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Genomic relationships between *Turnera orientalis* and *T. occidentalis* (Turneraceae)

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Aiming to differentiate *Turnera orientalis* (2n = 6x = 30) from *T. occidentalis* (2n = 6x = 30), traditional and molecular cytogenetic techniques were used. Numerous hybrids were obtained from controlled crosses between *T. orientalis* and *T. occidentalis*. An analysis of their pollen viability and the failure to obtain F2 provided evidence of reproductive isolation. The present study reports for the first time the karyotype 22 metacentric + 8 submetacentric for *T. occidentalis*, and confirms the karyotype of *T. orientalis*. These species differ in a chromosome pair with secondary constriction and satellite. A FISH analysis showed six rDNA sites located on different chromosome pairs. Although the meiotic chromosome pairing of hybrids suggested a close relationship of the two species, the karyotype and mapped location of rDNA sites were different between the taxa, supporting that *T. orientalis* and *T. occidentalis* are distinct species.

Key words: allopolyploid, FISH, fluorescent *in situ* hybridization, interspecific hybrids

Introduction

The subseries *Turnera* of the genus *Turnera* (Turneraceae) has 20 species distributed from southern USA to central Argentina, with ploidy levels ranging from diploid (2x) to octoploid (8x), the basic chromosome number being x = 5 (Arbo 1986, Solís Neffa & Fernández 2000).

Turnera orientalis of this subseries was recently (Arbo & Shore 2005) split into *T. orientalis* and *T. occidentalis*, based on morphological and phenological characters as well as on the geographic distributions of the two taxa. Both are yellow-flowered, but the flowers of *T. occidentalis* are brighter. The base of the petals is villous in *T. occidentalis* but glabrous in *T. orientalis*. *Turnera occidentalis* is endemic to Peru, whereas *T. orientalis* is widely distributed over eastern Brazil, southwestern Bolivia, eastern Paraguay and northeastern Argentina. Moreover, these species showed temporary reproductive isolation when cultivated in an experimental

garden, with *T. occidentalis* entering *anthesis* earlier (mid morning) than *T. orientalis* (noon) (Arbo 2005).

There are about 100 species in *Turnera*. The chromosome number has been determined for 35 species, but only 21 karyotypes have been reported so far (Solís Neffa & Fernández 2000). No molecular cytogenetic studies on this group have yet been published.

The aim of the present work was to analyze the genomic relationships between *T. orientalis* and *T. occidentalis* using traditional and molecular cytogenetic techniques. The karyotype of *T. occidentalis* is described for the first time and the previously reported karyotype of *T. orientalis* is re-investigated. In addition, mapping of rDNA sites was performed for both species using fluorescence *in situ* hybridization (FISH). Interspecific hybrids were obtained from controlled crosses for further analysis of meiotic pairing and pollen viability. The integrated analysis of all these data should contribute to the understanding of the mechanisms involved in the reproductive isolation of the taxa, to clarify their genomic relationship, and to verify the idea of Arbo (2005) that *T. orientalis* and *T. occidentalis* are two distinct species.

Material and methods

Plant material

Samples were obtained from plants cultivated in the greenhouse at the Instituto de Botánica del Nordeste (IBONE). Specimens were deposited in the herbarium of the Institute (CTES). The following accessions were studied:

- *T. orientalis* (2n = 6x = 30) O2, Argentina, Misiones, Teyú Cuaré, *Cabral 358*.
- *T. occidentalis* (2n = 6x = 30) O10, Peru, Cajamarca, prov Contumaza, Jaguey, 600 m, 1.V.1993, *A. Sagástegui 14896*.
- *T. orientalis* × *T. occidentalis* (2n = 6x = 30), cultivated at the Instituto de Botánica del Nordeste, Corrientes, Argentina.
- *T. occidentalis* × *T. orientalis* (2n = 6x = 30), cultivated at the Instituto de Botánica del Nordeste, Corrientes, Argentina.

Interspecific crossings

Crossings were carried out as follows: emasculation of the maternal plant, pollination with the anthers from the paternal plant, and labeling of the maternal flower with a tag indicating the identity of the paternal progenitor. The seeds obtained from each cross were sown into individual pots and the hybrid seedlings were transplanted after the development of the first pair of leaves.

Karyotype analysis

Somatic chromosomes were studied in root tips pretreated in 0.002 M 8-hydroxyquinoline (Merck) for 3 h at room temperature, fixed in 3:1 ethanol:acetic acid for 24 h, and then stored in 70% ethanol.

At least eight metaphases of each species were measured. Measurements were made using MicroMeasure 3.3 program (Reeves 2001). Idiograms were based on mean centromeric index (CI = short arm × 100/total length of the chromosome) and arranged in order of decreasing size. Chromosome nomenclature followed Levan *et al*. (1964), the 'm' and 'sm' designate metacentric and submetacentric chromosomes, respectively.

