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Cytogenetic and molecular evidence suggest multiple origins and geographical parthenogenesis in *Nothoscordum gracile* (Alliaceae)

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• *Background and Aims* *Nothoscordum gracile* is an apomitic tetraploid widely distributed throughout the Americas and naturalized in many temperate regions of other continents. It has been suggested to form a species complex with sexual and apomictic *N. nudicaule* and *N. macrostemon*. Tetraploids of these species also share a structurally heterozygous chromosome complement $2n = 19$ (13M + 6A). In this work, the origin of *N. gracile* and its relationships with its related species was investigated based on cytological and molecular data.
• *Methods* Cytogenetic analyses were based on meiotic behaviour, CMA bands, localization of 5S and 45S rDNA sites, and genomic *in situ* hybridization (GISH). Nuclear ITS and plastidial *trn*L-*trn*F sequences were also obtained for most individuals.
• *Key Results* Proximal CMA bands were observed in the long arms of all acrocentrics of $2x$ and $4x$ *N. macrostemon* but not in diploid and some tetraploid cytotypes of *N. nudicaule*. Samples of *N. gracile* showed a variable number of CMA bands in the long arms of acrocentrics. Analysis of ITS sequences, dot-blot, GISH, and 5S and 45S rDNA sites, revealed no differentiation among the three species. The *trn*L-*trn*F cpDNA fragment showed variation with a trend to geographical structuring irrespective of morphospecies and fully congruent with karyotype variation.
• *Conclusions* The $2n = 19$ karyotype was probably formed by a centric fusion event occurring in *N. nudicaule* and later transmitted to tetraploid cytotypes of *N. macrostemon*. Diploids of *N. nudicaule* and *N. macrostemon* appeared as consistent recently diverged species, whereas tetraploid apomicts seem to constitute an assemblage of polyploid hybrids originating from multiple independent hybridization events between them, part of which are morphologically recognizable as *N. gracile*.

Key words: *Nothoscordum gracile*, CMA bands, rDNA sites, haplotype network, Robertsonian translocations.

INTRODUCTION

Taxonomical decisions in apomictic complexes may particularly affect the outcome of evolutionary and phylogenetic studies. The level of intraspecific variability is dependent on the circumscription of the entity under study which, in extreme cases, may constitute a monoclonal apomictic microspecies (Gornall, 1999). The broader distribution of some asexual taxa relative to their sexual relatives is referred to as 'geographic parthenogenesis' (Vandel, 1928). The success of an apomictic clone is attributed to its ability to colonize new environments where homogenous abiotic conditions limit the spread of sexual relatives. New populations may be efficiently established from a single propagule, or by the indefinite propagation of a particular genotype that is able to adapt to a wide range of environmental conditions, and which is maintained due to the absence of recombination (reviewed in Bierzychudek, 1985; Verduijn *et al.*, 2004). Range expansions are also attributed to the effect of polyploidy irrespective of breeding systems (Lowry and Lester, 2006). In polyploid/agamic complexes, the effects of asexual reproduction, hybridity and polyploidy on range expansion are confounded, given that frequently the polyploid components are in turn of hybrid origin and reproduce by apomixis (Bierzychudek, 1985; Hörandl, 2006).

The genus *Nothoscordum* Kunth (Gilliesioideae, Alliaceae) is native to South America and comprises >20 described species (Guaglianone, 1972; Crosa, 1996). *Nothoscordum gracile* (Aiton) Stearn, also referred to as *N. inodorum* (Aiton) G.Nicholson or *N. fragrans* (Vent.) Kunth (Stearn, 1986), is the only *Nothoscordum* species that is widely distributed throughout the Americas and naturalized in many temperate regions of other continents. It belongs to the section *Inodorum* Guag. and it is closely related morphologically and cytologically to *N. nudicaule* (Lehm.) Guagl. and *N. macrostemon* Kunth (Guaglianone, 1972; Nuñez *et al.*, 1974), which have previously been classified as varieties of *N. gracile* (Guaglianone, 1972). Nuñez *et al.* (1974), based mainly on mitotic and meiotic chromosome analyses, concluded that these three species constitute a closely related group forming the *Inodorum* complex.

The chromosomes of *Nothoscordum*, as well as those of other Alliaceae taxa, are easily amenable to detailed cytogenetic analysis and may provide a wealth of information in evolutionary studies. All representatives of the section *Inodorum* have large metacentric (M) or acrocentric (A) chromosomes, some

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>20 μm long (Nuñez *et al.*, 1974). The basic number of the section is $x = 5$ (3M + 2A) and the number of chromosome arms, or fundamental number (FN), is always eight or a multiple of eight. Diploids have $2n = 10$ (6M + 4A) and FN = 16, and tetraploids display $2n = 19$ (13M + 6A) or rarely $2n = 18$ (14M + 4A), both with FN = 32 (Crosa, 1972, 1996). Dyer (1967) and Nuñez *et al.* (1974) observed seedlings with $2n = 20$ obtained from seeds of individuals of *N. gracile* with $2n = 19$, suggesting that the perfectly doubled karyotype is occasionally formed. This variation in chromosome numbers among tetraploids ($2n = 18$, 19 and 20) seems to be due to centric fission/fusion events, which frequently occur in the genus *Nothoscordum* (Crosa, 1996; Jones, 1998; Souza *et al.*, 2009).

Nothoscordum gracile, *N. macrostemon* and *N. nudicaule* were initially known as strictly polyploid (Nuñez *et al.*, 1974), but the finding of diploid cytotypes of the two latter species provided some insight into the evolutionary history of the complex *Inodorum* (Crosa, 1996). Meiotic, morphological and reproductive biology studies have confirmed that the diploid cytotypes of both taxa are allogamous and self-incompatible (Crosa, 1972, 1996). They are easily distinguishable from one another due to their morphological and phenological differences. Both display a more restricted geographical distribution than their respective tetraploid cytotypes, diploid *N. nudicaule* having a relictual distribution along the river banks of eastern Uruguay. On the other hand, tetraploids of *N. macrostemon* and *N. nudicaule* are facultatively apomictic, self-pollinating and more widespread, the former being the dominant species in the south of Uruguay, colonizing roadside lands and cultivated areas (Crosa, 1996).

Nothoscordum gracile is an invasive species found exclusively in disturbed habitats. It is a strictly tetraploid, facultative apomictic, showing high variability in the shapes of its ovaries, staminate filaments and tepals (Guaglianone, 1972). Some of these variant forms are intermediate between *N. nudicaule* and *N. macrostemon*, which led Ravenna (1978) to propose a hybrid origin for this species. The species *N. arenarium* Herter, included in section *Inodorum*, is strictly diploid (Crosa, 1972; Souza *et al.*, 2009).

Cytologically, *N. gracile* is the most studied species of the genus, although the origin of the number $2n = 19$ is still controversial. Nuñez *et al.* (1974) suggested that $2n = 19$ arose from the union of a non-reduced gamete of a species with $2n = 10$ with a reduced gamete of a species with $2n = 18$. Alternatively, $2n = 19$ could have arisen from the fusion of two acrocentric chromosomes of a normal tetraploid with $2n = 20$ (12M + 8A) (Crosa, 1996). Nuñez *et al.* (1974) and Jones (1998) suggested that *N. gracile* could be an allotetraploid involving the two basic numbers of the genus, $x = 4$ (4M) and $x = 5$ (3M + 2A), with three sets of $n = 5$ and one of $n = 4$. Whatever its origin, the maintenance of this singular chromosome number is ensured by adventitious nucellar embryony and bulb offsets, as well as by sexual reproduction (Dyer, 1967).

Karyotypic analyses of *N. gracile* using chromosome C-banding techniques have shown small bands on the short arms of the six acrocentric chromosomes and in the proximal regions of the long arms of five of them, one of the proximal bands being duplicated. This same heterozygotic banding pattern was observed in plants from Japan (Kurita and Kuroki, 1963; Sato *et al.*, 1979) and from Argentina (Canelada and Fernandez, 1985), suggesting the occurrence of a single widespread apomictic clone. The heterochromatin on the short arms of acrocentric chromosomes is associated with nucleolus organizer regions (NORs), as first reported in *N. gracile* by Levan and Emsweller (1938) and later confirmed by a number of other authors (see also Kurita and Kuroki, 1963; Sato *et al.*, 1982; Canelada and Fernandez, 1985). The presence of 45S rDNA sequences on these short arms was first demonstrated by Crosa (1996) in *N. nudicaule* using fluorescent *in situ* hybridization (FISH). 45S rDNA sites were also observed on the short arms of the acrocentric chromosomes of *N. arenarium*, *N. hirtellum* (Kunth) Herter, *N. felipponei* Beauverd and *N. pulchellum* Kunth (Guerra and Felix, 2000; Souza *et al.*, 2009, 2010).

C-banding in chromosomes of *N. arenarium* and *N. pulchellum* revealed exactly the same banding pattern observed after double staining with the fluorochromes chromomycin A3 (CMA) and 4',6-diamidino-2-phenylindole (DAPI) (Guerra and Felix, 2000; Souza *et al.*, 2009). The heterochromatic bands, including the 45S rDNA sites, were always positively stained with CMA and negatively stained with DAPI (CMA bands). As the CMA/DAPI technique is simpler than C-banding, does not alter the chromatin structure, and the best slides can be reutilized for FISH analyses, it became the most suitable method for sequential banding and FISH analyses in these species.

