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Karyological characterization and identification of four repetitive element groups (the 18S – 28S rRNA gene, telomeric sequences, microsatellite repeat motifs, *Rex* retroelements) of the Asian swamp eel (*Monopterus albus*)

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Abstract

Among teleost fishes, Asian swamp eel (*Monopterus albus* Zuiew, 1793) possesses the lowest chromosome number, 2n = 24. To characterize the chromosome constitution and investigate the genome organization of repetitive sequences in *M. albus*, karyotyping and chromosome mapping were performed with the 18S – 28S rRNA gene, telomeric repeats, microsatellite repeat motifs, and *Rex* retroelements. The

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18S – 28S rRNA genes were observed to the pericentromeric region of chromosome 4 at the same position with large propidium iodide and C-positive bands, suggesting that the molecular structure of the pericentromeric regions of chromosome 4 has evolved in a concerted manner with amplification of the 18S – 28S rRNA genes. (TTAGGG)n sequences were found at the telomeric ends of all chromosomes. Eight of 19 microsatellite repeat motifs were dispersedly mapped on different chromosomes suggesting the independent amplification of microsatellite repeat motifs in *M. albus*. *Monopterus albus Rex1* (*MALRex1*) was observed at interstitial sites of all chromosomes and in the pericentromeric regions of most chromosomes whereas *MALRex3* was scattered and localized to all chromosomes and *MALRex6* to several chromosomes. This suggests that these retroelements were independently amplified or lost in *M. albus*. Among *MALRexs* (*MALRex1*, *MALRex3*, and *MALRex6*), *MALRex6* showed higher interspecific sequence divergences from other teleost species in comparison. This suggests that the divergence of *Rex6* sequences of *M. albus* might have occurred a relatively long time ago.

Keywords
Asian swamp eel, C-band, dispersion, microsatellite repeat, retroelement

Introduction

Teleost fishes possess high morphological and physiological variation with nearly 30,000 extant species (Nelson 2016). The Asian swamp eel (*Monopterus albus* Zuiew, 1793) is a commercially important, air-breathing fish (Synbranchidae, Synbranchiformes) which is a protogynous hermaphrodite native in freshwaters of East and Southeast Asia and invasive elsewhere in the world including North America (Liem 1963, Chan et al. 1972, Cheng et al. 2003). The diploid chromosome number of *M. albus* is 24, comprising 12 pairs of acrocentric chromosomes (Yu et al. 1989, Ji et al. 2003). This is considered to be the lowest chromosome number known in teleosts (genome sizes 0.6–0.8 pg), while common chromosome numbers of teleosts are 2n = 40–50 and genome sizes around 0.8–2 pg (Zhou et al. 2002). The Asian swamp eel is, therefore, a good model to investigate genome evolution and the developmental process in teleosts.

Synbranchids are freshwater eel-like fishes which include four genera (*Macrotrema* Cantor, 1849, *Monopterus* Lacépède, 1800, *Ophisternon* McClelland, 1844, and *Synbranchus* Bloch, 1795) and *Monopterus* is phylogenetically located at the basal position except for the *Macrotrema* (Perdices et al. 2005, Betancur et al. 2013). This phylogenetic relationship suggests that the Asian swamp eel might retain the ancestral karyotype of Synbranchidae. When compared to other synbranchids, it has a unique karyotype with very few chromosomes. For example, the diploid chromosome numbers of *Monopterus cuchia* Hamilton, 1822, a closely related species, is 42 and those of *Synbranchus* and *Ophisternon* species are 42 and 46, respectively (Rishi and Haobam 1984, Foresti et al. 1992, Nirchio et al. 2011, Carvalho et al. 2012, Utsunomia et al. 2014). An investigation of *M. albus* chromosome constitution to compare it with other synbranchid fishes could shed light evolutionary scenarios of chromosomal rearrangements and genome organization within Synbranchidae.

Vertebrate genomes are commonly characterized by a large copy number of repetitive sequences, belonging to two main classes: the site-specific type (such as satellite DNA, microsatellite repeats, ribosomal RNA genes and telomeric sequences), and the interspersed type (transposable elements, TEs) (Jelinek and Schmid 1982). Although most repetitive DNAs do not code for proteins, repetitive sequences can also play important role in the function, dynamics, and evolution of genomes (Csink and Henikoff 1998, Henikoff et al. 2001). Microsatellites, which are tandem repeats of small stretches of DNA motifs, are widespread in the genomes. Amplification of microsatellite repeat motifs has often been observed on sex chromosomes (Cioffi et al. 2011, Matsubara et al. 2015) or several autosomes (Schneider et al. 2015) of vertebrates. Microsatellite repeat motifs have been widely used as cytogenetic markers for chromosome identification, particularly for map-poor species (Srikulnath 2010). TEs are also thought to play an important role in genome evolution (Kidwell and Lisch 2000) acting as a substrate for homologous recombination resulting in chromosomal rearrangements. Additionally, TEs can be transmitted by both vertical and horizontal transfers being present in genomes of phylogenetically distant species (Tang et al. 2015). Retrotransposons (retroelements) are a class of TEs which have RNA as an intermediate, and the *Rex* retroelements (*Rex1*, *Rex3*, and *Rex6*) were active during teleost evolution (Volff et al. 1999, 2000, 2001). These retroelements are widely used as markers for molecular evolution and physical mapping, which allow to understand the role of repetitive elements in genome organization and evolution of teleosts (Ferreira et al. 2011, Schneider et al. 2013).

In this study, karyotyping was performed with conventional Giemsa staining, 4', 6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) fluorescent staining, C-banding, and fluorescence *in situ* hybridization (FISH) with four repetitive elements; namely, the 18S – 28S ribosomal RNA genes, telomeric (TTAGGG)n sequences, *Rex* retroelements and 19 microsatellite repeat motifs. Partial DNA fragments of *Rex* retroelements (*Rex1*, *Rex3*, and *Rex6*) were molecularly characterized and the evolutionary processes responsible for these retroelements in teleost genomes were discussed, together with the organization of synbranchid genomes.

Materials and methods

Specimens and chromosome preparation

Ten specimens of the Asian swamp eel were purchased from an animal pet shop in Bangkok, Thailand. Animal care and all experimental procedures were approved by the Animal Experiment Committee, Kasetsart University, Thailand (approval no. ACKU00958), and conducted according to the Regulations on Animal Experiments at Kasetsart University, Thailand. Mitotic chromosomes were obtained from gill and kidney cells using the air drying method. Briefly, after intraperitoneal injection of

0.01% colchicine (Sigma, St. Louis, Missouri, USA) in the proportion of 0.7 ml per 100 g of fish weight for 2 h, fishes were anesthetized in ice-cold water, and the anterior portion of the gill and kidney were removed and used for mitotic chromosome preparation. After hypotonic treatment of gill and kidney in 0.075 M KCl for 50 min at room temperature, the organs were minced and placed in the first fixative solution (3:1 methanol/acetic acid) for 5 min and in the second fixative solution (2:1 methanol/acetic acid) for 5 min on ice. The cells were collected by filtration using gauze, and then fixed with 3:1 methanol/acetic acid. The cells in suspension were dropped onto clean glass slides and air-dried. The slides were kept at -80°C until use. For karyotyping with conventional Giemsa staining, the chromosome slides were stained with 4% Giemsa solution (pH 7.2) for 10 min.

C-banding

To examine the chromosomal distribution of constitutive heterochromatin, C-banding was performed using the standard barium hydroxide/saline/Giemsa method (Sumner 1972) with slight modification as follows: chromosome slides were treated with 0.2 N HCl at room temperature for 60 min and then with 5% $Ba(OH)_2$ at 50°C for 15 s, followed by 2× SSC at 65°C for 60 min.

Polymerase chain reaction (PCR) amplification and molecular cloning

Genomic DNA was extracted from liver and muscle tissue following the standard salting-out protocol as described previously (Supikamolseni et al. 2015), and used as templates for polymerase chain reaction (PCR). Partial DNA fragments of the 18S – 28S rRNA genes, and *Rex* retroelements (*Rex1*, *Rex3*, and *Rex6*) were amplified using following PCR primers (see Suppl. material 1). PCR amplification was performed using 20 µl of 1× ExTaq buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5.0 µM the primers, and 0.25 U of TaKaRa Ex Taq (TaKaRa Bio, Otsu, Japan), and 25 ng of genomic DNA. PCR conditions were as follows: an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 53–59°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min. The PCR products were cloned using the pTG19-T vector (Vivantis Technologies Sdn Bhd, Selangor Darul Ehsan, Malaysia), and nucleotide sequences of the DNA fragments were determined using DNA sequencing service (First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor, Malaysia). Nucleotide sequences of three to five DNA clones, and their consensus sequences were searched for homologies with annotated sequences in the National Center for Biotechnology Information (NCBI) database to identify the amplified DNA fragments, using the BLASTx and BLASTn programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). They were then deposited in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/index-e.html) (Suppl. material 2).

Sequence analysis

Multiple sequence alignments of the three data sets (*Rex1*, *Rex3*, and *Rex6*) were performed with those of other teleosts taken from the NCBI database (Suppl. material 2), using the default parameters of Molecular Evolutionary Genetics Analysis 6 (MEGA6) software (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA) (Tamura et al. 2013). Numbers of indels (insertions and deletions) for each data set of *Rex* retroelements were calculated using the multiallelic mode of DNAsp 5.0 (Librado and Rozas 2009). All unalignable and gap-containing sites were carefully removed from the data sets. Interspecific sequence divergence was estimated using uncorrected pairwise distances (*p*-distances), and for the *Rex* reverse transcriptase region, synonymous (K_s) and nonsynonymous (K_a) substitution rates (±standard error) were calculated using the Nei-Gojobori method (Nei and Gojobori 1986) with Jukes-Cantor correction (Jukes and Cantor 1969). Phylogenetic analyses were then performed using Bayesian Inference (BI) using MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001) and the optimal model of DNA substitution was determined for each data set using Kakusan4 (Tanabe 2011). The Markov Chain Monte Carlo (MCMC) process was set to run four chains simultaneously for one million generations. After the log-likelihood value plateaued, a sampling procedure was performed every 100 generations to obtain 10,000 trees, and subsequently to provide a majority-rule consensus tree with average branch lengths. All sample points were discarded as burn-in prior to reaching convergence, and the Bayesian posterior probability in the sampled tree population was obtained in percentage terms. All phylogenetic trees were midpoint-rooted due to the absence of suitable outgroup in *Rex3* data set. However, additional phylogenetic tree based on *Rex1* and *Rex6* sequences were constructed with using outgroup method from other *Rex* sequences.

FISH mapping

Chromosomal locations of the 18S – 28S rRNA genes, *Rex* retroelements (*Rex1*, *Rex3*, and *Rex6*), telomeric (TTAGGG)n sequences, and 19 microsatellite repeat motifs: $(CA)_{15}$, $(GC)_{15}$, $(GA)_{15}$, $(AT)_{15}$, $(CAA)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GAG)_{10}$, $(AAT)_{10}$, $(AAGG)_8$, $(AATC)_8$, $(AGAT)_8$, $(ACGC)_8$, $(AAAT)_8$, $(AAAC)_8$, $(AATG)_8$, $(AAATC)_6$, and $(AAAAT)_6$ were determined using FISH, as described previously (Matsuda and Chapman 1995, Srikulnath et al. 2009). We used a 1,366-bp genomic DNA fragment of *M. albus* 18S – 28S rRNA genes (LC151290), a 533-bp genomic DNA fragment of *M. albus Rex1* (LC110446), a 415-bp genomic DNA fragment of *M. albus Rex3* (LC110447), a 471-bp genomic DNA fragment of *M. albus Rex6* (LC110448), biotin-labeled 42-bp TTAGGG repeat, and 19 biotin-labeled oligonucleotide microsatellite repeat probes, respectively. We labeled 250 ng of DNA fragments with biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) by nick translation, according to the manufacturer's protocol and ethanol-precipitated with salmon sperm DNA and *Escherichia coli* tRNA. After hybridization of biotin-labeled probes to *M. albus* chro-

mosomes, the probes were stained with avidin labeled with fluorescein isothiocyanate (avidin-FITC; Invitrogen, CA, USA). Slides were subsequently stained with 0.75 μg/ml PI or 1 μg/ml DAPI. Fluorescence hybridization signals were captured using a cooled CCD camera mounted on a ZEISS Axioplan2 microscope and processed using MetaSystems ISIS v.5.2.8 software (MetaSystems, Alltlussheim, Germany).

For dual-color FISH, two probes differentially labeled with either biotin-16-dUTP or digoxigenin-11-dUTP (Roche Diagnostics) were mixed in hybridization buffer and co-hybridized to one slide. After hybridization, digoxigenin- and biotin-labeled probes were stained with anti-digoxigenin-rhodamine Fab fragments (Roche Diagnostics) and avidin labeled with fluorescein isothiocyanate (avidin-FITC; Invitrogen), respectively.

Results

Karyotype of *Monopterus albus*

Over 10 Giemsa-stained metaphase spreads were examined for each *M. albus* individual. Diploid chromosome number is 24 (FN = 24) comprising twelve pairs of acrocentric chromosomes (Fig. 1a). The size difference of chromosome pairs was sequential, but most pairs were identified by size and banding pattern with DAPI and PI fluorescent staining. Large DAPI-positive bands were observed at the pericentromeric region of chromosome 9 (Fig. 1b), and large PI-positive bands were found at the pericentromeric region of chromosome 4 (Fig. 1c) coincident with a large C-positive heterochromatin bands (Fig. 1d).

Chromosomal location of the 18S – 28S rRNA genes and $(TTAGGG)_n$ sequences

Fluorescence hybridization signals for the 18S – 28S rRNA genes were also detected at the pericentromeric region of chromosome 4 co-localizing with both PI-positive bands and large C-positive heterochromatin blocks (Fig. 2a, c, d, e). Hybridization signals of TTAGGG repeats were observed at telomeric ends of all chromosomes, but no interstitial signal was found (Fig. 2b, c).

Chromosomal localization of microsatellite repeat motifs

Eight of the 19 microsatellite repeat motifs were dispersedly mapped onto most chromosomes (Fig. 3). Notably, strong hybridization signals of trinucleotide $(CGG)_{10}$ were localized to chromosomes 2, 4, and 6, tetranucleotide $(AAAT)_8$ to chromosomes 3 and 5, $(AGAT)_8$ to chromosomes 5 and 9, $(ACGC)_8$ to chromosomes 1, 2, 4, 7, 8 and 9, and pentanucleotide $(AAATC)_6$ to chromosomes 1 and 8. No signal was observed from the other 11 microsatellite repeat motifs ($(CA)_{15}$, $(GC)_{15}$, $(GA)_{15}$, $(AT)_{15}$, $(CAA)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(GAG)_{10}$, $(AAT)_{10}$, $(AAGG)_8$, $(AATC)_8$, $(AAAC)_8$, $(AATG)_8$, and $(AAAAT)_6$).

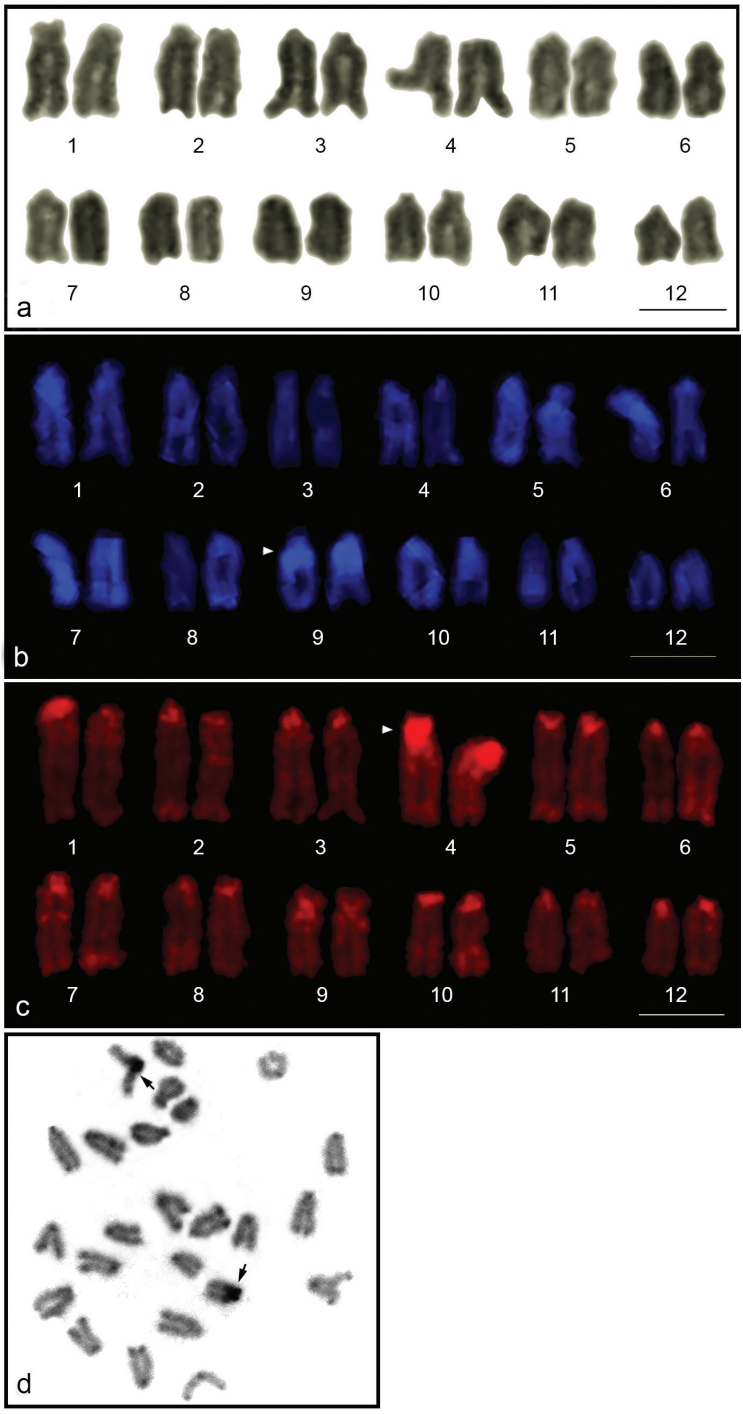

Figure 1. Giemsa-stained (**a**), DAPI-stained (**b**), PI-stained karyotype (**c**), and C-banded metaphase spread (**d**) of *Monopterus albus*. Arrowheads indicate the large DAPI-stained and large PI-stained regions. Arrows indicate C-positive heterochromatin blocks. Scale = 10 μm.

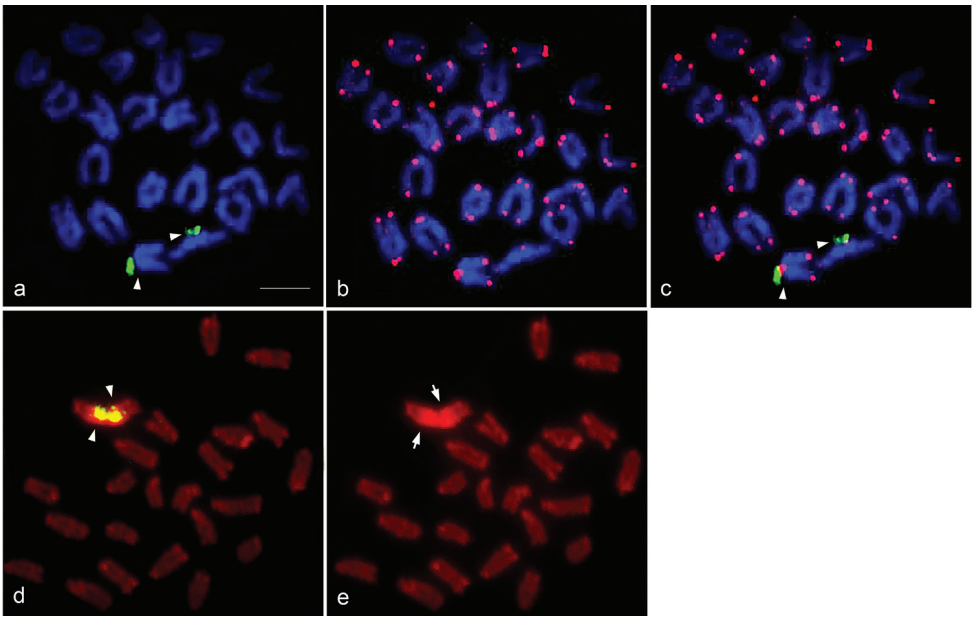

Figure 2. Chromosomal locations of the 18S – 28S rRNA genes and (TTAGGG)n sequences in *Monopterus albus*. Hybridization pattern of FITC-labeled 18S – 28S rRNA genes (green) (**a**) and rhodamine-labeled TTAGGG repeats (red) (**b**) on DAPI-stained chromosomes, and their co-hybridization pattern (**c**). Hybridization pattern of FITC-labeled 18S – 28S rRNA genes (green) (**d**) on PI-stained chromosomes. PI-stained patterns of the same metaphase spreads of (**d**) is shown in (**e**). Arrowheads indicate FISH signals of the 18S – 28S rRNA genes. Arrows indicate the large PI-stained region. Scale =10 μm.

Chromosomal distribution of *Rex* retroelements (*Rex1*, *Rex3*, and *Rex6*)

M. albus Rex1 (*MALRex1*) obtained from a single *M. albus* individual was localized to the pericentromeric region and interstitial sites of all chromosomes, except for chromosomes 4 and 9 where *MALRex1* was found only at interstitial sites (Fig. 4a). *MALRex3* was located scattered in all chromosomes with strong hybridization signals observed on chromosomes 1–4 and 8 and weak signals on chromosomes 5–7 and 9–12 (Figs 4b, 5b, d). FISH signals of *MALRex6* were found on chromosomes 1, 2, 5, 6, 8, and 10 as dispersion along the chromosomes (Figs 4c, 5c, d).

Molecular evolutionary dynamics of *Rex* retroelements

The nucleotide sequence of a 533 bp-fragment of *MALRex1* was used in multiple sequence alignment with 28 other teleosts, evidencing 32 indel sites. Sequence divergence among species varied from 0 to 50.13% with an average of 29.56±1.13% (Suppl. material 3). *MALRex1* sequences in *M. albus* showed the minimum interspecific sequence divergence of 1.88% from nototheniids *Dissostichus mawsoni* Norman, 1937

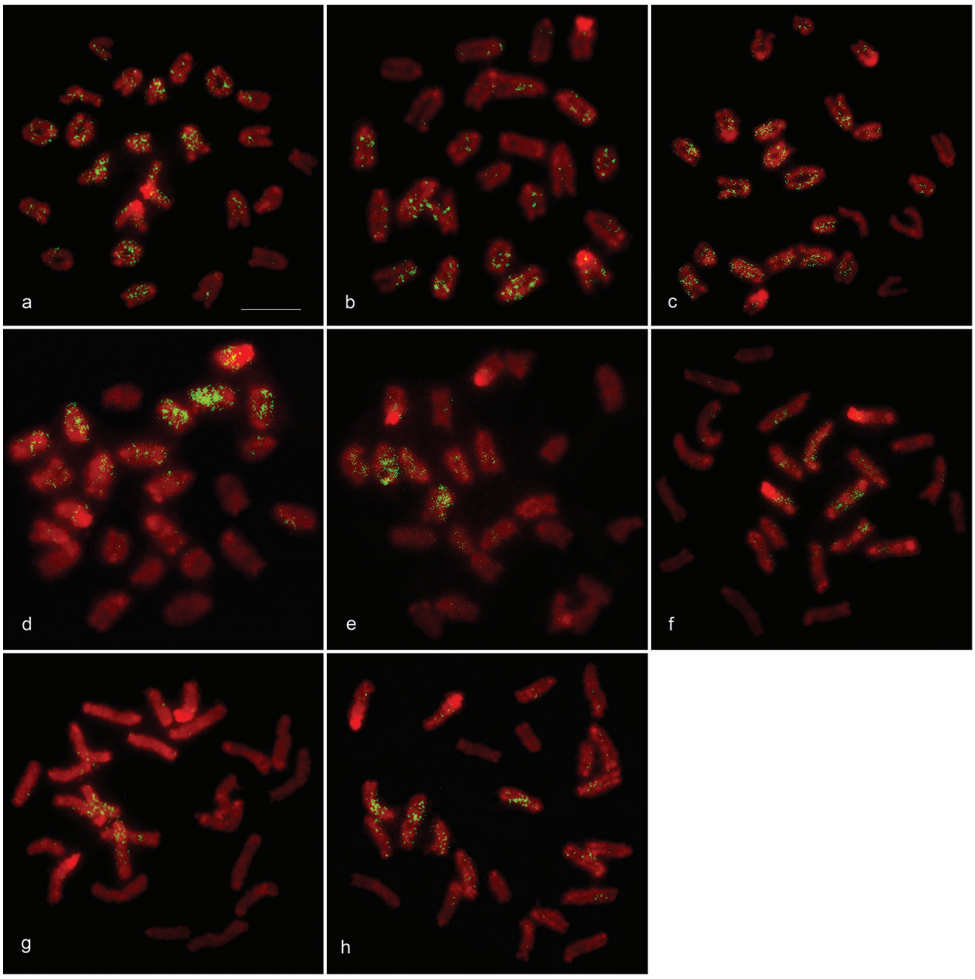

Figure 3. Chromosomal locations of microsatellite repeat motifs in *Monopterus albus*. Hybridization pattern of FITC-labeled $(CAA)_{10}$ (**a**), $(CAG)_{10}$ (**b**), $(CGG)_{10}$ (**c**), $(GAG)_{10}$ (**d**), $(AGAT)_{8}$ (**e**), $(ACGC)_{8}$ (**f**), $(AAAT)_{8}$ (**g**), and $(AAATC)_{6}$ (**h**) on PI-stained chromosomes.

and *Notothenia coriiceps* Richardson, 1844 (Perciformes) and the maximum divergence of 41.95% to *Poeciliopsis gracilis* Heckel, 1848 (Cyprinodontiformes); the average is 24.51±8.14%. The phylogenetic placement of *Rex1* sequences showed that most species were grouped in their respective orders (Fig. 6, Suppl. material 6). The average K_s/K_a value of *Rex1* sequences was 2.19±0.08 (Table 1). The nucleotide sequence of a 415 bp-fragment of *MALRex3* was used in multiple sequence alignment with 24 other teleosts, showing 23 indels. The average sequence divergence among species was 33.94±17.24%, ranging from 2.65% to 69.54% (Suppl. material 4). *MALRex3* sequences showed the minimum interspecific sequence divergence of *M. albus*, 18.54%, from *Esox lucius* Linnaeus, 1758 (Esociformes) and the maximum divergence, 66.65%, from *Astyanax fasciatus* Cuvier, 1819 (Characiformes); average 31.84±12.74%. The phylogenetic

Table 1. Synonymous substitution site (K_s) per nonsynonymous substitution sites (K_a) of *Rex1* retroelement among twenty eight teleosts.

	AJA	PTI	HLE	HNI	OFL	CAL	OLA	FUN	GAF	PME	PAM	PGR	XMA	MAL
Anguilla japonica (AJA)														
Pseudotocinclus tietensis (PTI)	2.09													
Hisonotus leucofrenatus (HLE)	1.97	2.97												
Hypostomus nigromaculatus (HNI)	2.71	3.07	3.14											
Otocinclus flexilis (OFL)	2.18	2.82	2.69	3.43										
Coregonus albula (CAL)	3.20	3.28	2.78	2.75	3.22									
*Oryzias latipes (*OLA)	1.93	2.37	2.14	2.67	2.19	2.62								
Fundulus sp. (FUN)	2.05	2.06	1.76	2.38	2.39	2.15	1.62							
Gambusia affinis (GAF)	1.75	1.87	1.62	1.81	1.97	2.20	1.56	1.74						
Poecilia mexicana (PME)	2.06	1.83	1.79	1.76	1.70	2.33	1.62	1.83	4.03					
Phallichthys amates (PAM)	1.82	1.52	1.51	1.80	1.79	2.01	1.53	1.71	2.22	3.31				
Poeciliopsis gracilis (PGR)	1.55	1.67	1.57	1.72	1.81	1.64	1.71	1.53	2.43	2.10	2.17			
Xiphophorus maculatus (XMA)	1.85	1.72	1.54	1.86	1.99	2.16	1.66	1.78	3.23	3.20	2.11	2.26		
Monopterus albus (MAL)	2.65	2.52	1.80	3.08	1.97	3.18	2.45	2.56	1.93	2.20	1.88	1.50	1.91	
Lates calcarifer (LCA)	1.34	2.05	1.44	3.01	2.60	2.45	2.21	1.65	1.62	1.84	1.64	1.44	1.75	1.75
Astronotus ocellatus (AOC)	2.63	2.27	1.90	2.60	2.77	2.55	2.27	2.47	1.82	1.89	1.80	1.58	1.84	1.46
Cichla monoculus (CMO)	2.69	2.30	1.93	2.65	2.83	2.60	2.30	2.43	1.82	1.89	1.80	1.60	1.84	1.49
Cichlasoma labridens (CLA)	2.24	2.59	2.55	2.92	2.70	2.82	2.07	2.49	1.80	1.80	1.69	1.41	1.77	2.24
Geophagus proximus (GPR)	2.63	2.27	1.90	2.60	2.77	2.55	2.27	2.47	1.82	1.89	1.80	1.58	1.84	1.46
Heterandria bimaculata (HBI)	2.01	2.72	2.69	2.44	2.67	2.57	2.15	1.94	1.64	1.89	1.72	1.57	1.63	2.32
Oreochromis niloticus (ONI)	1.82	2.60	2.44	2.86	2.67	2.59	2.11	2.35	1.70	1.91	1.73	1.64	1.68	2.24
Pterophyllum scalare (PSC)	2.69	2.30	1.93	2.65	2.83	2.60	2.30	2.43	1.82	1.89	1.80	1.60	1.84	1.49
Symphysodon discus (SDI)	2.13	2.55	2.47	2.74	2.57	2.63	2.08	2.43	1.72	1.89	1.74	1.58	1.68	2.02
Dissostichus mawsoni (DMA)	2.86	2.58	1.99	2.83	2.23	3.34	2.16	2.05	1.64	1.96	1.67	1.52	1.70	1.89
Notothenia coriiceps (NCO)	3.22	3.14	2.26	3.02	2.41	3.70	2.28	2.30	1.84	2.19	1.74	1.53	1.88	2.59
Trematomus newnesi (TNE)	2.75	2.73	2.20	2.86	2.18	3.37	2.10	2.37	1.70	2.04	1.67	1.61	1.76	2.39
Gymnodraco acuticeps (GAC)	2.71	2.67	2.00	2.84	2.09	3.18	2.10	2.04	1.71	1.98	1.63	1.54	1.75	2.06
Battrachocottus baikalensis (BBA)	2.51	2.30	2.39	2.91	2.35	4.24	2.17	2.11	1.73	1.92	1.64	1.81	1.73	2.75

n/c indicate that number is uncountable value.

Table 1. Continued.

	LCA	AOC	CMO	CLA	GPR	HBI	ONI	PSC	SDI	DMA	NCO	TNE	GAC	BBA
Anguilla japonica (AJA)														
Pseudotocinclus tietensis (PTI)														
Hisonotus leucofrenatus (HLE)														
Hypostomus nigromaculatus (HNI)														
Otocinclus flexilis (OFL)														
Coregonus albula (CAL)														
Oryzias latipes (OLA)														
Fundulus sp. (FUN)														
Gambusia affinis (GAF)														
Poecilia mexicana (PME)														
Phallichthys amates (PAM)														
Poeciliopsis gracilis (PGR)														
Xiphophorus maculatus (XMA)														
Monopterus albus (MAL)														
Lates calcarifer (LCA)														
Astronotus ocellatus (AOC)	1.91													
Cichla monoculus (CMO)	1.91	0.00												
Cichlasoma labridens (CLA)	2.14	2.31	2.26											
Geophagus proximus (GPR)	1.91	n/c	0.00	2.31										
Heterandria bimaculata (HBI)	2.04	2.39	2.35	3.02	2.39									
Oreochromis niloticus (ONI)	2.02	2.35	2.30	3.80	2.35	1.86								
Pterophyllum scalare (PSC)	1.91	0.00	n/c	2.26	0.00	2.35	2.30							
Symphysodon discus (SDI)	2.02	2.06	2.02	1.35	2.06	2.37	3.56	2.02						
Dissostichus mawsoni (DMA)	1.90	2.20	2.24	2.40	2.20	2.00	2.22	2.24	2.23					
Notothenia coriiceps (NCO)	2.32	2.47	2.52	2.74	2.47	2.27	2.53	2.52	2.65	3.78				
Trematomus newnesi (TNE)	2.14	2.29	2.33	2.79	2.29	2.24	2.58	2.33	2.62	3.75	3.20			
Gymnodraco acuticeps (GAC)	2.16	2.16	2.20	2.41	2.16	2.03	2.25	2.20	2.27	1.76	1.79	1.45		
Battrachocottus baikalensis (BBA)	2.38	2.62	2.66	2.22	2.62	2.18	2.07	2.66	2.30	2.57	2.72	2.86	2.50	

n/c indicate that number is uncountable value.