The following parameters were estimated in each metaphase plate to characterize the karyotype numerically: (1) total haploid chromosome length (HCL); (2) total chromosome length (TCL); (3) mean chromosome length (ML); (4) mean centromeric index (CI); (5) intrachromosomal asymmetry index (A1) = $1 - \Sigma(b/B)/n$; and (6) inter-chromosome asymmetry index (A2) = s/x, where b and B are the mean lengths of short and long arms of each pair of homologues, n is the number of homologous chromosome, s is the standard deviation, and x is the mean chromosome length.

Karyotype asymmetry was estimated using the mean centromeric index, the ratio of the shortest/longest pair, and the A1 and A2 indices (Romero Zarco 1986).

Meiotic chromosomes were examined in pollen mother cells (PMC) of young buds, fixed in 3:1 absolute ethanol–acetic acid for 24 h at 4 °C and stored in 70% ethanol at 4 °C.

Anthers were stained with 3% aceto-orcein. Slides were made permanent in Euparal using Bowen's (1956) method. In order to estimate pollen viability, pollen stainability was also estimated using carmine–glycerin (1:1). At least 300 grains per flower were counted.

Fluorescent *in situ* hybridization

In situ hybridization was performed following Poggio *et al*. (1999a, 1999b) with minor modifications. The pTa71 plasmid, containing the 18S–5.8S–25S ribosomal sequences from *Triticum aestivum* (Gerlach & Bedbrook 1979), was used as a probe. The probe was labeled with Biotin Nick Translation Kit (Boehringer Mannheim, Germany), according to the manufacturer's procedures.

Root tips were pretreated in 0.002 M 8-hydroxyquinoline (Merck) for 3 h at room temperature, and fixed in 3:1 ethanol:acetic acid for 24 h. Fixed root tips were washed in 0.01 M citric acid–sodium citrate buffer (pH 4.6) to remove the fixative. They were then transferred to an enzyme solution containing 2% cellulase Onozuka R10 (Merck) and 20% liquid pectinase (Sigma), and squashed in a drop of 45% acetic acid. Slides were selected using phase-contrast light microscopy. After removal of coverslips by freezing, the slides were air dried.

Slide preparations were incubated in 100 μg ml^{-1} DNAse-free RNAse in 2 × SSC for 1 h at 37 °C in a humified chamber and washed three times in 2 × SSC at room temperature for 5 min. The slides were post-fixed in freshly prepared 4% (w/v) paraformaldehyde in water for 10 min, washed in 2 × SSC for 15 min, dehydrated in a graded ethanol series and air dried. The hybridization mixture consist of 50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) SDS, followed by adding 100 ng of labeled probe to 30 μl of hybridization mixture for each slide. The hybridization mixture was denatured for 15 min at 75 °C, loaded onto the slide preparation and covered with a plastic coverslip. The slides were placed in a thermocycler at 75 °C for 7 min (denaturation), 10 min at 45 °C and 10 min at 37 °C. The slides were incubated overnight at 37 °C for hybridization. Following hybridization, the coverslips were carefully floated off by placing the slides in 2 × SSC at 42 °C for 3 min and then given a stringent wash in 20% formamide in 0.1 × SSC at 42 °C for 10 min. The slides were washed in 0.1 × SSC at 42 °C for 5 min; 2 × SSC for 5 min at 42 °C and 4 × SSC, 0.2% (v/v) Tween20 for 5 min at 42 °C.

To detect biotin-labeled probes the slides were treated with streptavidine–Cy3 conjugate (Sigma). Slides were then treated with 2.5% (w/v) BSA for 5 min and then incubated in a 1:40 solution in a buffer containing 2.5% BSA for 1 h at 37 °C.

Slides were counterstained with 1 μg ml^{-1} of 4´,6-diamidino-2-phenolindole (DAPI) in 4 × SSC-Tween buffer, for 10 min at room temperature and then mounted in anti-fade solution (Vector Lab). Slides were examined with a Carl Zeiss Axiophot epifluorescence microscope. Photographs were taken using Kodak Gold 400 color print film.

Results

Karyotype analysis

Turnera occidentalis is hexaploid, with 30 chromosomes. Twenty-two of these are metacentric and eight submetacentric, carrying a satellite on the short arm of pair 5. Mean chromosome length (ML) was 2.51 μm, total haploid chromosome length (HCL) was 58.33 μm. Mean centromeric index (CI) and asymmetry indices (A1 = 0.31 and A2 = 0.18) were also estimated. All the results of the chromosome measures for *T. occidentalis* are provided in Table 1. Our results for *T. orientalis* were consistent with the data previously reported by Solis Neffa and Fernández (1993).