The relationships between *N. gracile* and the other species of the *Inodorum* complex may be further investigated by the comparison of DNA sequences. Analyses of the ITS (intergenic transcribed spacer) nuclear region of the 45S rDNA of *Allium* species revealed divergences among species greater than those detected with other DNA sequences, suggesting that this region could be adequate for the evolutionary studies of other members in the family Alliaceae (Friesen *et al.*, 2006; Nguyen *et al.*, 2008; Li *et al.*, 2010). Sequences of plastid DNA (cpDNA) are also important in phylogenetic analyses as they show uniparental inheritance. As a consequence, taxa of different ploidy levels can be included in the analyses irrespectively of the auto- or allopolyploid nature of the species involved, and their phylogenies can be directly inferred by conventional methods (Rua *et al.*, 2010). The combined use of nuclear ITS and plastid sequences has allowed us to analyse the relationships among some of the representatives of Gilliesioideae (Fay *et al.*, 2006).

The present work investigates the origin of *N. gracile* and its relationships with *N. macrostemon* and *N. nudicaule*, based on cytological data including chromosome number variability, meiotic behaviour, CMA bands, 5S and 45S rDNA sites, and genomic *in situ* hybridization (GISH). Additionally, evolutionary relationships between species and cytotypes were compared using ITS and *trn*L-*trn*F sequences and the *N. gracile* intraspecific variability was investigated searching for clonal diversification.

MATERIALS AND METHODS

Plant material

Eighty-five plants of *Nothoscordum gracile*, *N. macrostemon* and *N. nudicaule* collected from natural populations were analysed. A sample of *N. arenarium* was also used for comparison

in the sequence analysis. The collection sites and numbers of all examined individuals are shown in Table 1. Vouchers were deposited in the Bernardo Rosengurtt Herbarium at the Facultad de Agronomía, Universidad de la República, Montevideo, Uruguay (MVFA). Plants of *Ipheion uniflorum* (Lindl.) Raf. ($2n = 12$) and *I. recurvifolium* (C.H.Wright) Traub ($2n = 20$) were used as controls in GISH and slot-blot experiments, respectively (see Souza *et al.*, 2010 for details of samples and karyotypes).

Diagnostic characters to identify species followed Guaglianone (1972). The principal traits defined by this author to separate these three species were as follows: *N. gracile* and *N. macrostemon* show a turbinate-evident perianth; stamens upright and closely spaced, sometimes forming a cylinder around the gynoecium during anthesis, and staminate filaments curved at their apices. *Nothoscordum gracile* differs from *N. macrostemon* by the former having an ellipsoidal to obovate sessile ovary, while the latter has an oblong and stipulate ovary. On the other hand, *N. nudicaule* has a cupuliform-subrotate perianth and lanceolate-subulate staminate filaments joined at their bases. In general, the flowers of *N. macrostemon* are nocturnal and those of *N. nudicaule* are diurnal, while those of *N. gracile* are morphologically and phenologically intermediate between these two types.

Meiotic analyses, pollen-grain mitosis and pollen-grain germination

For meiotic analysis, young inflorescences from *N. gracile*, *N. macrostemon* and *N. nudicaule* were directly fixed in ethanol : acetic acid (3 : 1, v/v) for 2–24 h at room temperature and stored at −20 °C. Prior to slide preparation, young flower

TABLE 1. *Species cytologically investigated with provenance, accession number in the living collection, number of individuals analysed, chromosome number (*2n*) and number of acrocentric chromosomes without CMA bands (A^0), with one CMA band (A^1) or two CMA bands (A^2)*

Species	Provenance (locality, state/department/province, country)	Accession no.	No. of individuals	2*n*	Karyotype formulae	CMA bands		
						A^0	A^1	A^2
Nothoscordum gracile (Aiton) Stearn	Blumenau, Santa Catarina, Brazil	GRA1832	2	19	13M + 6A	1	4	1
	Botucatu, São Paulo, Brazil	GRA1817	2	19	13M + 6A	1	4	1
	Buenos Aires, Argentina	GRA1849	3	19	13M + 6A	1	4	1
	Gramado, Rio Grande do Sul, Brazil	GRA1718	1	19	13M + 6A	1	4	1
	Lima, Peru	GRA1813	3	19	13M + 6A	1	4	1
	Londrina, Paraná, Brazil	GRA1846	3	19	13M + 6A	1	4	1
	Montevideo, Montevideo, Uruguay	GRA1851	1	19	13M + 6A	1	4	1
	Passo Fundo, Rio Grande do Sul, Brazil	GRA1843	5	19	13M + 6A	1	4	1
	Santa Maria, Rio Grande do Sul, Brazil	GRA1845	6	19	13M + 6A	1	4	1
	São Paulo, São Paulo, Brazil	GRA1706	4	19	13M + 6A	1	4	1
	Lavras, Minas Gerais, Brazil	GRA1770	3	19	13M + 6A	3	3	–
	Londrina, Paraná, Brazil	GRA1716	10	19	13M + 6A	3	3	–
	Piracicaba, São Paulo, Brazil	GRA1848	2	19	13M + 6A	3	3	–
	Tucumán, Tucumán, Argentina	GRA1847	3	19	13M + 6A	3	3	–
	Maldonado, Uruguay	GRA1711	4	18	14M + 4A	–	4	–
N. macrostemon Kunth	Ruta 5, Rivera, Uruguay	MAC1391	1	10	6M + 4A	–	4	–
	Ruta 9, Arroyo Solís Grande, Canelones, Uruguay	MAC1358	4	10	6M + 4A	–	4	–
	Ruta 9, Arroyo Solís Grande, Canelones, Uruguay	MAC1712	2	19	13M + 6A	–	6	–
N. nudicaule (Lehm.) Guagl.	Uruguay	NUD1082	1	10	6M + 4A	4	–	–
	Ruta 7 Km 323, Durazno, Uruguay	NUD1392	1	10	6M + 4A	4	–	–
	Ruta 8, Arroyo Convoy, Treinta y Tres, Uruguay	NUD1363	1	10	6M + 4A	4	–	–
	Ruta 8, Arroyo Otazo, Treinta y Tres, Uruguay	NUD1361	1	10	6M + 4A	4	–	–
	Paso Aguiar, Rio Negro, Tacuarembó, Uruguay	NUD1386	1	10	6M + 4A	4	–	–
	Bella Unión, Artigas, Uruguay	NUD0104	2	10	6M + 4A	4	–	–
	Canelones, Uruguay	NUD1717	1	18	14M + 4A	2	2	–
	Ruta 9, Solís Grande, Canelones, Uruguay	NUD1714	5	18	14M + 4A	2	2	–
	Artigas, Uruguay	NUD1751	2	19	13M + 6A	6	–	–
	Bella Unión, Artigas, Uruguay	NUD1750	1	19	13M + 6A	6	–	–
	Ruta 3, Arroyo Malo, Flores, Uruguay	NUD1752	1	19	13M + 6A	6	–	–
	Canelones, Uruguay	NUD1717	1	19	13M + 6A	4	2	–
	Porto Alegre, Rio Grande do Sul, Brazil	NUD1842	4	19	13M + 6A	4	2	–
Nothoscordum sp. 1	Punta Ballena, Maldonado, Uruguay	**1707**	3	15	9M + 6A	–	6	–
Nothoscordum sp. 2	Punta Ballena, Maldonado, Uruguay	**1030**	1	19	13M + 6A	–	6	–

buds were washed in distilled water, anthers were squashed in a drop of 45 % acetic acid, the coverslip was removed after freezing in liquid nitrogen, and the chromosomes were stained with CMA/DAPI as indicated below. For pollen-grain mitosis fixed anthers were directly squashed in 2 % acetocarmine. For pollen-tube germination, anthers of opened flowers were gently squeezed in a drop of 15 % sucrose solution and maintained during 12–24 h in a wet chamber.

CMA/DAPI banding and FISH

Root tips obtained from bulbs were pretreated with 0·05 % colchicine for 24 h at 10 °C, fixed and stored as described before. CMA/DAPI banding and FISH procedures were performed according to Souza *et al.* (2009). Fixed root tips were washed in distilled water and digested in a 2 % (w/v) celullase (Onozuka)/20 % (v/v) pectinase (Sigma) solution, at 37 °C, for 90 min. Meristem was macerated in a drop of 45 % acetic acid and the coverslip was later removed in liquid nitrogen.

The CMA/DAPI double-staining technique was used for fluorochrome banding. Slides were aged for 3 d, stained with CMA (0·1 mg mL^{-1}) for 60 min and re-stained with DAPI (1 μg mL^{-1}l) for 30 min. Slides were mounted in glycerol : McIlvaine buffer pH 7·0 (1 : 1) and aged for 3 d before analysis in an epifluorescence Leica DMLB microscope. Images were captured with a Cohu CCD video camera using the Leica QFISH software and later edited in Adobe Photoshop CS3 version 10·0.

To localize the rDNA sites, 5S rDNA from *Lotus japonicus* labelled with Cy3-dUTP (Amersham) and 45S rDNA from *Arabidopsis thaliana* labelled with digoxigenin-11-dUTP were used as probes (Souza *et al.*, 2009, 2010). Both labellings were done by nick translation. The 45S rDNA probe was detected with sheep anti-digoxigenin FITC conjugate (Roche) and amplified with rabbit anti-sheep FITC conjugate (Dako). The hybridization mixture contained formamide 50 % (v/v), dextran sulfate 10 % (w/v), 2× SSC and 5 ng μL^{-1} of each probe. The slides were denaturated at 75 °C for 3 min. Stringent washes were performed reaching a final stringency of approx. 76 %. Images of the best cells were captured as previously described.