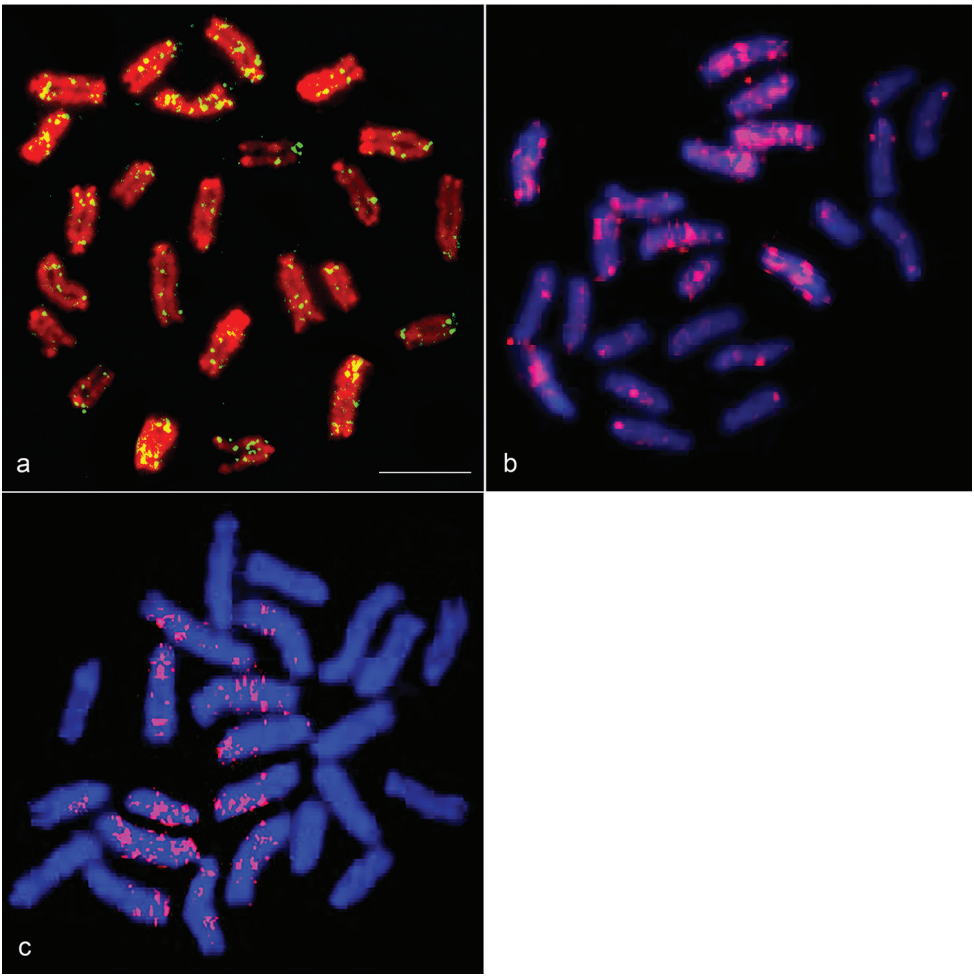

Figure 4. Chromosomal locations of *Rex1*, *Rex3*, and *Rex6* in *Monopterus albus*. Hybridization pattern of FITC-labeled *Rex1* (green) (**a**) on PI-stained chromosomes, and rhodamine-labeled *Rex3* (red) (**b**) and *Rex6* (red) (**c**) on DAPI-stained chromosomes. Scale =10 μm.

placement of *Rex3* sequences showed a clade for each order except for Perciformes fishes (Fig. 7). The average K_s/K_a value of *Rex3* sequences was 1.05±0.05 (Table 2). The nucleotide sequences of a 471 bp-fragment of *MALRex6* was used in multiple sequence alignment with 17 other teleosts showing 15 indels. The sequence divergences among species varied from 3.13 to 65.546% (average 27.94±19.53%). *MALRex6* sequences showed the minimum interspecific sequence divergence of *M. albus*, 60.31%, from *Geophagus proximus* Castelnau, 1855 (Perciformes) and the maximum divergence, 65.54%, from *Oreochromis niloticus* Cuvier, 1832 (Perciformes,); average 62.60±1.14% (Suppl. material 5). The phylogenetic placement of *Rex6* sequences showed a clade for each order (Fig. 8, Suppl. material 7). The average K_s/K_a value of *Rex6* sequences was 0.85±0.04 (Table 3).

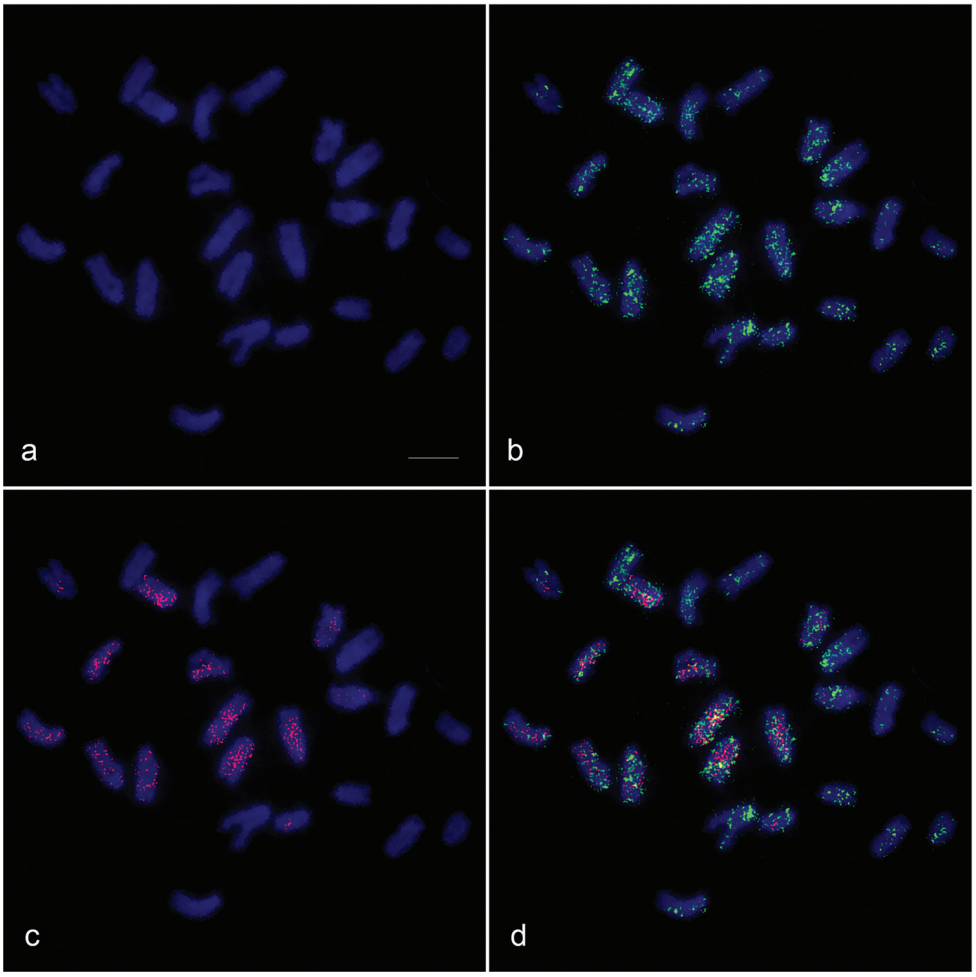

Figure 5. Chromosomal locations of *Rex3* and *Rex6* in *Monopterus albus*. Hybridization pattern of FITC-labeled *Rex3* (green) (**b**) and rhodamine-labeled *Rex6* (red) (**c**) on DAPI-stained chromosomes, and their co-hybridization pattern (**d**). DAPI-stained patterns of the same metaphase spreads of (**b, c,** and **d**) is shown in (**a**). Scale =10 μm.

Discussion

Karyotype and chromosomal localization of rRNA gene clusters, telomeric sequences, and microsatellite repeat motifs in *Monopterus albus*

The karyotype of *M. albus* (2n = 24, FN = 24) composed of 12 acrocentric chromosome pairs was found to be similar to that reported by Yu et al. (1989) and Ji et al. (2003). The chromosome number of *M. albus* is the lowest among synbranchids, e.g., *M. cuchia* (2n = 42, FN = 46) (Rishi and Haobam 1984), *Synbranchus marmoratus* Bloch, 1795 (2n = 42–46, FN = 46–54) (Carvalho et al. 2012; Utsunomia et al. 2014),

Table 2. Synonymous substitution site (K_s) per nonsynonymous substitution sites (K_a) of *Rex3* retroelement among twenty four teleosts.

	AAN	CCA	DRE	AFA	CCU	PTI	ELU	OLA	FUN	GAF	HBI	PFO
Anguilla anguilla (AAN)												
Cyprinus carpio (CCA)	1.40											
Danio rerio (DRE)	1.33	1.56										
Astyanax fasciatus (AFA)	1.02	1.07	0.90									
Corumbataia cuestae (CCU)	0.94	1.16	0.97	1.62								
Pseudotocinclus tietensis (PTI)	0.98	1.09	0.96	1.63	2.29							
Esox lucius (ELU)	1.05	1.52	1.80	0.98	1.08	0.91						
Oryzias latipes (OLA)	1.35	0.81	1.31	0.88	0.97	0.91	1.01					
Fundulus sp.(FUN)	1.35	1.26	1.49	0.93	1.12	1.09	1.71	1.03				
Gambusia affinis (GAF)	1.13	0.84	1.19	0.88	1.09	0.96	1.11	0.81	0.80			
Heterandria bimaculata (HBI)	1.35	1.47	1.53	0.87	1.06	1.02	1.59	0.87	0.94	0.87		
Poecilia formosa (PFO)	1.19	0.94	1.45	0.83	1.01	0.96	1.15	1.19	0.91	0.83	1.07	
Phallichthys amates (PAM)	1.17	1.08	1.44	0.82	1.02	0.97	1.23	0.89	0.65	1.01	0.76	0.75
Xiphophorus hellerii (XHE)	1.30	1.14	1.48	0.82	1.04	0.99	1.36	0.84	0.72	0.94	0.97	1.55
Monopterus albus (MAL)	1.06	1.25	1.66	0.94	0.90	0.85	1.48	0.81	1.24	0.93	1.27	1.07
Siniperca chuatsi (SCH)	1.12	0.95	1.36	0.90	0.96	0.97	0.97	0.63	1.54	0.97	1.32	1.28
Astronotus ocellatus (AOC)	1.00	1.39	1.63	1.00	0.89	0.90	1.20	1.01	1.11	1.01	1.22	1.27
Cichla monoculus (CMO)	0.92	0.95	1.28	0.99	1.01	0.98	0.92	0.75	0.85	0.84	0.89	0.87
Cichlasoma labridens (CLA)	0.97	1.20	1.63	1.04	1.04	1.01	0.96	0.64	0.93	0.89	0.96	0.96
Geophagus surinamensis (GSU)	1.02	1.13	1.07	1.50	1.84	2.27	1.15	0.96	1.15	1.07	1.10	1.10
Oreochromis niloticus (ONI)	1.35	1.53	1.80	0.98	1.03	1.07	1.47	0.74	0.99	0.79	0.90	0.98
Pterophyllum scalare (PSC)	1.24	1.56	1.37	1.01	0.88	0.90	1.55	1.04	1.22	1.11	1.02	1.25
Symphysodon discus (SDI)	0.97	1.11	1.51	1.03	1.01	1.01	0.93	0.60	0.94	0.80	0.92	0.94
Battrachocottus baikalensis (BBA)	1.16	0.92	1.42	0.98	0.94	0.93	0.93	0.70	1.07	0.89	0.92	0.87

Table 2. Continued.

	PAM	XHE	MAL	SCH	AOC	CMO	CLA	GSU	ONI	PSC	SDI	BBA
Anguilla anguilla (AAN)												
Cyprinus carpio (CCA)												
Danio rerio (DRE)												
Astyanax fasciatus (AFA)												
Corumbataia cuestae (CCU)												
Pseudotocinclus tietensis (PTI)												
Esox lucius (ELU)												
Oryzias latipes (OLA)												
Fundulus sp.(FUN)												
Gambusia affinis (GAF)												
Heterandria bimaculata (HBI)												
Poecilia formosa (PFO)												
Phallichthys amates (PAM)												
Xiphophorus hellerii (XHE)	1.03											
Monopterus albus (MAL)	1.00	1.01										
Siniperca chuatsi (SCH)	1.02	1.22	0.92									
Astronotus ocellatus (AOC)	1.18	1.09	0.81	1.15								
Cichla monoculus (CMO)	0.79	0.74	0.71	0.74	1.03							
Cichlasoma labridens (CLA)	0.82	0.76	0.78	0.90	1.22	0.15						
Geophagus surinamensis (GSU)	1.07	1.05	0.92	1.01	0.94	1.04	1.06					
Oreochromis niloticus (ONI)	0.87	0.84	1.06	1.12	1.15	0.69	0.71	1.02				
Pterophyllum scalare (PSC)	1.25	1.17	0.98	1.21	1.65	1.51	1.26	0.97	0.99			
Symphysodon discus (SDI)	0.77	0.75	0.73	0.85	1.05	0.16	0.00	1.03	0.74	1.17		
Battrachocottus baikalensis (BBA)	0.74	0.78	0.84	0.54	0.91	0.70	0.77	1.07	0.71	1.06	0.70	

Table 3. Synonymous substitution site (K_s) per nonsynonymous substitution sites (K_a) of *Rex6* retroelement among seventeen teleosts.

	OLA	GAF	PFO	PGR	XMA	MAL	AOC	CLA	CMO	CRE	GPR	HBI	MAU	ONI	PSC	SDI	RSO
Oryzias latipes (OLA)																	
Gambusia affinis (GAF)	0.34																
Poecilia formosa (PFO)	0.28	0.71															
Poeciliopsis gracilis (PGR)	0.18	0.98	0.59														
Xiphophorus maculatus (XMA)	0.23	0.73	0.55	1.10													
Monopterus albus (MAL)	0.94	1.02	0.96	0.99	1.03												
Astronotus ocellatus (AOC)	0.71	0.68	0.64	0.60	0.62	0.88											
Cichlasoma labridens (CLA)	0.89	0.78	0.99	0.77	0.80	0.93	1.69										
Cichla monoculus (CMO)	0.55	0.42	0.54	0.41	0.33	0.86	0.90	1.35									
Crenicichla sp. (CRE)	0.33	0.40	0.49	0.33	0.29	0.85	0.51	1.18	0.73								
Geophagus proximus (GPR)	0.72	0.72	0.84	0.73	0.69	0.84	1.05	1.37	1.15	0.91							
Hemichromis bimaculatus (HBI)	0.27	1.16	0.00	0.85	0.82	0.95	0.74	1.02	0.59	0.57	0.90						
Melanochromis auratus (MAU)	0.64	0.58	0.68	0.67	0.59	0.81	0.83	1.20	1.32	0.66	1.14	0.79					
Oreochromis niloticus (ONI)	0.61	0.57	0.68	0.64	0.57	0.85	0.86	1.36	1.07	0.54	1.08	0.77	0.89				
Pterophyllum scalare (PSC)	0.75	0.73	0.91	0.73	0.69	0.90	1.03	1.83	1.54	1.20	1.33	1.00	1.27	1.01			
Symphysodon discus (SDI)	0.80	0.59	0.82	0.52	0.56	1.01	1.27	1.60	0.89	1.04	1.24	0.85	1.40	1.30	1.61		
Rexea solandri (RSO)	0.92	0.93	0.86	0.87	0.88	1.08	0.97	1.12	0.92	0.97	0.94	0.91	0.98	0.96	0.97	1.04	

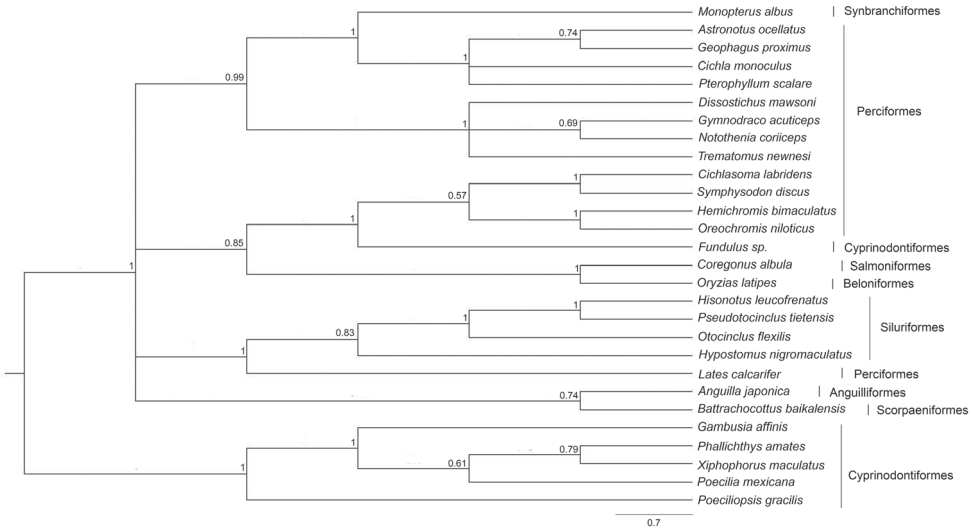

Figure 6. Phylogenetic placements of partial nucleotide sequences of *Rex1* from 28 teleosts. Support values at each node are Bayesian posterior probability.

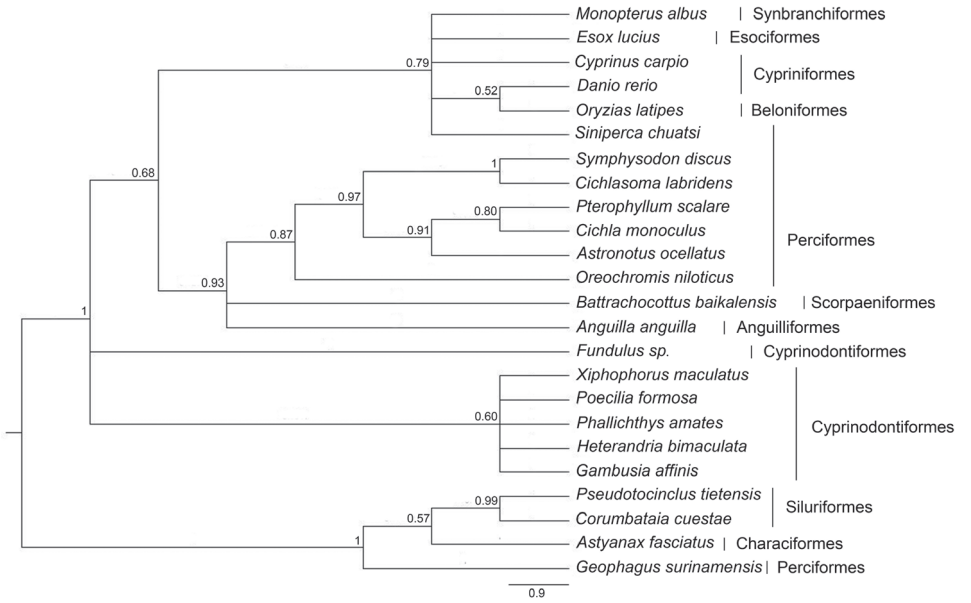

Figure 7. Phylogenetic placements of partial nucleotide sequences of *Rex3* from 24 teleosts. Support values at each node are Bayesian posterior probability.

Ophisternon aenigmaticum Rosen & Greenwood, 1976 (2n = 46, FN = 52) (Nirchio et al. 2011), and *O. bengalense* McClelland, 1844 (2n = 46, FN = 52) (Carvalho et al. 2012), as well as the species of family Mastacembelidae of the same order (2n = 48,

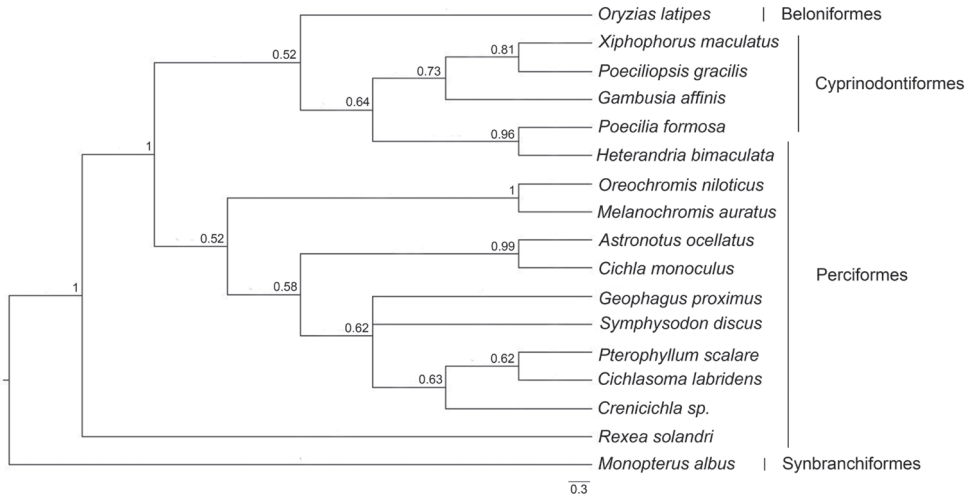

Figure 8. Phylogenetic placements of partial nucleotide sequences of *Rex6* from 17 teleosts. Support values at each node are Bayesian posterior probability.

FN = 58–88) (Khuda-Bukhsh and Barat 1987). The fundamental numbers of *M. albus* is reduced to 50% of norm in synbranchid fishes and teleosts, which suggests that the acrocentric chromosomes of *M. albus* may have been formed by repeated tandem fusion of the ancestral acrocentric chromosomes contained in the ancestral karyotype of Synbranchidae. However, the hybridization signal of (TTAGGG)n at interstitial telomeric sites (ITSs) that appears to be remnants of fusion or inversion (Srikulnath et al. 2009, 2011, 2015) was not found in any chromosomes of *M. albus* in this study (Fig. 2). Comparative chromosome mapping of Asian swamp eel with zebrafish (*Danio rerio* Hamilton, 1822) using human bacterial artificial chromosome (BAC) probes revealed the Asian swamp eel retains a number of gene copies found in tetrapods, while other teleosts underwent the third genome duplication (GD), leading to multiple copies of the genes (Yi et al. 2001, Zhou et al. 2002). This suggests that Asian swamp eel retained the genome composition before the event of the third GD that occurred in teleosts (Zhou et al. 2002). Molecular structure of the pericentromeric regions of chromosome 4 which were high GC-rich have evolved in a concerted manner with amplification of the 18S – 28S rRNA genes. However, the chromosomal locations of the 18S – 28S rRNA genes varied in *M. albus* individuals (Fig. 2d, e), a phenomanon also observed in Chinese population on pair of chromosome 3 and/or chromosome 7 (Ji et al. 2003). In other synbranchid fishes, the 18S – 28S rRNA genes are generally located on a pair of chromosome 1 and on a pair of medium-sized acrocentric chromosomes in *O. aenigmaticum* (Nirchio et al. 2011), as well as on several other chromosome pairs in various pattern of *S. marmoratus* (Utsunomia et al. 2014). These results suggest that chromosomal locations of the 18S – 28S rRNA genes considerably differ in Synbranchidae.

In this study, eight microsatellite repeat motifs [$(CAG)_{10}$, $(CAA)_{10}$, $(CGG)_{10}$, $(GAG)_{10}$, $(AGAT)_8$, $(ACGC)_8$, $(AAAT)_8$, and $(AAATC)_6$] were dispersedly mapped on different chromosomes (Fig. 3). This suggests that the amplification of several microsatellite repeat motifs has occurred independently in the genome of *M. albus*. Interestingly, the dispersion of the microsatellite repeat motifs signals was co-localized to *M. albus* chromosomes with *Rex* retroelements. A similar case was found in cichlid species *Cichla monoculus* Agassiz, 1831, *Pterophyllum scalare* Schultze, 1823, and *Symphysodon discus* Heckel, 1840 (Schneider et al. 2015). This suggests that both *Rex* retroelements and microsatellite repeat motifs have co-amplified in the evolutionary process of the genome of *M. albus*.

Organization of *Rex* retroelements (*MALRex1*, *MALRex3*, and *MALRex6*) on *Monopterus albus* chromosomes

The diversity of chromosomal distribution for *Rex* retroelements (*Rex1*, *Rex3*, and *Rex6*) was found in teleosts (Table 4). Two major distinctive patterns were observed: (1) compartmentalization as found in pericentromeric, centromeric, or telomeric regions, and (2) uniform dispersion throughout the genome or along the chromosomes (Ozouf-Costaz et al. 2004). Chromosomal distribution of *Rex1*, *Rex3*, and *Rex6* were generally located in the specific region together as compartmentalization within each family/order (Table 4). In this study, although *MALRex1* was dispersed throughout the genome, this element was predominantly localized to pericentromeric regions of all chromosomes except for chromosomes 4 and 9. By contrast, strong hybridization signals of *MALRex3* were dispersed on five chromosome pairs, with weak signals on seven chromosome pairs, which implies that *MALRex3* were specifically amplified in chromosomal regions of *M. albus*.

The differences in the copy number and chromosomal distribution of *MALRex1*, *MALRex3*, and *MALRex6* suggest that these retroelements were independently amplified or lost in the lineage of *M. albus*, where *MALRex3* is prone to retain a copy number higher than *MALRex1* and *MALRex6*. A similar case of copy number variation in *Rex* retroelements was also found in several Antarctic nototheniid species (Ozouf-Costaz et al. 2004).

Molecular diversity of *Rex* retroelements (*Rex1*, *Rex3*, and *Rex6*)

Three *Rex* retroelements were identified in the genome of *M. albus*, and the degree of sequence divergence for the three retroelements was high (14–67%) from other species in comparison. *MALRex1* and *MALRex3* showed high interspecific sequence divergences from Cyprinodontiformes and Characiformes, respectively, but low interspecific sequence divergences from Perciformes fishes for *Rex1* and Escociformes for *Rex3* (Suppl.

Table 4. Chromosomal distribution of *Rex1*, *Rex3*, and *Rex6* in teleosts. "n.d." means not described.

Order	Family	Species	Chrosmosomal distribution			Chromosome number	Reference
			Rex1	*Rex3*	*Rex6*		
Characiformes	Characidae	*Astyanax paranae*	dispersion	telomeric region	n.d.	2n = 50	Silva et al. 2014
		Astyanax fasciatus	n.d.	telomeric region	n.d.	2n = 46 – 48	Pansonato-Alves et al. 2013
Siluriformes	Loricariidae	*Hisonotus leucofrenatus*	dispersion	n.d.	n.d.	2n = 54	Ferreira et al. 2011
		Hypostomus nigromaculatus	dispersion	dispersion	dispersion	2n = 76	Pansonato-Alves et al. 2013
		Pseudotocinclus tietensis	dispersion	dispersion	n.d.	2n = 54	Ferreira et al. 2011
Salmoniformes	Salmonidae	*Coregonus albula*	pericentromeric region	n.d.	n.d.	2n = 80	Symonová et al. 2013
		Coregonus fontanae	pericentromeric region	n.d.	n.d.	2n = 80	Symonová et al. 2013
Synbranchiformes	Synbranchidae	*Monopterus albus*	pericentromeric region and insterstitial site	dispersion	dispersion	2n = 24	in this study
Perciformes	Latidae	*Lates calcarifer*	telomeric region	centromeric region	n.d.	2n = 48	Kuznetsova et al. 2014
	Cichlidae	*Astronotus ocellatus*	centromeric region	telomeric region	telomeric region	2n = 48	Schneider et al. 2013
		Cichla kelberi	centromeric region	centromeric region	dispersion	2n = 48	Teixeira et al. 2009
		Cichla monoculus	telomeric region	telomeric region	telomeric region	2n = 48	Schneider et al. 2013
		Geophagus proximus	telomeric region	telomeric region	telomeric region	2n = 48	Schneider et al. 2013
		Hemichromis bimaculatus	pericentromeric region	pericentromeric region	centromeric region	2n = 44	Valente et al. 2011
		Melanochromis auratus	pericentromeric region	pericentromeric region	pericentromeric region	2n = 44	Valente et al. 2011
		Oreochromis niloticus	pericentromeric region	pericentromeric region	pericentromeric region	2n = 44	Valente et al. 2011
		Pterophyllum scalare	centromeric region	telomeric region	telomeric region	2n = 48	Schneider et al. 2013
		Oreochromis niloticus	pericentromeric region	pericentromeric region	pericentromeric region	2n = 44	Valente et al. 2011
		Symphysodon discus	dispersion	telomeric region	telomeric region	2n = 60	Schneider et al. 2013
	Nototheniidae	*Dissostichus mawsoni*	dispersion	dispersion	n.d.	2n = 48	Ozouf-Costaz et al. 2004
		Notothenia coriiceps	dispersion	dispersion	n.d.	2n = 22	Ozouf-Costaz et al. 2004
		Trematomus newnesi	dispersion	dispersion	n.d.	2n = 46	Ozouf-Costaz et al. 2004
	Bathydraconidae	*Gymnodraco acuticeps*	dispersion	dispersion	n.d.	2n = 48	Ozouf-Costaz et al. 2004

materials 3 and 4). This suggests that *M. albus* and Perciformes or Escociformes shared relatively recent activity of *Rex1* or *Rex3*, respectively. The average K_s/K_a value of *Rex1* was higher than 1 between all compared species and between *M. albus* and other species (Table 1). These results suggest that *Rex1* evolved under purifying selection and that retrotranspositions occurred during the evolution of teleosts. By contrast, the average K_s/K_a value of *Rex3* was closer to 1, which suggests that after retrotransposition, *Rex3* was influenced by pseudogene-like evolution (Table 2) (McAllister et al. 1997).

Only few data of *Rex6* sequences were available because specific PCR primers were not feasibly effective to detect this element in the genome of teleosts (Volff et al. 2001, Ozouf-Costaz et al. 2004, Schneider et al. 2013). The absence of *Rex6* was observed in several Antarctic nototheniid species, but *Rex6* exists in some other species of the same order Perciformes (Volff et al. 2001, Ozouf-Costaz et al. 2004, Schneider et al. 2013). This suggests that *Rex6* might have rapidly diverged in teleosts. *MALRex6* showed high interspecific sequence divergences (approximately 60%) of *M. albus* from other teleosts (Suppl. material 5). This may indicate that the divergence of *Rex6* sequences of *M. albus* (or Synbranchidae in general) and other teleosts was rather ancestral. The average K_s/K_a value of *Rex6* was less than 1 (Table 3). This suggests that *Rex6* has a more diverse function in teleosts.

The present results of chromosomal distribution and molecular diversity of four repetitive element groups (the 18S – 28S rRNA gene, telomeric sequences, microsatellite repeat motifs, and *Rex* retroelements) revealed the chromosome constitution and genome organization of Asian swamp eels. This enabled us to learn more about the chromosome constitution in synbranchid fishes and teleosts as a whole. Further work is required to investigate and compare synbranchid fishes, including *M. cuchia,* to better understand the process of karyotype and genome evolution in this lineage.

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Supplementary material 1

Supplementary Table 1

Authors: Aorarat Suntronpong, Watcharaporn Thapana, Panupon Twilprawat, Ornjira Prakhongcheep, Suthasinee Somyong, Narongrit Muangmai, Surin Peyachoknagul, Kornsorn Srikulnath

Data type: Table

Explanation note: Primers used molecular cloning in this study.

Link: https://doi.org/10.3897/CompCytogen.v11i3.11739.suppl1

Supplementary material 2

Supplementary Table 2

Authors: Aorarat Suntronpong, Watcharaporn Thapana, Panupon Twilprawat, Ornjira Prakhongcheep, Suthasinee Somyong, Narongrit Muangmai, Surin Peyachoknagul, Kornsorn Srikulnath

Data type: Table

Explanation note: Teleost species and nucleotide sequences of the *Rex1*, *Rex3*, and *Rex6* genes used in this study. "–" means no data.

Link: https://doi.org/10.3897/CompCytogen.v11i3.11739.suppl2

Supplementary material 3

Supplementary Table 3

Authors: Aorarat Suntronpong, Watcharaporn Thapana, Panupon Twilprawat, Ornjira Prakhongcheep, Suthasinee Somyong, Narongrit Muangmai, Surin Peyachoknagul, Kornsorn Srikulnath

Data type: Table

Explanation note: Pairwise comparison of nucleotide sequence divergences of *Rex1* among twenty eight teleosts.

Link: https://doi.org/10.3897/CompCytogen.v11i3.11739.suppl3

Supplementary material 4

Supplementary Table 4

Authors: Aorarat Suntronpong, Watcharaporn Thapana, Panupon Twilprawat, Ornjira Prakhongcheep, Suthasinee Somyong, Narongrit Muangmai, Surin Peyachoknagul, Kornsorn Srikulnath

Data type: Table

Explanation note: Pairwise comparison of nucleotide sequence divergences of *Rex3* among twenty eight teleosts.

Link: https://doi.org/10.3897/CompCytogen.v11i3.11739.suppl4

Supplementary material 5

Supplementary Table 5

Authors: Aorarat Suntronpong, Watcharaporn Thapana, Panupon Twilprawat, Ornjira Prakhongcheep, Suthasinee Somyong, Narongrit Muangmai, Surin Peyachoknagul, Kornsorn Srikulnath

Data type: Table

Explanation note: Pairwise comparison of nucleotide sequence divergences of *Rex6* among seventeen teleosts.

Link: https://doi.org/10.3897/CompCytogen.v11i3.11739.suppl5

Supplementary material 6

Supplementary Figure 1

Authors: Aorarat Suntronpong, Watcharaporn Thapana, Panupon Twilprawat, Ornjira Prakhongcheep, Suthasinee Somyong, Narongrit Muangmai, Surin Peyachoknagul, Kornsorn Srikulnath

Data type: Image

Explanation note: Phylogenetic placements of partial nucleotide sequences of *Rex1* from 28 teleosts and from *Physalaemus henselii*, Peters 1872 (KU842414) as the outgroup. Support values at each node are Bayesian posterior probability.

Link: https://doi.org/10.3897/CompCytogen.v11i3.11739.suppl6

Supplementary material 7

Supplementary Figure 2

Authors: Aorarat Suntronpong, Watcharaporn Thapana, Panupon Twilprawat, Ornjira Prakhongcheep, Suthasinee Somyong, Narongrit Muangmai, Surin Peyachoknagul, Kornsorn Srikulnath

Data type: Image

Explanation note: Phylogenetic placements of partial nucleotide sequences of *Rex6* from 17 teleosts and from *Podocnemis unifilis*, Troschel 1848 (KR336823) as the outgroup. Support values at each node are Bayesian posterior probability.

