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Interstitial telomeric sites and Robertsonian translocations in species of *Ipheion* and *Nothoscordum* (Amaryllidaceae)

Gustavo Souza[1] · Andre L. L. Vanzela[2] · Orfeo Crosa[3] · Marcelo Guerra[1]

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Abstract The genera *Nothoscordum* and *Ipheion* (Allioideae, Amaryllidaceae) are cytologically characterized by a dysploid series with variable numbers of metacentric and acrocentric chromosomes typical of karyotypes rearranged by Robertsonian translocations (RT). Since they have large chromosomes, low diploid numbers, and possess two telomeric motifs [the vertebrate-type $(TTAGGG)_n$ and the *Arabidopsis*-type $(TTTAGGG)_n$] they are suitable for investigating the occurrence and possible role of interstitial telomeric sites (ITS) associated with RT. We analyzed the distributions of telomeric sites in 12 species of *Nothoscordum* and *Ipheion* and found that both telomeric probes colocalized in all chromosome termini. Cloning and sequencing PCR products obtained using both telomeric primers simultaneously revealed long stretches of $(TTAGGG)_n$ and $(TTTAGGG)_n$ sequences together with degenerated telomeric sequences. Most acrocentric chromosomes have a 45S rDNA site at the terminal region of the short arms adjacent to the most distal telomeric sites. Telomeric signals were found at all chromosome ends, but ITS were also detected in a few proximal and subterminal regions in some *Nothoscordum* species. Although RT are common in this group of plants, our findings suggest that proximal positioning of telomeric motifs are not necessarily related to that kind of rearrangement. Rather, transposition of telomeric sequences followed by amplification, could better explain the presence of $(TTAGGG)_n$ and $(TTTAGGG)_n$ repeats at those sites. Furthermore, a few small interstitial sites found in some *Nothoscordum* species indicate that dispersion of these sequences was not restricted to the proximal region.

Keywords Asparagales · Interstitial telomeric sites · *Ipheion* · *Nothoscordum* · Robertsonian translocations · 45S rDNA

Introduction

Telomeres are DNA–protein complexes located at the ends of eukaryotic chromosomes whose main functions are: (1) the prevention of end-to-end chromosome fusions; (2) interactions with proteins that bind chromosome tips to the nuclear envelope; and, (3) preventing the shortening of the linear DNA molecule caused by incomplete replication of the 5′ end by DNA polymerase (Kupiec 2014). Telomeric DNA sequences consist of tandemly repeated motifs rich in guanine (G) and thymine (T), although the combinations of these bases vary among different plant groups. The most common motif in plants is $(TTTAGGG)_n$, which was originally isolated from *Arabidopsis thaliana* (Brasicaceae) (Richards and Ausubel 1988) and it has been found in most species (Cox et al. 1993; Fuchs et al. 1995). Other telomeric sequences have been reported for species of the order

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✉ Gustavo Souza
lgrsouza@hotmail.com

[1] Laboratory of Plant Cytogenetics and Evolution, Centro de Ciências Biológicas, Department of Botany, Federal University of Pernambuco, Rua Nelson Chaves S/N, Cidade Universitária, Recife, PE 50670-420, Brazil

[2] Laboratory of Cytogenetics and Plant Diversity, Department of General Biology, State University of Londrina, Londrina, Paraná, Brazil

[3] Laboratory of Genetics, Department of Plant Biology, Faculty of Agronomy, University of the Republic, Montevideo, Uruguay

Asparagales (monocots), however, mainly the $(TTAGGG)_n$ motif characteristic of vertebrates (Adams et al. 2001; Sýkorová et al. 2003a, b).

Although telomeric DNA repeats are normally found at chromosome ends, interstitial telomeric sites (ITS) are found in different chromosomal regions in some species but have no obvious functions (Meyne et al. 1990; Cox et al. 1993; He et al. 2012). In several species, including the plant species *Vicia faba* (Schubert et al. 1992) and *Eleocharis subarticulata* (da Silva et al. 2005), ITS appear to represent traces of chromosomal fusions and have been found in several plant and animal species unrelated to any type of chromosomal rearrangements (Meyne et al. 1990; He et al. 2012).

The karyotypic differentiation of South American genera of Allioideae (Amaryllidaceae) is strongly associated with Robertsonian rearrangements (Jones 1998; Souza et al. 2010, 2012). *Nothoscordum*, the largest genus of this subfamily, comprises about 20 species with variable numbers of metacentric (M) and/or acrocentric (A) chromosomes and two putative basic chromosome numbers: $x = 4$ and $x = 5$ (Crosa 1972). The number $x = 5$ (3M + 2A) seems to be a plesiomorphic condition, as it appears in both sections of the genus (*Inodorum* and *Nothoscordum*), as well as in the closely related genus *Leucocoryne* (Crosa 1972; Souza et al. 2015). In spite of the large variation in chromosome number, all species of *Nothoscordum* display the fundamental number NF = 16, or multiples of 16. In contrast, the three species of *Ipheion*, another South American genus of Allioideae, have smaller chromosomes with different basic chromosome numbers and karyotype formula. *Ipheion tweedieanum* ($2n = 14$; 14A) differs from *I. uniflorum* ($2n = 12$; 2SM + 10A) by a chromosome fusion/fission, whereas *I. recurvifolium* ($2n = 20$; 4SM + 16A) seems to be a tetraploid with a more complex disploid reduction (Souza et al. 2010). The high frequency of chromosomal rearrangements found in *Ipheion* and *Nothoscordum* make this plant group a good target for investigating the role of telomeric sequences in karyotype evolution by Robertsonian translocations.