GISH and dot-blot hybridization

Total nuclear DNA was extracted following the CTAB method of Doyle and Doyle (1987). Genomic DNA utilized as probe or block was broken into fragments of approx. 500 bp by incubation at 100 °C for approx. 30 min. In the two first experiments, the genomic DNA of *N. gracile* labelled with Cy3-dUTP (Amersham) by nick translation was used as a probe, while non-labelled genomic DNA of *N. nudicaule* or *N. macrostemon* was separately utilized as blocking DNA in concentrations of 1 × , 25 × , 50× and 75×. The hybridization mixture containing the genomic probe and the blocking DNA was tested on metaphases of *N. gracile* or a control species, following the same protocol used for FISH.

In other experiments, the genomic DNA of *N. macrostemon* labelled with digoxigenin-11-dUTP was used as probe and non-labelled genomic DNA of *N. nudicaule* was used as blocking DNA. Inverse labelling was also tested using *N. nudicaule* DNA as probe and *N. macrostemon* as blocking DNA. As an internal control of hybridization efficiency, in some cases, root tips of *Ipheion uniflorum*, *N. macrostemon* (2*x*) or *N. nudicaule* (4*x*) (without CMA bands) were macerated together with root tips of *N. gracile* or *N. nudicaule* 4*x* on the same slide for *in situ* hybridization.

Dot-blot hybridization was performed using whole-genome DNA (500 ng) from *N. gracile*, *N. macrostemon* and *N. nudicaule* loaded manually onto a nylon membrane (Hybond N^+; Amersham Biosciences, Amersham, Bucks, UK). *Ipheion recurvifolium* DNA was used as negative control. DNA of *N. macrostemon* and *N. nudicaule* used as probes was labelled with digoxigenin-11-dUTP as described above. The membranes were hybridized at 37 °C overnight. The genomic DNA was added to the hybridization buffer [1 % blocking reagent (Roche), 0·1 % sodium dodecyl sulfate (SDS), 5 % dextran sulfate, in 5× SSC, pH 7·0], according to Sambrook and Russell (2001). After hybridization, membranes were washed twice in 2× SSC, 0·1 % SDS for 5 min and in 0·5× SSC, 0·1 % SDS for 15 min, at 45 °C and 68 °C for low and high stringency, respectively. The genomic DNA probe was detected using anti-DIG alkaline phosphatase conjugate (Roche) and CDP-Star (Roche). The hybridization signals were captured on X-ray ECL film (Amersham Biosciences) and the intensity of probe signals was measured in the digital image using software QFISH version 2·1 (Leica). The highest relative signal intensity (100 %) was obtained when labelled genomic DNA of one species was hybridized with genomic DNA of this same species.

Sequence analyses

The non-coding DNA plastid region *trn*L-*trn*F was analysed in 33 samples, including representatives from *N. macrostemon* (2*x*), *N. nudicaule* (2*x* and 4*x*), *N. gracile* (4*x*) and *N. arenarium* (2*x*). Universal primers (C and F) described by Taberlet *et al.* (1991) were used to amplify *trn*L(UAA) and *trn*L(UAA)-*trn*F(GAA) regions. The nuclear ITS1-5·8S-ITS2 region of 16 samples of *N. gracile*, *N. macrostemon*, *N. nudicaule* and *N. arenarium* were amplified and sequenced using ITS4 and ITS5 universal primers (White *et al.*, 1990).

All PCR amplifications were carried out in 50-μL reactions containing 5 U of Taq polymerase, 1·75 mM $MgCl_2$, 0·5 μM of each primer and 0·1 mM of each dNTP in the manufacturer's buffer. The PCR programme for cpDNA amplification consisted of an initial step of 5 min at 95 °C, the first cycle consisted of 1 min at 94 °C, 1 min at 58 °C and 2 min 30 s at 72 °C, and then the annealing temperature was decreased by 1 °C for six cycles followed by 32 additional cycles with an annealing temperature of 52 °C and a final elongation step of 5 min at 72 °C. For ITS amplification, the PCR programme consisted of an initial denaturing step of 95 °C for 2 min, five cycles of 95 °C for 1 min, 53 °C for 1 min and 72 °C for 2 min, with a decrease of 1 °C per cycle in the annealing temperature, 35 cycles with an annealing temperature of 48 °C, and a final extension step of 72 °C for 12 min. All amplified fragments were sequenced in both directions at the Instituto Pasteur de Montevideo (Uruguay) or by Macrogen Inc. (Korea).

The sequences were edited manually using Sequencher™ (V4·1·4; Genecodes, AnnArbor, MI, USA) and all ambiguous end regions were removed. The resulting partial sequences were pre-aligned with the Clustal-W (Thompson *et al.*, 1994) algorithm included in BioEdit version 5·0·6 (Hall, 2001) and manually adjusted. All sequences and alignments were submitted to Genbank for *trn*L-*trn*F (accession nos JN591627–JN591651) and ITS (accession nos JN591597–JN591618). A haplotype network was constructed based on this matrix using the software NETWORK version 4·5·1·6 based on the standard function of maximum parsimony (Fluxus Technology Ltd).

RESULTS

Mitotic analyses

Chromosome counts of 85 individuals from different localities revealed the occurrence of diploid [$2n = 10$ (6M + 4A)] and tetraploid [$2n = 18$ (14M + 4A) or $2n = 19$ (13M + 6A)] cytotypes of both *N. macrostemon* and *N. nudicaule* while *N. gracile* showed exclusively tetraploid cytotypes ($2n = 18$ or $2n = 19$). Tetraploids with $2n = 18$ were only rarely found in any of these species (13·0 %; Table 1). Diploid and tetraploid plants of the same species were very similar morphologically, and could be distinguished by cytological analyses only. *Nothoscordum nudicaule* was typically found in wet areas along river banks, while *N. macrostemon* and *N. gracile* were collected along roadsides. Plants with B chromosomes or mosaics with $2n = 18/19$ were never observed. However, metaphases with two acrocentric chromosomes associated by the ends of their short arms, resembling a single metacentric chromosome, were often found (Fig. 1A). Multiple associations including three or more 45S rDNA sites were also common (Figs 1 and 2E).

$CMA^+/DAPI^-$ banding (CMA bands) revealed the presence of heterochromatin on the short arms of the acrocentric chromosomes, and one or two CMA bands on the proximal region of the long arms of most acrocentrics (Figs 1 and 2). No $DAPI^+$ heterochromatic blocks were observed. According to the number of CMA bands on the long arms, the acrocentric chromosomes were classified as A^0 (without bands), A^1 (with one band), and A^2 (with two bands). Sequential analyses with CMA/DAPI and FISH using 5S and 45S rDNA probes revealed a single 5S rDNA site per monoploid complement, always located on the terminal region of a metacentric chromosome, and 45S rDNA sites on the short arms of all acrocentric chromosomes co-localized with CMA bands. No variation in the number or position of these sites was detected.

The most frequent karyotype of *N. gracile* was $2n = 19$ ($13M + 1A^0 + 4A^1 + 1A^2$), observed in 57·7 % of the individuals and considered the standard karyotype of this species. This karyotype was found in most Brazilian populations and in the samples from Lima (Peru), Buenos Aires (Argentina) and Montevideo (Uruguay) (Fig. 1B). Other populations from Brazil (Lavras, Londrina and Gramado) and Argentina (Tucumán), totalling 34·6 % of the individuals, showed $2n = 19$ ($13M + 3A^0 + 3A^1$) (Fig. 1C), whereas a single sample from Uruguay (Canelones) had $2n = 18$ ($14M + 4A^1$) (Fig. 1A). Four out of six individuals from Santa Maria (Brazil) displayed an aberrant $2n = 19$ karyotype ($13M + 2A^0 + 4A^1$) bearing a CMA band on the long arm of a metacentric chromosome, in the same position as that of the long arm of A^1 acrocentrics. Noteworthy, this karyotype also had one A^0 chromosome without a 45S rDNA site (Fig. 1D), suggesting a reciprocal translocation between an M and an A^1 chromosome. The other two individuals from this locality showed the standard karyotype. For the distribution of the different cytotypes of *N. gracile* see below (Fig. 5).

Diploid samples of *N. nudicaule* always showed acrocentric chromosomes without proximal CMA bands ($6M + 4A^0$) (Fig. 2A). Likewise, tetraploid plants from northern (Departamentos de Artigas and Salto) and south-eastern (Departamento de Flores) Uruguay (Fig. 2B) always showed $2n = 19$ ($13M + 6A^0$). A mixed population with $2n = 19$ ($13M + 4A^0 + 2A^1$) and $2n = 18$ ($14M + 2A^0 + 2A^1$) was found in southern Uruguay (Departamento de Canelones). The diploid and tetraploid samples of *N. macrostemon* examined had exclusively A^1 chromosomes, with karyotype formulae $2n = 10$ ($6M + 4A^1$) (Fig. 2C) or $2n = 19$ ($13M + 6A^1$) (Fig. 2E). Vigorous triploid individuals bearing many bulbils, referred to as *Nothoscordum* sp. 1 [$2n = 15$ ($9M + 6A^1$); Fig. 2D], were only found in Punta Ballena, southern Uruguay, together with the closely related tetraploid *Nothoscordum* sp. 2 [$2n = 19$ ($13M + 6A^1$)].