Link: https://doi.org/10.3897/CompCytogen.v11i3.11739.suppl7

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RESEARCH ARTICLE

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Chromosomal organization of four classes of repetitive DNA sequences in killifish *Orestias ascotanensis* Parenti, 1984 (Cyprinodontiformes, Cyprinodontidae)

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Abstract

Orestias Valenciennes, 1839 is a genus of freshwater fish endemic to the South American Altiplano. Cytogenetic studies of these species have focused on conventional karyotyping. The aim of this study was to use classical and molecular cytogenetic methods to identify the constitutive heterochromatin distribution and chromosome organization of four classes of repetitive DNA sequences (histone H3 DNA, U2 snRNA, 18S rDNA and 5S rDNA) in the chromosomes of *O. ascotanensis* Parenti, 1984, an endemic species restricted to the Salar de Ascotán in the Chilean Altiplano. All individuals analyzed had a diploid number of 48 chromosomes. C-banding identified constitutive heterochromatin mainly in the pericentromeric region of most chromosomes, especially a GC-rich heterochromatic block of the short arm of pair 3. FISH assay with an 18S probe confirmed the location of the NOR in pair 3 and revealed that the minor rDNA cluster occurs interstitially on the long arm of pair 2. Dual FISH identified a single block of U2 snDNA sequences in the pericentromeric regions of a subtelocentric chromosome pair, while histone H3 sites were observed as small signals scattered in throughout the all chromosomes. This work represents the first effort to document the physical organization of the repetitive fraction of the *Orestias* genome. These data will improve our understanding of the chromosomal evolution of a genus facing serious conservation problems.

Keywords
Orestias, molecular cytogenetics, multigene families

Introduction

Cytogenetic analysis is a useful tool for describing evolutionary patterns and the histories of closely-related species or species complexes. *Orestias* Valenciennes, 1839 is a genus of freshwater fish endemic to the South American Altiplano. The genus includes 45 species, grouped into four complexes: *O. cuvieri*, *O. mulleri*, *O. gilsoni* and *O. agassii* (Costa 1999, Parenti 1984). Conventional karyotyping studies involving the seven species of *O. agassii* complex found in the Chilean Altiplano (17°and 22°S) have revealed variations in the chromosome number (2n=48-55) and the presence/absence of microchromosomes, suggesting that Robertsonian rearrangements may play a role in the karyotypic evolution of these species (Arratia 1982, Vila et al. 2007, 2010, 2011, Habit et al. 2006, Villwock and Sienknecht 1996).

The most commonly-used approaches for comparative cytogenetic analysis of fish include characterizing the distribution and composition of constitutive heterochromatin and fluorescence *in situ* hybridization (FISH) mapping of molecular landmarks such as 18S and 5S ribosomal DNA. New markers of repeated elements such as histone H1, H3 and H4 genes and the U2 snRNA gene have recently been incorporated into these studies (Hashimoto et al. 2011, Utsunomia et al. 2014a, Silva et al. 2015, Utsunomia et al. 2016). The repetitive nature of these sequences makes them useful markers for chromosomal mapping as they provide insight into the structure and organization of the genome and facilitate detection of karyotype rearrangements (Kavalco et al. 2013). However, studies involving chromosomal mapping of repetitive sequences in fish are scarce and typically focus exclusively on the location of ribosomal DNA sites. Studies involving physical mapping of histone genes and mobile elements are also limited, and data is available for only a few species (Pendas et al. 1994, Hashimoto et al. 2011, Ferreira et al. 2011).

Orestias ascotanensis Parenti, 1984 is an endemic species restricted to the small isolated freshwater springs of the Salar de Ascotán. This fish is on the Chilean Endangered Species List (MINSEGPRES, 2008). Major threats to conservation of this species include global climate change and intense regional mining activity. Both situations contribute to a gradual lowering of the water level in the springs, potentially making the salinity of the water incompatible with life for these populations (Vila et al. 2007, Morales et al. 2011). The *O. ascotanensis* karyotype consists of 48 chromosomes, which is the most common diploid number among species in the order Cyprinodontiformes. The chromosomal formula is (2M + 4SM + 4 ST + 38T) (Vila et al. 2010).

The aim of this study was to identify for the first time the constitutive heterochromatin distribution and chromosome organization of four classes of repetitive DNAs (histone H3 DNA, U2 snRNA and 18S and 5S rDNA) in the chromosomes of *O. ascotanensis*. This data will shed light on the physical organization of the repetitive fraction of

the genome of *O. ascotanensis*, a species endemic to the Chilean Altiplano that is facing serious conservation problems. In addition, application of these cytogenetic tools will allow for comparisons among *Orestias* species, facilitating the identification of genomic modifications underlying the chromosomal variations observed in these species.

Materials and methods

Sampling and mitotic chromosome isolation

Eight *O. ascotanensis* individuals, 3 male and 5 female, were obtained from Salar de Ascotán (21°31'S 68°15'W), Region de Antofagasta, Chile, under Scientific Collection Permit Number 1103 issued by SERNAPESCA. The fish were transported to the laboratory and maintained alive in aquaria until processing. Mitotic chromosomes were obtained from kidney cell suspensions according to a modified version of the protocol established by Foresti et al. (1993). Approximately 20 metaphase spreads from different individuals were analyzed to confirm the diploid number and karyotype structure of *O. ascotanensis*. The chromosomes were measured and classified as metacentric (m), submetacentric (sm), subtelocentric (st) or telocentric (t) (Levan et al. 1964), and the karyotype was arranged according to Vila et al. (2010). The images were captured with a digital camera (Nikon D60) attached to an epifluorescence photomicroscope (Nikon Optiphot). Karyotype mounting and image brightness and contrast adjustments were performed in Adobe Photoshop CS6.

Chromosome banding: C- banding and CMA_3

The constitutive heterochromatin (HC) distribution pattern was visualized according to a modified version of the protocol established by Sumner (1972); briefly, chromosomes were subjected to hydrolysis with HCL 0.2 N for 45 min at room temperature, denatured with 5% barium hydroxide at 60°C for 8 min and incubated in saline buffer 2× SSC, and stained with propidium iodide (50 ug/mL) (Lui et al. 2009). Chromomycin A_3 staining was then performed using the method described by Sola et al. (1992). Metaphase plates were observed using a Nikon (Optiphot) microscope with the appropriate filter.

Repetitive sequence probes and FISH experiments

U2 snRNA, 5S rDNA, 18S rDNA and histone H3 DNA probes were obtained from the genomic DNA of *O. ascotanensis*. DNA was collected from a piece of fin tissue with the Wizard Genomic DNA Purification Kit (Promega) according to manufacturer instructions, using previously-described primers (Table 1). The U2 snRNA and

Table 1. Primers used to PCR amplification for gene fragments 5S rDNA, 18S rDNA, U2 snRNA and Histone H3.

Gene	Primers sequences	References
5S rDNA	5SA 5'-TCAACCAACCACAAAGACATTGGCAC-3'	Pendás et al. 1994
	5SB 5'-TAGACTTCTGGGTGGCCAAAGGAATCA-3'	
18S rDNA	18SF 5'-GTAGTCATATGCTTGTCTC-3'	White et al. 1990
	18SR 5'-TCCGCAGGTTCACCTACGGA-3'	
U2snRNA	U2 F 5'-ATCGCTTCTCGGCCTTATG-3'	Bueno et al. 2013
	U2 R 5'-TCCCGGCGGTACTGCAATA-3'	
Histone H3	H3F 5'- ATATCCTTRGGCATRATRGTGAC-3'	Colgan et al. 1998
	H3R 5'- ATGGCTCGTACCAAGCAGACVGC-3'	

5S rDNA probes were labeled by PCR with biotin-16-dUTP, and the 18S rDNA and histone H3 DNA probes were labeled by PCR with digoxigenin-11-dUTP. FISH was performed under high-stringency conditions using the method described by Pinkel et al. (1986). Slides were incubated with RNAse (50µg/ml) for 1 h at 37°C. Next, the chromosomal DNA was denatured in 70% formamide/2× SSC for 5 min at 70°C, and the slides were taken through an ice-cold ethanol series (70°-80°-100°). For each slide, 30µl of hybridization solution containing 200 ng of each labeled probe, 50% formamide, 2× SSC and 10% dextran sulfate was denatured for 10 min at 95°C, dropped onto the slides and hybridized overnight at 37°C in a 2× SSC moist chamber. After hybridization, slides were washed in 0.2× SSC/15% formamide for 20 min at 42°C, followed by a second wash in 0.1× SSC for 15 min at 60°C and a final wash at room temperature in 4× SSC/0.5% Tween for 10 min. Probe detection was carried out with Avidin-FITC (Sigma) or anti-digoxigenin-rhodamine (Roche). Chromosomes were counterstained with DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories).

Results

All *O. ascotanensis* individuals analyzed had a diploid number of 48 chromosomes, consistent with the chromosome formula defined by Vila et al. (2010) (Fig. 1A). No morphologically differentiated sex chromosomes were found when metaphase plates from males and females were compared. C-banding revealed that the constitutive heterochromatin was mainly distributed in the pericentromeric regions of most chromosomes (Fig. 1B). Submetacentric pair 3 was noteworthy due to the presence of conspicuous HC blocks extending along the entire short arm. An interstitial C-band was present on the long arm of chromosome pair 2, proximal to the centromeric region. Additionally, in all observed metaphases, CMA_3-banding revealed that the short arm of pair 3 was strongly stained, reflecting a greater abundance of GC bases in this heterochromatic region (see box, Fig. 1A).

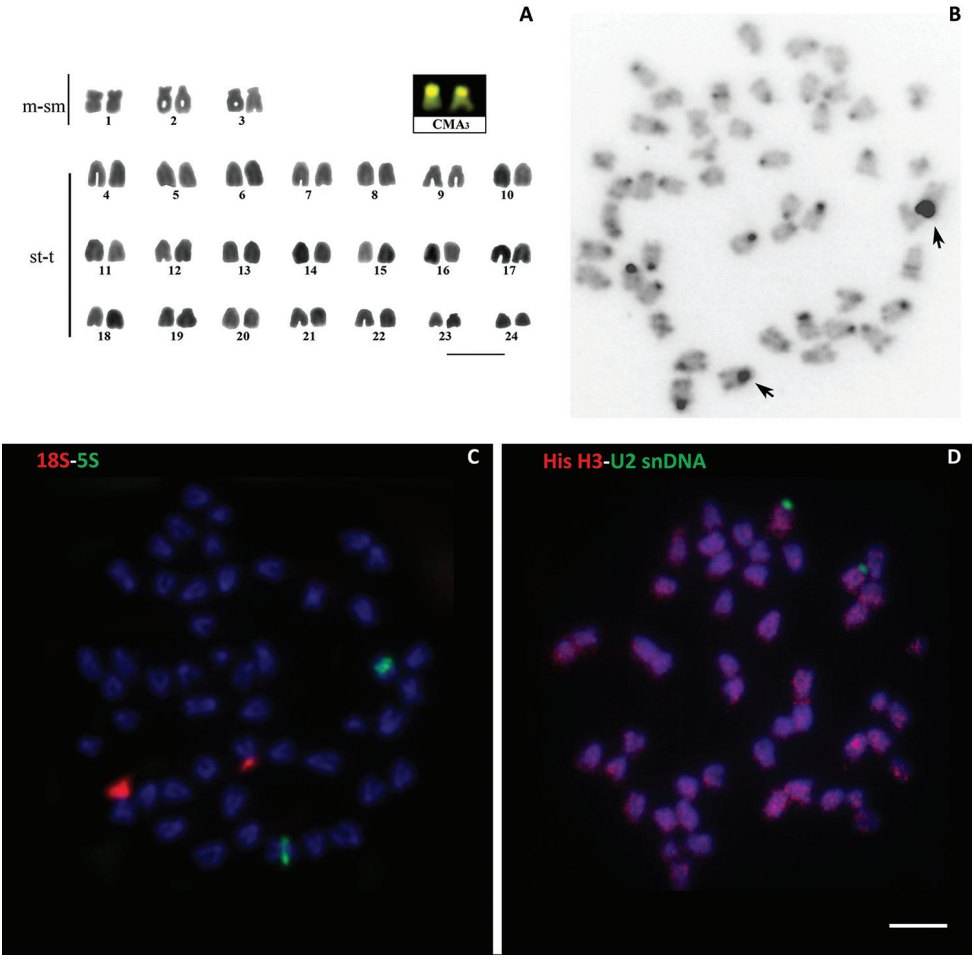

Figure 1. Karyotype of *O. ascotanensis* (female), 2n=48. A Giemsa-stained karyotype and CMA_3 positive bands (box) B C-banded somatic metaphase C metaphases counterstained with DAPI after FISH treatment using 5S and 18S rDNA probes D metaphases counterstained with U2 snDNA/histone H3 DNA probes. The arrows show a block of HC on the short arm of pair 3. Bar = 10μm.

Dual FISH detected 18S and 5S rDNA probes on different chromosome pairs (Fig. 1C). The major rDNA cluster (18S) was located on the short arm of pair 3, with a size polymorphism between the bearing arms of these sequences. 5S rDNA sequences were detected in the region proximal to the centromere of the long arm of pair 2, coincident with the HC band described above. Dual FISH (Fig. 1D) identified a single block of U2 snDNA sequences in the pericentromeric region of a subtelocentric chromosome pair, while histone H3 sites were detected as scattered signals throughout the *O. ascotanensis* chromosomes.

Discussion

Previous cytogenetic studies involving the seven species of *O. agassii* complex of the Chilean Altiplano were limited to characterizing the chromosome number and morphology of the species. The diploid number has been reported to vary between 48 and 55 chromosomes and the fundamental number of chromosome arms (FN) between 54 and 56 (Arratia 1982, Vila et al. 2010, Vila et al. 2011)

Characterization of the repetitive fraction of the genome is a useful tool for identifying recent genomic changes during the evolutionary process as well as possible hotspots associated with chromosomal rearrangements (Valente et al. 2011, Ozouf-Costaz et al. 2004, Yano et al. 2014). The organization of the repetitive fraction of the genome in Cyprinodontiformes fish has remained relatively unexplored, with prior studies focusing primarily on the distribution and composition of constitutive heterochromatin and physical mapping of 18S rDNA genes. Noteworthy studies include reports on: *Fundulus* (Lacépède, 1803) (Kornfield 1981); *Austrolebias* Costa, 1998 (García et al. 1993, 1995, 2001, 2014, 2015); *Aphanius* Nardo, 1827 (Vitturi et al. 2005, Gaffaroglu et al. 2014) and *Hypsolebias* Costa, 2006 (Do Nascimento et al. 2014), with constitutive heterochromatin found to be distributed mainly in centromeric, telomeric and interstitial regions. In addition, in some species of *Chromaphyosemion* Myers, 1924, conspicuous blocks of HC have been identified in the short arm of bi-armed chromosomes (Völker et al. 2005, Völker et al. 2006, Volker et al. 2007, Völker et al. 2008).

In *O. ascotanensis*, the C-band regions were found mainly in the pericentromeric regions, unlike other Cyprinodontiformes that have been studied. CMA_3 also revealed that the conspicuous blocks of HC found in the short arm of pair 3 have a higher proportion of GC bases than previously-analyzed fish. Moreover, the presence of 18S rDNA sequences in this chromosome arm defines this pair as the carrier of the NOR. An association between 18S and 28S rDNA sequences and heterochromatin has been found in other fish, such as salmonids (Fujiwara et al. 1998, Pendas et al. 1994), species of the genera *Epinephelus* Bloch, 1793 (Sola et al. 2000), *Imparfinis* Eigenmann & Norris, 1900 and *Pimelodella* Eigenmann & Eigenmann, 1888 (Gouveia et al. 2013) and sturgeon species (Fontana et al. 2003), suggesting that the repeated HC sequences play an important role and exercise diverse functions in the eukaryotic genome (Grewal and Jia 2007). It has even been postulated that heterochromatin is involved in maintaining the structure of the nucleolus and the integrity of ribosomal DNA repeats (McStay and Grummt 2008).

The single 18S rDNA sequence-bearing chromosome pair in *O. ascotanensis* (Fig. 1C) is a feature observed in most teleosts (Pisano and Ghigliotti 2009, Gornung 2013). However, varied numbers of chromosomes carrying the major ribosomal DNA cluster have been reported in Cyprinodontiformes, with findings ranging from one to seven pairs of chromosomes (Völker et al. 2005, Völker et al. 2006, Volker et al. 2007, Völker et al. 2008). Data on the chromosomal location of the minor ribosomal sites are almost non-existent for Cyprinodontiformes. In *O. ascotanesis*, pair 2 is the 5S-bearing pair, with submetacentric morphology (Fig. 1C). The hybridization signal was detected on the long arm, proximal to the centromere region, associated with the interstitial

heterochromatic band of this pair. 5S and 18S rDNA are typically localized on different chromosomes in vertebrates, including teleosts (Scacchetti et al. 2015, Sánchez-Romero et al. 2015). However, in the Cyprinodontiform *Lebias fasciata* (Valenciennes, 1821), FISH mapping has shown that the 28S and 5S ribosomal DNA probes co-localize on a pair of telocentric chromosomes, conserving the 5S locus on the medial position of the chromosome (Tigano et al. 2004). In general, these sequences vary among teleosts in relation to the chromosomal distribution due to their association with transposable elements, typically within the internal spacer regions (Martins and Galetti 2001, Cabral-de-Mello et al. 2011, Scacchetti et al. 2012, Sene et al. 2015).

Data on the physical location of U2 snRNA sites in fish is also scarce. Two general configurations are recognized: (I) clustered on a single pair of chromosomes, as in the present case and (II) scattered throughout the genome (Ubeda-Manzanaro et al. 2010, Utsunomia et al. 2014, Scacchetti et al. 2015, Silva et al. 2015). According to Medrano et al. (1988), teleosts show low levels of genomic compartmentalization, suggesting that the configuration observed for the U2 snRNA, 5S rDNA and 18S rDNA in *O. ascotanensis* represents a relatively simple genomic organization.

The finding of scattered histone H3 sites distributed throughout the *O. ascotanensis* chromosomes diverges strongly from data reported for other fish, such as Characiformes (Hashimoto et al. 2011, Pansonato-Alves et al. 2013a, Silva et al. 2015), Siluriformes (Hashimoto et al. 2013, Pansonato-Alves et al. 2013b) and Perciformes (Lima-Filho et al. 2012), which generally have large blocks of these sequences in specific chromosome pairs. The histone H3 DNA site distribution found in *O. ascotanensis* chromosomes is similar to the organization described for *Synbranchus marmoratus* Bloch, 1795, suggesting that H3 sequences may be organized in small, abundant copies throughout the genome, as has been proposed by Utsunomia et al. (2014b). Further studies are necessary to confirm that this scattered distribution of H3 DNA is conserved among *Orestias* species.

To understand the relationship of these repeated genomic elements to the chromosomal evolution of these fish and to historical changes in the fishes' environment, further studies are needed to physically map the repetitive DNA in other *Orestias* representatives. These findings would enhance our understanding of native wildlife species facing serious conservation problems.

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RESEARCH ARTICLE

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Comparison of the genetic relationship between nine Cephalopod species based on cluster analysis of karyotype evolutionary distance

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Abstract

Karyotype analysis was carried out on gill cells of three species of octopods using a conventional air-drying method. The karyotype results showed that all the three species have the same diploid chromosome number, 2n=60, but with different karyograms as 2n=38M+6SM+8ST+8T, FN (fundamental number)=104 (*Cistopus chinensis* Zheng et al., 2012), 2n=42M+6SM+4ST+8T, FN=108 (*Octopus minor* (Sasaki, 1920)) and 2n=32M+16SM+12T, FN=108 (*Amphioctopus fangsiao* (d'Orbigny, 1839–1841)). These findings were combined with data from earlier studies to infer the genetic relationships between nine species via cluster analysis using the karyotype evolutionary distance (D_e) and resemblance-near coefficient (λ). The resulting tree revealed a clear distinction between different families and orders which was substantially consistent with molecular phylogenies. The smallest intraspecific evolutionary distance (D_e=0.2013, 0.2399) and largest resemblance-near coefficient (λ=0.8184, 0.7871) appeared between *O. minor* and *C. chinensis*, and *Sepia esculenta* Hoyle, 1885 and *S. lycidas* Gray, 1849, respectively, indicating that these species have the closest relationship. The largest evolutionary gap appeared between species with complicated karyotypes and species with simple karyotypes. Cluster analysis of D_e and λ provides information to supplement traditional taxonomy and molecular systematics, and it would serve as an important auxiliary for routine phylogenetic study.

Keywords

octopods, cytogenetics, chromosome, genetic relationship, evolutionary distance

Introduction

Cephalopoda is an old and evolutionarily successful molluscan group with a worldwide distribution (Jazayeri et al. 2011, Adachi et al. 2014). It includes several species that are precious marine resources but are difficult to manage due to their short life span and sensitivity to environmental conditions (Emery et al. 2016). Extant cephalopods are divided into two subclasses: Nautiloidea and Coleoidea. Members of Coleoidea are main catch targets and are common in fish markets (Lu 2000). Approximately 134 cephalopod species (Lu et al. 2012), including commercially important marine species such as *Octopus minor* (Sasaki, 1920), *Amphioctopus fangsiao* (d'Orbigny, 1839–1841), *Cistopus chinensis* Zheng et al., 2012 and *Sepia esculenta* Hoyle, 1885, are found in Chinese waters. According to the China fishery statistical yearbook (Zhao 2016), cephalopod landings totalled nearly 0.7 million tonnes in 2015, with an increase of 3.42% over the previous year. Because of the high economic benefits surrounding octopods, many intensive studies have investigated their population genetics (Zheng et al. 2009, Meriam et al. 2015, Gao et al. 2016), behaviour (Meisel et al. 2013, Polese et al. 2015, Levy et al. 2015, Richter et al. 2016), neurology (Nixon and Young 2004, Zarrella et al. 2015), and reproductive biology (Wada et al. 2006, Ebisawa et al. 2011, Wang et al. 2015b). However, while significant genetic knowledge is required for effective breeding and aquaculture of octopods, modern cytogenetic studies of these species are scarce.

Karyotype analysis is the foundation of cytogenetic studies, playing an important role in understanding the origin and evolution of organisms by studying the variation in the number or structure of their chromosomes (Chung et al. 2012). Despite the importance of understanding the role of chromosomes in cephalopod evolution, chromosome research in these species is poorly developed because of their huge diploid chromosomes and the lack of good split phases. The most reliable karyotype information comes from Gao and Natsukari (1990), who studied two octopods *O. ocellatus* Gray, 1849 (*A. fangsiao*) (Jereb 2014) and *O. vulgaris* Cuvier, 1797, two sepiids (*S. esculenta* and *S. lycidas* Gray, 1849) and three loliginids (*Heterololigo bleekeri* Natsukari, 1984, *Sepioteuthis lessoniana* Blainville, 1824 and *Photololigo edulis* (Hoyle, 1885)) (Table 1). Earlier studies led by Inaba and Vitturi reported the chromosome number of *O. vulgaris*, *O. minor* and *S. officinalis* Linnaeus, 1758 (Inaba 1959, Vitturi et al. 1982), but included no detailed karyotype description. In the last three decades, only a scant few publications have been focused on cephalopod karyotype research. Bonnaud et al. (2004) reported the *Nautilus macromphalus* Sowerby, 1849 karyotype, with 52 chromosomes, and other studies revealed the chromosome number of Gulf cuttlefish (*S. arabica* Massy, 1916 and *S. pharaonis* Ehrenberg, 1831) via examination of the blood cells (Papan et al. 2010, Jazayeri et al. 2011). However, the findings of these follow-up studies remain uncertain since they lacked ideal division phases and basic chromosome parameters. Similarly, recent karyotype analyses of *S. esculenta* and *O. areolatus* de Haan, 1839–1841 (*A. fangsiao*) (Jereb 2014) have been revealing but were not sufficiently thorough (Wang et al.

Table 1. Basic karyotype information of nine species of cephalopods.

Species	Origin		Karyotype			References
	Locations	Materials	2n	FN	Formulas	
O. minor	Weihai, Shandong Province, China	gills	60	108	42M+6SM+4ST+8T	This study
O. vulgaris	Nagasaki, Japan	embryos	60	76	14M+2SM+8ST+36T	Gao and Natsukari (1990)
A. fangsiao	Qingdao, Shandong Province, China	gills	60	108	32M+16SM+12T	This study
C .chinensis	Ningde, Fujian Province, China	gills	60	104	38M+6SM+8ST+8T	This study
S. lycidas	Ohmura, Nagasaki, Japan	wild eggs	92	172	66M+14SM+10ST+2T	Gao and Natsukari (1990)
S. esculenta	Shimabara, Nagasaki, Japan	wild eggs	92	164	48M+24SM+14ST+6T	Gao and Natsukari (1990)
S. lessoniana	Nomozaki, Nagasaki, Japan	wild eggs	92	156	54M+10SM+24ST+4T	Gao and Natsukari (1990)
P. edulis	Nagasaki, Japan	embryos	92	160	50M+18SM+16ST+8T	Gao and Natsukari (1990)
H. bleekeri	Nagasaki, Japan	embryos	92	166	54M+20SM+18ST	Gao and Natsukari (1990)

2011, Adachi et al. 2014). In general, to obtain satisfactory split phases, embryos are better; however, this method is severely constrained by the availability and accessibility of material during the cephalopod breeding season. In addition, the use of germ cells is also restricted by season, and chromosomes are short during this period, which is not conducive to routine karyotype analysis (Zhang et al. 2007). Gills provide an alternative source for karyotyping which is convenient, fast, and not subject to seasonal restrictions; however, due to the slow metabolism of adults, there is little cell division in this tissue. Together, these factors act to limit cephalopod chromosome studies.

Karyotype evolutionary distance has been used as an important parameter in studying the classification and evolution of animals. In this approach, the distance of karyotype evolution (D_e) and resemblance-near coefficients (λ) are estimated from the karyotype data by mathematical statistics based on the principles of numerical taxonomy and similar analysis theory, and these parameters accurately reflect the interspecific or intraspecific relationship at the cytological level. While the classification and genetic relationships of cephalopods is a continuing topic of interest and has been addressed using molecular systematics tools, such as mitochondrial DNA (Cheng et al. 2013, Zhang et al. 2015), without reaching a consensus, evidence from chromosome morphology is still seldom used to analyse the relationships and evolution of cephalopod taxa (Thiriot-Quiévreux 2003). Determining the genetic relationships between species based on cellular characteristics would be an effective supplement to traditional taxonomy and molecular systematics, and would serve as an important auxiliary means of routine analysis.

Here, we use a cytogenetic approach to study the genetic relationships of cephalopods at the chromosome level. We used gills to obtain good metaphase mitotic plates, and then calculated the D_e and λ in order to construct a cluster analysis diagram among nine species cephalopods. These findings enrich our knowledge of cephalopod chromosome structure and provide a new and important index for cephalopod taxonomic classification and the determination of genetic relationships at the cytological level.

Material and methods

Specimens

We obtained ten live *O. minor* specimens from the Rongcheng coastal waters of the Bohai Sea (37°13'N, 122°33'E), Shandong Province, China, and ten specimens of *A. fangsiao* were from the Qingdao coastal waters of the Yellow Sea (36°06'N, 120°32'E), Shandong Province, China. Another ten *C. chinensis* was transported to laboratory in plastic bags with oxygenation, at a low temperature, from the Ningde coastal waters of the East Sea (27°18'N, 119°32'E), Fujian Province, China. All individuals were about 40g and were identified based on morphological characteristics.

Chromosome preparation

Chromosome preparation followed the method of Gao and Natsukari (1990) with some modifications. Briefly, the octopods were cultured in a 0.01% colchicine solution for 12h. In keeping with the PETA protocols, the gills were rapidly immersed in a 0.075M KCl solution for 1 hour, then the conventional air-drying method was applied. After indoor drying, the slides were stained with a 5% Giemsa solution for 10 min following the protocol used by Okumura et al. (1995). They were then observed under a light microscope with an oil lens (Leica MC170 HD, Germany).

Construction of karyo-idiograms

Microphotographs of the chromosomes were used for karyotype analysis with Image-Pro Plus 6.0 (Wang et al. 2015a). Chromosomes were extracted from the original images, with homologous chromosome pairing and sorting based on visual observation. Chromosomes were classified adhering to Levan et al. (1964), and the length index was calculated according to Kobayashi (1986). Using these criteria, we automatically generated a schematic showing the long and short arms with different colours based on the measured values. A notch to represent the centromere was added to each chromosome using SmoothDraw. Finally, homologous chromosomes were arranged below the diagrams with Image-Pro Plus 6.0.

Cluster analysis

We used the chromosome relative length as karyotype parameter of nine species (three from this study) for the analysis of evolutionary relationships (Table 1). D_e and λ values were calculated with preliminary statistical analysis according the proposed criterion (for details, see Supplemental formulae). Further data analysis through SPSS 19.0 and Microsoft Excel 2007, the D_e data matrix was then incorporated into a MEGA5.0 (Tamura et al. 2011) genetic distance operation document (.meg), and the karyotype evolution distance cluster tree was constructed.

Results

Karyotype analysis

Karyological analysis of Giemsa-stained chromosomes was successfully obtained from at least seven well- divided metaphase plates from the studied populations of *O. minor*, *A. fangsiao* and *C. chinensis* (Fig. 1), and measurements of the chromosomes are shown in Table 2. All three octopods had a diploid chromosome number of 2n=60. The *O. minor* karyotype was 2n=42M+6SM+4ST+8T (FN=108), composed of 21 pairs of metacentric (1st-21st), 3 pairs of submetacentric (22nd-24th), 2 pairs of subtelocentric (25th-26th), and 4 pairs of telocentric (27th-30th) chromosomes. The relative length of each chromosome ranged from 1.15 to 4.99. In all metaphases we observed, the arm ratio (AR) of the 22nd pair chromosomes was greater than or equal to 1.70, making it a submetacentric chromosome pair according the centromeric index (CI). The *A. fangsiao* karyotype was 2n=32M+16SM+12T (FN=108), consisting of 16 pairs of metacentric (1st-16th), 8 pairs of submetacentric (17th-24th), and 6 pairs of telocentric (25th-30th) chromosomes. The relative length of each chromosome ranged from 0.90 to 6.88. Finally, the *C. chinensis* karyotype was 2n=38M+6SM+8ST+8T (FN=104), consisting of 19 pairs of metacentric (1st-19th), 3 pairs of submetacentric (20th-22nd), 4 pairs of subtelocentric (23rd-26th), and 4 pairs of telocentric (27th-30th) chromosomes. The relative length of each chromosome ranged from 1.56 to 8.28. From the karyotype formulas, we found that *A. fangsiao* had no subtelocentric chromosomes, while *O. minor* and *C. chinensis* had quite close karyotypes, with differences only in the (sub)metacentric chromosomes. It is obvious that the metacentric and submetacentric chromosomes account for most of the chromosomes (>73.3%) (Fig. 4), indicating that they are derived with a higher classification status.

We compared the relative chromosome length of the nine species of cephalopods and plotted a detailed chromosome distribution diagram to show the number and proportion of the different types of chromosome in the different species (Fig. 4). *S. lycidas* had the highest proportion of metacentric chromosomes (M, up to 71.7%), while the lowest appeared in *A. fangsiao* (below 23.5%). They correspondingly had the lowest and highest proportion of telocentric chromosomes (T, 2.2% and 60.0%).