Species of Allioideae (Asparagales group) also show an uncommon variability of telomeric motifs, including the *Arabidopsis*-type $(TTTAGGG)_n$ and the vertebrate-type $(TTAGGG)_n$ (Sýkorová et al. 2003b, 2006). In *Allium* (Allioideae), these sequences were substituted by 45S rDNA, or by a satellite DNA (Pich et al. 1996). In the present, work we compared chromosomal distributions of telomeric DNA using fluorescent in situ hybridization (FISH) in nine species of *Nothoscordum* and three of *Ipheion*. We further analyzed the organization of both telomeric motifs by DNA sequencing of five telomeric segments of *Nothoscordum gracile* obtained by PCR.

Materials and methods

Plant material

Karyotype features as well as the voucher numbers and provenance of all of the specimens of *Nothoscordum* and *Ipheion* analyzed are presented in Table 1. Vouchers were deposited in the MVFA herbarium (Faculdad de Agronomía, Universidad de la República, Uruguay). Further karyotype details of the species have been described by Crosa (1972), Guerra and Felix (2000), and Souza et al. (2009, 2010, 2012).

Root tips obtained from bulbs were pretreated with 0.05 % colchicine, during 24 h at 10 °C, fixed in ethanol-acetic acid (3:1; v/v) for 2 to 24 h at room temperature, and stored at −20 °C until use. Fixed root tips were washed in distilled water and digested in a 2 % celullase (Onozuka)-20 % pectinase (Sigma) solution at 37 °C for 90 min. The meristems were squashed in a drop of 45 % acetic acid and the cover slips removed in liquid nitrogen.

Fluorescent in situ *hybridization*

The vertebrate-type telomeric motif was localized with a protein nucleic acid (PNA) probe labeled with FITC (Kit PNA/Telomere probe, Dako), following the instructions of the manufacturer. The telomere *Arabidopsis*-type $(TTTAGGG)_n$ and the 45S rDNA sites were detected using a 400 bp length clone (p*At*T4) labeled with Cy3-dUTP (GE) and a 6.5 kb 18S–5.8S–25S clone (R2) labeled with digoxigenin-11-dUTP as probes, respectively, both from *Arabidopsis thaliana*. The probes were labeled by nick translation and FISH was performed as previously described (Souza et al. 2010). The hybridization mix contained 30 % formamide (v/v), 10 % dextran sulphate (w/v), 2 × SSC, and 5 ng/μL of each probe. The slides were denaturated at 75 °C for three minutes. The 45S rDNA probe was detected with sheep anti-digoxigenin FITC conjugate (Roche) and amplified with rabbit anti-sheep FITC conjugate (Dako). Post-hybridization washes used SSC with astringency of 76 %. The slides were counterstained with 2 μg/mL DAPI/Vectashild® and examined using a Leica DMLB epifluorescence microscope. The images were captured with a Cohu CCD video camera using Leica QFISH software and later edited using Adobe Photoshop CS3 version 10.0.

Telomeric sequence analysis

Telomere sequences were amplified by PCR (as described by Ijdo et al. 1991, with small modifications) using *Arabidopsis* [$(TTTAGGG)_5$ and $(CCCTAAA)_5$] and vertebrate

Table 1 *Nothoscordum* and *Ipheion* species analyzed with respective voucher number, provenance, chromosome number (2*n*), karyotype formula, fundamental number (FN), and number and position of interstitial telomeric sites (ITSs) and 45S rDNA sites

Taxon	Voucher	Number of individuals	Provenance	2*n*	Karyotype formula[a]	FN	ITSs[b]	45S rDNA sites
Ipheion Rafinesque								
I. recurvifolium (Wright) Traub	MVFA 33781	1	Paso Rondán, Depto. Florida, Uruguay	20	18A + 2SM	22	–	10A
I. tweedieanum (Griseb) Traub	MVFA 21953	1	Ruta 24, km. 45.5, Depto. Río Negro, Uruguay	14	14A	14	–	14A
I. uniflorum (Raf) Traub	MVFA 33773	2	Ruta 26, km. 191, Depto. Tacuarembó, Uruguay	12	10A + 2SM	14	–	4A
Nothoscordum Kunth								
Section *Inodorum* Guag								
N. arenarium Herter	MVFA 7447	2	Colônia Winston, Depto. San José, Uruguay	10	6M + 4A	16	6P	4A
N. gracile (Ailton) Stearn	MVFA 2152	4	São Paulo-SP, Brazil	19	13M + 6A	32	–	
			Punta Ballena, Depto. Maldonado, Uruguay	18	14M + 4A	32	2P	4A
N. macrostemon Kunth	MVFA 1710	2	Arroyo Solís Grande, Depto. Canelones, Uruguay	10	6M + 4A	16	2P	4A
N. nudicaule (Lehm.) Guagl.	MVFA 104	3	Depto. Canelones, Uruguay	19	13M + 6A	32	–	
				18	14M + 4A	32	1P + 3ST	4A
Section *Nothoscordum* Guag								
N. bonariense Beauverd	MVFA 2730	4	Montevideo, Uruguay	26	22M + 4A	48	–	4A
				26	21M + 5A	47		4A
N. felipponei Beauverd (= *N. dialystemon* (Guagl.) Crosa)	MVFA 33778	1	Cerro Verdún, Depto. Lavalleja, Uruguay	10	6M + 4A	16	–	4A
N. montevidense Beauverd	MVFA 0004	2	Montevideo, Uruguay	8	8M	16	–	6M
			Arroyo Quebracho, Depto. Paysandú, Uruguay	16 + 1B	16M	32	–	6M
N. pulchellum Kunth	EAN 8425	3	Caruarú-PE, Brazil	10	6M + 4A	16	–	2A
Nothoscordum sp.1	MVFA IAN	1	Santiago, Chile	18	14M + 4A	32	–	2A + 4A