Meiotic analyses

Meiosis was regular in the diploid samples of *N. macrostemon* and *N. nudicaule*, with five bivalents and a predominance of interstitial chiasmata (Fig. 3A). On the other hand, the tetraploid cytotypes showed irregular meiotic pairing, with univalents, bivalents and multivalents, but only rare anaphase bridges or lagging chromosomes. A predominance of tetravalents formed by metacentric chromosomes was observed in tetraploid *N. macrostemon* ($2n = 19$) (Fig. 3B). In addition, one heteromorphic trivalent and multivalents with up to six acrocentric and one metacentric chromosomes were often observed (Fig. 3B). The heteromorphic trivalent was formed by one metacentric paired with two acrocentric chromosomes by their long arms (Fig. 3C). In tetraploid *N. nudicaule* the most common meiotic arrangement was $2^I + 3^{II} + 1^{III} + 2^{IV}$ (Fig. 3C), with an identical heteromorphic trivalent. The meiotic pairing of *N. gracile* was similar to that of the other tetraploid species, with the heteromorphic trivalent being particularly noticeable (Fig. 3C, insert).

In spite of their meiotic irregularities, the tetraploids of the *Inodorum* complex had apparently normal and viable pollen grains. When pollen grains of any tetraploid sample were immersed in a drop of 15 % sucrose solution, all or almost all of the grains germinated within 24 h. Chromosome counts in 38 cells in anaphase II (Fig. 3D) and 96 first pollen-grain mitosis (Fig. 3E, F) revealed a predominance of haploid chromosome complements of $n = 9$ (46·8 %) or $n = 10$ (45·7 %) with different combinations of metacentric and acrocentric chromosomes. Cells with $n = 8$ and $n = 11$ were also observed (7·4 %).

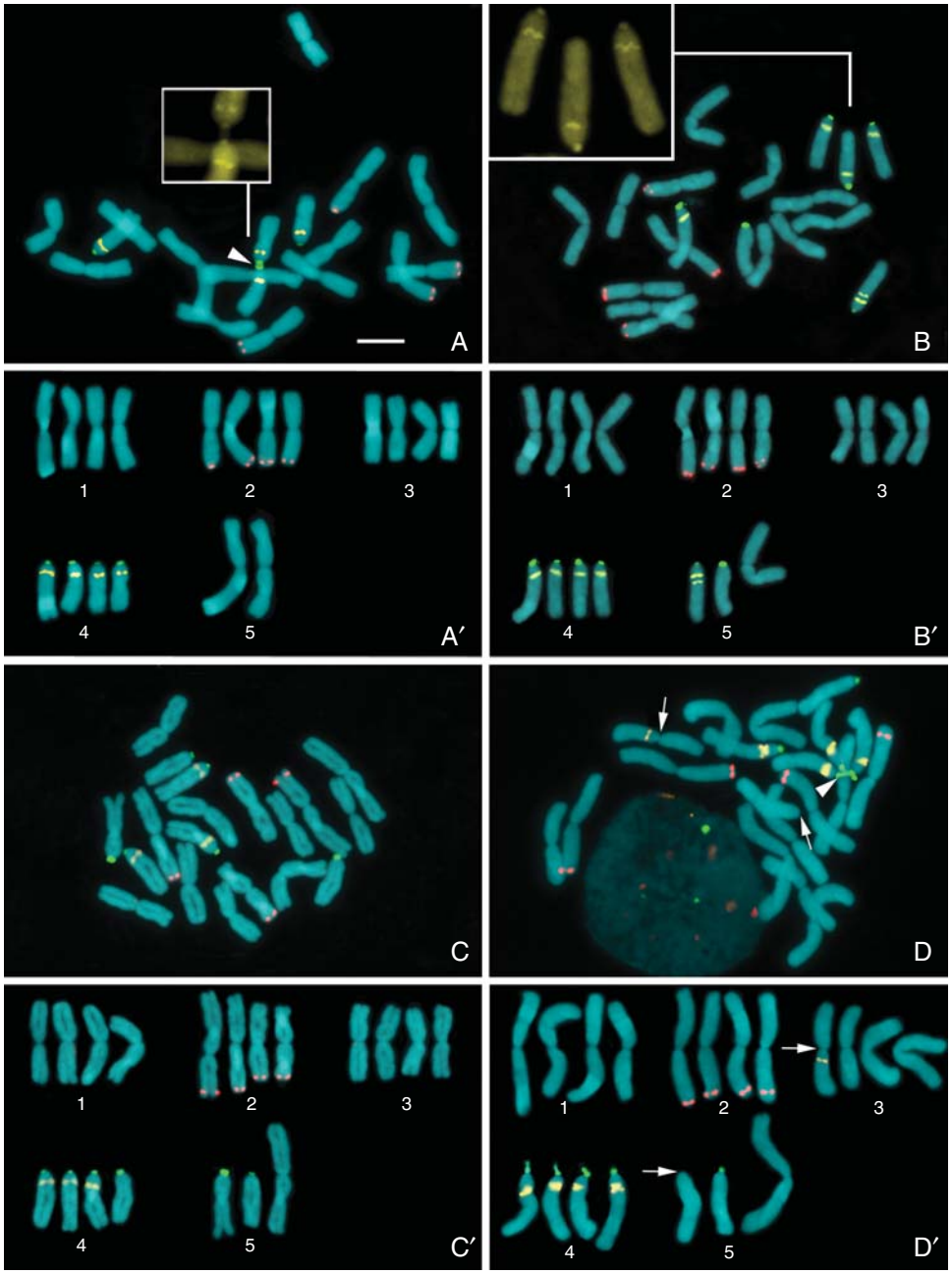

FIG. 1. Karyotype variability in *Nothoscordum gracile*: (A) $2n = 18$ ($14M + 4A^1$); (B) $2n = 19$ ($13M + 1A^0 + 4A^1 + 1A^2$); (C) $2n = 19$ ($13M + 3A^0 + 3A^1$); (D) $2n = 19$ ($13M + 2A^0 + 4A^1$) with a reciprocal translocation between the long arms of chromosomes 3 and 5 (arrows). (A'–D') Karyograms based on metaphases in (A–D). Observe the variable number of CMA bands on the proximal region of the long arms of acrocentrics. The arrowhead in (A) indicates an association between 45S rDNA sites of two acrocentric chromosomes. Inserts in (A) and (B) show higher magnification of short arms of acrocentric chromosomes stained with CMA. CMA = yellow, DAPI = blue, 5S rDNA = red, 45S rDNA = green. Scale bar in (A) = 10 μm.

GISH and dot-blot experiments

None of the GISH experiments using *N. gracile* metaphases and total nuclear DNA of *N. macrostemon* or *N. nudicaul*e as probe with or without blocking DNA could differentiate between the diploid genomes of *N. gracile*. All experiments showed uniform labelling of the chromosomes, whereas the chromosomes of the internal control (*Ipheion uniflorum*) were consistently weakly labelled (Fig. 4A, B). Moreover, after subsequent increments in the concentration of blocking DNA (25*x*, 50*x* and 75*x*) in the hybridization mixture, the labelling intensity of all chromosomes remained uniform. In the dot-blot analyses, the *N. macrostemon* probe hybridized with equal intensity on both *N. gracile* and *N. nudicaule* DNA, as well as on its own DNA. Similar results were observed when genomic DNA of *N. nudicaule* was used as probe (Fig. 4C). Only