Table 2. Comparison of karyotype parameters obtained among *O. minor*, *A. fangsiao* and *C. chinensis*. (SA, short arm relative length; LA, long arm relative length; AR, arm ratio=LA/SA; CI, centromeric index=SA/(SA+LA) ×100; M, metacentric, 1.0 < AR < 1.7; SM, submetacentric, 1.7 < AR < 3.0; ST, subtelocentric, 3.0 < AR < 7.0; T, telocentric, 7.0 < AR. Values as mean ± SE)

Chromosome no.	*Octopus minor* (42M+6SM+4ST+8T)						*Amphioctopus fangsiao* (32M+16SM+12T)						*Cistopus chinensis* (38M+6SM+8ST+8T)					
	SA	LA	SA+LA	AR	CI	Type	SA	LA	SA+LA	AR	CI	Type	SA	LA	SA+LA	AR	CI	Type
1	2.43	2.54	4.97±0.17	1.05±0.15	48.89	M	3.33	3.55	6.88±0.18	1.06±0.08	48.46	M	4.13	4.15	8.28±0.05	1.00±0.10	49.88	M
2	2.28	2.49	4.77±0.11	1.09±0.12	47.80	M	2.99	3.46	6.45±0.70	1.16±0.12	46.36	M	3.64	3.75	7.39±0.11	1.03±0.02	49.26	M
3	2.22	2.51	4.73±0.05	1.13±0.08	46.93	M	3.04	3.15	6.19±0.47	1.03±0.02	49.17	M	3.60	3.78	7.38±0.07	1.05±0.02	48.78	M
4	2.30	2.43	4.73±0.13	1.06±0.12	48.63	M	2.86	2.87	5.73±0.19	1.00±0.11	49.91	M	3.15	3.44	6.59±0.12	1.09±0.03	47.80	M
5	2.36	2.36	4.72±0.06	1.00±0.13	50.00	M	2.53	2.96	5.49±0.07	1.17±0.17	46.01	M	3.33	3.35	6.68±0.09	1.01±0.11	49.85	M
6	2.28	2.36	4.64±0.01	1.04±0.05	49.14	M	1.94	2.12	4.06±0.20	1.09±0.04	47.78	M	3.14	3.18	6.32±0.15	1.01±0.04	49.68	M
7	2.21	2.38	4.59±0.05	1.08±0.01	48.15	M	1.97	2.09	4.06±0.10	1.06±0.01	48.52	M	2.97	3.19	6.16±0.04	1.07±0.01	48.21	M
8	2.19	2.40	4.59±0.11	1.10±0.14	47.71	M	1.66	2.18	3.84±0.21	1.31±0.01	43.23	M	2.83	2.98	5.81±0.20	1.05±0.07	48.71	M
9	2.16	2.38	4.54±0.05	1.12±0.02	47.58	M	1.82	1.91	3.73±0.22	1.05±0.02	48.79	M	2.42	2.48	4.91±0.17	1.02±0.11	49.29	M
10	2.13	2.15	4.28±0.12	1.01±0.10	49.78	M	1.63	2.07	3.70±0.06	1.27±0.01	44.05	M	2.29	2.44	4.73±0.06	1.07±0.06	48.41	M
11	2.12	2.16	4.28±0.03	1.01±0.07	49.53	M	1.55	1.95	3.50±0.31	1.26±0.08	44.29	M	2.28	2.45	4.73±0.15	1.07±0.08	48.20	M
12	2.03	2.17	4.20±0.14	1.07±0.13	48.33	M	1.68	1.76	3.44±0.11	1.05±0.04	48.84	M	2.24	2.27	4.51±0.11	1.01±0.03	49.67	M
13	1.92	2.04	3.96±0.07	1.06±0.09	48.48	M	1.42	1.79	3.21±0.27	1.26±0.21	44.24	M	1.99	2.31	4.40±0.07	1.16±0.01	45.23	M
14	1.91	1.98	3.89±0.15	1.04±0.14	49.10	M	1.56	1.64	3.20±0.04	1.05±0.13	48.75	M	1.96	2.27	4.23±0.10	1.16±0.03	46.34	M
15	1.88	1.98	3.86±0.09	1.05±0.10	48.70	M	1.47	1.69	3.16±0.32	1.15±0.32	46.52	M	2.03	2.10	4.13±0.16	1.03±0.12	49.15	M
16	1.36	1.90	3.26±0.11	1.40±0.13	41.72	M	1.32	1.69	3.01±0.05	1.28±0.13	43.85	M	1.75	1.99	3.74±0.05	1.14±0.07	46.79	M
17	1.42	1.54	2.96±0.12	1.08±0.18	47.97	M	2.48	4.39	6.87±0.13	1.77±0.28	36.10	SM	1.58	1.96	3.54±0.03	1.24±0.08	44.63	M
18	1.28	1.29	2.57±0.05	1.01±0.10	49.81	M	2.37	4.30	6.67±0.07	1.81±0.14	35.53	SM	1.55	1.55	3.10±0.02	1.00±0.04	50.00	M
19	1.24	1.27	2.51±0.13	1.02±0.11	49.40	M	1.92	3.59	5.51±0.24	1.87±0.29	34.85	SM	1.02	1.23	2.25±0.04	1.21±0.06	45.33	M
20	0.93	1.19	2.12±0.08	1.28±0.10	43.87	M	2.02	3.46	5.48±0.09	1.71±0.12	36.86	SM	1.52	4.53	6.05±0.08	2.98±0.06	25.12	SM
21	0.95	0.96	1.91±0.10	1.01±0.05	49.74	M	1.53	2.66	4.19±0.26	1.74±0.20	36.52	SM	1.23	2.46	3.69±0.16	2.00±0.20	33.33	SM
22	1.85	3.14	4.99±0.05	1.70±0.05	37.07	SM	1.12	2.50	3.62±0.18	2.23±0.11	30.94	SM	1.02	2.42	3.44±0.08	2.37±0.08	29.65	SM
23	1.01	2.87	3.88±0.10	2.84±0.11	35.19	SM	1.05	2.53	3.58±0.19	2.41±0.01	29.33	SM	1.15	4.63	5.78±0.09	4.03±0.04	19.90	ST
24	0.97	2.90	3.87±0.09	2.99±0.02	25.06	SM	0.74	2.13	2.87±0.03	2.88±0.30	25.78	SM	1.10	3.89	4.99±0.03	3.54±0.01	22.04	ST
25	1.03	3.52	4.55±0.13	3.42±0.13	22.64	ST	0.32	2.86	3.18±0.66	8.90±0.13	10.10	T	0.55	3.35	3.90±0.16	6.09±0.13	14.10	ST

Chromosome no.	*Octopus minor* (42M+6SM+4ST+8T)						*Amphioctopus fangsiao* (32M+16SM+12T)						*Cistopus chinensis* (38M+6SM+8ST+8T)					
	SA	**LA**	**SA+LA**	**AR**	**CI**	**Type**	**SA**	**LA**	**SA+LA**	**AR**	**CI**	**Type**	**SA**	**LA**	**SA+LA**	**AR**	**CI**	**Type**
26	0.83	3.17	4.00±0.01	3.82±0.04	20.75	ST	0.22	2.88	3.10±0.40	12.90±0.32	7.20	T	0.73	3.15	3.88±0.10	4.32±0.13	18.81	ST
27	-	3.02	3.02±0.01	∞	-	T	-	2.71	2.71±0.42	∞	-	T	-	2.71	2.71±0.02	∞	-	T
28	-	3.02	3.02±0.05	∞	-	T	-	2.54	2.54±0.16	∞	-	T	-	2.74	2.74±0.06	∞	-	T
29	-	1.77	1.77±0.01	∞	-	T	-	1.90	1.90±0.03	∞	-	T	-	1.69	1.69±0.07	∞	-	T
30	-	1.15	1.15±0.07	∞	-	T	-	0.90	0.90±0.03	∞	-	T	-	1.56	1.56±0.10	∞	-	T

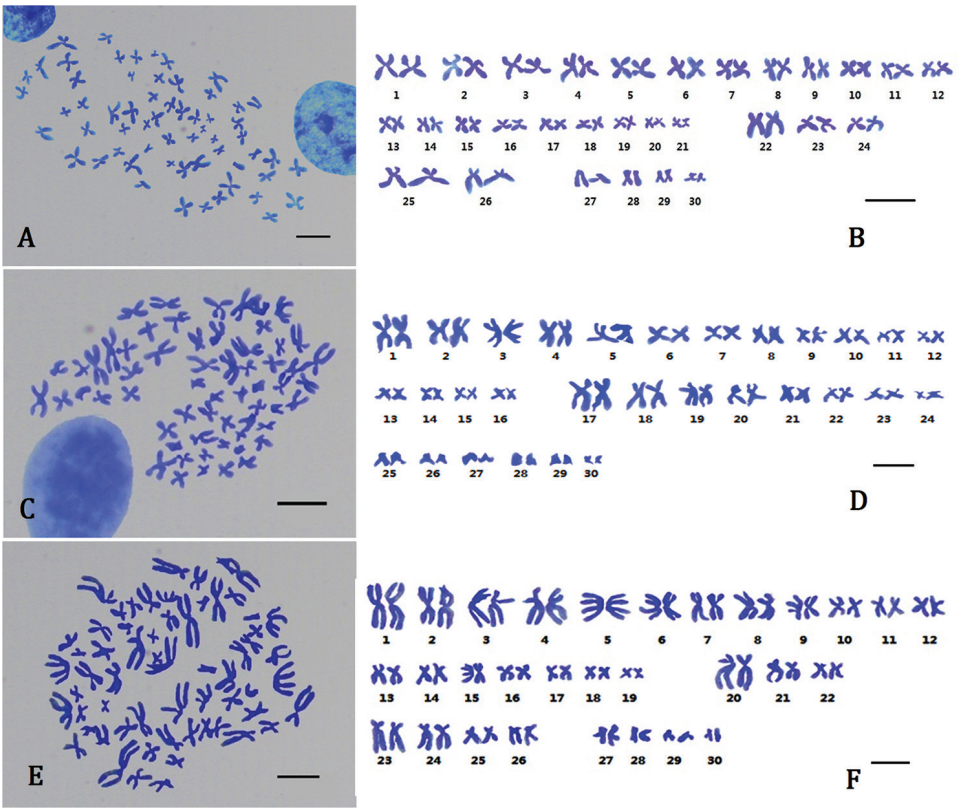

Figure 1. Photomicrographs of somatic diploid metaphase plates and karyotypes from three species of octopod gills. **A** The metaphase plate of *O. minor* **B** Karyogram of *O. minor* from (**A**) showing the karyotype composition: 42 metacentric (#1–#21), 6 submetacentric (#22–#24), 4 subtelocentric (#25–#26), and 8 telocentric (#27–#30) chromosomes **C** The metaphase plate of *A. fangsiao* **D** Karyogram of *A. fangsiao* from (**C**) showing the karyotype composition: 32 metacentric (#1–#16), 16 submetacentric (#17–#24), and 12 telocentric (#25–#30) chromosomes **E** The metaphase plate of *C. chinensis* **F** Karyogram of *C. chinensis* from (**E**) showing the karyotype composition: 38 metacentric (#1–#19), 6 submetacentric (#20–#22), 8 subtelocentric (#23–#26), and 8 telocentric (#27–#30) chromosomes. Scale bar 5 μm.

The four chromosome types (M, SM, ST and T) made up 56.9%, 16.6%, 14.5% and 12.0%, respectively, of the total chromosomes in the cephalopod karyotypes. Metacentric and submetacentric chromosomes were the major components of the karyotypes of Octopodiformes and Decapodiformes, accounting for 65.0% and 77.8% of the chromosomes, respectively. In almost all nine species, M was the largest proportion of chromosome types (with a minimum of 52.2%), followed by SM, while the other two types had variable proportions. The only exception was *O. vulgaris*, in which the highest proportion was T chromosomes (up to 60.0%), followed by M (23.3%), ST (13.3%), and SM (3.4%). These differences suggest that *O. vulgaris* may have experienced comparatively large chromosomal rearrangements, such as translocations or inversions, during its evolution.

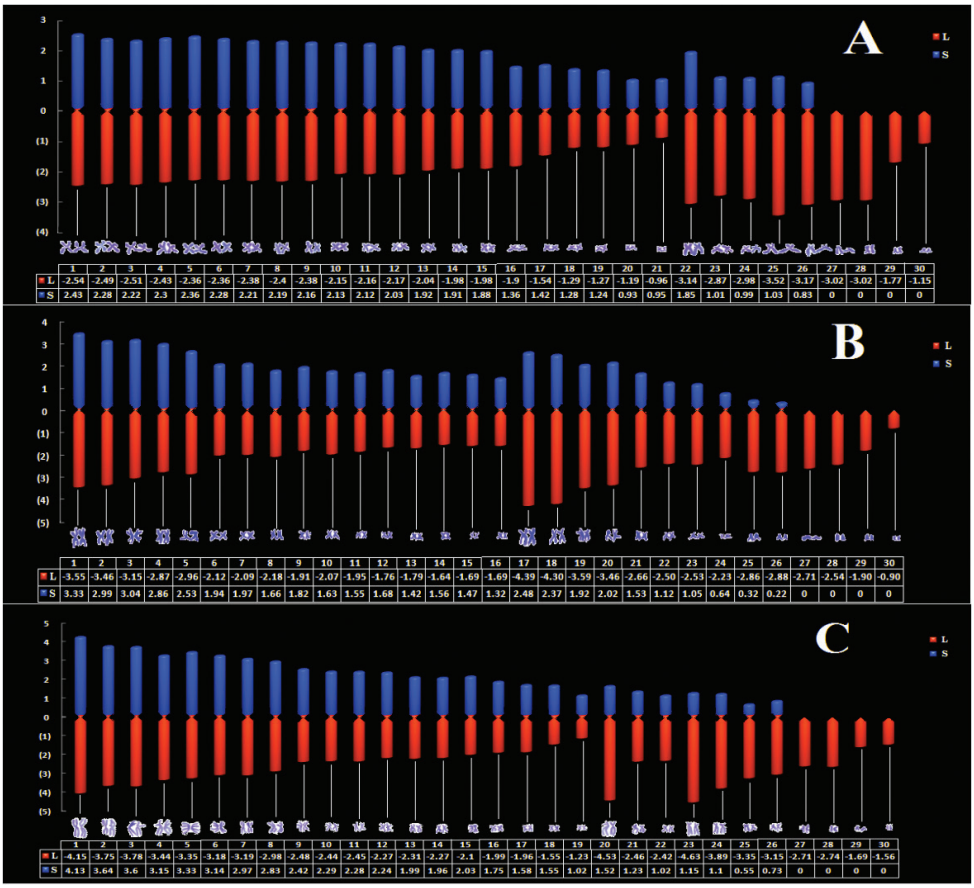

Figure 2. A novel display method of karyo-idiograms. Three octopods are shown: **A** *O. minor* **B** *A. fangsiao* **C** *C. chinensis*. The blue columns are the short arms and the red columns are long arms. Nicks mark the centromeres.

Construction of karyo-idiograms

We developed a novel method to create normative karyo-idiograms of the three species based on the karyotype parameters (Fig. 2). The diagrams vividly and intuitively show the basic characteristics of each chromosome. The zero point in the diagram is the location of the centromere, and the chromosomes are arranged according to their type and size.

Genetic relationship analysis

Karyotypes vary greatly between species, with greater karyotype evolutionary distance (D_e) and smaller resemblance-near coefficients (λ) between distantly related species.

Table 3. The karyotype evolutionary distance and resemblance-near coefficient among nine species of cephalopods.

Species	*O. minor*	*O. vulgaris*	*A. fangsiao*	*C. chinensis*	*S. lycidas*	*S. esculenta*	*S. lessoniana*	*P. edulis*	*H. bleekeri*
O. minor		0.5894	0.7594	0.8184	0.5244	0.3747	0.4401	0.4839	0.2742
O. vulgaris	0.5291		0.5495	0.5540	0.4183	0.3765	0.4725	0.4392	0.3057
A. fangsiao	0.2760	0.6000		0.7976	0.5423	0.5075	0.4515	0.4963	0.2640
C. chinensis	0.2013	0.5912	0.2262		0.5343	0.4744	0.5467	0.3846	0.3663
S. lycidas	0.6460	0.8722	0.6122	0.6271		0.7871	0.6328	0.6297	0.5990
S. esculenta	0.9809	0.9782	0.6776	0.7471	0.2399		0.5809	0.5594	0.6051
S. lessoniana	0.8214	0.7494	0.7960	0.6034	0.4570	0.5431		0.5280	0.3650
P. edulis	0.7265	0.8230	0.7011	0.9550	0.4620	0.5822	0.5904		0.5984
H. bleekeri	1.2954	1.1845	1.3323	1.0101	0.5120	0.5030	0.6940	0.5140	

Note: The evolutionary distance is in the left lower quadrant, and the resemblance-near coefficient is in the right upper quadrant.

Likewise, the karyotype evolutionary distance within a family is generally smaller than that between different families. To make an integrative analysis of the genetic relationships, the D_e and λ values of the nine cephalopods were calculated (Table 3). D_e measures ranged from 0.2013 to 1.3323, with an average of 0.6742. The largest D_e was between *A. fangsiao* and *H. bleekeri* (Keferstein, 1866), whereas the smallest distance was between *O. minor* and *C. chinensis*. Correspondingly, the largest estimate for λ was between *O. minor* and *C. chinensis*, whereas the smallest estimate was between *A. fangsiao* and *H. bleekeri*. Overall, the λ values ranged from 0.2640 to 0.8184, with an average of 0.5283. In the Decapodiformes (Sepioidea and Teuthoidea), *S. esculenta* and *S. lycidas* had the closest relationship, with the smallest D_e (0.2399).

In order to shed further light on phylogenetic divergence within the clades Octopoda, Sepiida and Teuthida, a cluster analysis was applied (Fig. 3A). The results showed clear distinctions between the different families and orders which were not quite concordant with the phylogenetic analysis at the molecular level. Decapodiformes and Octopodiformes (Octopoda) were definitely classified as two major clades. The four species in the order Octopoda clustered together as clade I, with D_e=0.1418, while species from the orders Sepiida and Teuthida form a second clade, with D_e=0.1429. Within clade I, *O. minor* and *C. chinensis* clustered as a monophyletic group with the smallest D_e (0.0249), indicating the closest relationship, while *A. fangsiao* appeared as a sister group with D_e=0.1612; *O. vulgaris* formed a sister to the three other octopod species. In clade II, formed by five species of the Decapodiformes, *S. esculenta* and *S. lycidas* formed one monophyletic group and *H. bleekeri* and *P. edulis* formed a second, sister monophyletic group, with D_e=0.1338 and 0.0073, respectively, while *S. lessoniana* was as a sister to the two monophyletic groups.

Discussion

In previous reports, germ cells, blood cells, and embryos (Inaba 1959, Gao and Natsukari 1990, Papan et al. 2010, Jazayeri et al. 2011, Adachi et al. 2014) have been used in cephalopod karyological studies, but this is the first study to use gill cells as a source of chromosomes, from which we were able to obtain positive metaphase plates.

The chromosome number of the three species in the present study was 2n=60, which is consistent with previous karyotype studies of octopods (Gao and Natsukari 1990, Adachi et al. 2014). However, in the present study, the *A. fangsiao* karyotype (32SM+16SM+12T) had twelve telocentric chromosomes, which disagrees with Gao and Natsukari 1990 (32M+28SM) and Adachi et al. 2014 (48M+8M/SM+4SM), who contend that this species has only M and SM chromosomes. Furthermore, the karyotype formula we found for this species was different from previous reports, which may be due to differences in sampling and preparation methods causing chromosome polymorphism, which is common in shellfish (Wang et al. 2015a). Arslan and Zima (2015) also emphasized that a cytotype may include several populations with different karyotypes despite having the same diploid number of chromosomes. Similarly, chro-

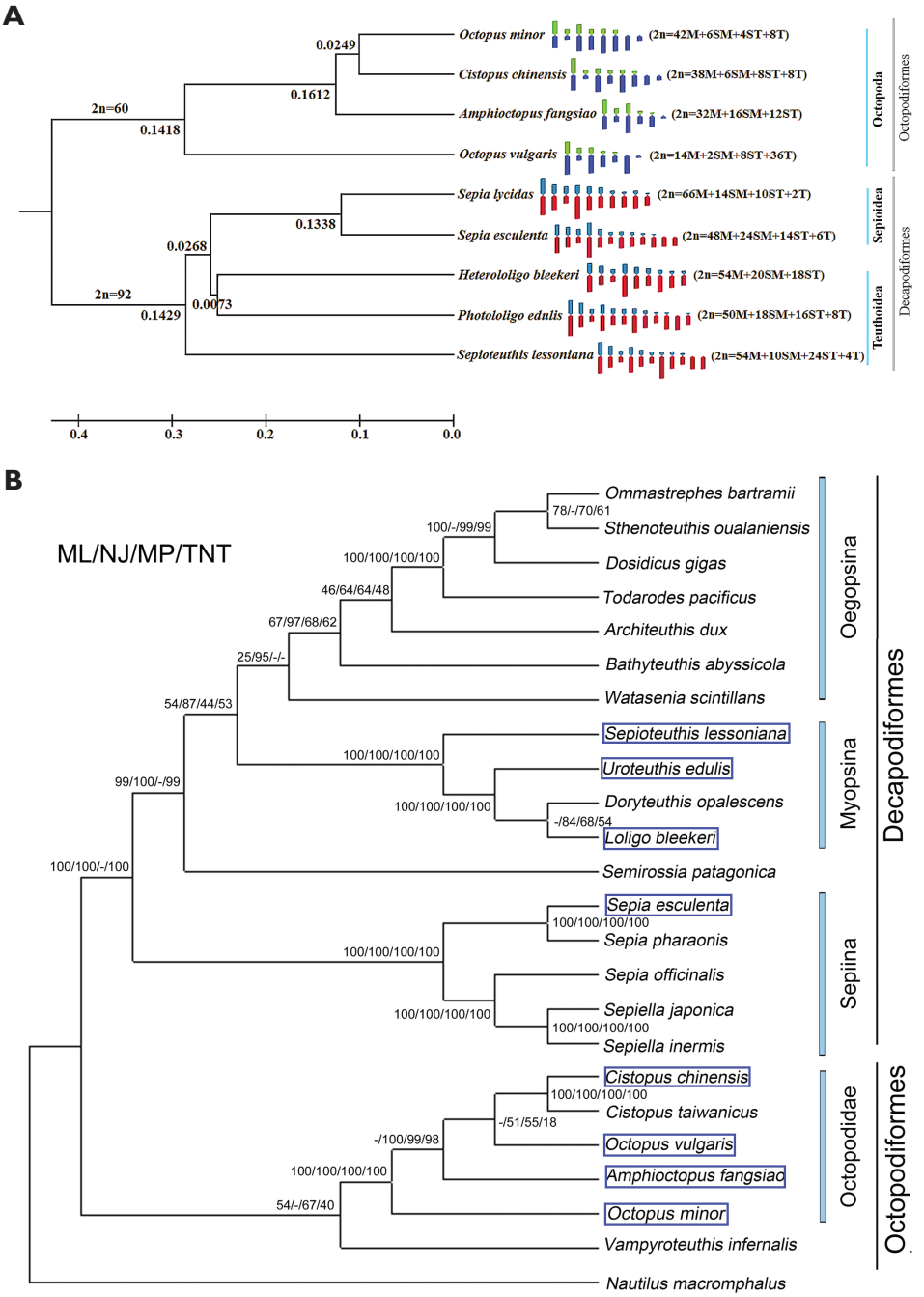

Figure 3. A Relationships between chromosome number and UPGMA clustering of nine species of cephalopods by evolutionary distance with simplified karyo-idiogram and karyotype formulas. Chromosome numbers and D_e values are shown on the corresponding branches **B** Phylogenetic relationships among the cephalopods based on mitochondrial DNA sequences including the nine species of this study (Cheng et al. 2013).

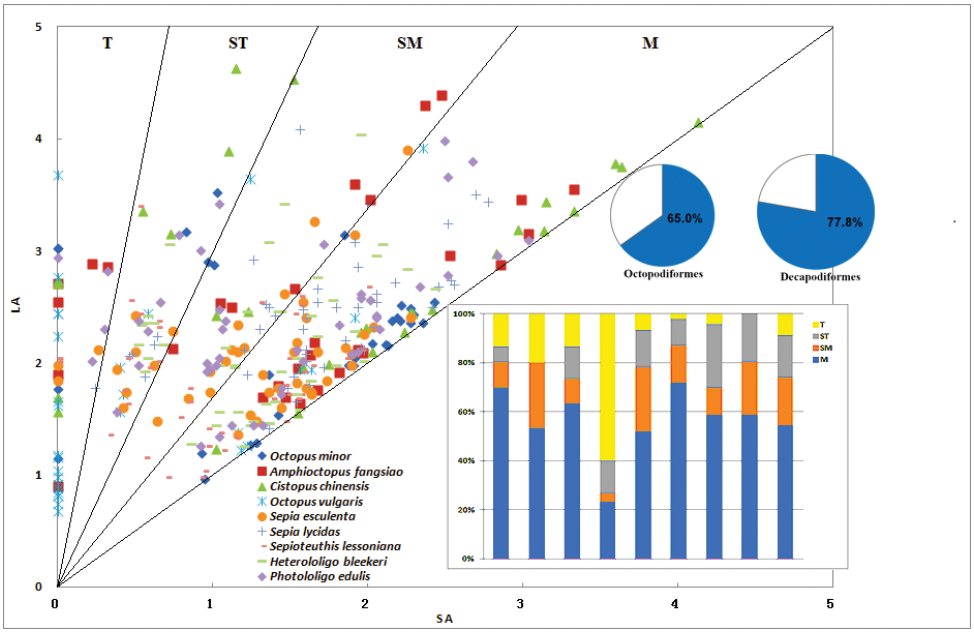

Figure 4. Chromosome distribution diagram of nine species of cephalopods. The slopes of the four lines are 1, 1.7, 3 and 7, dividing the diagram into four zones which represent four types of chromosome. SA, short arm relative length; LA, long arm relative length; M, metacentric; SM, submetacentric; ST, subtelocentric; T, telocentric. The blue area of pie charts and bar charts means M+SM and M, respectively.

mosomal diversity and differentiation has been confirmed in creepers (Manthey et al. 2015). In addition, the present study revealed a new karyotype of *O. minor* which was clearly different from results of Inaba (1959), who reported a diploid chromosome number of 56 in spermatogonia and primary spermatocytes. However, the lack of dependable metaphase division and detailed chromosomal parameters leads us to doubt the earlier result and favour the current study. Zhang et al.(2007) also pointed out that chromosomes obtained from sperm cells were too small to observe.

Despite the three octopods having the same number of chromosomes, the karyotypes were remarkably different from each other. Compared with *O. minor* and *C. chinensis*, *A. fangsiao* had a specialized karyotype without ST, while the former two had almost the same karyotype, with only slight differences in M and ST (Fig. 4). Based on the findings reported in the present study and in Gao and Natsukari (1990), the three species of order Teuthida and the two species of the order Sepiida should have a higher classification status than the four species of the order Octopoda because they have a significantly greater diploid chromosome number (92 vs. 60), which is consistent with the results of the cluster analysis (Fig. 3). Together, M and SM were the main components of karyotype, suggesting that the cephalopods have a higher classification status. Similar observations have been made in bivalve shellfish, where karyotypes with a majority of metacentric-submetacentric chromosomes were characteristic of most

bivalve species (Thiriot-Quiévreux 2002). Interestingly, *S. lycidas* and *A. fangsiao* contained the highest and lowest proportion of M and the lowest and highest proportion of T chromosomes.

Earlier analyses of cephalopod genetic relationships mainly concentrated on phylogenetic reconstruction via specific rDNA sequences (Bonnaud et al. 2004, Cheng et al. 2013, Zhang et al. 2015). Comparison of the karyological characters of the nine species of cephalopods for which data are available at the cytological level with a cluster analysis using karyotype evolutionary distance yielded substantial agreement with the phylogeny based on mitochondrial genes (Fig. 3B). Within clade II, the two species of the order Sepiida and three species of the order Teuthoidea formed different groups, which is concordant with the reported of Cheng et al. (2013), while larger divergence appeared in the order Octopoda. *C. chinensis* and *C. taiwanicus* Liao & Lu, 2009 formed a monophyletic group, with *O. vulgaris* and *A. fangsiao* appeared as sisters to the above group, while *O. minor* was a sister to the combined group. A subsequent publication validated these phylogenetic relationships based on an analysis of the complete mitochondrial genome (Zhang et al. 2015). In both cases, there was a closer genetic relationship between *C. chinensis* and *O. vulgaris* than with *A. fangsiao* and *O. minor*. However, the present study found the closest relationship between *C. chinensis* and *O. minor*, which formed a clade with the smallest D_e (Table 3 and Fig. 3A). The taxonomic status of *O. minor* has been in dispute, with a recent study assigning it to the genus *Callistoctopus* Taki, 1964 based on CO1 and CO3 phylogenetic analyses (Kaneko et al. 2011). From the present analysis, we concluded that chromosome number and type played a leading role in clustering, since some species grouped together as a clade based on chromosome number, while others clustered separately into different branches based on karyotype similarity. For example, *C. chinensis* and *O. minor* readily clustered together based on the similarity of their karyotype, while *O. vulgaris* had a special karyotype which deviated from the other three species. This may explain the difference between the present study and the conclusions of molecular analysis methods. Furthermore, without quantization of gene mutation effects, using only formulas to describe the karyotype structure creates limitations in our ability to fully determine the genetic relationship. Ideally, genetic and karyological information should be combined in phylogenetic analyses.

In view of this, more detailed cephalopod chromosome information is urgently needed to facilitate comprehensive analyses of genetic relationships at the cytological level. Fluorescence in situ hybridization (FISH), which enables visualization of target DNA sites on chromosomes through a signal display using probes, has been widely applied in chromosomal localization (Colomba et al. 2002, Zhang et al. 2007, Wang et al. 2015, Escudero et al. 2016) and gene mapping (Ishizuka et al. 2016, Yasukochi et al. 2016) for many years; however, there is only one report of its use in cephalopods, which was based on the localization of telomere sequence (Adachi et al. 2014). In order to improve our understanding of cephalopod karyotypes, the development of

chromosomal markers with higher resolution is needed to identify chromosome gene structure (Amar-Basulto et al. 2011). For example, if the complete telomere sequence positioning of *O. vulgaris* was available, we could determine whether chromosome translocation or rearrangements have taken place during its evolution.

In this study, we revealed the karyotypes of three octopods, bringing the total to nine reliable cephalopod karyotypes. Furthermore, this is the first study to determine the genetic relationship among these nine species at the cytological level by cluster analysis based on the karyotype evolutionary distance and resemblance-near coefficient. Our results demonstrated the feasibility of D_e cluster analysis for cephalopod taxonomic classification, which could serve an important auxiliary means of routine phylogenetic analysis and provide insights into chromosome evolution.

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Supplementary material 1

Chromosome relative length, supplemental formulae

Authors: Jin-hai Wang, Xiao-dong Zheng

Data type: statistical data

Explanation note: Chromosome relative length, supplemental formulae and all of the original images are made available under the online digital repository Figshare, and it is free to access, in adherence to the principle of open data, more details in https://figshare.com/s/8d21a0db9ffe1f17d279

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RESEARCH ARTICLE

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Diversification of the P genome among *Agropyron* Gaertn. (Poaceae) species detected by FISH

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Abstract

The genomes of five *Agropyron* Gaertner, 1770 species were characterized using all potential di- or trinucleotide simple sequence repeat (SSR) motifs and four satellite DNA repeats as fluorescence in situ hybridization (FISH) probes. The sites of 5S and 45S rDNA were relatively conserved among the diploid and tetraploid species. A number of sites for the dinucleotide SSRs AC, AG, and pSc119.2 was detected in all investigated species except *A. mongolicum* Keng, 1938. Several different trinucleotide SSRs were identified in different tetraploid species. All *Agropyron* species were suggested to include the basic P genome, although genome differentiation was still observed. The P genome of *A. mongolicum* was distinct from that of the diploid *A. cristatum* (Linnaeus, 1753) Gaertner, 1770. and other tetraploid species, with no hybridizations for AC, AG, or pSc119.2 observed. This finding supports designation of the P genomes of *A. cristatum* and *A. mongolicum* as P^c and P^m, respectively. An exceptional 5S rDNA site revealed in one set of homoeologous chromosomes strongly supports the allopolyploid origin of *A. desertorum* (Fischer ex Link, 1821) Schultes, 1824. However, the diploid donors to *A. desertorum* need further investigation. Similarly, the unique FISH pattern of a pair of 5S rDNA-carrying chromosomes was indicative of a potential allopolyploid origin for *A. fragile* (Roth, 1800) Candargy, 1984. The conserved distribution of 5S and 45S rDNA suggests *A. cristatum* (4x) and *A. michnoi* Roshevitz, 1929 are closely related. Two forms of B chromosomes were identified among individuals *A. mongolicum* and *A. desertorum* plants.

Keywords
Agropyron, FISH, P genome, rDNA, repetitive sequence

Introduction

The genus *Agropyron* Gaertner, 1770, also referred to as the crested wheatgrass complex, includes 10–15 species (Asay and Jensen 1996). The genus is distributed in Eurasia and comprises a series of diploid (2n = 14), tetraploid (2n = 28), and hexaploid (2n = 42) species (Löve 1982, 1984, Dewey 1984). The diploid species are less common and distributed from Europe to Mongolia, whereas tetraploids commonly occur in central Europe, the Middle East, and central Asia. Hexaploids are rare in Turkey and Iran (Dewey and Asay 1982). The spike morphology is important for identification for the species, and varies in a continuous fashion from broad, pubescent, pectinate spikes to narrow, linear, glabrous spikes (Dewey and Asay 1982). The species taxonomy is confounded by overlapping geographic distributions and high cross-compatibility among species. The taxonomy of *Agropyron* spp. has been characterized as "a multitude of taxonomic binomials" (Dewey and Pendese 1967). Although morphologically diverse, all crested wheat grasses are considered to share the same basic genome; this was previously termed the C genome and is currently referred to as the P genome (Dewey and Pendese 1967).

Inter-specific differentiation in *Agropyron* has been extensively studied using morphology, cytology and molecular markers. *Agropyron mongolicum* Keng, 1938 is a diploid species indigenous to northern China. It is distinguished from the diploid species *A. cristatum* (Linnaeus, 1753) Gaertner, 1770 based on its narrow, linear spikes (Dewey 1981). Hsiao et al. (1986, 1989) revealed *A. cristatum* and *A. mongolicum* share the same basic P genome but differ as a result of structural rearrangement of some chromosomes. These findings were based on a karyotype analysis and meiotic analysis of interspecific hybrids. Data from amplified fragment length polymorphism (AFLP) markers showed that *A. mongolicum* clusters separately from other *Agropyron* species, including *A. cristatum* and *A. fragile* (Roth, 1800) Candargy, 1984 (Mellish et al. 2002). These findings suggest that the P genomes of *A. cristatum* and *A. mongolicum* should be designated "P^c" and "P^m", respectively (Mellish et al. 2002). The origin of *A. desertorum* (Fischer ex Link, 1821) Schultes, 1824 has been extensively discussed. Taylor and McCoy (1973) concluded that *A. desertorum* originated from amphidiploids between *A. imbricatum* Roemer & Schultes, 1817 and *A. pectiniforme* Roemer & Schultes, 1817 on the basis of chromatographic and karyotypic analysis. Both *A. imbricatum* and *A. pectiniforme* have been reclassified subsequently as subspecies of *A. cristatum* (Tzvelev 1976). On the basis of morphology and meiotic relationship in hybrids between *A. mogolicum* × *A. cristatum* amphiploid and *A. desertorum*, Asay et al. (1992) suggested that the ancestral germplasm of *A. desertorum* was derived from hybridization between *A. mongolicum* and *A. cristatum*, followed by chromosome doubling. In the same study, the authors further proposed that *A. fragile* is an autotetraploid derivative of *A. mongolicum*, and that variants of *A. desertorum* could be generated from hybrids between *A. fragile* and

tetraploid *A. cristatum* (Asay et al. 1992). Molecular data from AFLP markers suggest that *A. desertorum* is an alloploid incorporating the P genomes of *A. mongolicum* and *A. cristatum*, and thus the genome of *A. desertorum* could be designated "$P^cP^cP^mP^m$" (Mellish et al. 2002). However, Vogel et al. (1999) concluded that the genome length of *A. desertorum* was more consistent with that of an autoploid of *A. cristatum* than that of an allotetraploid between *A. cristatum* and *A. mongolicum*. Moreover, Che et al. (2015) distinguished populations of *A. desertorum*, *A. mongolicum* and *A. michnoi* Roshev. from populations of *A. cristatum* by means of principal coordinate analysis. The results suggest that *A. desertorum*, A. *mongolicum* and *A. michnoi* Roshevitz, 1929 are derivative species of *A. cristatum* that share the same basic genome as the ancestral species.

Fluorescence *in situ* hybridization (FISH) is a powerful tool for characterization of genome composition based on a few repetitive sequences. Species-level phylogenies across species can be derived from repeat-based comparative FISH karyotyping (Jiang and Gill 2006, Heslop-Harrison and Schwarzacher 2011). Comparative FISH may allow to detect genome differentiation between closely related species (Carmona et al. 2013, Dou et al. 2016).