[a] *M* metacentric, *SM* submetacentric, *A* acrocentric

[b] *P* proximal, *ST* subterminal

$[(TTAGGG)_5$ and $(CCCTAA)_5]$ telomeric primers. To check for possible contiguous distributions of these telomere motifs in *Nothoscordum*, PCR with alternate forward and reverse primers $[(TTTAGGG)_5 + (CCCTAA)_5$ or $(TTAGGG)_5 + (CCCTAAA)_5]$ was performed using the *N. gracile* genome as the template. PCR reactions were performed in 100 μL containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.1 μM of each primer, and 2 units of *Taq* polymerase. Amplification consisted first of 10 cycles of 1 min at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, followed by 30 cycles of 1 min at 94 °C, 30 s at 60 °C, and 90 s 72 °C, with one final step of 5 min at 72 °C. After amplification, 5 μL of the PCR products were analyzed by electrophoresis at 3 V/cm in 1.0 % ultrapure agarose (Invitrogen™, USA), and then stained with 3 μL of 10 g/mL ethidium bromide.

The PCR products were cloned in pMOS Blue vector in Dh5α *E. coli* competent cells using a Blunt-Ended PCR cloning kit (GE Healthcare, USA). Recombinants were

tested using PCR with M13 universal primers. All amplified fragments were sequenced in both directions by Macrogen Inc. (Korea). The chromatograms and sequences were edited using Geneious version 7.1.4 (http://www.geneious.com, Kearse et al. 2012) for checking the *Arabidopsis* and vertebrate telomeric repeats. The sequences were deposited in the NCBI genebank (KP845244–KP845248). The fragments were analyzed with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to check the identities of non-telomeric inserts in PNG-Tel3 clone.

Results

Telomeric DNA cloning and sequence analysis

PCR using primers for both *Arabidopsis* and alternately positioned vertebrate motifs [$(TTTAGGG)_5$ + $(CCCTAA)_5$ or $(TTAGGG)_5$ + $(CCCTAAA)_5$] in the genome of *N. gracile*, amplified 254 to 444 bp length fragments (Fig. 1). The negative controls did not amplify any of the PCR reactions, indicating that the *Arabidopsis* and vertebrate alternated primers did not concatenate, and that the amplified fragment consisted of a segment present in the *N. gracile* genome. All of the clones showed both motifs organized in tandem repeats (Fig. 1). When the sequences were concatenated, the clones exhibited 77.9 % *Arabidopsis*-like sequences, 14.8 % vertebrate-like sequences, and 7.3 % degenerate sequences. The most important substitutions were related to T-slippage and substitution at poly-T or poly-G regions. The clones p*Ng*-tel3 and p*Ng*-tel6, with 338 and 83 bp lengths, respectively, showed non-telomeric fragments. Analysis using the BLAST tool revealed that the 338 bp insert of the p*Ng*-tel3 clone had ~69 % similarity (Fig. 1) with the 5S rDNA sequences of other angiosperms (Appendix S1).

Karyotypes and chromosomal distributions of 45S rDNA and telomeric motifs

All 12 species of *Nothoscordum* and *Ipheion* exhibited chromosome numbers and morphologies coincident with previous studies (Table 1). One tetraploid individual of *N. montevidense* showed a relatively small metacentric B-chromosome (Fig. 2b). FISH using a 45S rDNA probe showed that all rDNA sites were located in the terminal regions of metacentric or acrocentric chromosomes (Figs. 2, 4; Table 1) adjacent to the telomeric sites (Fig. 2c). An extra pair of 45S rDNA signals were observed in the proximal region of a metacentric pair in *Nothoscordum* sp. (Fig. 2e, f).

In situ hybridization using *Arabidopsis* and vertebrate-type telomeric probes showed signals in all chromosome termini of all *Nothoscordum* (Figs. 2, 4) and *Ipheion* (Fig. 3) species. Although the probes hybridized differentially in some termini, the sizes of their ectopic signals were similar (Fig. 2e, f). Interestingly, no telomeric hybridization signals were found at the chromosome ends of one metacentric chromosome of *N. montevidense* and two metacentrics of *N. bonariense* (Fig. 2d). These chromosome termini also did not hybridize with the 45S rDNA probe (arrowheads in Fig. 2a, d).