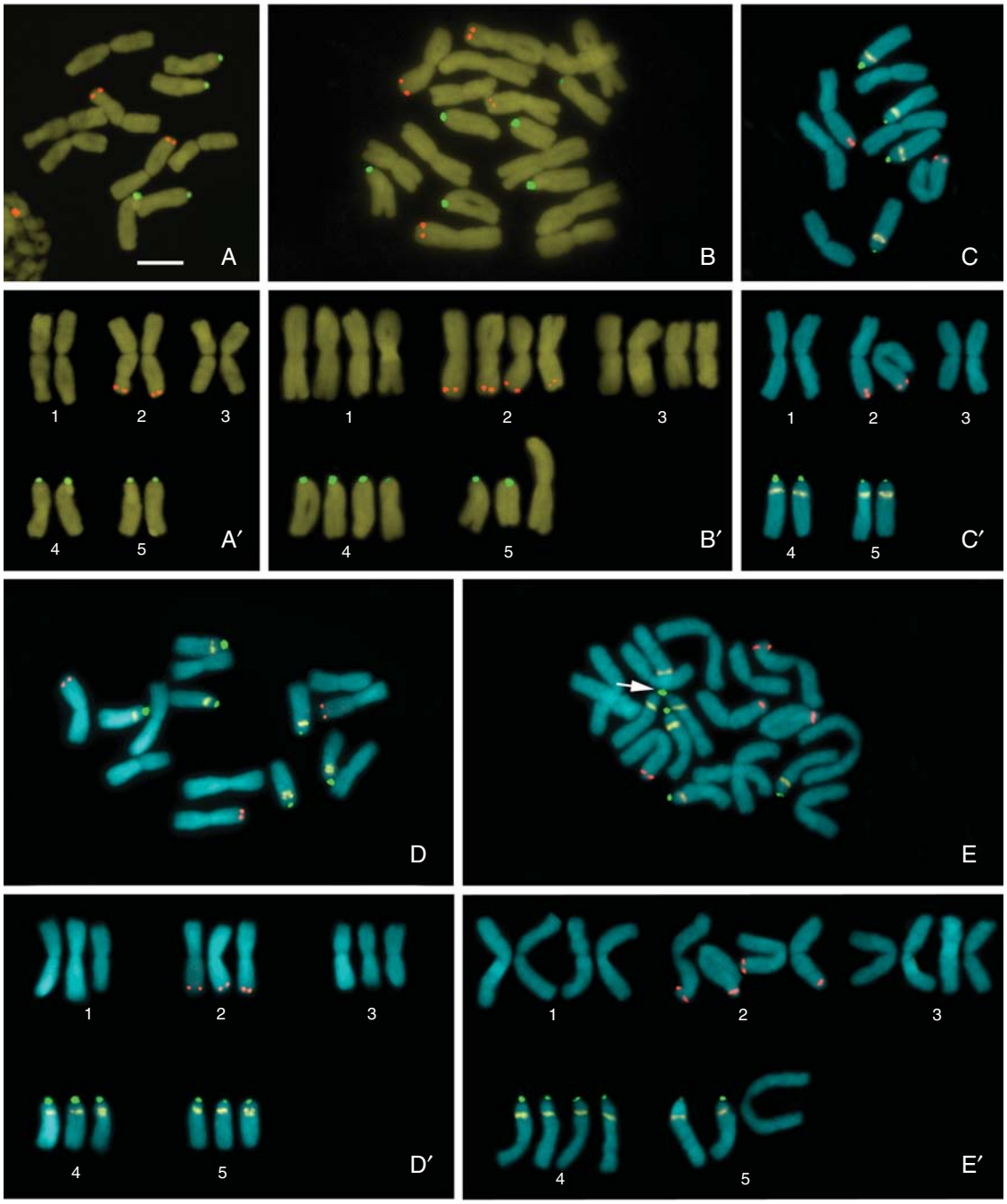

FIG. 2. Cytotypes of *Nothoscordum nudicaule*, *N. macrostemon* and *Nothoscordum* sp. 1: (A, B) *N. nudicaule* with $2n = 10$ ($6M + 4A^0$) (A) and $2n = 19$ ($13M + 6A^0$) (B); (C, E) *N. macrostemon* with $2n = 10$ ($6M + 4A^1$) (C) and $2n = 19$ ($13M + 6A^1$) (E); (D) *Nothoscordum* sp. 1 $2n = 15$ ($9M + 6A^1$). (A'–E') Karyograms based on metaphases in (A–E). The arrow in (E) indicates an association between three 45S rDNA sites. CMA = yellow, DAPI = blue, 5S rDNA = red, 45S rDNA = green. Scale bar in (A) = 10 μm.

when DNA of *N. macrostemon* or *N. nudicaule* was hybridized with genomic DNA of an external group (*Ipheion recurvifolium*) was a much lower labelling intensity (approx. 17 %) obtained.

Sequence analyses

The ITS1-5·8S-ITS2 nuclear region was highly conserved among the species analysed here, including *N. arenarium*. The aligned ITS matrix was 655 bp long, with only three parsimony-informative loci. All samples investigated were quite similar to each other, with the exception of two plants of *N. nudicaule* (JN591604 and JN591605) collected near Rio Quaraí, Bella Unión (Uruguay), which exhibited eight variable sites in relation to the remaining samples of the *Inodorum* complex. The samples of *N. macrostemon* JN591608 and JN591609, both from North Uruguay, shared the same A → G transition which distinguished

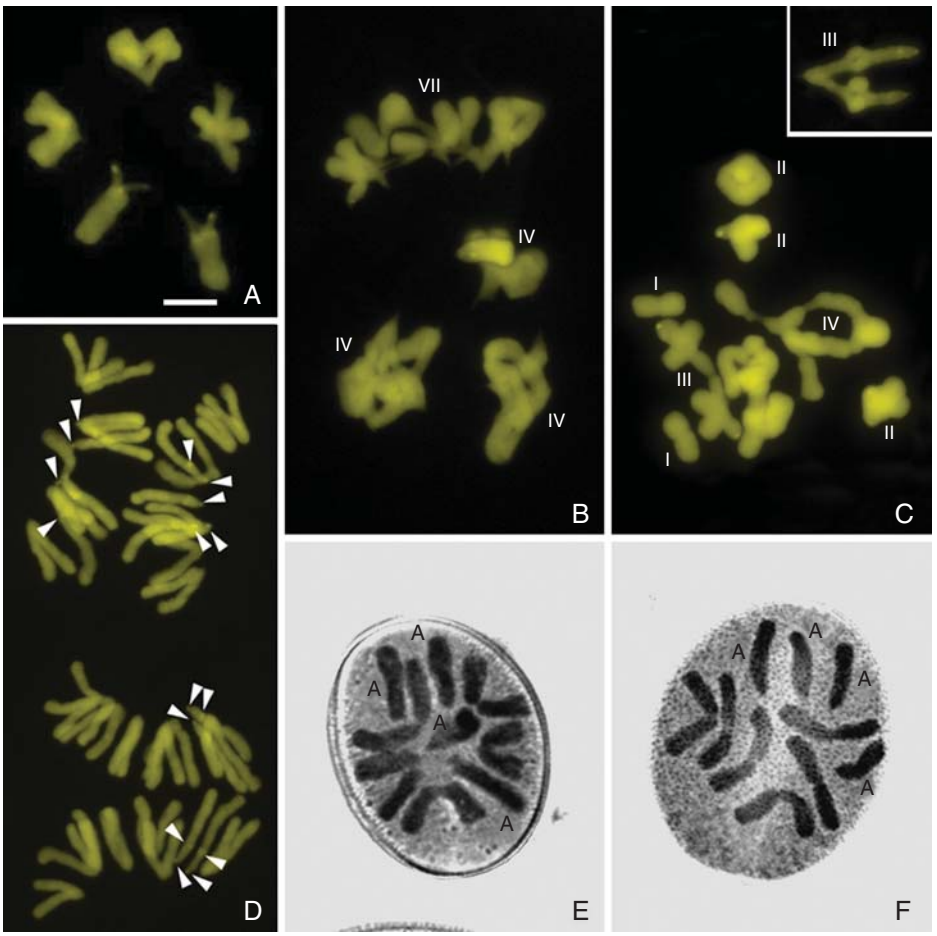

FIG. 3. Meiosis in *Nothoscordum macrostemon* (A, D) and *N. nudicaule* (B, C) and first pollen-grain mitosis in *N. gracile* (E, F). (A–C) Metaphase I with five bivalents in *N. macrostemon* $2n = 10$ (A) and with multivalents in *N. macrostemon* $2n = 19$ (B) and *N. nudicaule* $2n = 19$ (C). (D) Anaphase II in *N. macrostemon* $2n = 19$ showing chromosome sets with 6M + 4A (above) and 7M + 2A (below). (E, F) First pollen-grain mitosis of *N. gracile* with $n = 9$ (5M + 4A) (E) and $n = 10$ (6M + 4A) (F). Inset in (C) shows a heteromorphic trivalent of another cell oriented to the poles. Univalent, bivalent, trivalent, quadrivalent and heptavalent are indicated by Roman numerals. Arrowheads in (D) point to CMA bands of some acrocentric short arms and proximal ones. Scale bar in (**A**) = 10 μm.

them from the remaining *N. macrostemon* sample from South Uruguay.

The plastid DNA sequence *trn*L-*trn*F showed greater variability. When aligned, the *trn*L-*trn*F matrix was 849 bp long, with 831 constant and 17 parsimony-informative characters. Sequence variation included two substitutions (one autapomorphic), one single base-pair insertion, and sequence-length variations in repetitive regions of one AT microsatellite and in two poly-T regions. The analysis of haplotypes resulted in an incompletely resolved network. Plastid lineages did not appear clearly diverging and some haplotypes were shared by different morphospecies. The network separated most diploids of *N. macrostemon* (haplotypes in green; Fig. 5) from diploids of *N. nudicaule* (in orange) but the same did not occur with tetraploid accessions of those species or *N. gracile*. The triploid *Nothoscordum* sp. 1 and the tetraploid *Nothoscordum* sp. 2 (represented as empty circles) were more closely related to a less-common haplotype of *N. gracile* (black) and to *N. arenarium* (yellow). Three distinct haplotypes of *N. gracile* were detected: two widely distributed (red and black haplotypes) and one restricted to Uruguay (orange).The latter was also found in some diploid samples of *N. macrostemon* and *N. nudicaule*. The red and black haplotypes corresponded to the two most frequent cytotypes of *N. gracile*, $2n = 19$ ($13M + 1A^0 + 4A^1 + 1A^2$) and $2n = 19$ ($13M + 3A^0 + 3A^1$), respectively (Fig. 5).

Tetraploid apomicts did not show a strict correspondence between morphospecies and cpDNA haplotypes. A geographical trend was apparent instead with tetraploid samples of both morphospecies possessing the same haplotype (orange in Fig. 5) towards south-western Uruguay and another haplotype (blue) towards the north-east. This trend was also apparent in Rio Grande do Sul (Brazil) where one haplotype (red) of *N. gracile* was found along the Atlantic coast and a different haplotype (black) was distributed further inland (Fig. 5).