The genomes of Triticeae species are large, of which 75% of the genome comprises repetitive sequences (Flavell et al. 1974, 1977). The genome composition in Triticeae species can be characterized using different microsatellite or satellite repetitive sequences via FISH (Cuadrado et al. 2013; Dou et al. 2016). In the present study, comparative FISH was conducted using all potential dinucleotide and trinucleotide simple sequence repeats (SSRs), 5S and 45S ribosomal DNA (rDNA), pSc119.2, and pAs1 repeats on mitotic chromosomes of five *Agropyron* species. The objective was to examine the genome differentiation among species within the genus *Agropyron* and infer genetic relationships among the investigated *Agropyron* species.

Material and methods

Plant material

Six accessions belonging to *A. mongolicum*, *A. cristatum*, *A. desertorum*, *A. fragile*, and *A. michnoi* were used in this study (Table 1). Seeds of each accession were randomly selected for cytogenetic investigation.

Table 1. Plant materials used in the study.

No.	Species	Provenance
1	*Agropyron mongolicum* Keng, 1938	Inner Mongolia, China
2	*Agropyron cristatum* (2x) (Linnaeus, 1753) Gaertner, 1770 'Fairway'	USA
3	*Agropyron cristatum* (4x) (Linnaeus, 1753) Gaertner, 1770	Qinghai, China
4	*Agropyron desertorum* (Fischer ex Link, 1821) Schultes, 1824 'Nordan'	USA
5	*Agropyron fragile* (Roth, 1800) Candargy, 1984	Inner Mongolia, China
6	*Agropyron michnoi* Roshevitz, 1929	Inner Mongolia, China

Slide preparation

The seeds were germinated on moist filter paper in Petri dishes at room temperature. Root tips of of 1–2 cm length were excised and pretreated with nitrous oxide at a pressure of 8 atm for 2 h at room temperature following the method of Kato (1999). Subsequently, the root tips were fixed in ethanol–glacial acetic acid (3:1, v/v) for at least 30 min at room temperature. Each root tip was squashed in a drop of 45% acetic acid.

DNA probes and labelling

All potential dinucleotide and trinucleotide SSRs and four satellite DNA sequences, namely pAs1 (Rayburn and Gill 1986), pSc119.2 (Bedbrook et al. 1980), 5S and 45S rDNA, were used to generate DNA probes. The procedure for labeling the above sequences was performed in accordance with the method of Dou et al. (2016).

FISH and microphotometry

The FISH experiments were conducted following the method of Dou et al. (2009; 2016). The images were captured with a Photometrics CoolSNAP CCD camera (Roper Scientific, Trenton, NJ, USA) using a fluorescence microscope (model DM R HC, Leica Microsystems, Wetzlar, Germany) and processed with the Meta imaging system (Universal Imaging Corporation, West Chester, PA, USA). Finally, the images were adjusted with Adobe Photoshop 6.0 for contrast and background optimization.

Results

Karyotype features of the P genome among species

A chromosome number of 2n = 14 was detected in *A. mongolicum* (Fig. 1a–c) and *A. cristatum* (Fig. 1d–h), whereas a chromosome number of 2n = 28 was detected in most individuals of the other *Agropyron* species (Figs 1i–r, 2a–r). Both diploid and tetraploid species were detected. However, monosomic chromosomes were identified in many cases in tetraploid species (Figs 1o, r 2k, r). The repetitive sequence pAs1 showed multiple subtelomeric, intercalary, or pericentromeric hybridization sites on all chromosomes. The hybridization pattern of pAs1 was much more useful for distinguishing each chromosome of the P genome in all species compared with the other repetitive sequences. The tentative karyotypes of all individuals analyzed were determined using pAs1 as a land marker and the chromosome arm ratio and relative length as references (Fig. 3). Four pairs of metacentric chromosomes and three pairs of submetacentric chromosomes were identified in both *A. cristatum* and *A. mongolicum*. In addition,

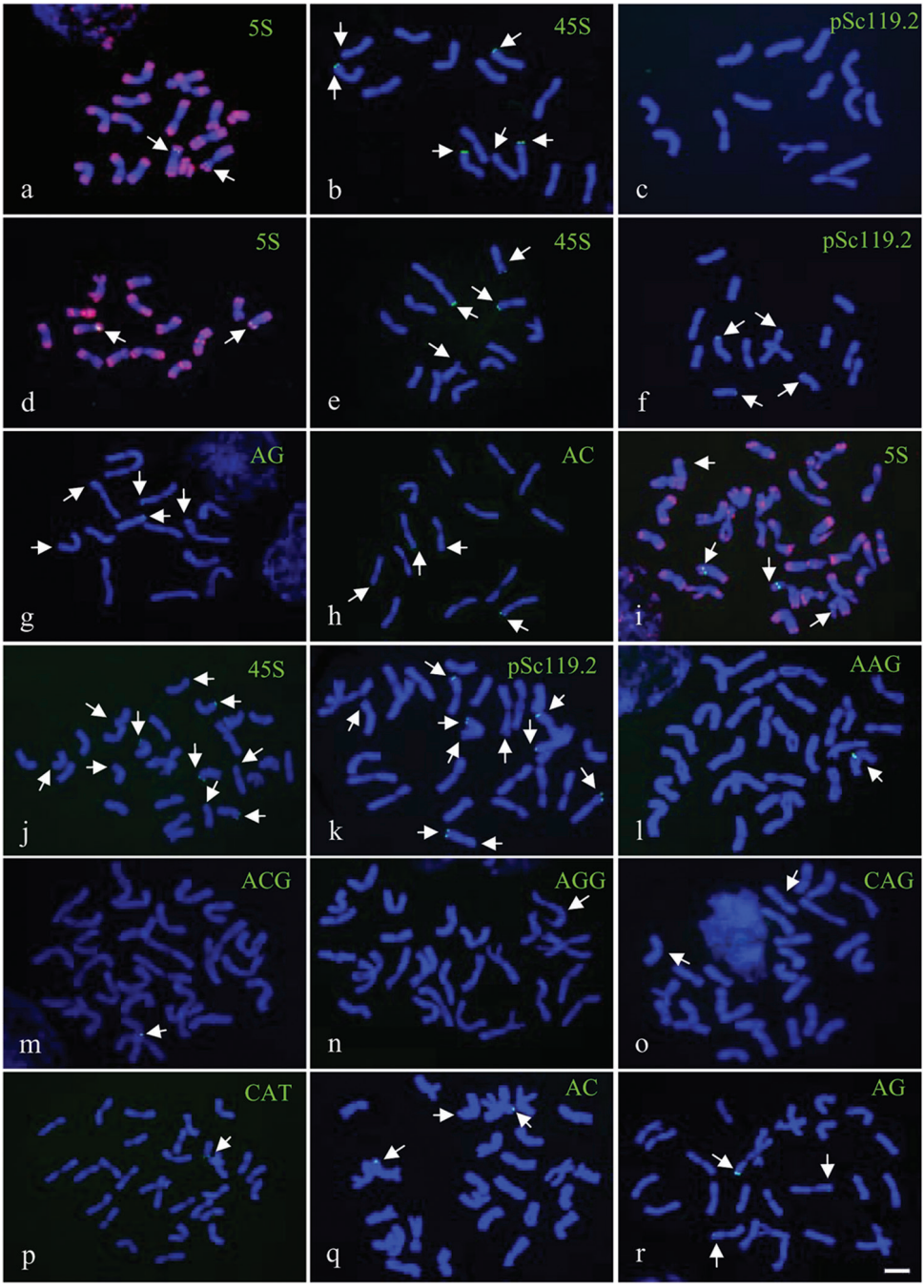

Figure 1. FISH patterns of mitotic metaphase chromosomes detected using pAs1 probes (red) combined with 5S rDNA, 45S rDNA, and pSc119.2 probes in *A. mongolicum* (**a–c**); 5S rDNA, 45S rDNA, pSc119.2, AG, and AC in *A. cristatum* (2x) (**d–h**); 5S rDNA, 45S rDNA, pSc119.2, AAG, ACG, AGG, CAG, CAT, AG, and AC in *A. cristatum* (4x) (**i–r**). The pAs1 signals (red) were removed artificially in all images except in (**a, d, i**). Arrows indicate the target signals (green). Bar = 10μm.

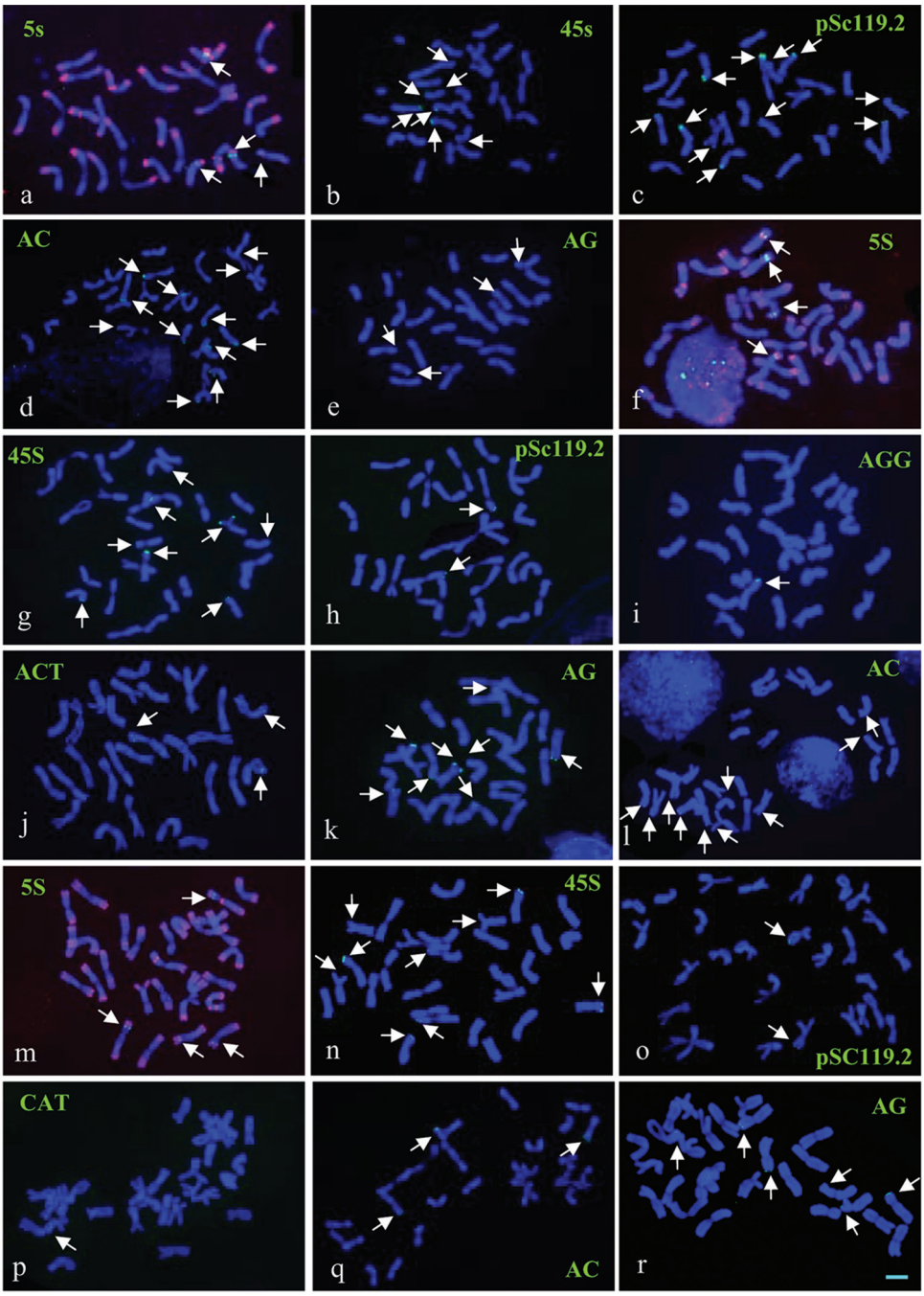

Figure 2. FISH patterns of mitotic metaphase chromosomes detected using pAs1 probes (red) combined with 5S rDNA, 45S rDNA, pSc119.2, AG, and AC probes in *A. desertorum* (**a–e**); 5S rDNA, 45S rDNA, pSc119.2, AAG, ACT, AG, and AC in *A. fragile* (**f–l**); 5S rDNA, 45S rDNA, pSc119.2, CAT, AC, and AG in *A. michnoi* (**m–r**). The pAs1 signals (red) were removed artificially in all pictures except in (**a, f, m**). Arrows indicate the target signals (green). Scale bar = 10 μm.

eight pairs of metacentric chromosomes and six pairs of submetacentric chromosomes were identified in the other tetraploid species. Although a high level of intra- and inter-species chromosomal polymorphisms were detected, homoeologous chromosomes among species were tentatively identified and designated 1-7 (1^-7^) (Fig. 3). In this designation, the numerals do not correspond with those of the homoeologous groups used in the nomenclature system of the chromosomes of wheat and barley. Seven homoeologous chromosomes were identified:

- Chromosome 1 is a metacentric chromosome with pAs1 hybridization signals in pericentromeric regions in most cases.
- Chromosome 2 is a metacentric chromosome lacking pAs1 hybridization signals in the subtelomeric region of the short arm in most cases.
- Chromosome 3 is a metacentric chromosome with pAs1 hybridization signals distributed from the intercalary to subtelomeric regions of both arms.
- Chromosome 4 is the smallest metacentric chromosome.
- Chromosome 5 is the largest submetacentric chromosome.
- Chromosome 6 is a submetacentric chromosome with the smallest arm ratio (short arm to long arm).
- Chromosome 7 is a submetacentric chromosome lacking pAs1 hybridization signals in the subtelomeric region of the short arm.

Chromosomal distribution of 5S rDNA, 45S rDNA and pSc119.2 among species

The hybridization sites of 5S rDNA and 45S rDNA were stably detected in all analyzed species. The 5S rDNA hybridization signals were detected in one pair of chromosomes in the diploid species *A. mongolicum* and *A. cristatum* (Fig. 1a, d) and in two pairs of chromosomes in the other tetraploid species (Figs 1i, 2a, f, m). The physical position of the 5S rDNA sites were the most conserved, which were stably detected in the intercalary regions of the short arms of homoeologous chromosome 6 (6 and 6^ chromosomes). Notably, additional 5S rDNA sites in the pericentromeric regions of the long arms of chromosome 6^ and additional 5S rDNA sites in the intercalary regions of the short arms of chromosome 6^ were detected in *A. desertorum* and *A. fragile*, respectively (Fig. 3).

Four highly conserved 45S rDNA sites were stably revealed in subtelomeric regions in both diploid species (Fig. 1b and e). However, variable numbers of 45S rDNA sites (from four to six) were identified in tetraploid species: six for *A. cristatum* (Fig. 1j), five for *A. desertorum* (Fig. 2b), four for *A. fragile* (Fig. 2j) and five for *A. michnoi* (Fig. 2n). Most of the 45S rDNA sites were physically mapped to the subtelomeric regions of homoeologous chromosomes 2, 5, 6, and 7 among the tetraploid species. Occasionally, 45S rDNA sites were detected on chromosome 1 and in the subtelomeric regions of the long arms of chromosomes 4 and 6 in *A. desertorum* (Fig. 3). The 45S rDNA sites on homoeologous chromosome 6 were the most conserved, which were detected on

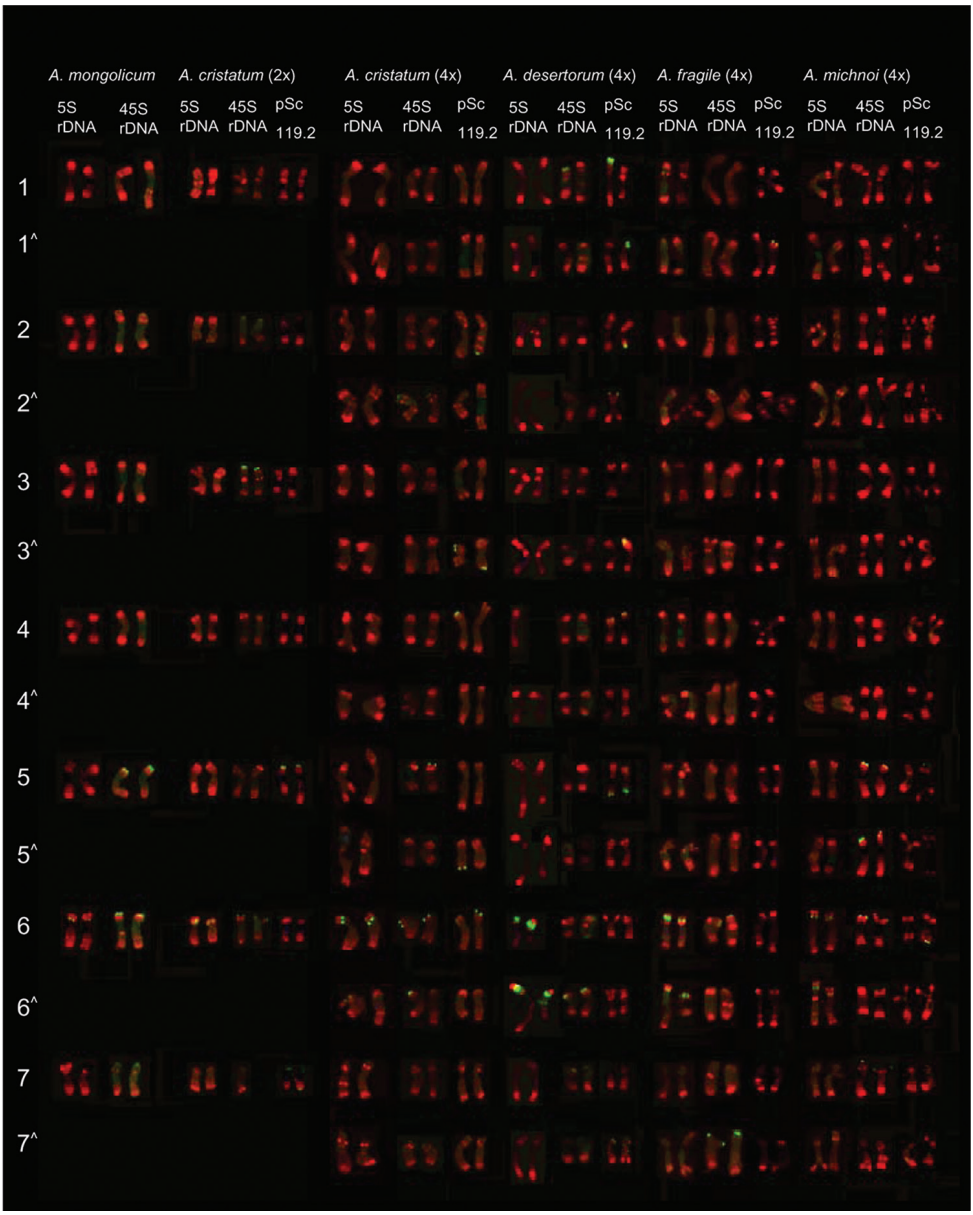

Figure 3. Molecular karyotypes of six *Agropyron* species probed using pAs1 (red) combined with 5S rDNA (green), 45S rDNA (green) or pSc119.2 (green) sequences. The numbers 1 to 7 designate seven different homologous chromosomes, whereas the same number followed by "^" indicates the respective homoeologous chromosomes in each genome of tetraploid species.

both chromosomes 6 and 6^ in all tetraploid species. In addition, the 45 S rDNA sites on homoeologous chromosome 7 were only detected on one set of the homoeologous chromosomes (7 or 7^) (Fig. 3, Table 2).

Table 2. Number of hybridization sites and chromosomal distribution of the repetitive sequences for each *Agropyron* species.

Repeats	Species					
	A. mongolicum	***A. cristatum* (2x)**	***A. cristatum* (4x)**	***A. desertorum***	***A. fragile***	***A. michnoi***
5S rDNA	1" (6)	1" (6)	2" (6, 6^)	2" (6, 6^)	2" (6, 6^)	2" (6, 6^)
45S rDNA	4" (2, 5, 6, 7)	4" (2, 3, 6, 7)	6" (2^, 5, 5^, 6, 6^, 7^)	2"+3' (6^, 7^+1, 4^, 6)	4" (2^, 6, 6^, 7^)	4"+1' (5, 5^, 6, 7+6^)
pSc119.2	–	2" (5, 7)	4"+1" (3^, 4, 5^, 6^, +2)	3"+5' (4, 5, 7^+1, 1^, 2, 2^, 3^)	2' (1^, 6^)	2' (5, 6)
AC	–	1' (1)	1"+1' (1+1^)	2"+4' (1, 1^+ 2, 2^, 3, 4)	4"+2' (1, 1^, 2, 2^+6, 7)	1"+1' (4+ 6^)
AG	–	1"+1' (5+2)	1"+1' (1+3')	4' (2, 2^, 5, 7)	2"+4' (3, 4^+ 2^, 3^, 5, 6)	2"+2' (6, 2+2^, 3)
AAG	–	–	1' (2)	–	–	–
ACG	–	–	1' (2)	–	–	–
ACT	–	–	–	–	1"+1' (4+4^)	–
AGG	–	–	1' (5)	–	1' (2)	–
CAG	–	–	1" (2)	–	–	–
CAT	–	–	1' (2)	–	–	1' (5)

A highly variable number of pSc119.2 hybridization sites was identified at either or both ends of the chromosomes in all diploid and tetraploid species, except *A. mongolicum*. The tetraploid species *A. cristatum* (Fig. 1k) and *A. desertorum* (Fig. 2c) harbored a high number of pSc119.2 hybridization sites (5–8; Table 2), whereas the other species carried as few as two sites. No conserved pSc119.2 sites were identified among the species.

Chromosomal distribution of dinucleotide and trinucleotide SSRs among species

Among the four potential dinucleotide SSR probes, $(AC)_{15}$, $(AG)_{15}$, $(AT)_{15}$, and $(GC)_{15,}$ only $(AC)_{15}$ and $(AG)_{15}$ produced detectable hybridization signals, which were observed in subtelomeric regions, in all species analyzed, except *A. mongolicum* (Figs 1 and 2, Table 2). The number of hybridization signals for both $(AC)_{15}$ and $(AG)_{15}$ was highly among the deferent species. *Agropyron desertorum* (Fig. 2d) and *A. fragile* (Fig. 2l) carried a high number of $(AC)_{15}$ hybridization signals (8–10), whereas the other species carried a low number of $(AC)_{15}$ hybridization signals (1–3) (Table 2).

Although the $(AC)_{15}$ hybridization signals were frequently detected in homoeologous 1 chromosomes in most species, the conserved distribution of the $(AC)_{15}$ signal among all investigated species was not observed. *Agropyron fragile* (Fig. 2k) and *A. michnoi* (Fig. 2r) carried a high number of $(AG)_{15}$ hybridization signals (6–8), whereas the others carried low number of $(AG)_{15}$ hybridization signals (3–4; Table 2).

Numbers followed by " or ' are the number of hybridization sites of the respective sequence. " Indicates a pair of homologous chromosomes; ' indicates one of the homologous chromosomes. Numbers in parentheses indicate the chromosome designation shown in Fig. 3

All ten potential trinucleotide SSRs probes, $(AAG)_{10}$, $(AAC)_{10}$, $(AAT)_{10}$, $(ACG)_{10}$, $(ACT)_{10}$, $(AGG)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, and $(GGC)_{10}$, were used to characterize the chromosomes of all species. No hybridization signals for any trinucleotide SSR probes were detected in the diploid species *A. mongolicum* and *A. cristatum* 'Fairway' or in the tetraploid species *A. desertorum*. A small number of SSR hybridization signals were identified in *A. cristatum* (4x), *A. fragile*, and *A. michnoi*, although these signals were not commonly shared among the species. *Agropyron cristatum* (4x) harbored the greatest number of trinucleotide SSR hybridization sites. One hybridization site of $(AAG)_{10}$, $(ACG)_{10}$, $(AGG)_{10}$, $(CAG)_{10}$ and $(CAT)_{10}$ was well identified in the individuals of *A. cristatum* (4x) (Fig. 1l–p). All $(AAG)_{10}$, $(ACG)_{10}$, $(CAG)_{10}$ and $(CAT)_{10}$ sites were physically mapped to the pericentromeric regions of the long arms of chromosome 2. This finding implies the co-localized distribution of $(AAG)_{10}$, $(ACG)_{10}$, $(CAG)_{10}$, and $(CAT)_{10}$ in *A. cristatum* (4x). However, $(AGG)_{10}$ was physically mapped to the pericentromeric region of the short arm of chromosome 5 (Table 2). Two trinucleotide SSRs $(ACT)_{10}$ and $(AGG)_{10}$, gave rise to hybridization signals in *A. fragile* (Fig. 2i and j). The SSR $(ACT)_{10}$, which was not detected in the other species analyzed, was localized in the intercalary regions of the short arms of chromosome 4 in *A. fragile* (Fig. 2j).The $(AGG)_{10}$ was hybridization site was physically mapped to subtelomeric regions in *A. fragile* (Fig. 2i) rather than to pericentromeric regions as in *A. cristatum* (4x) (Fig. 1n). Only one trinucleotide SSR, $(CAT)_{10}$, was detected in *A. michnoi*. The $(CAT)_{10}$ was hybridizatin site was localized to intercalary regions in *A. michnoi* (Fig. 2p) rather than to pericentromeric regions as in *A. cristatum* (4x) (Fig. 1p).

B chromosome in *A. mongolicum* and *A. desertorum*

B chromosomes were identified in a few individuals of *A. mongolicum* (Fig. 1a) and *A. desertorum* (Fig. 2b) but not in the other *Agropyron* species analyzed. The B chromosomes in *A. mongolicum* and *A. desertorum* were intensely stained by the pAs1 probe. A distinct primary constriction was identified in the B chromosomes of both species. On the basis of the position of the primary constriction, the B chromosome in *A. mongolicum* was submetacentric, whereas that of *A. desertorum* was of metacentric type (Fig. 4).

Figure 4. FISH patterns of B chromosomes detected by pAs1 (red). **a** submetacentric B chromosomes in *A. mongolicum* **b** metacentric B chromosomes in *A. desertorum.*

Discussion

Genomic characterization of the P genome in Agropyron using repetitive sequences

In this study, all potential dinucleotide and trinucleotide SSRs and four satellite DNA repeats were used to characterize the P genome of five *Agropyron* species. Unlike other genomes such as the H, I, A, B, and D genomes in Triticeae (Cuadrado et al. 2007; 2008; Dou et al. 2016), which include a number of SSR hybridization signals based on FISH, the P genome in *Agropyron* harbored only the dinucleotide SSRs AG and AC in all species, except *A. mongolicum*, and fewer trinucleotide SSRs in the different species. The distribution of AAG corresponded to the distribution of N bands, which are composed of heterochromatin in Triticeae (Pedersen et al. 1996). In the P genome, only one AAG site was detected in the intercalary regions in one accession, *A. cristatum* (4x). The pAs1 in the P genome was detected more frequently in subtelomeric or pericentromeric regions than in intercalary regions, and the repeatedly detected 45S rDNA, pSc119.2, AC, and AG repeats were localized to subtelomeric regions. Forty-eight P-genome-specific sequences have been identified from the DOP-PCR product of the 6P chromosome of *A. cristatum*, and 14 of 48 sequences have been physically mapped to chromosomes, centromeres, pericentromeric regions, or subtelomeric regions throughout the P genome (Han et al. 2017). Taken together, the results of the present study suggest that the tandem-repetitive sequences in the P genome are much more intensively localized to the pericentromeric and subtelomeric regions rather than the intercalary regions.

The lack of tandem-repetitive sequences in intercalary regions makes the accurate discrimination of each chromosome of the P genome more difficult. In this study, 5S rDNA, 45S rDNA, and pAs1 repeats were more stable and informative. Four to five pairs or homoeologous chromosomes could be accurately identified in each diploid and tetraploid species based on the above-mentioned chromosomal markers, the chromosome length and the arm ratio.

P genome differentiation and genetic relationships of the *Agropyron* species

The P genome of *A. mongolicum*, which includes no AG, AC or pSc119.2 repeats in the subtelomeric regions of any chromosome, is distinct from the P genome of the other

Agropyron species analyzed. Structural rearrangements of some chromosomes were revealed between the two diploid species *A. cristatum* and *A. mongolicum* (Hsiao et al. 1986, 1989). In the present study, genome rearrangements were difficult to identify accurately, reflecting the low resolution of the chromosomal markers. However, discrepancies in the localization of the 45S rDNA sites were revealed on chromosomes 5 and 3 in *A. mongolicum* and *A. cristatum*, respectively. This observation strongly suggested that the chromosome structure arrangements involved these two chromosomes. The differentiation of both diploid species revealed in the present study suggests that the P genome of *A. cristatum* should be designated P^c, and the P genome of *A. mongolicum* should be designated P^m, consistent with the suggestions of Mellish et al. (2002). *Agropyron mongolicum* differs from other *Agropyron* species in producing a narrow, linear, glabrous spike (Dewey 1981). Data from AFLP markers discriminated *A. mongolicum* from the other *Agropyron* species (Mellish et al. 2002). Taken together, these results are strongly suggestive of a distant relationship between *A. mongolicum* and the other *Agropyron* species.

The tetraploid *A. cristatum* is considered to be an autopolyploid originating from the diploid species *A. cristatum*. However, the molecular karyotype of this tetraploid was not consistent with a doubled diploid karyotype. High variation in the number of hybridization sites and localization of 45S rDNA, AC, AG, and pSc119.2 repeats was revealed in the present study. Notably, a few trinucleotide SSRs were detected in tetraploid species rather than in diploid species. These observations indicate that high genomic variation, including DNA sequence deletion, amplification and genomic rearrangement, might have occurred in the transition from diploid to tetraploid species.

Agropyron desertorum is considered to be an allotetraploid between diploid *A. mongolicum* and *A. cristatum* (Asay et al. 1992, Mellish et al. 2002). The localization of a numer of 45S rDNA sites in *A. desertorum* was highly variable compared with the other species analyzed. Notably, one homoeologous set of chromosome 6 (6^) contained additional 5S rDNA sites in the pericentromeric regions of the long arm. The homoeologous chromosome 6 stably carried 5S rDNA sites in the short arms of all *Agropyron* species studied. The distinct differences in the FISH patterns between chromosomes 6 and 6^ strongly support the allopolyploid origin of *A. desertorum*. However, the FISH pattern of chromosome 6^ in *A. desertorum* was not similar to either *A. mongolicum* or *A. cristatum*.

Agropyron fragile has been suggested to be an autotetraploid derivative of *A. mongolicum* (Asay et al. 1992). The chromosomes of *A. fragile* include a few AG, AC, and pSc119.2 hybridization sites. The genomic characters of *A. fragile* are different from those of *A. mongolicum*. The present results did not support the suggestion that *A. fragile* is an autotetraploid derivative of *A. mongolicum*. Moreover, additional 5S rDNA sites were revealed in the intercalary regions of the short arms of one homoeologous set of chromosome 6 (6^). Similar to *A. desertorum*, the unique FISH pattern revealed in chromosome 6^ is supportive of a potential allopolyploid origin for *A. fragile*.

Agropyron michnoi exhibited a FISH pattern more similar to that of *A. cristatum* (4x). Specifically, the chromosomal co-linearity of the detected 45S rDNA sites was well retained between the two species, suggesting that *A. michnoi* might be more closely related to *A. cristatum* (4x) than to other *Agropyron* species studied.

Variability of the repetitive sequences in the P genome

The repeats, such as AC, AG, and pSc119.2, that are localized in subtelomeric regions, showed a highly varying number of hybridizations among species with the P genome and within populations, particularly in tetraploid species. The presence or absence of hybridization signals might reflect the deletion or duplication of the repetitive sequences. Unequal crossing over is a type of gene duplication or deletion event (Graur et al. 2000). Highly variable repeats of the above-mentioned sequences in tetraploid species, which include two sets of the basic P genome, suggests that variability of repeats may be strongly driven by unequal crossing over during the interfered meiosis process in tetraploid species with two similar genomes. The small number of trinucleotide SSRs hybridization sites and aneuploids frequently detected in tetraploid species might further confirm interference of meiosis in tetraploid species.

Specific FISH patterns of trinucleotide SSRs were detected in three different *Agropyron* species. Extensive intra- or inter-population genetic variation has been detected in *Agropyro*n species (Mellish et al. 2002, Che et al. 2011, Chen et al. 2013). However, limited materials of each species were analyzed in the present study. Whether the FISH patterns of trinucleotide SSRs also exhibit high intraspecific variation needs further investigation.

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Cestrum strigilatum (Ruiz & Pavón, 1799) B chromosome shares repetitive DNA sequences with A chromosomes of different *Cestrum* (Linnaeus, 1753) species

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Abstract

Species of *Cestrum* (Linnaeus, 1753) have shown large diversity in the accumulation and distribution of repetitive DNA families, and B chromosomes have been described in seven species. Some types of repetitive DNA were identified in A and B chromosomes in species of this plant group, such as AT-rich SSR, 35S and 5S rDNA, C-Giemsa and C-CMA/DAPI bands and retrotransposons. To increase our understanding of the relationships of A and B chromosomes, the B of *C. strigilatum* Ruiz & Pavón, 1799 was microdissected, amplified and hybridized *in situ* against chromosomes of this species, and in six other species of this genus. FISH signals were observed in whole the B of *C. strigilatum*, including stretches of A chromosomes, as well as in some A chromosomes of all tested species. A strong FISH signal was seen adjacent to the 5S rDNA in the proximal region of pair 8 of all species and, due to this, we have searched for 5S rDNA fragments in the microdissected B chromosome. PCR and sequencing data evidenced 5S rDNA deletion along evolutionary pathways of the B of *C. strigilatum*. Although A and B chromosomes displayed redundancy in the repetitive DNA families in different species, the B of *C. strigilatum* seemed to differ from those Bs of other *Cestrum* species by the loss of rDNA fractions. A possible origin of Bs in *Cestrum* was discussed.