The diploids *N. macrostemon* and *N. arenarium* ($2n = 10$, 6M + 4A) showed interstitial telomeric sites in the proximal regions of one and three metacentric pairs respectively (Fig. 4b, c). The tetraploid sample of *N. gracile* ($2n = 18$; 14M + 4A) displayed ectopic sites in the proximal region of one metacentric pair (Fig. 4d). In *N. nudicaule* ($2n = 18$; 14M + 4A), minor telomeric signals were observed in the subterminal regions of one acrocentric and two metacentric chromosomes (Fig. 4f). Sequential hybridization with plant- and vertebrate-type telomeric probes showed colocalization in practically all sites, including the ITS. In some sites, however, the signal intensities were different for each probe (Fig. 2e, f); and one or both probes were not detected in a few cases (Fig. 2d).

The telomeric signals were distributed in Rabl orientations in all anaphases, telophases, and most interphase nuclei. It was notable that most telomere signals of species with 3M + 2A were present on one side of the nucleolus, with just four on the opposite side (Fig. 5a–c). The proximity between these telomeres and centromeres may have played a role in the putative transposition of telomere sequences from the telomeres of acrocentrics to the proximal regions of other chromosomes (Fig. 5d).

Discussion

Telomeric diversity in Nothoscordum and Ipheion

The results of FISH and PCR indicated that the chromosome ends of *Nothoscordum gracile* were composed of sets of different sequences. The *Arabidopsis*-like sequence was the most common motif, and it often alternated with vertebrate-type sequences and degenerated sequences associated with telomeric sequences. The presence of canonical and variant motifs were previously described for *Ipheion*, *Nothoscordum,* and other Asparagales (Weiss-Schneeweiss et al. 2004; Sýkorová et al. 2006). Terminal and subterminal chromosome regions are often found to harbor repeats of different natures, such as the 5S rDNA-like insert identified here in a telomeric clone of *N. gracile*, as well as satDNA, SSR, transposable elements, and others (Mizuno et al. 2006).