DISCUSSION

Karyotype variability of the Inodorum *complex*

The chromosome numbers and morphologies of the species analysed here were similar to those previously described (Crosa, 1972, 1996; Nuñez *et al.*, 1974). Intra-individual

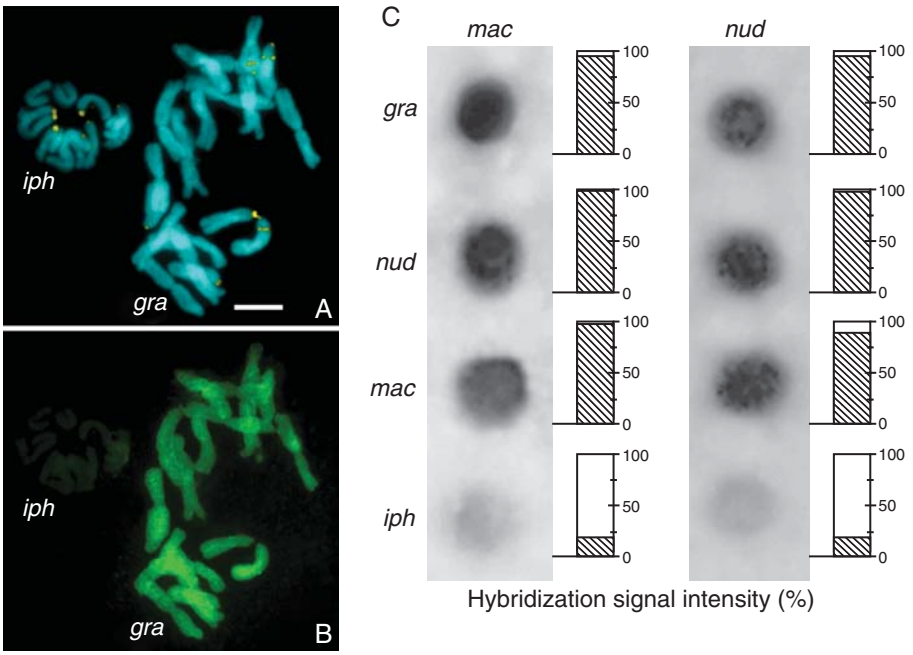

FIG. 4. GISH (A, B) and dot-blot hybridization (C) among species of the complex *Inodorum* and the genus *Ipheion*. (A, B) Metaphase of *N. gracile* (*gra*) and prometaphase of *I. uniflroum* (*iph*) stained with CMA/DAPI (A) and *in situ* hybridized with *N. nudicaule* genomic probe (B). (C) Labelled genomic DNA of *N. macrostemon* (*mac*) and *N. nudicaule* (*nud*) hybridized in membrane with non-labelled genomic DNA of *N. gracile*, *N. macrostemon* and *N. nudicaule*. Relative hybridization intensity based on the 100 % value for homologous hybridization is indicated below. Scale bar in (A) = 10 μm.

variation with $2n = 18$ and 19 was not observed here, although several cells displayed two acrocentric chromosomes associated by the NORs on their short arms, resembling a metacentric chromosome. Associations between acrocentric chromosomes with terminal NORs have been described in a number of species, including *Nothoscordum gracile* (Nuñez *et al.*, 1974) and *Allium sativum* (Sato and Kawamura, 1981) and might be the cause of the apparent mosaicism ($2n =$ 18/19) reported by Nassar and Aguiar (1978) for *N. gracile*.

The presence of CMA bands in the proximal region of the long arms of the acrocentric chromosomes of $2x$ and $4x$ *N. macrostemon*, and their absence in the diploids and most tetraploids of *N. nudicaule*, appears to be the most prominent cytological difference between these two species. The occurrence of acrocentric chromosomes with and without CMA bands in all of the samples of *N. gracile* constitutes a strong indication of their hybrid origin and the probable independent formation of different cytotypes. In spite of the high variability observed in the CMA banding pattern in the tetraploids of the *Inodorum* complex, little intrapopulational variation was observed. Moreover, the occurrence of karyotypic heterozigosity suggests that propagation of tetraploids is predominantly asexual.

The diversity of banding patterns and chromosome numbers was higher in localities in Uruguay where different cytotypes of the *Inodorum* complex occur. The largest variability occurred at the contact zone between tetraploid apomictics and related sexual diploids, whereas only some fixed genotypes may show considerably expanded geographic ranges (Daurelio *et al.*, 2004; Hörandl, 2006). Consequently, our data confirm that the centre of diversity of the *Inodorum* complex is located in Uruguay (Crosa, 1996) because in that area the largest karyotypic and haplotypic variability occur and it is where only diploid cytotypes of *N. macrostemon* and *N. nudicaule* were found. Throughout its range, *N. gracile* was found in disturbed roadside environments, a pattern that has been found for apomictic hybrids in other complexes (Bierzychudek, 1985; Hörandl, 2006).

The production of viable pollen and effective pollination are necessary for endosperm formation in *N. gracile* because it is pseudogamous (Tandon and Kapoor, 1963). In the case of the tetraploids of the *Inodorum* complex, in spite of irregularities observed in their meiotic pairing the pollen grains were generally viable, with a high frequency of microspores with $n = 10$ and $n = 9$, thus indicating that sexual reproduction is also possible (see also Dyer, 1967; Nuñez *et al.*, 1974). The occurrence of facultative sexually reproducing individuals in apomictic complexes is not rare (Akins and Dijk, 2007). Residual female sexuality or the production of functional male gametes seems to be sufficient to allow cross-fertilization between apomictic tetraploids as well as between tetraploids and diploids, which can account for the karyotype variability observed and the formation of triploids and individuals with rare translocations. The karyotype variability of the *Inodorum* complex, as in other apomictic complexes, may have been generated by multiple *de novo* origins of the apomictic polyploids, backcrossing to sexually related species or by somatic mutations (Hörandl *et al.*, 2009). On the other hand, extensive asexual reproduction ensured by apomixis and the abundant production of bulbils may explain the stabilization and dissemination of a few cytotypes (Akins and Dijk, 2007).

Origin of the 2n = *19 karyotype*

Considering the stability of the diploid chromosome number of the section *Inodorum* ($2n = 10$; $6M + 4A$), the number $2n = 20$ ($12M + 8A$) should be expected in the tetraploids. Indeed, tetraploids with $2n = 20$ may have been formed a