Keywords

FISH, karyotypes, microdissection, rDNA, Solanaceae, supernumerary chromosomes

Introduction

B chromosomes have been described as additional and dispensable components of genomes, especially because they show few or no essential genes, abundance of repetitive DNA families, and independent meiotic behavior, without pairing with A chromosomes (Puertas 2002, Jones and Houben 2003). Although new information from the genomic sequencing has improved the knowledge on the B chromosomes of plants (Houben et al. 2014), the exact origin of these chromosomes is difficult to know, and it can be different for each genomic history. It is widely accepted that Bs arise from portions of the A complement by different pathways (Camacho et al. 2000, Jones and Houben 2003), such as chromosome rearrangements and sequence amplifications (Teruel et al. 2010, Valente et al. 2014), or after interspecific hybridizations (Navabi et al. 2011). Since B chromosomes do not exhibit Mendelian inheritance pattern, it is possible that they are maintained in populations by drive mechanisms. Such events are favored by mitotic and/or meiotic instabilities, which can provide an accumulation of Bs in different somatic and germ tissues (Burt and Trivers 2006, Jones 2012).

Approximately 24,000 species of angiosperms (4% of them) have Bs (Levin et al. 2005), and apparently, large genomes could favor or influence the occurrence of B chromosomes (Trivers et al. 2004). This appears to be true for some Solanaceae, since in the genus *Cestrum*, for example, species have 16 chromosomes with up to 12 µm and 20 pg of DNA per diploid set (Paula et al. 2015), and show the highest number of taxa with Bs. These chromosomes have been reported in *C. diurnum* Linnaeus, 1753 (Sobti et al. 1979), *C. parqui* L'Héritier, 1788 × *C. aurantiacum* Lindley, 1844 (Sýkorová et al. 2003), *C. intermedium* Sendtner, 1846, *C. strigilatum* (Fregonezi et al. 2004), *C. parqui, C. euanthes* Schlechtendal, 1832 and *C. nocturnum* Linnaeus, 1753, with 1-2, 1-3 and 1-10 B chromosomes, respectively (Urdampilleta et al. 2015). B chromosomes have also been reported for *Nierembergia aristata* David Don, 1833 (Solanaceae), which also has large chromosomes (Acosta and Moscone 2011).

Species of *Cestrum* exhibit a large variation in the occurrence and distribution of repetitive DNA families (Paula et al. 2015), and some of them have already been identified and associated with B chromosomes (Fregonezi et al. 2004). In the hybrid *C. parqui* × *C. aurantiacum*, for instance, the B chromosome contains 35S and 5S rDNA and SSR AT-rich motifs (Sýkorová et al. 2003). Sequences of rDNA were also identified in Bs of *C. parqui, C. euanthes* and *C. nocturnum* (Urdampilleta et al. 2015). In *C. intermedium* and *C. strigilatum*, besides C-Giemsa$^+$/CMA$^+$/DAPI$^+$ bands (Fregonezi et al. 2004), the Bs also display hybridization signals with the *Gypsy*-like retrotransposons probe but not with rDNA probes (Fregonezi et al. 2007). The accumulation of data on the Bs of *Cestrum* (Sýkorová et al. 2003, Fregonezi et al. 2004, 2007, Fernandes et al. 2008 and Urdampilleta et al. 2015) have constantly prompted us to extend studies on the composition and behavior of these chromosomes, since this genus seems to be the main model for studies of Bs in the scope of the Solanaceae. The aim of this study

was to understand the relationships between the B and A chromosomes of *C. strigilatum,* as well as with A chromosomes of other *Cestrum* species. Additionally, we have also searched any indication on presence of rDNA sequences in this B, in view of the existence of rDNA in Bs of other species of this genus. The discussion addresses aspects about the origin and differentiation of Bs in *Cestrum*.

Material and methods

Samples of *Cestrum strigilatum*, *C. bracteatum* Link & Otto, 1828, *C. corymbosum* Schlechtendal, 1832, *C. laevigatum* Schlechtendal, 1832, *C. mariquitense* Kunth, 1818, *C. sendtnerianum* Martius, 1846, and *C. schlechtendalii* George Don, 1838 from Brazil are maintained in the greenhouse of the Laboratory of Cytogenetics and Plant Diversity, State University of Londrina, and vouchers are kept at the FUEL herbarium.

Root tips were pre-treated with 0.05% colchicine at room temperature for 4 h, fixed in a solution of ethanol/acetic acid (3:1,v:v) for up to 12 h and stored at -20°C. For conventional staining, samples were softened in 2% cellulase plus 20% pectinase (w:v) at 37°C, hydrolyzed in 1 M HCl for 10 min at 60°C, dissected in a drop of 45% acetic acid, and then squashed. Meiotic cells of *C. strigilatum* samples containing a B chromosome were obtained from young anthers, which were collected, directly fixed and dissected as described above. For both cases, after coverslips removal using freezing in liquid nitrogen, samples were stained in 2% Giemsa for conventional analysis and mounted with Entellan (Merck), or stored in 70% ethanol without staining when samples were for microdissection.

B chromosomes were isolated using an inverted Olympus IX71 microscope, equipped with Narishige micromanipulator. The B chromosome of *C. strigilatum* is three times smaller than those chromosomes of A set, being easily recognized and differentiated. We have dissected only those Bs that were far enough from the As, taking care to avoid contaminants. Five microdissections were made, and in each, 15 chromosomes were transferred to sterile tubes. Samples were treated with 1 μg/mL proteinase K at 60°C for 1 h and 30 min and the product was purified using phenol:chloroform (1:1, v:v), and quantified in a Nanodrop 2000 (Thermo). Afterwards, DNA of Bs was amplified using a Random Priming® kit (Invitrogen) with biotin-14-dATP for B probe production.

Sequences of 5S rDNA were amplified by PCR using as template the genomic DNA of *C. strigilatum*, and also a pool of 15 microdissected and purified B chromosomes of the same species. To test the reliability of reactions, three PCR repetitions were done using three different samples of microdissected B chromosomes. For this, two set of primers were used to amplify different fragments: 5S-plant-F 5'CACCGGATCCCATCAGAAACT and 5S-plant-R 5'TTAGTGCTGGTATGATCGCA, for NTS region, and UP46-F 5'GTGCGATCATACCAGCRYTAATGCACCGG and UP47-R 5' GAGGTGCAACACGAGGACTTCCCAGGAGG, for gene coding. PCR were done using a mix containing 2 mM $MgCl_2$, 0.4 μM of each primer,

0.2 mM dNTP, 0.2 mM DNA template, 2 U of *Taq* polymerase and ultrapure water to complete 25 μL. To probe labeling, 0.2 mM dNTP was changed by solution containing dGTP (25%), dCTP (25%), dTTP (25%), dATP (17.5%) and bio- or dig-dATP (7.5%). When UP46 and UP47 primers were used, thermal cycler was adjusting to the following conditions: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 30 sec at 60°C (genome the template) or 30 sec at 50°C (B chromosome) and 1 min at 72°C, and one end of step 5 min at 72°C. When 5S-plant primers were used the conditions were: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 40 sec at 50°C (genome and B chromosome as template) and 1 min at 72°C, and one end of step 5 min at 72°C. To check the presence of 5S rDNA fragments in the B chromosomes, both PCR products were used for FISH. The PCR products were used in a second reaction to produce templates for a Sanger sequencing, using the 3500xL Automatic Sequencer (Applied Biosystems), according to the manufacturer's procedures. For the sequencing three distinct reactions for each primer (F and R) were done, and repeated once. The consensus sequences were obtained after alignment of 12 sequences, in which quality were tested with Phred/Phrap/Consed software, and consensus sequences were contrasted against GenBank (http://www.ncbi.nlm.nih.gov/blast) to check similarities with other 5S rDNA sequences. For FISH, a mixture of 30 μL containing 100% formamide (15 μL), 50% polyethylene glycol (6 μL), 20× SSC (3 μL), 100 ng calf thymus DNA (1 μL), 10% SDS (1 μL) and 100 ng probes (4 μL), was treated at 70 °C for 10 min, placed on ice and immediately applied to the samples. B and 35S rDNA probes were labeled with biotin by random primers and nick translation, respectively. 5S rDNA probes were labeled with biotin or digoxigenin by PCR. Denaturation/hybridization was performed at 95 °C, 50 °C and 38 °C, ten minutes each, followed by 37 °C overnight in a humidified chamber. Post-hybridization washes were carried out in 6× SSC and 4× SSC/0.2% Tween 20 (> 60% stringency), and the probes were detected using avidin-FITC (green) and anti-dig-rhodamine (red) conjugated. Post detection washes were carried out in 4× SSC/0.2% Tween 20 at room temperature. Slides were mounted in 23 μL antifade solution (90% glycerol, 2.3% DABCO, 2% 20 mM Tris–HCl, pH 8.0, plus 1 μL of 2 μg/mL DAPI and 1 μL of 2.5 mM $MgCl_2$).

We have analyzed at least five preparations for each species and of these, at least five metaphases. All the chromosome images were acquired in gray-scale mode using a Leica DM 4500 B microscope equipped with a DFC 300FX camera and overlapped with blue for DAPI, greenish-yellow for FITC and red for rhodamine, using Leica IM50 4.0 software. The images were optimized for contrast and brightness using the GIMP 2.8 Image Editor. To ensure hybridization of the maximum possible sequences in B and A chromosomes, we did not use pre-hybridization or blocking DNA containing excess unlabeled repetitive DNA fractions, which is generally indicated for chromosome painting procedures. This allowed us to ascertain the participation of some A complement DNA fractions in the B composition. Additionally, this allowed us to determine the presence or absence of repetitive sequences in the genomes of other closely related species.

Results and discussion

B of *Cestrum strigilatum*

The screening for B chromosomes in the meiosis of *Cestrum strigilatum* showed that B always appears as univalent, without any kind of pairing with A chromosomes (Fig. 1a). In general, Bs of *C. strigilatum* appear displaced from the other chromosomes at metaphase I/anaphase I (Fig. 1a–b, see also Fregonezi et al. 2004), and more centrally located in the second stage of meiosis (Fig. 1c). The small size of the Bs (about three times less than A chromosomes), as well as the displacement from the A chromosomes, provided preparations of sufficient quality to isolate Bs using the microdissection technique, without any contamination with stretches of other chromosomes.

Chromosome painting using the microdissected B as probe was successfully employed in the *Cestrum* species. This procedure was previously used for chromosome painting in species of *Secale* Linnaeus, 1753*, Allium* Linnaeus, 1753 and *Brachyscome* Cassini, 1816 (see Houben et al. 2001), but when we compare data from literature with our case, the absence of blocking with repetitive fractions in FISH made it possible to see many signals on A chromosomes of *C. strigilatum.* The B chromosome of *C. strigilatum* was totally hybridized, and almost all A chromosomes of this species showed hybridization signals in different positions, such as: i) signals adjacent to C-DAPI bands, ii) lightly dispersed signals throughout the chromosomes, and iii) an intense hybridization signals associated with 5S rDNA sites, in the proximal region of long arm of pair 8 (Fig. 1d-e and 2b). Of all the signals produced by the B chromosome-specific probe, the strong hybridization signal in the 5S rDNA region (Fig. 1e) drew our attention, because previous reports indicate the absence of 5S rDNA in the Bs of *C. strigilatum* and *C. intermedium* (Fregonezi et al. 2004). Some repetitive sequences have been described for Bs of different species of *Cestrum*, such as C-Giemsa$^+$/CMA$^+$/DAPI$^+$ bands, rDNA, SSR and *Gypsy*-like retrotransposons, and all of them also appeared in parts of A chromosomes (Sýkorová et al. 2003, Fregonezi et al. 2004, 2007, Urdampilleta et al. 2015). According to Houben et al. (2014), B chromosomes can be more common in species or groups of species whose genomes are involved in intense chromosomal rearrangements. Moreover, large genomes, as well as those involved with chromosome rearrangements and DNA sequence amplifications could favor the arising of B chromosomes (Trivers et al. 2004, Teruel et al. 2010, Valente et al. 2014). This seems to be the case of *Cestrum* species, since they exhibit large genomes and karyotypes with wide diversity in the distribution of repetitive DNA fraction (Paula et al. 2015).

Detecting 5S rDNA fragments on B chromosome

Previous studies using FISH technique have shown that B of *C. strigilatum* carry no rDNA sequences (Fregonezi et al. 2004). On the other hand, in the B chromosomes

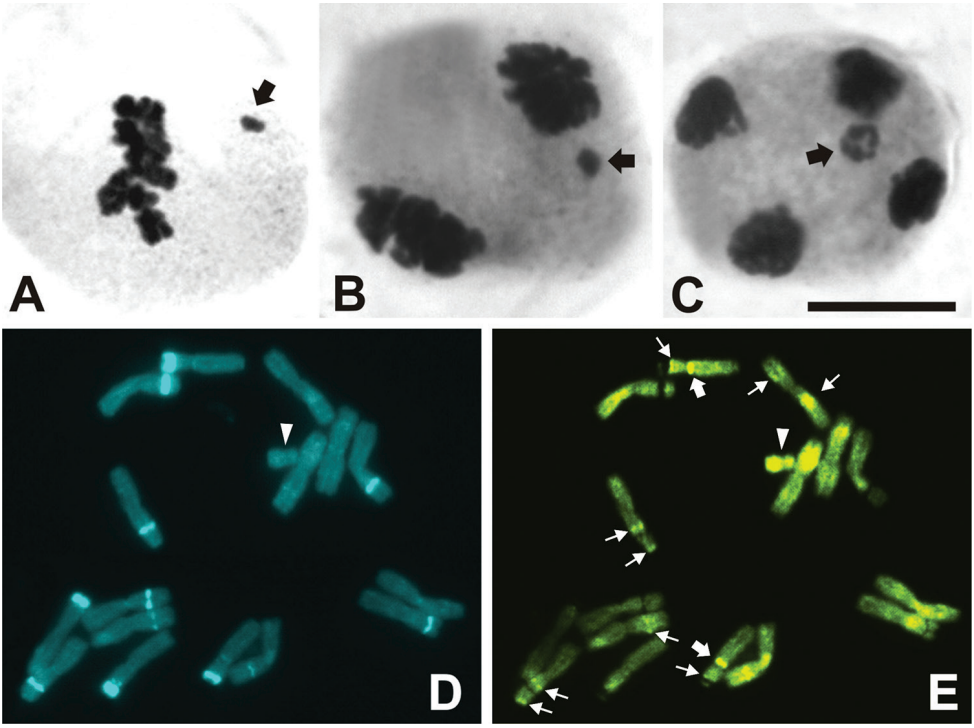

Figure 1. Details of B chromosome of *Cestrum strigilatum* at meiosis, and FISH using the B probe against the karyotype. **A** Metaphase I showing the B as univalent, laterally located (arrow) **B** Anaphase I showing the lagging B univalent moved to one of the complements (arrow) **C** Late anaphase II close to one of the four complements. Note that the chromatids are partially separated (arrow) **D** Mitotic metaphase stained with DAPI. Arrowhead indicates the B **E** Mitotic metaphase hybridized with B probe. Note that some DAPI bands in D appear negative in E, but others do not. Large arrows indicate FISH signal colocalized with 5S rDNA (see also Fig. 2), small arrows point out intercalary and terminal FISH signals, and arrowhead indicates the B completely stained by probe. Bar = 10 μm.

of *C. parqui* × *C. aurantiacum* (Sýkorová et al. 2003) and in *C. parqui, C. euanthes* and *C. nocturnum* (Urdampilleta et al. 2015), 35S and 5S rDNA signals were detected. To test the colocalization of B probe signals with rDNA clusters, a double FISH using p*Ta*71 and p*Ta*794 clones (containing 35S and 5S rDNA of *Triticum* Linnaeus, 1753, respectively) was done after FISH with the B probe in chromosomes of *C. bracteatum, C. corymbosum*, *C. laevigatum*, *C. mariquitense, C. sendtnerianum*, *C. schlechtendalii* and also with *C. strigilatum* with one B. Results showed that there was no association between hybridization signals produced by the B and 35S rDNA probes in any species analyzed. Although the B probe signal appeared colocalized with the signal of 5S rDNA in *C. strigilatum* and in the other six species (Fig. 4a–l), the p*Ta*794 probe containing the 5S rDNA fraction did not hybridize with the B chromosome of *C. strigilatum* (Fig. 2b, f, e). The presence of ribosomal sites on B chromosomes is not uncommon, having been reported for Bs of *Crepis capillaris* (Linnaeus, 1753) Wallroth, 1840, Aster-

aceae (Maluszynska and Schweizer 1989), *Nierembergia aristata,* Solanaceae (Acosta and Moscone 2011) and *Plantago lagopus* Linnaeus, 1753*,* Plantaginaceae (Dhar et al. 2002). This latter species is remarkable because a large amplification of 35S and 5S rDNA sequences contributed to the origin of B chromosome. In addition, the B chromosome of *P. lagopus* exhibits one satDNA (named PLsatB), which represents approximately 3.3% of the genome of individuals with B, that was originated from amplification of 5S rDNA sequences (Kumke et al. 2016). At least for *Cestrum*, the occurrence of rDNA sequences in the Bs seems to be a plesiomorphic feature, and accordingly, the absence of 35S and 5S rDNA signals in the B of *C. strigilatum* raised an issue: Were rDNA sequences lost during B chromosome differentiation in these two species or do these Bs have an independent origin, without any contribution of rDNA sequences?

PCR was conducted using DNA templates of the genome and microdissected B chromosome of *C. strigilatum*, and two distinct primer sets for amplification of different internal 5S rDNA sequences were used (Fig. 2a). The UP46-F and UP47-R primers were used to amplify the coding region, and the 5S-Plant-R and 5S-Plant-F primers were used for amplification of the less-conserved NTS fragment, including a small stretch of the coding region (Fig. 3). The PCR using the UP primers and the *C. strigilatum* genome as template amplified a fragment with 122 bp length, but when the B chromosome was used as template, the PCR product was a 97 bp fragment (Fig. 2a). For the PCR using the 5S-Plant primers, only the genome template produced amplification, with a fragment containing 513 bp (Fig. 2a). Results of Sanger sequencing have shown three scenarios (Table 1): i) that fragment of 122 bp length of UP primers+genome exhibited high identity with a conserved 5S gene coding of *Vigna angularis* (Willdenow) Ohwi & Ohashi, 1969 and *Lilium tsingtauense* Gilg, 1904, ii) in the second, using UP primers+B template, the fragment of 97 bp length showed a partial coverage with the conserved 5S gene coding of *V. angularis* and *L. tsingtauense*. When these two fragments were alingned, they exhibited 86% identity, but the B chromosome fragment showed two internal deletions (18 and 11 bp in length) separated by a short AGA(A/G)C motif, which seems to indicate a degeneration in the middle of the sequence (Figs 3 and S1). In the third case (5S-Plant primers+genome template), the fragment of 513 bp presented high identity with 5S non transcribed sequences (NTS) of the hybrid *C. aurantiacum* × *C. parqui* and *Cestrum psittacinum* (Stapf, 1828) (Fig. 3 and Suppl. material 1). PCR using 5S-Plant primers using B chromosome as template produced no bands. When these three fragments were biotin-labeled and used in FISH reactions, only hybridization signals in the proximal region of the large arm of pair 8 were detected, i.e., the B chromosome of *C. strigilatum* did not exhibit any FISH signals (Fig. 2b–e).

The most reasonable explanation for the positive FISH signals with the B probe in the 5S rDNA region of A chromosomes, would be the presence of one or more repetitive DNA sequences associated with the 5S rDNA. As mentioned above, there is evidence of the involvement of 5S rDNA in the origin of satDNA in Bs of *P. lagopus* (Kumke et al. 2016), but as we still isolate no the satDNA fraction in *C. strigilatum*, aspects on the intern organization of this B is to be answered in future research. Besides

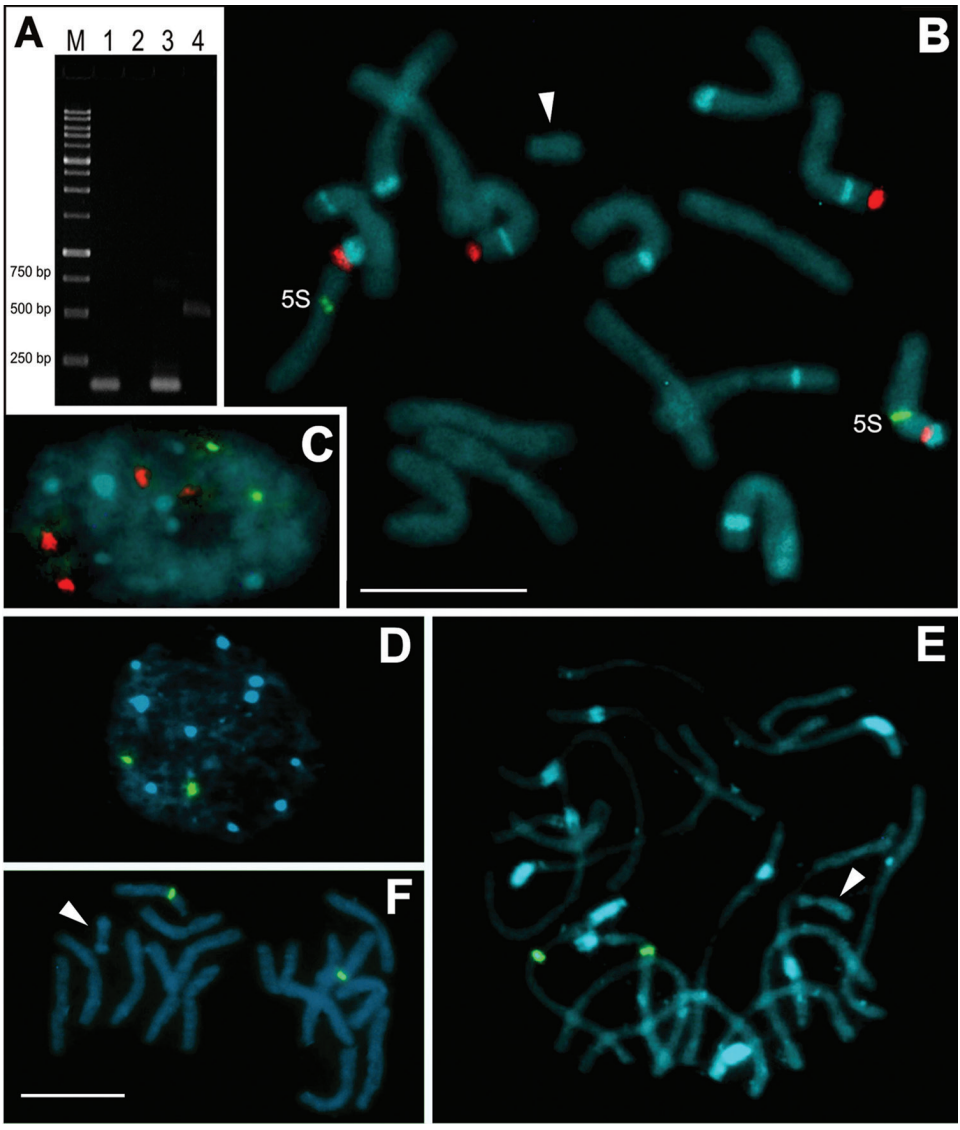

Figure 2. Detection of 5S rDNA by PCR and FISH using probes produced with genomic and B chromosome DNA templates. **A** Electrophoresis gel containing: M = ladder with 250, 500, 750, 1000 bp. Lanes 1 and 3 represent fragments of about 120 bp using the UP primers using the microdissected B chromosome as template (1) and the genome as template (3). Lanes 2 and 4 represent the PCR using 5S-plant primers with microdissected B chromosome as template (see the absence of fragment in lane 2) and with genome as template (see a fragment with about 500 bp length in lane 4 **B–C** Double FISH showing four hybridization sites for 35S rDNA using the p*Ta*71 probe (red) and two sites for 5S rDNA using the UP primer probe of the genome (green) in the chromosomes (**B**) and nucleus (**C**) of *C. strigilatum* **D–F** FISH using the 5S rDNA fragment amplified, using the UP primers and the microdissected B DNA as template. Note only two signals in the nucleus and prometaphase chromosomes of *C. strigilatum* (**D, E** respectively). Note also the absence of hybridization signals in the Bs (arrowheads). Bands in blue color represent AT-rich regions identified by DAPI staining. Bar = 10 μm.

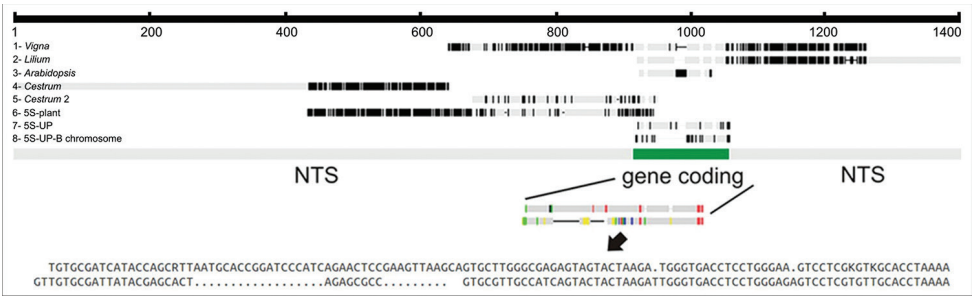

Figure 3. Diagram representing the partial alignment of 5S rDNA sequences of *Vigna* (AP017185.1), *Lilium* (KM117262.1), *Arabidopsis* (AY130622.1), *Cestrum aurantiacum* × *C. parqui* (AY135508.1), *Cestrum psittacinum* (AF495752.1), 5SplantCestrum, 5SUPCestrum and 5SUP-BChrom, for location of gene coding and NTS regions. Note the two internal deletions in the 5S rDNA gene coding of B chromosome sequence (arrow).

Table 1. Results of Blast*n* alignment using 5S-Plant and UP primers PCR products against B chromosomes and genome of *Cestrum strigilatum*

Fragment	5S region	Subject	Accession	Cover	Ident.	*E-value*
5S-plant×Genome	NTS	*Cestrum aurantiacum* × *C. parqui*	AY135508.1	69%	76%	1e-60
5S-plant×Genome	NTS	*Cestrum psittacinum*	AF495752.1	42%	83%	2e-51
UP×Genome	gene coding	*Lilium tsingtauense*	KM117262.1	95%	96%	5e-44
UP×Genome	gene coding	*Vigna angularis*	AP017185.1	95%	96%	5e-44
UP×5S rDNA	gene coding	*Arabidopsis thaliana*	AY130622.1	100%	83%	2e-33
UP×B-chrom	gene coding	*Vigna angularis*	AP016873.1	92%	82%	6e-12
UP×B-chrom	gene coding	*Lilium tsingtauense*	KM117262.1	51%	92%	1e-07

that, 5S rDNA genes can be linked with different repeated sequences, in either coding or non-coding regions, as reviewed by Roa and Guerra (2015). Our results showed that although the conserved 5S rDNA fragment was amplified by PCR using a B chromosome as template, this DNA fraction could be in small copy numbers in the Bs. Besides, since the NTS region was not amplified by PCR using the isolated B as template, it is possible that 5S rDNA has been degraded in B, preventing the visualization of FISH signals. According to Houben et al. (1999), ribosomal DNAs are very dynamic sequences and cistron number can vary between B and A chromosomes.

Detecting repetitive DNA families on A chromosomes

The B chromosome probe of *C. strigilatum* was also used to find complementarities in A chromosomes of six other *Cestrum* species (*C. bracteatum*, *C. corymbosum, C. laevigatum*, *C. mariquitense, C. sendtnerianum*, and *C. schlechtendalii*). FISH signals were detected in terminal, interstitial and proximal chromosome regions of these six species, but they varied in intensity, size and positioning between them. Intercalary

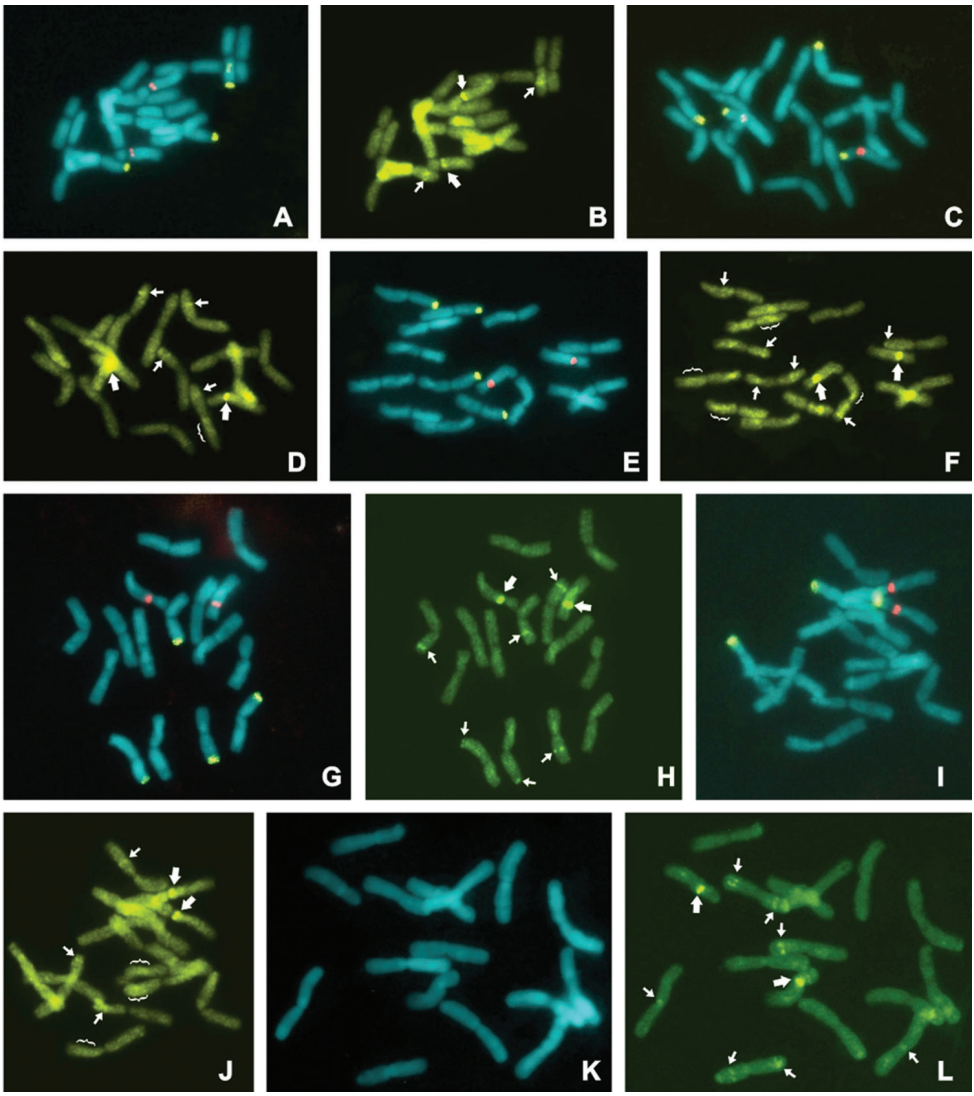

Figure 4. FISH showing the location of the 35S and 5S rDNA sites, and the diversity in the distribution of B probe signals in different species of *Cestrum*. All species showed four terminal 35S rDNA signals (green) and two proximal 5S rDNA signals (red). Note that in all cases, the 35S probe signals did not colocalize with B probe signals, but the 5S rDNA signals did with strong signals with B probe (large arrows). **A–B** *C. sendtnerianum*: the B probe also showed intercalary signals in one chromosome pair, without evidence of scattered hybridization signals. For other species **C–D** *C. corymbosum* **E–F** *C. laevigatum*, (Fig. 2D) **G–H** *C. schlechtendalii* **I–J** *C. bracteatum* (Fig. 2H) and **K–L** *C. mariquitense*, there was a variability in the number of intercalary hybridization signals (small arrows), besides small subterminal and terminal signals. Although all species showed scattered hybridization signals, it was more evident in chromosomes of *C. laevigatum* and *C. bracteatum* (brackets). The **K** image is only a conventional DAPI staining, without FISH with rDNA probes. Bar = 10 μm.

signals were observed in one chromosome pair of *C. sendtnerianum* (Fig. 4b), two pairs of *C. corymbosum* (Fig. 4d), *C. schlechtendalii* (Fig. 4h) and *C. mariquitense* (Fig. 4l) and in three pairs of *C. laevigatum* (Fig. 4f) and *C. bracteatum* (Fig. 4j). Terminal hybridization signals were observed in one pair of *C. laevigatum* (Fig. 4f) and two pairs of *C. schlechtendalii* (Fig. 4h), and proximal signals were observed in one pair of *C. mariquitense* (Fig. 4l). All these FISH signals with the B probe support the idea that repetitive sequences present in different parts of A chromosomes may have been potentially adept to contributing for the formation and evolution of Bs. The example of Bs of *Brachycome dichromosomatica* (Asteraceae) is very illustrative, because the mini B chromosome contains a set of tandemly repetitive sequences are also found in A chromosomes, indicating that B can be involved in more than one event from the A complement (Houben et al. 2001, Jones and Houben 2003). Furthermore, the positive hybridization signals with this probe showed that although *Cestrum* species vary greatly in the distribution of repetitive DNA in karyotypes (Paula et al. 2015), some of these repetitive families are still conserved in the genus. In another example, rye B chromosomes share similar repeats with A chromosomes, differing in abundance retrotransposons (Klemme et al. 2013).

B chromosomes are generally under little or no selection pressure, and due to this, mobile elements and other repetitive DNA lineages may insert, spread, and amplify independently in these chromosomes (Houben et al. 2014). Possibly the dispensable nature of the Bs allowed the stronger chromosome diversification, such those reported for Bs of *C. parqui* × *C. aurantiacum*, *C. intermedium, C. strigilatum*, *C. parqui, C. euanthes* and *C. nocturnum* (Sýkorová et al. 2003, Fregonezi et al. 2004, Urdampilleta et al. 2015) for the presence and location of SSR, heterochromatin, *Gypsy* elements and rDNA between them. This feature is in accordance with the idea of specie-specific evolutionary pathways that were mentioned for the Bs (Houben et al. 2014). However, regardless of this diversification and the independent evolutionary history within each genome, this set of information could suggest that Bs have a recent origin in *Cestrum*, as they appear to retain similar sizes, and also because they share repetitive sequences with complement A chromosomes between different species, although we still do not know exactly the nature of this repetitive fraction. New investments in sequencing of isolated Bs should be next steps for provided us a better understand of B chromosomes organization, as well as to reveal details of the repetitive sequences roles in the arising of Bs.