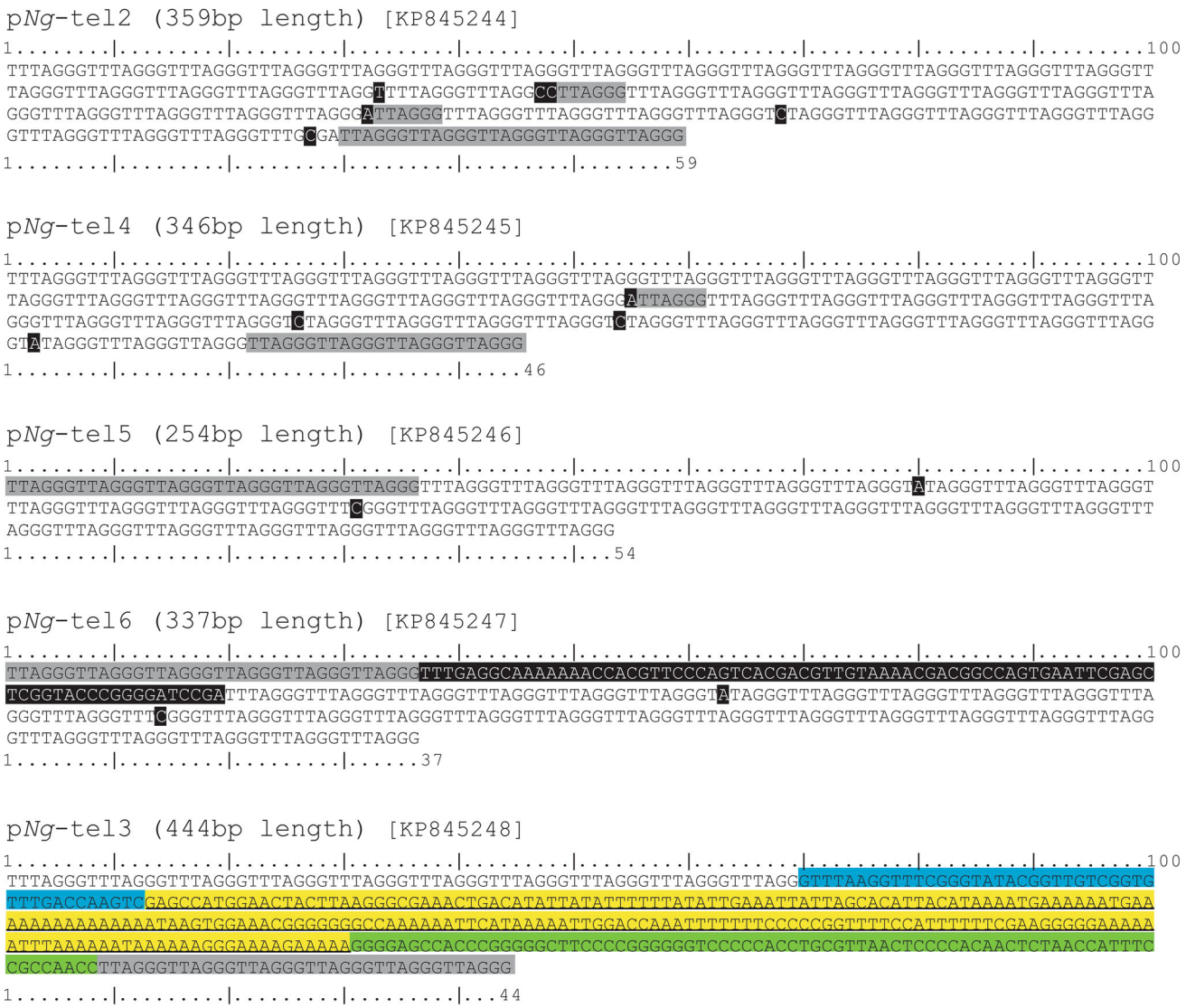

Fig. 1 Molecular and cytogenetic analysis of *Nothoscordum gracile* telomeric clones. Five telomeric clones (p*Ng*-tel) showing telomeric motifs *Arabidopsis*-type, vertebrate-type (*gray box*) and variant motifs (*black box*). The p*Ng*-tel3 clone differentiated into three segments (based in BLAST similarity): unknown, high identity with 5S rDNA intergenic spacer (*underlined*), and 5S rDNA gene

All of the 12 species of *Ipheion* and *Nothoscordum* investigated here showed hybridization signals with both telomeric probes (vertebrate- and *Arabidopsis*-types) in accordance with previous analyses of a few species of those genera (Sýkorová et al. 2003b). Both telomeric probes produced similar sized hybridization signals in most chromosome termini, although the signals were of different sizes or even absent in others. The apparent absence of signals could be due to telomeric sites below the detection capability of the FISH protocol used (Jiang and Gill 2006). Interestingly, in another species of Asparagales (*Othocallis siberica*), the *Arabidopsis*-type sequence seems to have been completely substituted by the vertebrate-type sequence in most telomeres (Weiss-Schneeweiss et al. 2004). Distal FISH signals more internally located than telomeric signals were observed using the 45S rDNA probe, but no cases were observed of 45S rDNA sequences substituting telomere-specific sequences, as was observed in *Allium cepa* (Pich et al. 1996).

Interstitial telomeric sites *versus* Robertsonian translocations

Chromosome number variations without changes in the numbers of chromosome arms, as observed in *Nothoscordum* and *Ipheion*, are commonly attributed to centric fusion-fission events (Guerra 2008). The presence of an ITS site in the proximal region of a bi-armed chromosome