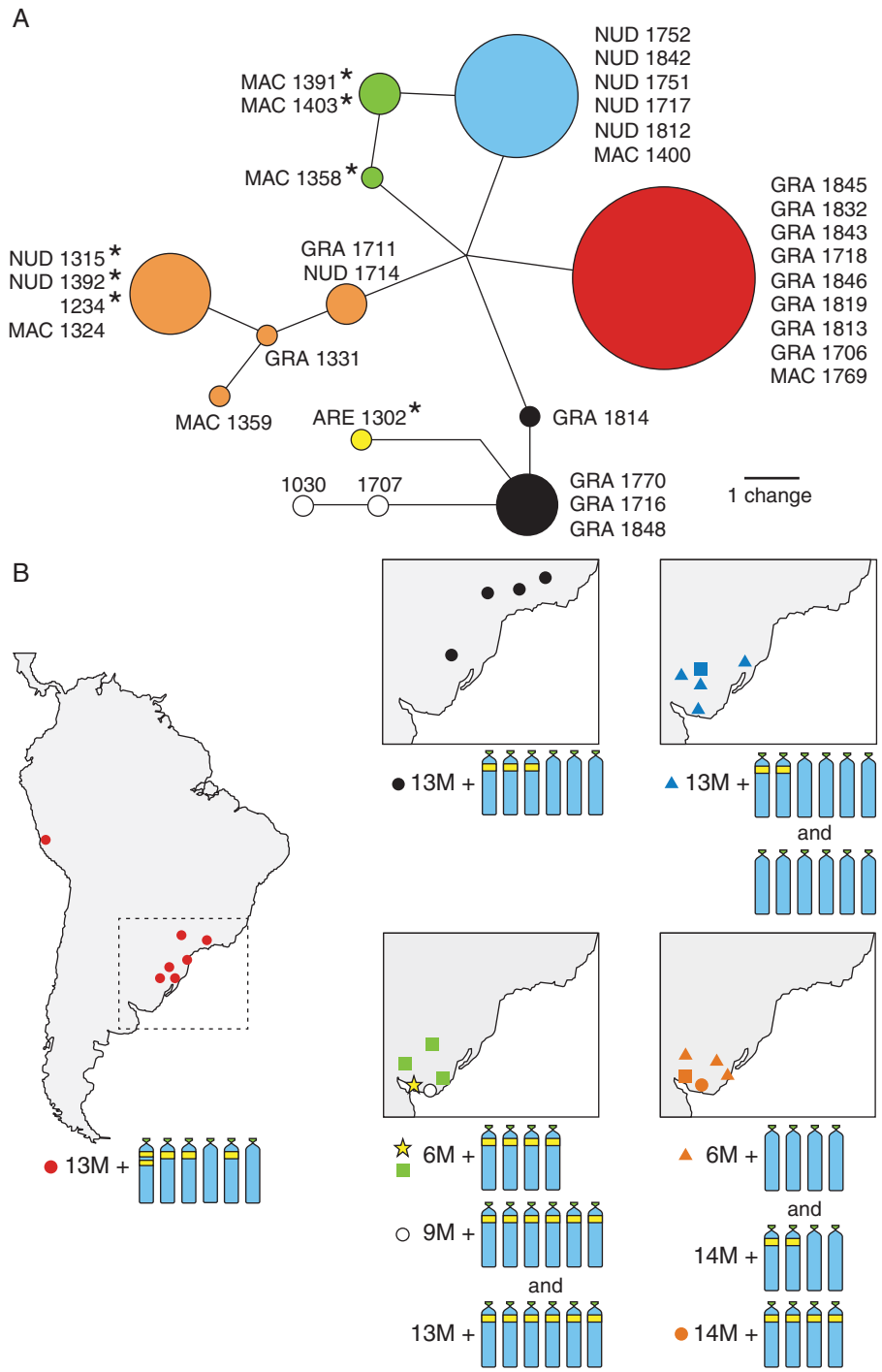

FIG. 5. Network connecting *trn*L-*trn*F of section *Inodorum* (A) and their relationships with geographic and cytogenetic data (B). Each haplotype is indicated by a different colour in both (A) and (B). Circle sizes in the network are proportional to the number of individuals observed for each haplotype. Idiograms of acrocentric chromosomes represent the predominant cytotype for each haplotype, with the same colours as in (A). Diploid samples in (A) are indicated by asterisks. In (B) closed circles indicate *N. gracile* (GRA), squares *N. macrostemon* (MAC), triangles *N. nudicaule* (NUD), stars *N. arenarium* (ARE) and open circles *Nothoscordum* sp. 1 and sp. 2.

number of times in the wild, as pollen grains of *N. gracile* with $n = 10$ and $n = 9$ are very frequent. Nuñez *et al.* (1974) reported $n = 10$ in 22 % of the pollen grains and meiotic dyad cells of *N. gracile*, most of them having 6M + 4A, as in the present sample. Seedlings with $2n = 20$ (14M + 6A) descendents of clones of *N. gracile* with $2n = 19$ were reported by Dyer (1967) and Nuñez *et al.* (1974).

The $2n = 19$ (13M + 6A) karyotype seems to be derived from a centric fusion in the ancestral karyotype with $2n = 20$ (Crosa, 1996). This assumption is supported by the maintenance

of the FN expected for $2n = 20$ (FN = 16) and by the presence of a heteromorphic trivalent formed by a metacentric and two acrocentric chromosomes during meiosis (see also Levan and Emsweller, 1938; Dyer, 1967; Nuñez *et al.*, 1974). This fusion must have occurred between the centromeres of two acrocentric chromosomes and resulted in the loss of their short arms, since no 45S rDNA signal were observed in the extra metacentric chromosomes. Fusion of acrocentric chromosomes bearing nucleolar organizer regions on their short arms, with the loss of ribosomal sites, has been reported in several other genera such as *Lillium* (Muratović *et al.*, 2005) and *Rumex* (Koo *et al.*, 2004). In *Nothoscordum*, Robertsonian translocations seems to be a common phenomenon and may also have occurred between acrocentric and metacentric chromosomes, as in *N. arenarium* (Souza *et al.*, 2009) and in four individuals of *N. gracile* from Santa Maria (Brazil) which have a metacentric chromosome with a proximal CMA band on one of its arms.

The occurrence of $2n = 19$ in tetraploid cytotypes of three closely related species suggests that the fusion probably occurred in a tetraploid plant with $2n = 20$, and the extra metacentric chromosome was later transmitted to the other tetraploids of the complex *Inodorum* through interspecific hybridization. The absence of a CMA band in the proximal region of the extra metacentric chromosome suggests that this fusion may have initially occurred in *N. nudicaule*, as it is the only species in this complex without proximal CMA bands in their acrocentric chromosomes.

The union of a balanced gamete with $n = 9$ ($7M + 2A^0$) from a $2n = 19$ *N. nudicaule* and a non-reduced gamete of *N. macrostemon* $2x$, with $n = 10$ ($6M + 4A^1$), could have originated the $2n = 19$ tetraploid hybrid *N. nudicaule* × *N. macrostemon*, which, by self-fertilization or further introgression, generated the diversity of banding patterns observed in the tetraploids of this complex. Analysis of *trn*L-*trn*F sequences showed that each tetraploid cytotype exhibited a single and exclusive haplotype, with a few exceptions, suggesting that each tetraploid lineage had an independent origin. A non-reduced gamete of diploid *N. macrostemon* or *N. arenarium* may also have contributed to form the triploid plants (*Nothoscordum* sp. 1) with $2n = 15$ ($9M + 6A^1$) without the extra metacentric. Dyer (1967) proposed that the maintenance of this fusion in heterozygosis was due to the presence of a balanced lethal system, as has been observed in other species with permanent heterozygotic translocations (reviewed by Crosa, 1996; Levin, 2002). However, some homozygous samples of *N. gracile* [$2n = 18$ ($14M + 4A^1$)] and *N. nudicaule* [$2n = 18$ ($14M + 2A^0 + 2A^1$)] were observed here, suggesting that they did not have a lethal allele system. It is interesting to observe that the combination $7M + 2A$ is by far the most frequent one both in dyad cells of meiosis and in pollen-grain mitosis (Levan and Emsweller, 1938; Dyer, 1967; Nuñez *et al.*, 1974), and it could have allowed the formation of eventual tetraploids ($14M + 4A$) bearing two copies of the extra metacentric chromosome.

The origin of Nothoscordum gracile *and its relationships with* N. macrostemon *and* N. nudicaule

The ability of GISH to differentiate among parental genomes in a hybrid depends mainly on the size of the genomes, on the degree of divergence of the principal families of repetitive DNA sequences, and on the phylogenetic distance between the ancestral species (Markova and Vyskot, 2009). The fact that GISH and dot-blot could not differentiate between genomes of the species of the complex *Inodorum* and that artificial hybrids between diploids *N. nudicaule* and *N. macrostemon* are viable (O. Crosa, unpubl. res.) suggest that this complex is very young. A recent origin is also compatible with the lack of differentiation of the ITS sequences among members of this complex and the morphological and cytogenetic similarities between their tetraploids.

The variation among *trn*L-*trn*F haplotypes was consistent with the CMA banding patterns. Most populations with the same haplotype share the same cytotype and all *N. gracile* samples with the dominant or standard cytotype had the same haplotype. Despite the low level of variation among plastid sequences, the strict correspondence between them and the cytological complements supports the interpretation that haplotype similarity is not due to homoplasy but rather inherited from the progenitors of each lineage together with karyotypic features.

At least three distinct lineages can be recognized in *N. gracile* both by sequence and karyotypic data. The plastid sequences of the polyploids are related to different degrees to distinct putative sexual sources, and their cytological constitution can be explained by different contributions from the different diploid sources. In addition, the presence of shared plastid sequences among different morphospecies and the lack of distinct lineages suggest both recent diversification and ongoing gene flow. The karyotypes of asexual polyploids can be explained by a combination, but not the simple addition, of those of the diploid sources. Mechanisms explaining such combinations and variability may include recombination at the polyploid level following multiple origins of the tetraploids (Soltis *et al.*, 2003) and/or repeated introgression from sexuals in a reticulate pattern (Lo *et al.*, 2010) including unilateral sexual polyploidization involving triploid bridges (Ramsey and Schemske, 1998).

In the *Inodorum* complex, polyploid apomicts appear more widespread than their sexual progenitors, which is congruent with the concept of geographic parthenogenesis. Each of the lineages identified by an individual cytotype/haplotype may represent a single apomictic clone. When individual clones can be identified, their geographic distributions can be asymmetrical, some clones being far more widespread than others (Richards, 2003). The most widespread cytotype/haplotype of *N. gracile* in South America is a structural heterozygote [$2n = 19$ ($13M + 1A^0 + 4A^1 + 1A^2$)], also found in Japan (Sato *et al.*, 1979). The maintenance of structural heterozygosity and the occurrence of this lineage in areas far removed from the distributional ranges of either diploid or tetraploid sexual partners, support the interpretation that it possibly represents the most vigorous and successful apomictic clone generated within the *Inodorum* complex. Because other apomictic lineages of the tetraploid *N. gracile* are reported here, its comparatively greater success cannot be attributed to the advantages of apomixis or polyploidy but must be explained by some inherent property of this individual clone. It has been proposed that some clones represent general-purpose genotypes (Lynch, 1984) that possess wide adaptation

capabilities to a variety of environmental conditions. Further evidence of the exclusively clonal nature of this karyotype/cytotype may be needed to support this interpretation.

The diploid cytotypes of *N. nudicaule* and *N. macrostemon* represent the extremes of morphological and cytogenetic (CMA bands) variability within this complex and appear to constitute closely related true biological species in spite of the putative occurrence of chloroplast capture, causing the incongruent distribution of cpDNA haplotypes. At the tetraploid level, a greater trend towards geographical structuring of chloroplast haplotypes and a close correspondence between chloroplast sequences and karyotypes, irrespective of morphological variation, was found. Cytological evidence is highly congruent with a hybrid origin of not only *N. gracile* but also polyploid cytotypes assigned to *N. macrostemon* and *N. nudicaule*. This pattern strongly suggests that the sexual diploid components of the *Inodorum* complex hybridize frequently, producing apomictic clones spanning the morphological extremes represented by their parental species. Some of those clones are morphologically recognizable as *N. gracile*.