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Supplementary material 1

Figure S1

Authors: André Luís Laforga Vanzela, Adriano Alves de Paula, Carolina Cristina Quintas, Thiago Fernandes, Joana Neres da Cruz Baldissera1, Thaíssa Boldieri de Souza

Data type: JPEG file

Explanation note: **A**) Alignment of partial sequences of 5S rDNA of PCR product using the UP primers with *C. strigilatum* genome as template and PCR product using *C. strigilatum* B chromosome as template. Note the two internal deletions in the B chromosome amplicon, and other indels indicating a difference of 26%. **B**) Alignment of partial sequences of 5S rDNA of *C. aurantiacum* × *C. parqui,* AY135508.1 against PCR products using the 5S-Plant primers with *C. strigilatum* genome as template. Note an identity of 76% among them.

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RESEARCH ARTICLE

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Chromosomal constitutions of five wheat – *Elytrigia elongata* partial amphiploids as revealed by GISH, multicolor GISH and FISH

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Abstract

A combination of meiotic pairing analysis and *in situ* hybridization (genomic *in situ* hybridization [GISH], multicolor GISH [mcGISH] and fluorescence *in situ* hybridization [FISH]) of five *Triticum aestivum* (Linnaeus, 1753) – *Elytrigia elongata* (Podpěra, 1902) (2n = 10x = 70) amphiploids was employed to investigate the genomic constitution and relationships between wheat and alien chromosomes. GISH, multicolor GISH and FISH patterns of mitotic chromosomes indicate that the genomic constitution of the five partial amphiploids (XY693, XY7430, SN19, SN20 and SN122) are 14A + 12B + 14D + 8Js + 8J, 12A + 16B + 14D + 2St + 8Js + 2J + 2 W-E, 14A + 14B + 14D + 4St + 8Js, 14A + 14B + 14D + 2St + 10Js + 2J, and 14A + 14B + 14D + 2St + 8Js + 4J, respectively. Analysis of meiotic chromosome pairing in the F_1 hybrids between these five partial amphiploids suggests that SN20 and SN122 are the most closely related amphiploids and are somewhat related with XY693 and XY7430. However, the alien chromosome constitutions of SN19 differed from the other four amphiploids. In addition, a new pairing between wheat and *E. elongata* chromosomes was distinguished in some cells of the hybrids SN19 × XY7430, SN20 × XY7430 and SN122 × XY7430.

Keywords

Elytrigia elongata, partial amphiploid, cytogenetic, *in situ* hybridization

* The two authors contributed equally to this paper.

Introduction

Elytrigia (Á. Löve, 1980) species, such as those in the Triticeae tribe of the family Poaceae, contain excellent perennial forages with resistance to cold, drought, salinity, and various diseases (Mao et al. 2010). Among these, *E. elongata* was determined to have many agronomically useful traits for wheat improvement, such as a high protein content (Garg et al. 2009); tolerance to salt (Zhong and Dvorak 1995) and drought (Roundy 1985); resistance to stripe rust (Yang and Ren 2001), leaf rust (Friebe et al. 1996), *Fusarium* head blight (Scoles et al. 2009; Shen et al. 2004) and wheat streak mosaic virus (Li and Wang 2009). These genes have been transferred into wheat and extensively used to improve resistance to various pests and tolerance to abiotic stresses.

Previous research indicated that *E. elongata* is an autodecaploid with the JJJJJ genomes proposed by Muramatsu (1990). Further probing experiments using the E genome for blocking and the St genome as a probe revealed that the genomic composition of *E. elongata* is JJJJsJs (Chen et al. 1998c). The J genome of *E. elongata* is homologous to the J genome of the diploid *Thinopyrum bessarabicum* (Săvulescu & Rayss, 1923) and harbors St signals in the telomeric regions, whereas the Js genome is a modified J genome of unknown origin characterized by the presence of a St genome-specific hybridization signal near the centromere. Thus, the St genome probe (plus the genomic DNA of common wheat, with or without the E genome DNA as a block) could be used as a diagnostic tool to distinguish the St-, Js-, and J-genome chromosomes from the wheat chromosomes in wheat - alien amphiploids.

Wheat - alien amphiploids, which are stable and highly fertile, represent a crucial intermediate step in the transfer of agronomically useful genes from wheatgrass species to wheat (Fedak et al. 2000; Fedak and Han 2005; Jiang et al. 1994). Over recent decades, several wheat - *E. elongata* amphiploids have been developed and identified as promising sources of multiple disease resistance in various laboratories throughout the world (Chen et al. 1998a; He et al. 2013; Li et al. 2005; Oliver et al. 2006; Sepsi et al. 2008b; Zhang et al. 1996b). These amphiploids, which contain complete genomic sets of wheat plus a set of alien chromosomes, were developed by backcrossing the wheat and *E. elongata* hybrids to wheat. Thus, they provide useful intermediate material for studying the genomic affinities among alien chromosomes. These amphiploids are also useful for producing wheat - alien addition, substitution and translocation lines, which possess biotic stress resistance not found in common wheat.

Developed approximately 30 years ago in China, XY693 and XY7430 are two wheat - *E. elongata* partial amphiploids with 56 chromosomes. They possess many useful genes. XY693 exhibits excellent resistance to leaf rust, powdery mildew and Wheat streak mosaic virus (Li et al. 2005). Three partial amphiploids (SN19, SN20 and SN122) were derived from the cross *T. aestivum* 'Shannongfu63'// *T. aestivum* 'Yannong15'/ *E. elongata* and exhibit resistance to powdery mildew (He et al. 2013) and stripe rust (F. He and H. G. Wang, unpublished data). Being stable and highly fertile, genetic material of these amphiploids could serve as a potential source for wheat improvement. Li et al (2003) and He et al (2013) reported that the alien genome com-

position of XY693 (Li et al. 2003), SN20 and SN122 (He et al. 2013). However, the wheat genome compositions of these amphiploids were ignored. Zheng et al. (2014) examined the chromosome compositions of XY693 and XY7430 using GISH, FISH and mcGISH. However, a few investigations have focused on the homoeologous and compensatory relationships among *E. elongata* and wheat chromosomes between these wheat - *E. elongata* amphiploids.

Genomic *in situ* hybridization (GISH) is a widely used and effective method for detecting alien chromatin in wheat-alien species amphiploids. Multicolor GISH (mcGISH), which employs several different genomic probes, can be used to simultaneously visualize two or more genomes in a polyploid species. Han et al. (2004) used mcGISH to analyze wheat - *Thinopyrum intermedium* (Host, 1805) derivatives and detected intergenomic rearrangements involving *Th. intermedium* chromosomes and the A and B genomes of wheat. Fu et al. (2012) successfully used mcGISH to differentiate the A, B, D and E genomes of wheat - *E. elongata* addition, substitution and translocation lines. Fluorescence *in situ* hybridization (FISH), which uses repetitive DNA clones, is a powerful tool for identifying chromosomes within a species (Gill et al. 1993; Komuro et al. 2013; Pedersen and Langridge 1997) or tracing intergenomic chromosome rearrangements in a polyploid species (Danilova et al. 2014). The combination of the mcGISH technique with sequential FISH on the wheat alien hybrids enables chromosomes belonging to different genomes to be detected and identified, such that intergenomic rearrangements within a polyploid species can be visualized (Nagy et al. 2002; Wang et al. 2005).

In the present study, we used a combination of GISH, mcGISH and FISH to examine the cytogenetic composition of five partial amphiploids. In addition, the relationships between wheat and the alien chromosomes of the wheat - *E. elongata* amphiploids were assessed via the combination of the meiotic behavior of the F_1 hybrids.

Material and methods

Plant materials

Plant materials used in this study included *E. elongata*, *Pseudoroegneria spicata* (Pursh, 1814) (StSt, 2n = 14), *Triticum urartu* (Gandilyan, 1972) (AA, 2n = 14), *Aegilops speltoides* (Gerlach & Dyer, 1980) (SS, 2n = 14), *Aegilops tauschii* (Cosson, 1850) (DD, 2n = 14), the common wheat Yannong15 and five partial amphiploids (XY693, XY7430, SN19, SN20 and SN122). *E. elongata, T. urartu*, *A. speltoides* and *A. tauschii* were provided by Prof. Zhensheng Li (formerly of the Northwest Institute of Botany at the Chinese Academy of Sciences, Yangling, China). *Ps. spicata* was provided by Prof. Lihui Li (Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China). XY693 was derived from the cross *T. aestivum* 'Xiao Yan 2'// *T. aestivum* 'Lin 7'/ *E. elongata*. Similarly, XY7430 was derived from the cross *T. aestivum* 'Xiang Yang 4'// *T. aestivum* 'mi sui zao'/ *E. elongata*. The partial amphiploids SN19, SN20 and

SN122 were novel germplasms developed from the hybridization of *E. elongata*, the common wheat Yannong15 and Shannongfu63 at the Agronomy College of Shandong Agricultural University, Tai'an, China. All plant materials are maintained in our laboratory through self-crosses.

Genomic *in situ* hybridization (GISH)

Seeds were germinated at 25°C on moist filter paper in Petri dishes, maintained at 4°C for approximately 24 h, and then transferred to 25°C. Roots 1- to 2-cm in length were cut and treated in ice water for approximately 24 h before fixation in Carnoy's solution. After fixation, the root tips were stained and squashed in carbol fuchsin, and their mitotic chromosomes were observed under a microscope. When the plants reached the flag leaf stage, spikes were sampled and anthers at metaphase I (MI) of meiosis were fixed in Carnoy's solution, dissociated in 1 M HCl at 60°C for 6 to 8 min, and homogenized in 1% acetocarmine. *E. elongata* and *Ps. spicata* DNA were labeled with fluorescein-12-dUTP by the nick translation method and used as probes. Sheared genomic DNA from YN15 (AABBDD, 2n = 42) was used as blocking DNA. The slides were counterstained with propidium iodide (PI, 0.25 mg/mL) in the Vectashield mounting medium (Vector Laboratories, USA).

Multicolor genomic *in situ* hybridization (mcGISH)

The total genomic DNA was isolated from young leaves of *E. elongata*, *T. urartu*, *A. speltoides* and *A. tauschii*. The total genomic DNA of diploid *E. elongata* and *T. urartu* was labeled with fluorescein-12-dUTP, and the total genomic DNA of *A. tauschii* was labeled with Texas-red-5-dUCP via the nick translation method. The total genomic DNA of *A. speltoides* was used for blocking (at a ratio of 1:160). After hybridization, the slides were washed in 2 × saline sodium citrate (SSC) and mounted in Vectashield mounting medium (containing 1.5 mg/mL DAPI; Vector Laboratories, USA).

Fluorescence *in situ* hybridization (FISH)

FISH was performed after mcGISH analysis using two probes, pTa535 labeled with fluorescein-12-dUTP, and pSc119.2 labeled with Texas-red-5-dUCP. The two probes were mixed at a ratio of 1:1 before hybridization. The slides were washed in 2 × SSC and mounted in Vectashield mounting medium (containing 1.5 mg/mL DAPI; Vector Laboratories, USA). The detailed procedures for the chromosome preparation and hybridization were previously described by Han et al. (2004). Photographs were captured with an Olympus BX-60 fluorescence microscope equipped with a CCD (charge-coupled device) camera.

Results

McGISH analysis of five partial amphiploids

To further determine the five partial amphiploids by mcGISH, the mitotic chromosomes were probed using D-genomic DNA (*A. tauschii*) with Texas-red-5-dCTP (red), and the total genomic DNA of *E. elongata* and *T. urartu* was probed with fluorescein-12-dUTP (green) and blocked by the S-genome (*A. speltoides*) DNA. Using this technique, the A-, B-, and D-genomes from common wheat and the genomes from *E. elongata* could be simultaneously distinguished. The A-, B-, and D-genome chromosomes were labeled with yellow, brown/ gray and red/ pink fluorescence, respectively, whereas the alien chromosomes of *E. elongata* were labeled with green fluorescence.

Chromosome constitutions of these five wheat - *E. elongata* amphiploids (XY693, XY7430, SN19, SN20 and SN122) were analyzed using mcGISH (Table 1). As illustrated in Fig. 4A, XY693 contained 16 alien chromosomes plus 40 wheat chromosomes, including 14 A-genome, 12 B-genome, and 14 D-genome chromosomes. XY7430 had 12 green-fluorescing chromosomes that originated from *E. elongata* and two interspecific translocation chromosomes with green terminal fragments on red short arms. Of the remaining 42 wheat chromosomes, 12 fluoresced yellow, 16 gray, and 14 red. Therefore, in addition to the 12 alien chromosomes and 2 wheat - *E. elongata* translocation chromosomes (Fig. 4B, asterisk), XY7430 also possessed 12 A-genome, 16 B-genome, and 14 D-genome chromosomes (Fig. 4B). Similarly, SN19 possessed 12 *E. elongata* chromosomes and 42 wheat chromosomes, including 14 A-genome, 14 B-genome, and 12 D-genome chromosomes (Fig. 1A). Both SN20 and SN122 carried 14 *E. elongata* chromosomes plus 42 wheat chromosomes, including 14 A-genome, 14 B-genome, and 14 D-genome chromosomes (Fig. 1C, E).

FISH analysis of five partial amphiploids

Although the mcGISH patterns of the mitotic chromosomes indicated that the wheat - *E. elongata* amphiploid SN19 has 54 chromosomes and the remaining four partial amphiploids have 56 chromosomes, the number of alien chromosomes ranged from 12 to 16, suggesting that chromosome deletion and substitution occurred in the wheat genome. Three-color FISH, with the simultaneous hybridization of the repetitive DNA probes pTa535 and pSc119.2, has been successfully employed on mitotic metaphase cells of these five partial amphiploids. Chromosome elimination and addition has been detected with these probes (Figs 4C, Dm 1B, D, F). The results of the mcGISH analysis revealed that a pair of B-genome chromosomes was absent from XY693 and a pair of B-genome chromosomes was added from XY7430 (Fig. 4A, B). Comparing their FISH results using probes pTa535 and pSc119.2 with those for common wheat, the missing and added chromosomes were determined to be 6B and 2B, respectively. In

Table 1. GISH analysis of five partial amphiploids.

Line	Chromosome no.	Origin of alien chromosomes	Genomic chromosome constitution*
XY693	2n=56	16 of *E. elongata*	14A + 12B + 14D + 8Js + 8J
XY7430	2n=56	12 of *E. elongata*	12A + 16B + 14D + 2St + 8Js + 2J + 2 W-E
SN19	2n=54	12 of *E. elongata*	14A + 14B + 14D + 4St + 8Js
SN20	2n=56	14 of *E. elongata*	14A + 14B + 14D + 2St + 10Js + 2J
SN122	2n=56	14 of *E. elongata*	14A + 14B + 14D + 2St + 8Js + 4J

* W-E, wheat - *E. elongata* translocation chromosomes

addition, the FISH results revealed that the wheat - *E. elongata* translocation chromosomes in XY7430 were wheat chromosome 5D with a terminal alien segment on the short arm.

GISH identification

The alien chromosome constitutions of five wheat - *E. elongata* amphiploids were analyzed using GISH (Table 1). The assignment of the alien chromosomes present on partial amphiploids were based on the differences in the type of signal obtained with wheat DNA blocking and St genome probing (Chen et al. 1998b) (Fig. 2F).

Probing the partial amphiploid XY693 with St genomic DNA and blocking with wheat DNA revealed that 16 chromosomes emitted greenish-yellow hybridization signals: a Js type of signal was detected on 4 pairs of chromosomes, a J type of signal on 8 chromosomes, and four translocation signals on the telomere of four wheat chromosomes (Fig. 2A). The partial amphiploid XY7430 had 12 chromosomes from *E. elongata*, including 2 St-genome chromosomes, 8 Js-genome chromosomes, 2 J-genome chromosomes, and 2 wheat - *E. elongata* translocated chromosomes (Fig. 2B). The results with SN20 were similar to those with XY693 and XY7430, except that the SN20 contained 2St-, 10 Js- and 2 J-genome chromosomes as well as 4 wheat - *E. elongata* translocated chromosomes (Fig. 2D). In addition, the partial amphiploids SN19 and SN122 did not contain wheat - *E. elongata* translocated chromosomes. The genomic constitution of these two alien chromosomes was 4 St + 8 Js (Fig. 2C) and 2 St + 8 Js + 4 J (Fig. 2E), respectively.

Meiotic analysis of the partial amphiploids

Five partial wheat - *E. elongata* amphiploids were crossed with each other, and their meiotic chromosome behavior was studied in all nine F_1 hybrids (except XY693 × XY7430). *E. elongata* chromatin was distinguished in the pollen mother cells (PMCs) at MI of the F_1 hybrids using GISH, and the values for each meiotic parameter are presented in Table 2.

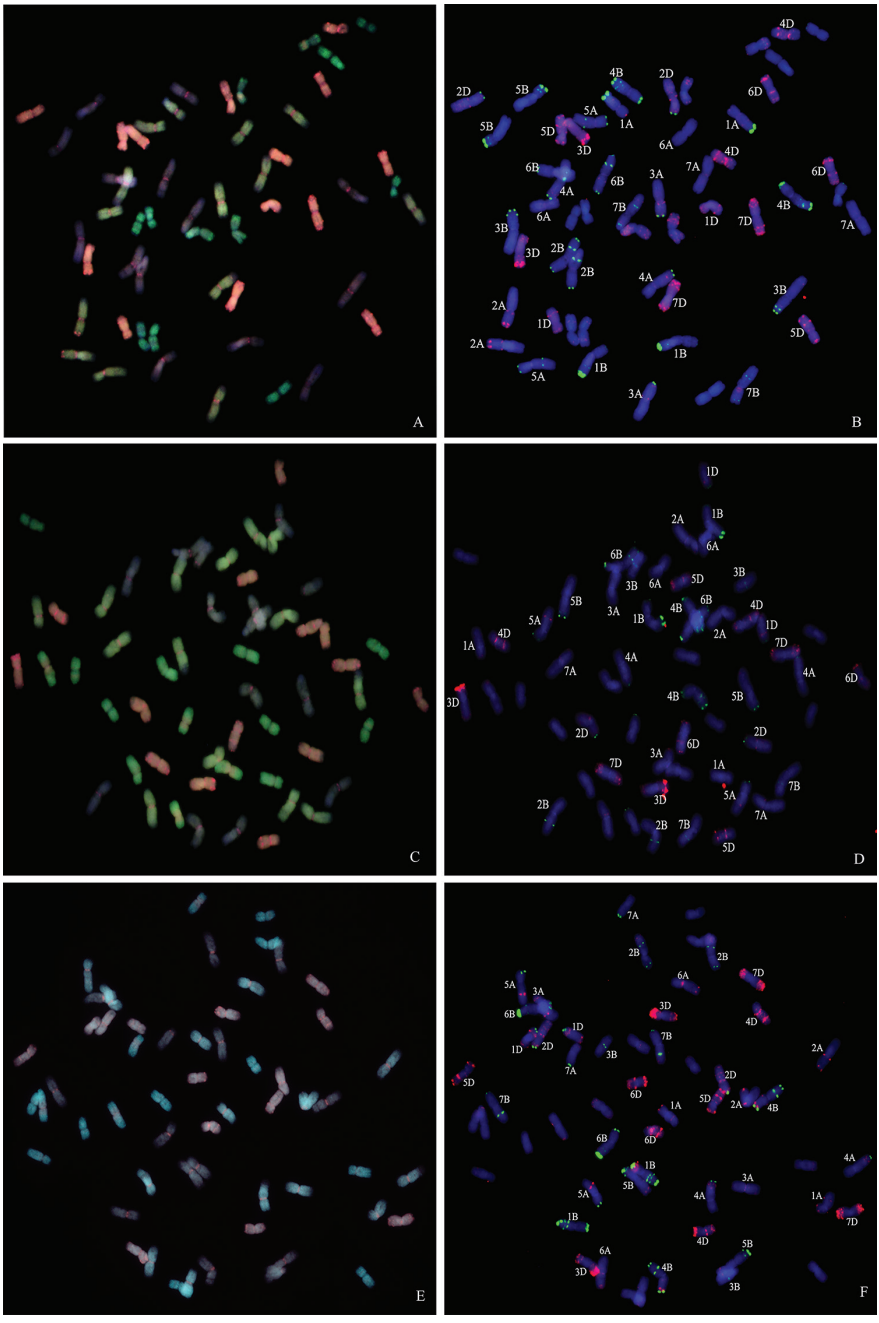

Figure 1. McGISH patterns and FISH analysis of chromosomes inSN19 (**A**), SN20 (**C**) and SN122 (**E**). Yellow denotes the A-genome chromosomes, gray indicates the B-genome chromosomes, red represents the D-genome chromosomes and green denotes the *E. elongata* chromosomes or chromosomal fragments. The asterisks indicate the wheat - *E. elongata* translocation chromosomes. FISH on the same metaphase chromosome spreads are simultaneously presented in lines SN19 (**B**), SN20 (**D**) and SN122 (**F**) by pTa535 (green) and pSc119.2 (red).

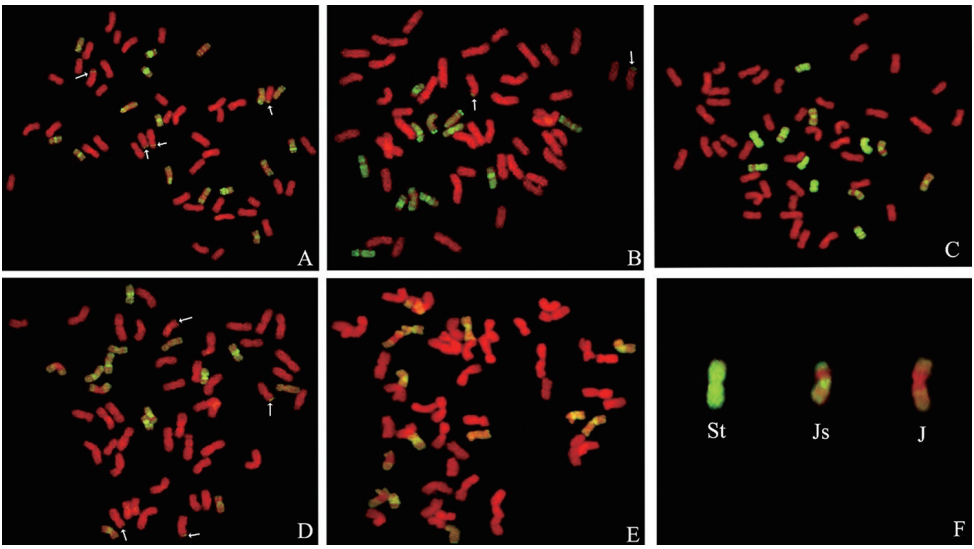

Figure 2. GISH patterns of the mitotic chromosomes probed with St-genomic DNA from *Ps. spicata* and blocked with ABD-genomic DNA from Yannong15 wheat. **A** XY693 **B** XY7430 **C** SN19 **D** SN20 **E** SN122 **F** Karyotype of chromosomes from Fig. 1C organized into three types. Arrows indicate translocated chromosomes.

As presented in Table 2, most of the nine F_1 hybrids exhibited high frequencies of univalents and low frequencies of multivalents, including trivalents and quadrivalents. All F_1 hybrids exhibited high frequencies of pairing between the wheat chromosomes, whereas the multivalents were absent in the *E. elongata* chromosomes, and univalents were observed for both the wheat and *E. elongata* chromosomes. In addition, pairing chromosomes between wheat and *E. elongata* were observed in some cells of the hybrids SN19 × XY7430, SN20 × XY7430 and SN122 × XY7430 (Fig. 3A, arrows). The F_1 hybrid between XY693 and XY7430 was not involved in this experiment considering that the configuration of these amphiploid hybrids and the relationship of the alien chromosomes between XY693 and XY7430 were analyzed in earlier studies.

In contrast to the regular meiotic behavior of the parents, the values for the F_1 hybrids were highly variable. Based on the lowest frequencies of unpaired chromosomes, the most closely related strains were SN20 and SN122. Only an average of 2.5 *E. elongata* chromosome univalents per cell was observed in the hybrid SN122 × SN20. Another grouping involved SN20 × XY7430 (Fig. 3B), SN122 × XY7430, XY693 × SN20 (Fig. 3C) and XY693 × SN122 (Fig. 3D). In this grouping, an average of 4.18 and 6.1 *E. elongata* chromosome univalents was observed in the hybrids of XY7430 with SN20 and SN122, respectively. Also, the F_1 hybrids of XY693 with SN20 and SN122 exhibited 5.26 and 5.68 *E. elongata* chromosome univalents, respectively. The strain SN19 appeared to be unrelated to the other partial amphiploids, as indicated by the high frequency of unpaired chromosomes in the hybrids. In addition, 9.1, 9.45, 9.32 and 10.24 unpaired *E. elongata* chromosomes were observed in the

Table 2. Average metaphase I configuration per meiocyte in the F_1 hybrids between partial amphiploids.

Hybrid	No. of plants	No. of cells	Meiotic configuration of amphiploids hybrids chromosomes					No. of alien chromosomes	Meiotic configuration of *E. elongata* chromosomes		
			I	II_{ring}	II_{rod}	III	IV		I	II_{ring}	III_{rod}
SN20 × SN19	6	83	11.81 (7-21)	17.54 (16-20)	3.88 (1-6)	0.04 (0-1)	0.06 (0-1)	13	9.1 (7-13)	1.03 (0-2)	0.92 (0-1)
SN19 × SN122	5	64	12.08 (9-17)	16.59 (16-18)	4.75 (2-6)	0	0.06 (0-1)	13	9.45 (5-13)	0.99 (0-3)	0.78 (0-1)
SN122 × SN20	5	79	3.44 (2-6)	21.49 (17-24)	4.78 (3-8)	0.01 (0-1)	0	14	2.5 (2-4)	3.96 (3-4)	1.79 (1-2)
SN19 × XY7430	5	66	10.93 (7-18)	16.37 (14-19)	5.51 (2-9)	0.05 (0-1)	0.04 (0-1)	12	9.32 (7-15)	0.77 (0-5)	0.57 (0-1)
SN20 × XY7430	6	76	5.98 (4-12)	17.2 (12-19)	6.37 (4-9)	0.11 (0-1)	0.64 (0-3)	13	4.18 (1-11)	3.06 (1-5)	1.35 (0-3)
SN122 × XY7430	7	88	8.38 (4-14)	15.7 (13-20)	7.29 (3-9)	0.27 (0-1)	0.21 (0-1)	13	6.1 (1-11)	2.76 (1-4)	0.69 (0-2)
XY693 × SN122	6	94	5.98 (2-12)	17.14 (16-19)	7.68 (7-9)	0.13 (0-1)	0	15	5.26 (3-11)	2.94 (1-4)	1.93 (0-3)
XY693 × SN19	7	69	11.61 (5-13)	15.96 (15-17)	5.05 (5-8)	0.14 (0-1)	0.24 (0-2)	14	10.24 (2-12)	0.87 (0-3)	1.01 (0-3)
XY693 × SN20	6	85	6.11 (2-10)	17.34 (15-19)	6.92 (4-9)	0.14 (0-1)	0.24 (0-2)	15	5.68 (3-9)	2.84 (2-4)	1.82 (1-3)

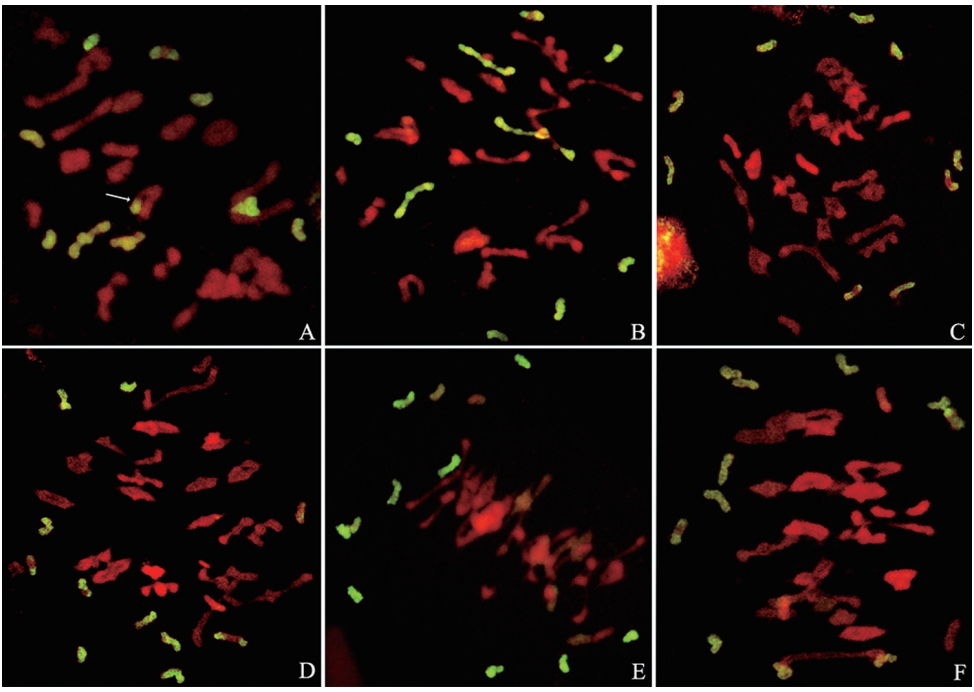

Figure 3. GISH patterns of meiotic chromosomes probed with *E. elongata* genomic DNA and blocked with Yannong15 wheat genomic DNA. **A** SN122 × XY7430 **B** SN20 × XY7430 **C** XY693 × SN20 **D** XY693 × SN122 **E** SN20 × SN19 **F** SN19 × SN122. Arrows indicate pairing between the wheat and *E. elongata* chromosomes.

F_1 hybrids obtained from crossing SN19 with SN20, SN122, XY7430 and XY693, respectively (Fig. 3E, F). In addition, a new pairing between the wheat and *E. elongata* chromosomes was distinguished in some cells of the hybrids SN19 × XY7430, SN20 × XY7430 and SN122 × XY7430 (Fig. 3A, arrows).

Discussion

To date, numerous studies have reported that many of the chromosomes contained in alien genomes are products of translocations either between *Elytrigia* chromosomes or with wheat chromosomes. Using GISH and mcGISH, Han et al. (2004) demonstrated that the wheat - *Th. intermedium* amphiploid Zhong 1 carried a translocation between a pair of A genome chromosomes and *Th. intermedium* chromosomes and that another wheat - *Th. intermedium* amphiploid Zhong 4 contained rearrangements between the A and D genomes of wheat. Similar results were obtained by Sepsi et al. (2008a), who found 4A and 7A chromosome pairs carrying telomeric translocations using mcGISH and FISH on the wheat - *E. elongata* amphiploid BE-1. Oliver et al. (2006) proved that wheat - *E. elongata* amphiploid SS363 carries 14 *E. elongata* chromosomes, 40 wheat

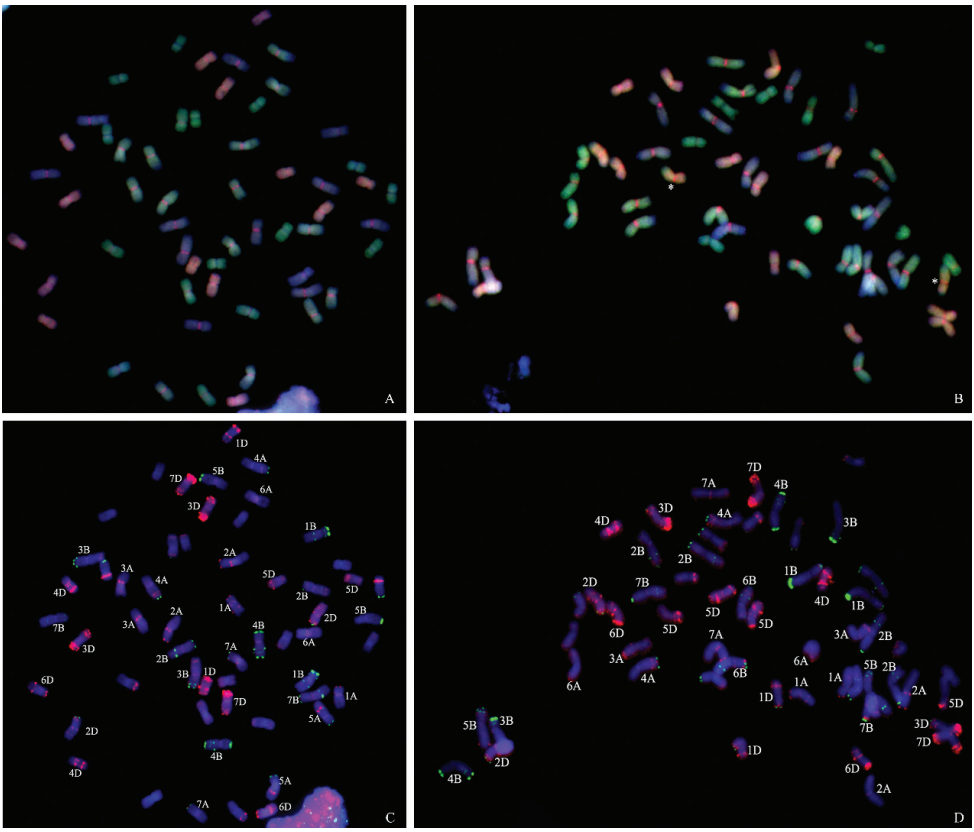

Figure 4. McGISH patterns and FISH analysis of chromosomes in XY693 (**A**), XY7430 (**B**). Yellow denotes the A-genome chromosomes, gray indicates the B-genome chromosomes, red represents the D-genome chromosomes and green denotes the *E. elongata* chromosomes or chromosomal fragments. The asterisks indicate the wheat - *E. elongata* translocation chromosomes. FISH on the same metaphase chromosome spreads are simultaneously presented in lines XY693 (**C**), XY7430 (**D**), by pTa535 (green) and pSc119.2 (red).

chromosomes, and two wheat *E. elongata* translocated chromosomes. The wheat - *E. elongata* partial amphiploids XY693 and XY7430 were developed approximately 30 years ago in China. Li et al. (2003) distinguished eight Js genomes and eight J genome chromosomes in line XY693. Zheng et al (2014) determined that the chromosome compositions of XY693 and XY7430 using GISH, FISH and mcGISH. In this study, we report similar results for XY693 but distinguished two pairs of translocated chromosomes. In addition, one pair of wheat - *E. elongata* translocated chromosomes in XY7430 and 4 translocated chromosomes in SN20 were also detected.