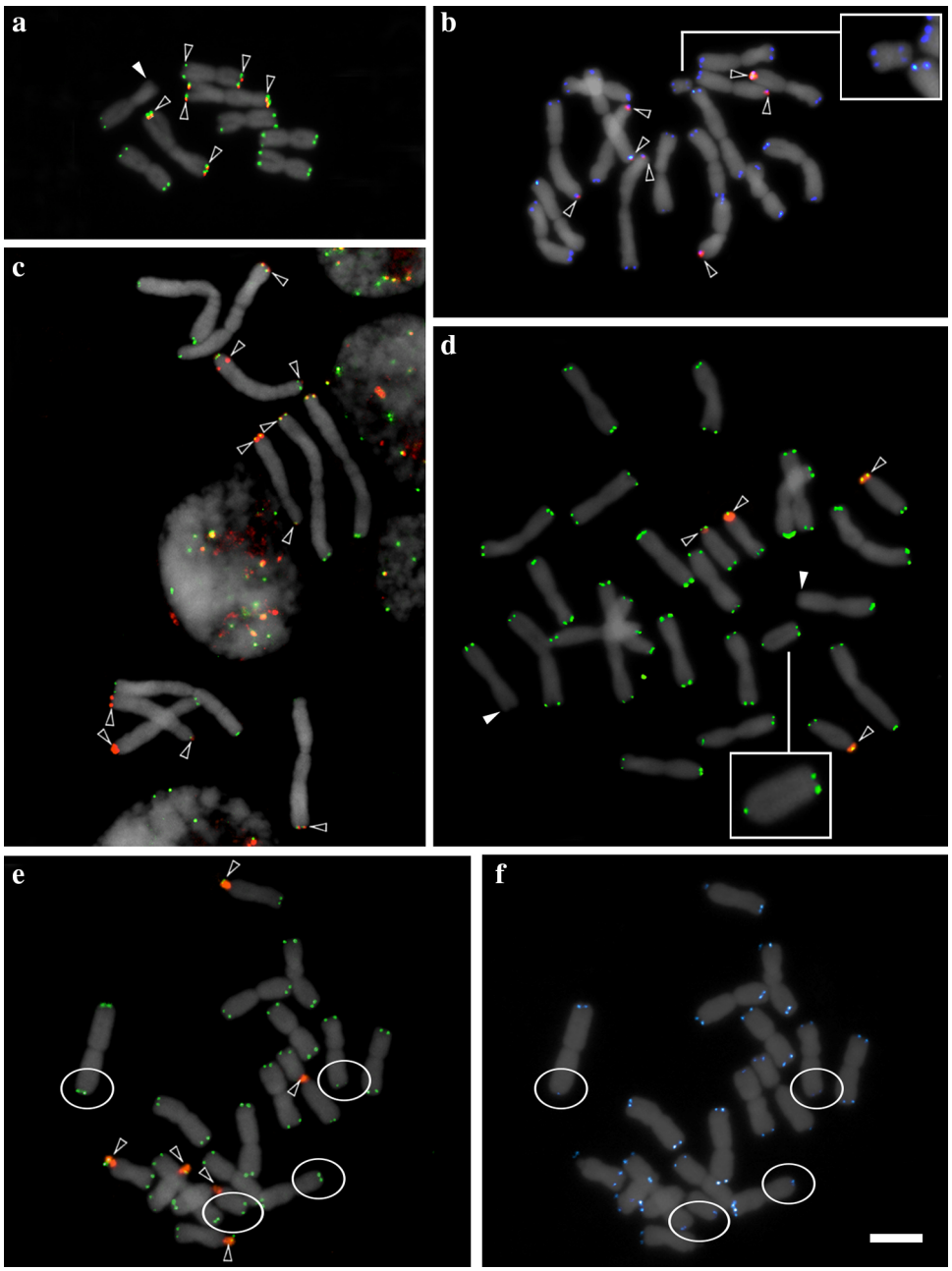

Fig. 2 Distribution of vertebrate-type (**a**, **c**, **d**, and **e**) and *Arabidopsis*-type (**b** and **f**) telomeres and 45S rDNA (*empty arrowhead* in **a**, **b**, **c**, **d**, and **e**) in species of *Nothoscordum* section *Nothoscordum*. **a**, **b** *N. montevidense* 2*x* (**a**) and 4*x* with a B chromosome (**b**); **c**, *N. felipponei*; **d**, *N. bonariense*; **e**, **f**, *Nothoscordum* sp. 1 sequentially in situ hybridized with vertebrate-type (**e**) and *Arabidopsis*-type (**f**) telomere. *Arrows* in **e** and **f** point to the proximal 45S rDNA sites. *Circles* in **e** and **f** show some chromosome termini with predominance of vertebrate-type telomere. *Bar* in **f** represents 10 μm

in species showing this type of chromosome number variation may represent a remnant of an end-to-end fusion between the short arms of acrocentric chromosomes (Ruiz-Herrera and Robinson 2008). The proximal ITS detected in some metacentric chromosomes of the $2n = 18$ cytotypes of *N. nudicaule* and *N. gracile*, therefore apparently derived from centric fusions ($2n = 18$, 19, instead of the expected $2n = 20$), and can be explained by the fusion of two non-homologous acrocentrics. Such sites are unstable, even considering the small samples of *N. nudicaule* and *N. gracile* investigated here. Similarly, ITS were found only in some cultivars of *Vicia faba*, indicating a very dynamic process of dispersion, amplification, and eventual losses of ITS (Schubert et al. 1992; Fuchs et al. 1995).

There are, however, some reasons to believe that centric fusion is not the most probable origin of proximal ITS in these species. Firstly, there is no indication that the metacentrics with interstitial sites were involved in the chromosome number reductions. Secondly, proximal ITS were also found in some metacentric chromosomes of the diploids *N. arenarium* and *N. macrostemon*, both of which have the basic karyotype of the genus ($n = 6M + 4A$) and no indication of centric fusions. Thirdly, none of the other karyotypes of *Ipheion* and *Nothoscordum* displayed ITS,

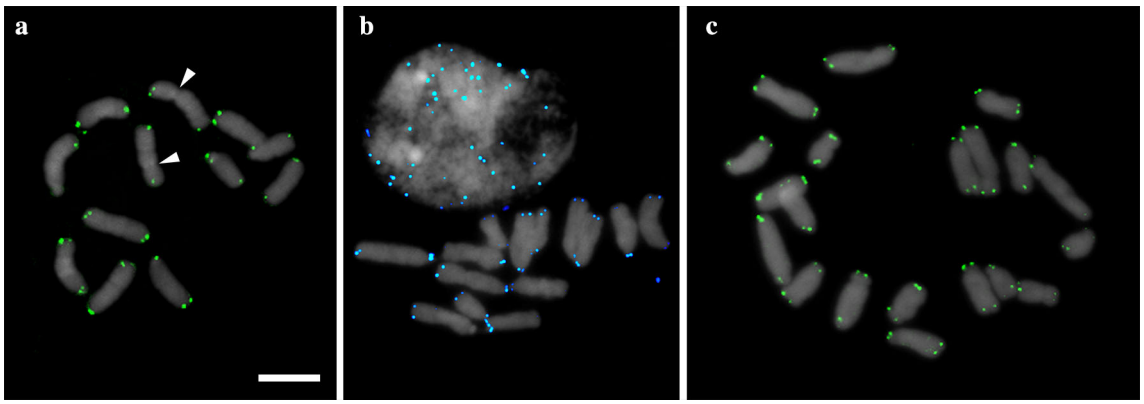

Fig. 3 Distribution of vertebrate-type (**a** and **c**) and Arabidopsis-type (**b**) telomere in *Ipheion* species. **a**, *I. uniflorum*; **b**, *I. tweedieanum*; **c**, *I. recurvifolium*. *Arrowheads* in **a** indicate the centromeric region of the submetacentric pair. *Bar* in **a** represents 10 μm