In this work we have shown that cytological and sequence information is congruent with the pattern suggested by morphological evidence that the *Inodorum* section is a species complex and that *N. gracile* is likely to be an assemblage of hybrids involving at least *N. macrostemon* and *N. nudicaule*. We have also shown that differentiation at the sequence level is incipient, suggesting a recent divergence among the sexual species of the complex. Analysis of ITS sequences points to possible gene flow constraints between the northern and southern populations of Uruguay, although no differences were observed in karyotype or *trn*L-*trn*F plastidial sequences between these populations. Further support for the evolutionary hypotheses and processes in this complex are likely to emerge from more intensive sampling and sensitive molecular markers.

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LITERATURE CITED

Akins PO, Dijk PJ. 2007. Mendelian genetics of apomixis in plant. *Annual Review of Genetics* **41**: 509–537.

Bierzychudek P. 1985. Patterns in plant parthenogenesis. *Experientia* **41**: 1255–1264.

Canelada MEL, Fernandez AMF. 1985. Bandeo cromosomico en *Nothoscordum inodorum* (Soland. Ex Aiton) Nich. var. *inodorum. Lilloa* **36**: 181–186.

Crosa O. 1972. Estudios cariología en el género *Nothoscordum* (Liliaceae). *Boletin de la Facultad de Agronomia de Uruguay* **122**: 3–8.

Crosa O. 1996. *Sistemática e evolução das espécies da seção Inodorum Guag. do gênero* Nothoscordum *Kunth (Allieae, Alliaceae)*. PhD Thesis Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Daurelio LD, Espinoza F, Quarin CL, Pessino SC. 2004. Genetic diversity in sexual diploid and apomictic tetraploid populations of *Paspalum notatum* situated in sympatry or allopatry. *Plant Systematics and Evolution* **244**: 189–199.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.

Dyer AE. 1967. The maintenance of structural heterozygosity in *Nothoscordum fragrans* Kunth. *Carylogia* **20**: 287–308.

Fay MF, Rudall PJ, Chase M. 2006. Molecular studies of subfamily Gilliesioideae (Alliaceae). *Aliso* **22**: 367–371.

Friesen N, Fritsch RM, Blattner FR. 2006. Phylogeny and new intrageneric classification of *Allium* (Alliaceae) based on nuclear ribosomal DNA ITS sequences. *Aliso* **22**: 372–395.

Gornall RJ. 1999. Population genetic structure in agamospermous plants. In: Hollingsworth PM, Bateman RM, Gornall RJ. eds. *Molecular systematics and plant evolution*. London: Taylor & Francis, 118–138.

Guaglianone EA. 1972. Sinopsis de las especies de *Ipheion* Raf. y *Nothoscordum* Kunth (Liliáceae) de Entre Ríos y regiones vecinas. *Darwiniana* **17**: 159–240.

Guerra M, Felix LP. 2000. O cariótipo de *Nothoscordum pulchellum (*Alliaceae) com ênfase na heterocromatina e sítios de DNAr. *Boletín de la Sociedad Argentina de Botánica* **35**: 283–289.

Hall T. 2001. *BioEdit*, version 5·0·6. Department of Microbiology, North Carolina State University. http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

Hörandl E. 2006. The complex causality of geographical parthenogenesis. *New Phytologist* **171**: 525–538.

Hörandl E, Greilhuber J, Klimova K, *et al*. 2009. Reticulate evolution and taxonomic concepts in the *Ranunculus auricomus* complex (Ranunculaceae): insights from morphological, karyological and molecular data. *Taxon* **58**: 1194–1215.

Jones K. 1998. Robertsonian fusion and centric fission in karyotype evolution of higher plants. *Botanical Review* **64**: 273–289.

Koo DH, Yoonkang H, Bang JW. 2004. Variability of rDNA loci in dioecious *Rumex acetosa* L. detected by fluorescence *in situ* hybridization. *Korean Journal of Genetics* **26**: 9–13.

Kurita M, Kuroki Y. 1963. Heterochromaty in *Nothoscordum* chromosomes. *Memoirs of Ehime University Section II* **4**: 493–500.

Levan A, Emsweller SL. 1938. sStructural hibridity in *Nothoscordum fragrans*. *Journal of Heredity* **29**: 291–294.

Levin DA. 2002. *The role of chromosomal change in plant evolution*. Oxford: Oxford University Press.

Li QQ, Zhou SD, He XJ, Yu Y, Zhang YC, Wei XQ. 2010. Phylogeny and biogeography of *Allium* (Amaryllidaceae: Allieae) based on nuclear ribosomal internal transcribed spacer and chloroplast *rps 16* sequences, focusing on the inclusion of species endemic to China. *Annals of Botany* **106**: 709–733.

Lo EYY, Stefanović S, Dickinson TA. 2010. Reconstructing reticulation history in a phylogenetic framework and the potential of allopatric speciation driven by polyploidy in an agamic complex in *Crataegus* (Rosaceae). *Evolution* **64**: 3593–608.

Lowry E, Lester SE. 2006. The biogeography of plant reproduction: potential determinants of species' range sizes. *Journal of Biogeography* **33**: 1975–1982.

Lynch M. 1984. Destabilizing hybridization, general-purpose genotypes and geographic parthenogenesis. *Quarterly Review of Biology* **59**: 257–290.

Markova M, Vyskot B. 2009. New horizons of genomic *in situ* hybridization. *Cytogenetic and Genome Research* **126**: 368–75.

Muratović E, Bogunié F, Šoljan D, Siljak-Yakovlev . 2005. Does *Lilium bosniacum* merit species rank? A classical and molecular-cytogenetic analysis. *Plant Systematics and Evolution* **252**: 97–109.

Nassar NMA, Aguiar MLR. 1978. Multiple karyotypes in individuals of *Nothoscordum fragrans* (Liliaceae). *Caryologia* **31**: 7–14.

Nguyen NH, Driscoll HE, Specht CD. 2008. A molecular phylogeny of the wild onions (*Allium*; Alliaceae) with a focus on the western North American center of diversity. *Molecular Phylogenetics and Evolution* **47**: 1157–1172.

Nuñez O, Frayssinet N, Rodriguez RH, Jones K. 1974. Cytogenetic studies in the genus *Nothoscordum* Kunt. I. The *N. inodorum* polyploid complex. *Caryologia* **27**: 403–441.

Ramsey J, Schemske DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics* **29**: 467–501.

Ravenna PF. 1978. Studies in the Genus *Nothoscordum*. *Plant Life* **34**: 136–145.

Richards AJ. 2003. Apomixis in flowering plants: an overview. *Philosophical Transactions of the Royal Society of London* **358**: 1085–1093.

Rua GH, Speranza PR, Vaio M, Arakaki M. 2010. A phylogenetic analysis of the genus *Paspalum* (Poaceae) based on cpDNA and morphology. *Plant Systematic and Evolution* **288**: 227–243.

Sambrook J, Russell DW. 2001. *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sato M, Sato S, Matsumoto E. 1982. Chromosome banding produced by UV-light exposure in the presence of Hoechst 33258. *Caryologia* **35**: 405–409.

Sato S, Kawamura S. 1981. Cytological studies on the nucleolus and the NOR-carrying segments of *Allium sativum*. *Cytologia* **46**: 781–790.

Sato S, Kuroki Y, Ohta S. 1979. Two types of color-diferentiated C-banding positive segments in chromosomes of *Nothoscordum fragrans*, Liliaceae. *Cytologia* **44**: 715–725.

Soltis DE, Soltis PS, Tate JA. 2003. Advances in the study of polyploidy since plant speciation. *New Phytologist* **161**: 173–91.

Souza LGR, Crosa O, Guerra M. 2009. The karyotype of *Nothoscordum arenarium* Herter (Gilliesioideae, Alliaceae): a populational and cytomolecular analysis. *Genetics and Molecular Biology* **32**: 111–116.

Souza LGR, Crosa O, Guerra M. 2010. Karyological circumscription of *Ipheion* Rafinesque (Gilliesioideae, Alliaceae). *Plant Systematics and Evolution* **287**: 119–127.

Stearn WT. 1986. *Nothoscordum gracile*, the correct name of *N. fragrans* and the *N. inodorum* of authors (Alliaceae). *Taxon* **35**: 335–338.

Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**: 1105–1109.

Tandon SL, Kapoor BM. 1963. Contributions to the cytology of endosperm in some Angiosperms II. *Nothoscordum fragrans* Kunth. *Caryologia* **16**: 377–395.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673–4680.

Vandel A. 1928. La parthénogenese geographique. Contribution a l'étude biologique et cytologique de la parthénogenese naturelle. *Bulletin Biologique de la France et de la Belgique* **62**: 164–182.

Verduijn MH, Van Dijk PJ, Van Damme JMM. 2004. Distribuition, phenology and demography of sympatric sexual and asexual dandelions (*Taraxacum officinale* s.l.): geographic parthenogenesis on a small scale. *Biological Journal of the Linnean Society* **82**: 205–218.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Gelfand D, Sminsky J, White T. eds. *PCR protocols: a guide to methods and applications*. San Diego, CA: Academic Press, 315–322.