The genomic composition of the decaploid species *E. elongata* has been a subject of interest for a considerable time (Chen et al. 1998c; Liu and Wang 1993; Zhang et al. 1996a). When a somatic chromosome preparation from this species is blocked with E genomic DNA and probed with St-genomic DNA, a “centromeric signal” was observed on 28 chromosomes, faint telomeric signals were observed on 42 chromosomes,

and uniform St signals were not noted in any genome. Thus, the genomic constitution of this species was designated as JJJJsJs (Chen et al. 1998c). In contrast, Zhang et al. (1996a) concluded that the St genome is also present in *E. elongata* and that the centromeric region is critical in distinguishing these chromosomes from E- (=Js-) or J-type chromosomes, which are also present in this species. In the current study, four of these five partial amphiploids exhibited one or two pairs of St-genomic chromosomes. This result indicates that the basic genomes of *E. elongata* were J, Js and St. The presence of the major discrepancy in the genome symbols may be explained by the varied origins of *E. elongata*. However, additional research is needed to confirm this hypothesis.

Chromosome counting on the metaphase spreads after GISH revealed 16 alien chromosomes in the partial amphiploid XY693 and 12 alien chromosomes in the partial amphiploid XY7430. However, the chromosome number of these two amphiploids was consistently 56. This observation suggests that the chromosome substitutions occurred between the *E. elongata* genome and wheat genome. Similar results were obtained in previous studies. Fedak et al. (2000) reported the genomic composition of six wheat - *E. elongata* amphiploids (PMW706, PMW206, PMW209, PMWIII, OK7211542 and an *Agropyron* - wheat hybrid) revealed by GISH using St-genomic DNA as a probe. The number of alien chromosomes varied from 12 to 18 among the studied amphiploids. Similarly, using the St genomic DNA as a probe, Sepsi et al. (2008a) demonstrated the genomic composition of a wheat - *E. elongata* amphiploid BE-1 included 16 alien chromosomes and 40 wheat chromosomes. FISH analysis identified the substituted wheat chromosome pair as 7D. Afterwards, other wheat - *E. elongata* amphiploids, such as Agrotana, ORRPX and SS660, were also found to contain 40 wheat and 16 alien chromosomes when applying GISH, but the substituted wheat chromosomes were not identified (Chen 2005; Fedak and Han 2005; Oliver et al. 2006). This observation suggested that in the preserved process of these amphiploids, a wheat chromosome substituted an alien chromosome and then duplicated spontaneously. However, the homologous group of the chromosomes involved in the substitution has not been identified. Further research on the internal mechanism for this spontaneous substitution would thus be helpful in understanding the genetic relationship among the genomes of *S. cereale*, *L. mollis* and wheat as well as in the development of alien substitution lines.

Zhang et al. (1995) implied that meiotic pairing control genes (*ph* genes) present in the decaploid species *E. elongata* genomes based on the homoeologous chromosome pairing were strongly promoted in such hybrids. The *ph* genes might be present in parts of the wheat - *E. elongata* amphiploids. However, no direct evidence is available to prove this deduction. Oliver et al. (2006) analyzed the chromosome pairing configurations of six F_1 hybrids from four partial wheat - *E. elongata* amphiploids hybridized with each other. Unfortunately, no pairing between the wheat and *E. elongata* chromosomes was observed in any of these hybrids. In this study, a new pairing between wheat and *E. elongata* chromosomes was distinguished in some cells of the hybrids SN19 × XY7430, SN20 × XY7430 and SN122 × XY7430. These results demonstrated that the *E. elongata* chromosomes in partial amphiploids XY7430 contain *ph* genes.

The data from the GISH and meiotic chromosome pairing analysis in the F_1 hybrids between different partial amphiploids were useful in identifying similar alien genomes. Based on the lowest frequency of *E. elongata* unpaired chromosomes, the two strains SN20 and SN122 would appear to be the most closely related of all the partial amphiploids examined. The highest frequency of the univalent *E. elongata* chromosome in the hybrids SN20 × SN19, SN19 × SN122, XY693 × SN19 and SN19 × XY7430 suggested that the alien chromosome constitutions of SN19 differed from the other four amphiploids. High *E. elongata* chromosome univalent and bivalent frequencies were observed in the other hybrids, including SN20 × XY7430, SN122 × XY7430, XY693 × SN122 and XY693 × SN20. These results indicate that the partial amphiploids SN20 and SN122 were related to XY693 and XY7430. Previous reports demonstrated that the alien chromosome constitutions of XY693 and XY7430 differed (Zhang and Dong 1994). Generally, these five partial amphiploids originated from the same alien parent and do not necessarily carry the same combination of alien chromosomes and the alien genomes in partial amphiploids, which are quite complex. The synthetic nature of the alien genomes in the partial amphiploids has been reported by several authors (Chen 2005; Chen et al. 1998b; Fedak and Han 2005; Sepsi et al. 2008a). These partial amphiploids are cytogenetically stable and highly fertile, perhaps because they are homologous and there is compensation between the *E. elongata* chromosomes and wheat chromosomes.

Partial wheat – *E. elongata* amphiploids, with a high cross-compatibility with wheat, are desirable "bridge" materials for transferring disease resistance genes from *E. elongata* to wheat. In previous studies, these five partial amphiploids demonstrated regular meiotic behavior and are highly fertile. Based on the chromosome constitution of these amphiploids, we manipulated *Elytrigia* chromosomes in these amphiploids to eliminate unwanted *Elytrigia* chromatin and to introduce useful agronomic traits into wheat. Due to the unpredictable and unstable meiotic behavior of the F_1 hybrids between the lines, the combining of traits by intercrossing partial amphiploids is not a promising alternative.

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RESEARCH ARTICLE

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Multiple sex chromosome system in penguins (*Pygoscelis*, Spheniscidae)

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Abstract

Penguins are classified in the order Sphenisciformes into a single family, Spheniscidae. The genus *Pygoscelis* Wagler, 1832, is composed of three species, *Pygoscelis antarcticus* Forster, 1781, *P. papua* Forster, 1781 and *P. adeliae* Hombron & Jacquinot, 1841. In this work, the objective was to describe and to compare the karyotypes of *Pygoscelis* penguins contributing genetic information to Sphenisciformes. The metaphases were obtained by lymphocyte culture, and the diploid number and the C-banding pattern were determined. *P. antarcticus* has 2n = 92, P. papua 2n = 94 and *P. adeliae* exhibited 2n = 96 in males and 2n = 95 in females. The difference of diploid number in *P. adeliae* was identified as a multiple sex chromosome system where males have Z1Z1Z2Z2 and females Z1Z2W. The C-banding showed the presence of a heterochromatic block in the long arm of W chromosome and Z2 was almost entirely heterochromatic. The probable origin of a multiple system in *P. adeliae* was a translocation involving the W chromosome and the chromosome ancestral to Z2. The comparison made possible the identification of a high karyotype homology in Sphenisciformes which can be seen in the conservation of macrochromosomes and in the Z chromosome. The karyotypic divergences in *Pygoscelis* are restricted to the number of microchromosomes and W, which proved to be highly variable in size and morphology. The data presented in this work corroborate molecular phylogenetic proposals, supporting the monophyletic origin of penguins and intraspecific relations.

Keywords
Sphenisciformes, karyotype, sex chromosomes, evolution

Introduction

In the class Aves, penguins are classified in the order Sphenisciformes in a single family Spheniscidae. The 18 extant species are divided in six genera, *Aptenodytes* Miller, 1778 (2 species), *Eudyptes* Vieillot, 1816 (6 species), *Pygoscelis* Wagler, 1832 (3 species), *Spheniscus* Brisson, 1760 (4 species), *Megadyptes* Milne-Edwards, 1880 (1 species) and *Eudyptula* Bonaparte, 1856 (2 species) (Stonehouse 1975). The monophyletic origin of penguins has been evidenced in different studies using morphological characters, DNA hybridization and more recently by molecular data with whole-genome analysis (Sibley and Ahlquist 1990, Giannini and Bertelli 2004, Baker et al. 2006, Jarvis et al. 2014).

Using mitochondrial and nuclear sequences, Baker and co-workers (2005) reconstructed the phylogeny of penguins and solved divergences observed in an intra genera relationship. The molecular dating estimated that ancestral penguins originated about 71 million years (Mya) ago in Gondwanaland while current species shows an Antarctic origin about 40 Mya. *Aptenodytes* (king and emperor penguins) was the first genus to diverge as basal lineage about 40 Mya. *Pygoscelis* branched about 38 Mya diversifying to *P. adeliae* Hombron & Jacquinot, 1841 in 19 Mya, *P. antarcticus* Forster, 1781 and *P. papua* Forster, 1781 at 14.1 Mya. The common ancestry of other genera was estimated at 27.8 Mya, followed by the division between genus *Spheniscus* and *Eudyptula* at approximately 25 Mya, followed by penguins *Megadyptes* and *Eudyptes,* about 15 Mya.

The karyotype information for Sphenisciformes is scarce, only five species have known diploid number. The black-footed penguin (*Spheniscus demersus* Linnaeus, 1758) has 2n = 72 (Jensen 1973), peruvian penguin (*S. humboldti* Meyen, 1834) has 2n = 78 (Takagi and Sasaki 1974) and magellanic penguin (*S. magellanicus* Forster, 1781) has 2n = 68 (Ledesma et al. 2003). Emperor penguin (*Aptenodytes forsteri* Gray, 1844) and king penguin (*A. patagonicus* Miller, 1778) have same diploid number 2n = 72 (Cardozo et al. 2003). Comparison of these karyotypes shows a numeric and morphological conservation of macrochromosomes among penguin species, indicating that differences observed in diploid number have their origin by fusion or fission involving in microchromosomes (Ledesma et al. 2003).

Related to sex chromosomes in Aves, it is known the chromosome system of type ZZ in males and ZW in females. The Z chromosome is relatively conserved among different orders, varying in size between the third and fourth pair of macrochromosomes (Takagi and Sasaki 1974), whereas the W chromosome presents significant differences, being of size similar to Z in Ratites to a small and heterochromatic chromosome in Passeriformes (Takagi and Sasaki 1974, Correia et al. 2009).

In this work, the goal was to describe the karyotype of *P. antarcticus*, *P. papua* and *P. adeliae,* contributing to the karyotypic knowledge about the order Sphenisciformes and to compare it with species already described.

Material and Methods

Location and sampling

The field work was carried out in the Potter peninsula (62°15'S, 58°40'W), King George Island (62°02'S, 58°21'W), South Shetland Island (60°18'S, 1°22'W). Blood samples were taken with heparin for the following species: *P. antarcticus* (8 males and5 females), *P. papua* (7 males and 5 females) and *P. adeliae* (8 males and 5 females).

Blood cultures

Blood samples were used for lymphocyte culture, according to Moorhead et al. (1960). Briefly, cultures were prepared using approximately 1ml of peripheral blood in 10ml of RPMI 1640 culture medium, supplemented with 20% fetal bovine serum, 0.25ml penicillin/streptomycin and 0.2ml phytohemaglutinin. Cultures were incubated at 39°C for 72 hours. One hour before the incubation end, cells were treated with 0.1ml of colchicine solution (0.05%). After incubation, samples were treated with 10ml of 0.075M KCL for 20 minutes and fixed in a methanol and acetic acid (3:1) solution.

Chromosomal analysis

For chromosomal analysis, the metaphases were stained with Giemsa solution. For each specimen, 40 metaphases were observed and photographed to assemble the karyotypes. Morphological classification of each chromosome pair was made according to Guerra (1986). CBG banding was conducted in sequential analysis, it was used slides preparations with a conventional staining and procedures of C-banding according to Sumner (1972).

Results

Pygoscelis antarcticus has 2n = 92 chromosomes (Fig. 1a). The karyotype is formed by seven pairs of macrochromosomes and the remaining were 38 pairs of microchromosomes. Sex chromosome Z was identified as submetacentric corresponding to the size of the 4th pair and W is acrocentric corresponding to size of the 8th pair.

Pygoscelis papua showed 2n = 94 (Fig. 1b). The karyotype exhibited the same morphological characteristic such as in *P. antarcticus*, with seven autosomal pairs of macrochromosomes and the divergence was observed in the number of microchromosomes, 39 pairs. The Z chromosome was submetacentric and W was metacentric.

For the *P. adeliae* it was observed that there is a difference in the diploid number between males and females. The males had 2n = 96, while the females showed 2n = 95 (Fig. 1c). The autosomal complement was formed by seven pairs of macrochromo-

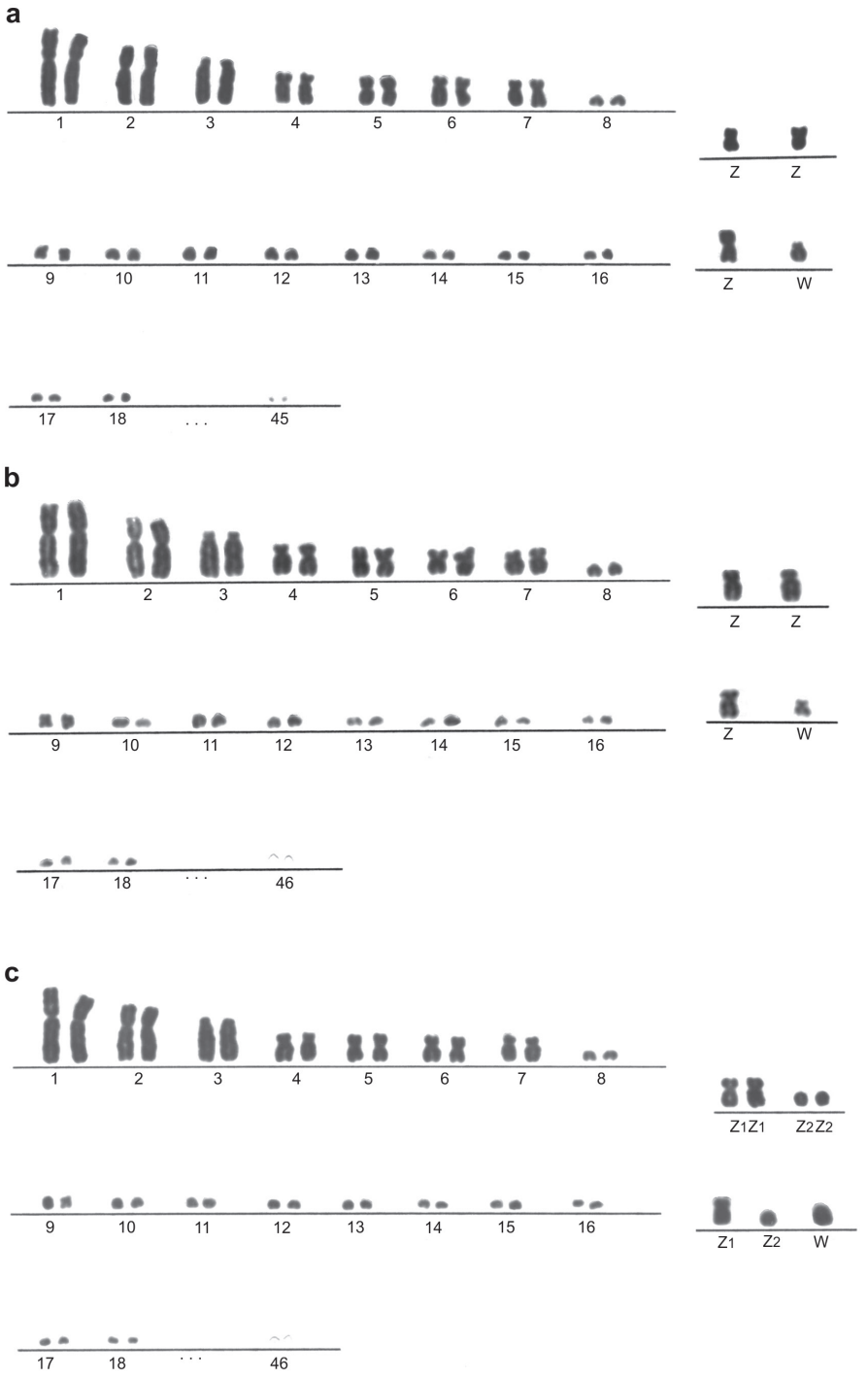

Figure 1. Partial karyotypes of *Pygoscelis* penguins **a** *P. antarcticus* 2n = 92 **b** *P. papua* 2n = 94 **c** *P. adeliae* 2n = 96 in males and 2n = 95 in females.

somes highly similar at *P. antarcticus* and *P. papua,* the remaining were microchromosomes. The sex chromosomes in males were identified as one pair of submetacentric chromosomes (Z_1Z_1) and one small pair of telocentric denominated as Z_2Z_2. In females *P. adeliae* the sex chromosome system $Z_1 Z_2W$ was identified, and the W chromosome was telocentric.

The classification of chromosome morphology in *Pygoscelis* confirmed the conservation of seven macrochromosomes and the Z chromosome between species. The pairs 1, 2, 4, 6, 7 and Z are submetacentric, pair 3 is acrocentric and pair 5 is metacentric. The W chromosome is acrocentric in *P. antarcticus*, metacentric in *P. papua* and telocentric in *P. adeliae* (Table 1).

The sequential Giemsa and C-banding analysis in *P. antarcticus* (Figs 2a–a') showed centromeric C-positive heterochromatin blocks in macro and microchromosomes; the Z chromosome was C negative. The W chromosome was slightly reactive to the banding. For the female *P. papua* (Figs 2b–b'), centromeric markings on macro and microchromosomes were observed. In the species *P. adeliae*, both sexes, female (Figs 2c–c') and male (Figs 2d–d') were analyzed. The macro and microchromosomes and the Z1 chromosome were little reactive and not showed marking in the centromere. The W chromosome has a characteristic heterochromatin block in the terminal region and the Z2 chromosome is completely heterochromatic.

Discussion

P. adeliae (2n = 95–96), *P. papua* (2n = 94) and *P. antarcticus* (2n = 92) showed typical avian karyotypes. When compared to ancestral species such as Paleognathae (Nishida-Umehara et al. 2007) and Passeriformes (Kretschmer et al. 2014), it is remarkable that the chromosomal organization in macrochromosomes and microchromosomes has been maintained throughout evolution.

The diploid number of *Pygoscelis* is slightly elevated in relation to the values observed in more than 60% of the known Avian karyotypes, which correspond to 2n = 74–86 according to Rodionov (1997). Nevertheless, the conservation of macrochromosomes morphology suggests that karyotypes differences among these species are due to variations which occurred in microchromosomes. Considering the proposed phylogeny by Baker et al. (2006), in which *P. adeliae* (2n = 95–96) was the first species of the genus to diverge 19 Mya, it is suggested that a diploid number reduction occurred in *P. papua* (2n = 94) and *P. antarcticus* (2n = 92). This reduction can be attributed to chromosomal fusions involving microchromosomes.

The numerical and morphological conservation of the macrochromosomes in *Pygoscelis* is shared with *Spheniscus* and *Aptenodytes* species (Table 1). Ledesma et al. (2003) was the first to identify that, despite slight variations in the diploid number, Sphenisciformes showed high chromosomal homology. This homology extends not only to autosomal macrochromosomes, but also to the Z chromosome which is observed as submetacentric in seven of the eight species with known karyotypes. The

Table 1. Comparison of the diploid number and morphology of the macrochromosomes and sex chromosomes of Sphenisciformes species.

Species	Common name	2n	1	2	3	4	5	6	7	Z	W	Z2	Reference
Spheniscus demersus	Black-footed	72	S	M	A	M	M	M	M	M	S	–	Jensen 1973
Spheniscus magellanicus	magellanic	68	S	S	A	S	M	S	S	S	S	–	Ledesma et al. 2003
Spheniscus humboldti	Peruvian	78	S	S	A	S	M	M	S	S	S	–	Takagi and Sasaki 1974
Pygoscelis antarcticus	chinstrap	92	S	S	A	S	M	S	S	S	A	–	This work
Pygoscelis papua	gentoo	94	S	S	A	S	M	S	S	S	M	–	This work
Pygoscelis adeliae	adelie	95–96	S	S	A	S	M	S	S	S	T	T	This work
Aptenodytes forsteri	emperor	72	S	S	A	S	S	M	M	S	*	–	Cardozo et al. 2003
Aptenodytes patagonica	king	72	S	S	A	S	M	S	S	S	S	–	Cardozo et al. 2003

M= Metacentric; S= Submetacentric; A= Acrocentric; T= Telocentric; - = absent; * Unanalyzed female.

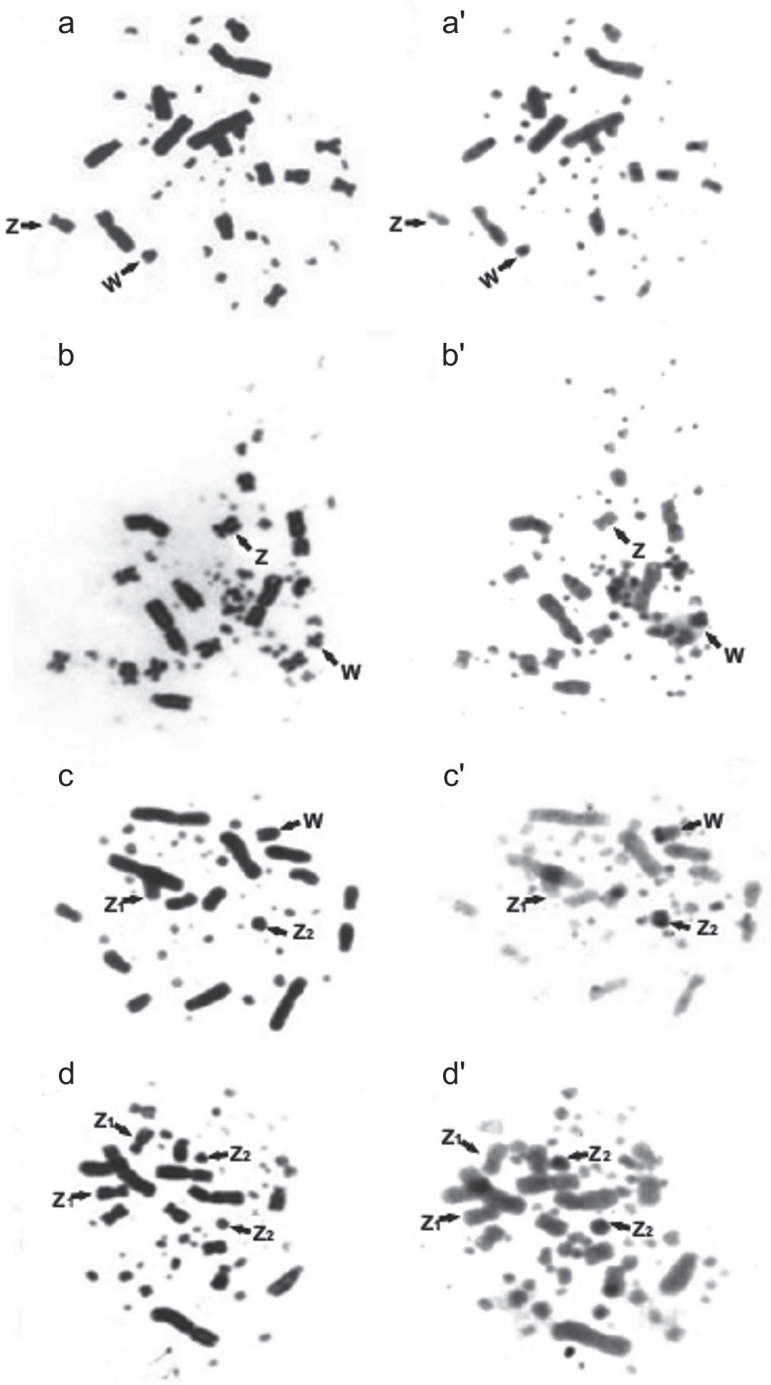

Figure 2. Metaphases in sequential Giemsa-C banding analysis **a-a'** female of the *P. antarcticus* **b-b'** female of the *P. papua,* **c-c'** female and **d-d'** male of *P. adeliae*. The arrows show the sex chromosomes Z, Z_2 and W.

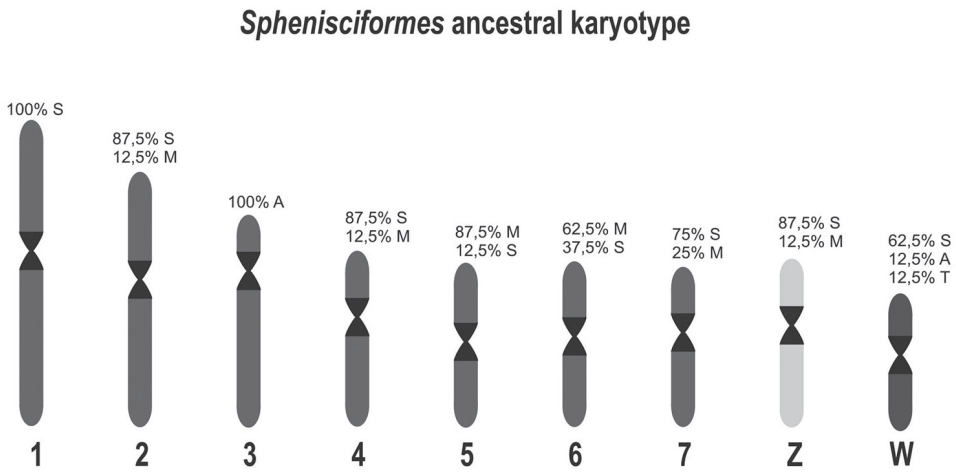

Figure 3. Hypothetical Sphenisciformes ancestral karyotype representing the conserved morphology of the macrochromosomes and ZW chromosomes. The frequencies of the morphologies (Metacentric= M; Submetacentric= S; Acrocentric= A; Telocentric= T) in the current species are represented above each pair.

small variations that can be observed in the morphology of the macrochromosomes correspond to a thin line dividing a chromosome to be classified as metacentric or submetacentric, such as in par 6 and 7 of the king and emperor penguins according to Cardozo et al. (2003). These homologies can be certainly confirmed by chromosome painting with fluorescent *in situ* hybridization experiments.

Furthermore, on the basis of these observations, in this work we propose an ancestral karyotype for the present penguin species (Figure 3), based on the frequency of macrochromosomes and the sex chromosomes Z and W (Table 1). According to Baker (2006) penguins from the genus *Spheniscus* diverged from the *Eudyptula* penguins, thus it is quite probable that karyotypes highly similar are observed in *Eudyptula minor* Forster, 1781 and *E. albosignata* Finsch, 1874, whose karyotypes are unknown.

The W chromosome seems to play an important role in chromosomal evolution of Sphenisciformes. It presented morphological variations (Table 1) especially in the species from the genus *Pygoscelis* ranging from to acrocentric in *P. antarcticus,* to metacentric in *P. papua* and telocentric in *P. adeliae*. The analysis of C-banding made possible the differentiation of *P. antarcticus* and *P. papua* (Figure 2 a' and b') karyotypes in relation to *P. adeliae* (Figure 2 c'). However, it was not possible to differentiate the karyotypes of the first two species. In fact, the male karyotypes can only be recognized by their different diploid number, while in females the distinction is facilitated by the presence of the W chromosome that presented variations in morphology.

Multiple sex chromosome system in Adelie penguin

The most interesting result of this work was seen in a multiple sex chromosome system in *P. adeliae*, where males have $Z_1Z_1Z_2Z_2$ and female has Z_1Z_2W. In birds this observation is unpublished, in higher animals multiple systems are rare exceptions and can be found in fishes, reptiles (some snakes), in some mammals Monotremes (platypus and echidna), in marsupials, in some Neotropical Primates, in eight species of bats, in two species of antelope (White 1977, Yonenaga et al. 1979, Tucker and Bickham 1986, Rens et al. 2007, Steinberg et al. 2014).

According to White (1973), multiple sex chromosome systems have arisen as a result of rearrangements involving sex chromosomes and autosomes, by centric fusion, reciprocal translocation, centric fission or tandem fusions. Cioffi et al. (2011) emphasizes the importance of repetitive sequences in multiple forms of sex chromosomes from fish, generating differences in morphology and size among them.

In the case of *P. adeliae* our hypothesis is that the multiple systems originated from a translocation involving the ancestral heterochromatic Z2 chromosome, with the terminal portion of the long arm of the W chromosome (Figure 4a). This hypothesis explains the presence of differentiated heterochromatic segment in the W chromosome (Fig. 2c'). In addition, the absence of this segment in the W chromosomes of *P. papua* and *P. antarcticus* suggests that this translocation occurred after their separation from *P. adeliae* or it was lost. A fairly similar heterochromatic labeling pattern was described by Toledo and Foresti (2001) on the X chromosome from the fish *Eigenmannia virescens* Valenciennes, 1842 and according to the authors these represent an early evolutionary stage of sex chromosome differentiation.

It is important to consider that the pairs Z2Z2 and Z1Z1 chromosomes, in males segregate normally during meiosis forming balanced gametes (Fig. 4b). In females, the formation of the trivalent Z1-W-Z2 must occur. The Z1 and Z2 segregate independently of the W chromosome. The W chromosome carries the translocated portion homologous to the Z2 chromosome (Fig. 4b). These hypotheses can be proven or refuted with the performance of meiotic analysis in females of *P. adeliae*.

In this work for the first time the karyotypes of *P. adeliae*, *P. papua* and *P. antarcticus* were presented. The presence of conserved macrochromosomes suggests that differences in diploid number 2n = 95-96, 94 and 92 are due to fusions between microchromosomes, reducing the diploid number. The results also point to a significant role of the W chromosome in speciation, with the first record of a multiple sex chromosome system in birds such as observed in *P. adeliae*. In addition, the comparison showed a high karyotype homology in Sphenisciformes which can be seen in the morphological conservation of macrochromosomes and in the chromosome Z.

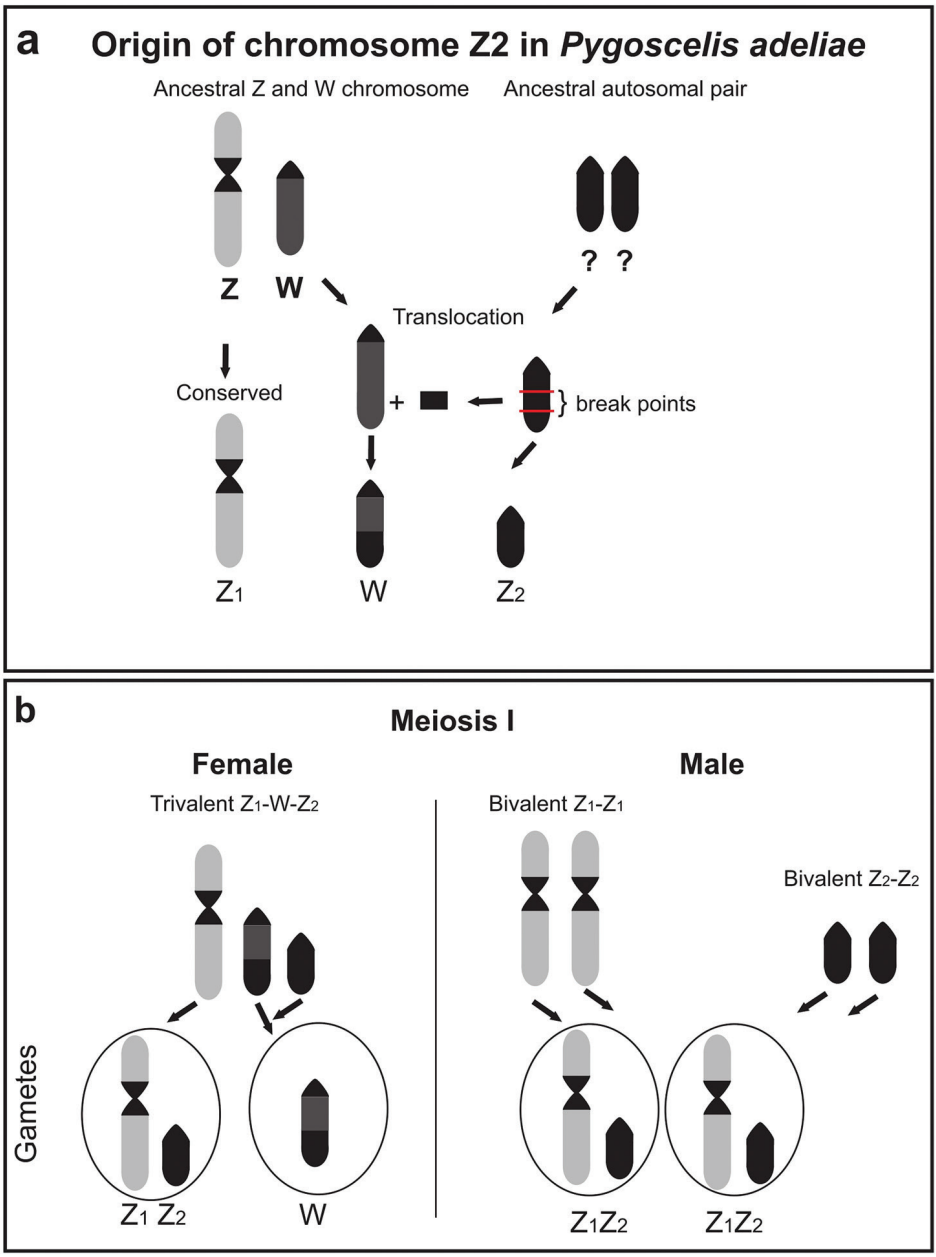

Figure 4. Schematic representation of the origin of the multiple sex chromosome system and a formation of the gametes during the meiosis I in *Pygoscelis adeliae* **a** The chromosomal translocation, which involving a heterochromatic segment of the ancestral chromosome Z2 with a terminal portion of the q arm of W chromosome **b** The meiosis in females and males of proposing the balanced gametes formation.

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