomina A, Golygina V, Gunderina L (2016) Karyotypes of Palearctic and Holarctic species of the genus *Chironomus.* Academic Publishing House GEO, Novosibirsk, 489 pp.

Kiknadze II, Gunderina L, Butler M, Wuelker W, Martin J (2008) Chromosomes and Continents. In: Dobretsov N, Kolchanov N, Rozanov A, Zavarzin G (Eds) Biosphere Origin and Evolution, 349–369. https://doi.org/10.1007/978-0-387-68656-1_25

Michailova P, Sella G, Petrova N (2012) Chironomids (Diptera) and their salivary gland chromosomes as indicators of trace metal genotoxicology. Italian Journal of Zoology 79: 218–230. https://doi.org/10.1080/11250003.2011.622084