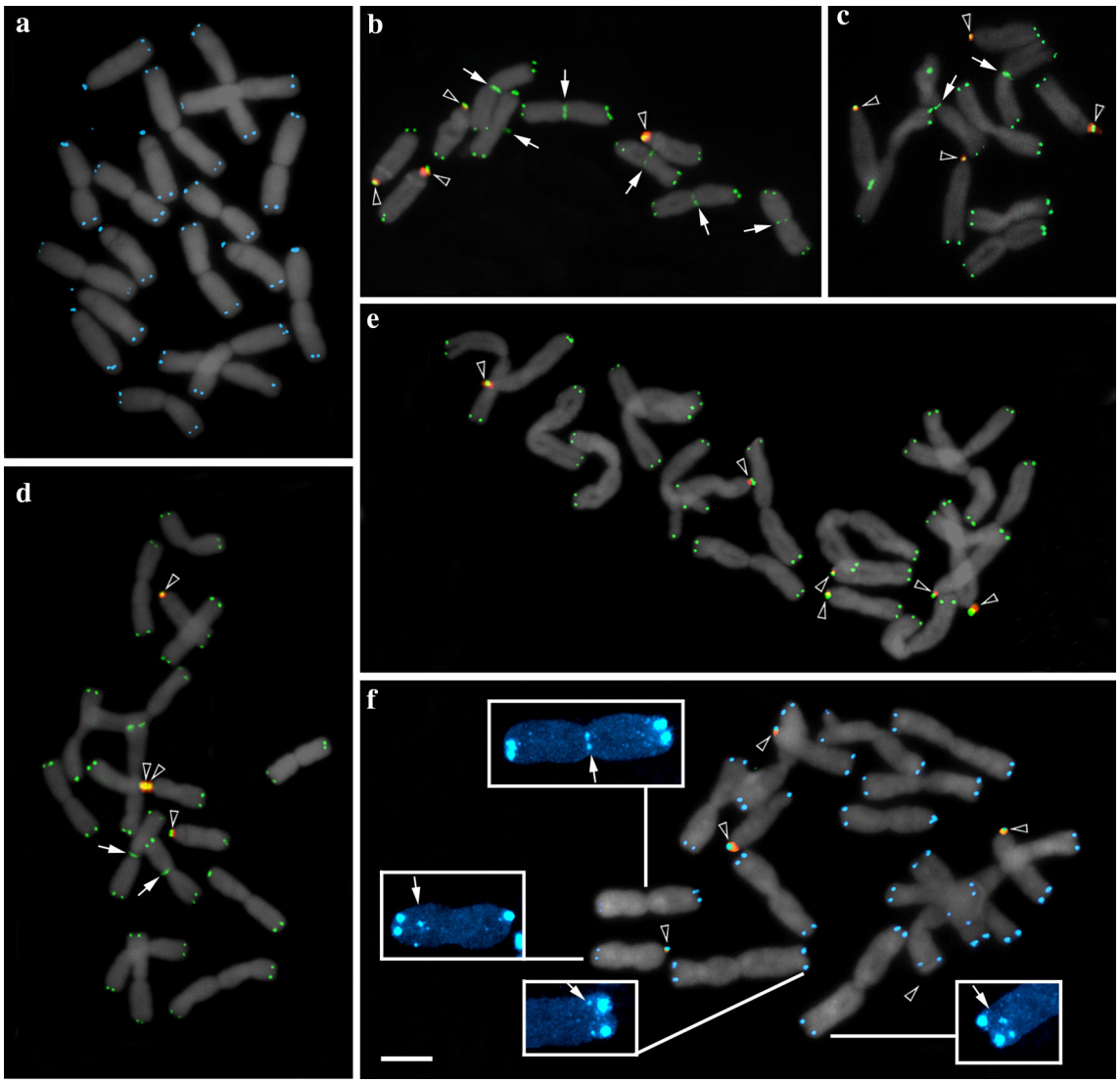

Fig. 4 Distribution of vertebrate-type (**b**, **c**, **d**, and **e**) and Arabidopsis-type (**a** and **f**) telomeres and 45S rDNA (*empty arrowhead* in **b**, **c**, **d**, **e**, and **f**) in species of *Nothoscordum* section *Inodorum*. **a**, **d** *N. gracile* with $2n = 19$ and $2n = 18$, respectively. **b** *N. arenarium*. **e**, **f** *N. nudicaule* with $2n = 19$ and $2n = 18$, respectively. *Arrows* indicate interstitial telomeric sites (ITS) and *empty-arrowheads* point to 45S rDNA sites. *Inserts* in **f** show ITS. *Bar* in **f** represents 10 μm

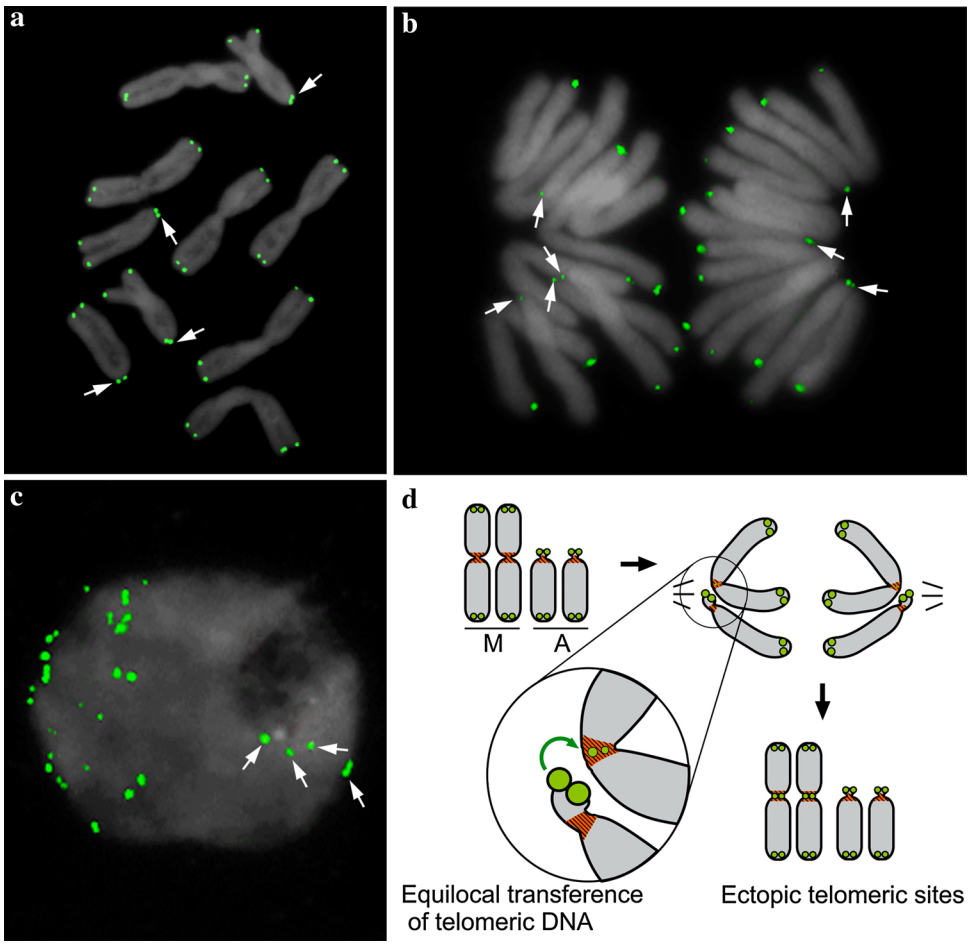

Fig. 5 Distribution of vertebrate-type telomere in metaphase (**a**), anaphase (**b**), and interphase (**c**) of *Nothoscordum pulchellum*. In **d** model of equilocal dispersion of telomeric repeats based on Schweizer and Loidl (1987). In **d** striped = pericentromeric regions; M = metacentrics, A = acrocentrics

although most of them show chromosome number reductions with conserved chromosome arm numbers (FN). Last, but not least, ITS were also found in subterminal positions in *N. nudicaule* ($2n = 18$), indicating that at least some ITS are not related to centric fusions. Proximal ITS and chromosome number reductions in *Nothoscordum* species, including those with $2n = 18$, may therefore be unrelated events, although the accidental maintenance of telomeric arrays during fusion events should be considered (Ocalewicz 2012).

If interstitial telomeric sites did not originate from centric fusion, why are they mostly restricted to proximal regions? The answer may be found in the different mechanisms of ITS origin. Small ITS may have been originated through different mechanisms of DNA double-strand break (DSB) repair (reviewed by Lin and Yan 2008), whereas larger ITS may have arisen by transposition from telomeric region arrays to other regions of the chromosome, either by mobile elements, non-homologous recombination, or other classical rearrangements such as inversions or tandem fusions (Azzalin et al. 2001; Lin and Yan 2008; Ruiz-Herrera and Robinson 2008; Ocalewicz 2012). Since the ITS of *Nothoscordum* species hybridized with TTAGGG and TTTAGGG probes, their most probable origin was by transposition of relatively large telomeric DNA arrays. In this case, Rabl chromosome orientation during interphase may have facilitated telomere-centromere transpositions, since it puts the telomeres of the acrocentric short arms and centromeres of the metacentric chromosomes in close proximity (Schweizer and Loidl 1987). Likewise, the subterminal ITS found in *N. gracile* may have originated by equilocal dispersion of telomeric arrays between long arms of similar sizes. Once transferred to proximal regions, the complex mixture of telomere sequences could have spread to other centromeric regions, as in *N. arenarium* and in several other species with widespread proximal ITS (He et al. 2012; Scacchetti et al. 2015).

Noteworthy, the occurrence of ITS in *Nothoscordum* species was restricted to the section *Inodorum*, which comprises *N. arenarium, N. gracile, N. macrostemon*, and *N. nudicaule*. Neither ITS nor intraspecific RTs have been reported in the remaining species belonging to the section *Nothoscordum*. Robertsonian translocations between acrocentric and metacentric chromosomes, but not between

metacentrics, has been demonstrated in *N. gracile* and *N. arenarium* (Souza et al. 2009, 2012), suggesting that the short arms of those acrocentric chromosomes may function as hotspots for RTs. In addition to telomere sequences, the short arms of acrocentrics usually contain pericentromeric heterochromatin, and are preferential sites for 45S rDNA repeats (Roa and Guerra 2012). Thus, being highly enriched in repetitive sequences, they are considered good candidates for hotspots for non-homologous recombinations (Raskina et al. 2008; Ruiz-Herrera and Robinson 2008; Cazaux et al. 2011; Jarmuz-Szymczak et al. 2014). Therefore, the proximal ITS of *Nothoscordum* species are most likely the cause rather than the consequence of the many RTs.

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