In situ hybridization

In *T. orientalis*, the physical mapping of rDNA loci using probes from hexaploid wheat (pTa71) shows three pairs of signals (Figs. 1A and 2A) located on the short arm of chromosome pairs 4, 14 and 15. The signal of chromosome pair 14 is co-located on the satellite. In *T. occidentalis*

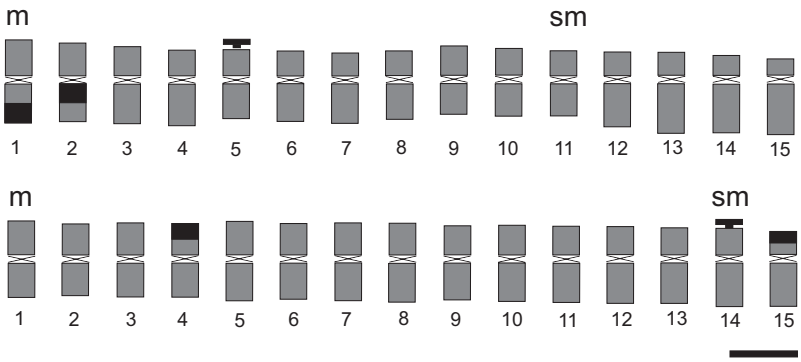

Fig. 1. Idiogram of *Turnera occidentalis* (top) and *T. orientalis* (bottom). The distribution of 45S rDNA loci is represented (black boxes). Bar = 5 μm.

three pairs of signals (Figs. 1B and 2B) were located on the long arm of chromosome pair 1, in the pericentromeric region of chromosome pair 2, and on the short arm of chromosome pair 5 at its secondary constriction, respectively.

Meiotic behavior of artificial hybrids

Hybrids were obtained from reciprocal crosses between *T. orientalis* and *T. occidentalis*. The progenitors *T. orientalis* and *T. occidentalis* (2n = 6x = 30) showed regular meiosis, consistently forming 15II in metaphase I.

The hybrids *T. orientalis* × *T. occidentalis* (2n = 6x = 30) exhibited four different pairing configurations (15II; 2I + 14II; 4I + 13II and 12II + 2III). The most frequent configuration was 15II (Fig. 2C) observed in 91.6% of the PMC, with up to two III per cell (Fig. 2D). Bridges with fragments (Fig. 2E) and laggards (Fig. 2F) were detected in anaphase I.

The reciprocal hybrids *T. occidentalis* × *T. orientalis* (2n = 6x = 30), exhibited eight different configurations (15II; 2I + 14II; 4I + 13II; 6I + 12II; 13II + 1IV; 1I + 13II + 1III; 3I + 12II + 1III and 2I + 12II + 1IV), the most frequent being 15II formed in 58.33% of the PMC and 2I + 14II (Fig. 2G) formed in 18.75% of the PMC. Trivalents and tetravalents (Fig. 2H) were observed in 0.25% PMC.

Pollen viability was 36.21% in the hybrids *T. orientalis* × *T. occidentalis* and 53.08% in the hybrids *T. occidentalis* × *T. orientalis* (Table 2). Early fruit abortion was observed in the hybrids *T. occidentalis* × *T. orientalis* as well as in the

Table 1. *Turnera occidentalis* chromosome morphology and measurements based on statistic measurements from metaphases analyzed. *B* = long arm length (± SD) (μm); *b* = short arm length (± SD) (μm); TCL = total chromosome length (μm); CI = centromeric index; m = metacentric; sm = submetacentric.

Pair	*B*	*b*	TCL	CI	Nomenclature
1	1.15 (± 0.47)	1.06 (± 0.45)	2.21	47.94	m
2	1.12 (± 0.17)	0.97 (± 0.15)	2.09	46.21	m
3	1.17 (± 0.16)	0.86 (± 0.09)	2.04	42.56	m
4	1.23 (± 0.16)	0.76 (± 0.14)	1.99	38.11	m
5	1.03 (± 0.35)	0.92 (± 0.35)	1.94	46.92	m
6	1.12 (± 0.23)	0.74 (± 0.11)	1.87	39.78	m
7	1.17 (± 0.20)	0.68 (± 0.15)	1.85	36.65	m
8	1.05 (± 0.13)	0.73 (± 0.09)	1.78	41.09	m
9	0.91 (± 0.17)	0.87 (± 0.15)	1.78	48.96	m
10	0.96 (± 0.15)	0.79 (± 0.12)	1.75	45.06	m
11	0.95 (± 0.12)	0.74 (± 0.11)	1.70	43.79	m
12	1.28 (± 0.21)	0.70 (± 0.14)	1.98	35.13	sm
13	1.47 (± 0.52)	0.68 (± 0.15)	2.15	31.83	sm
14	1.44 (± 0.39)	0.59 (± 0.13)	2.04	29.52	sm
15	1.50 (± 0.61)	0.48 (± 0.08)	1.98	25.45	sm

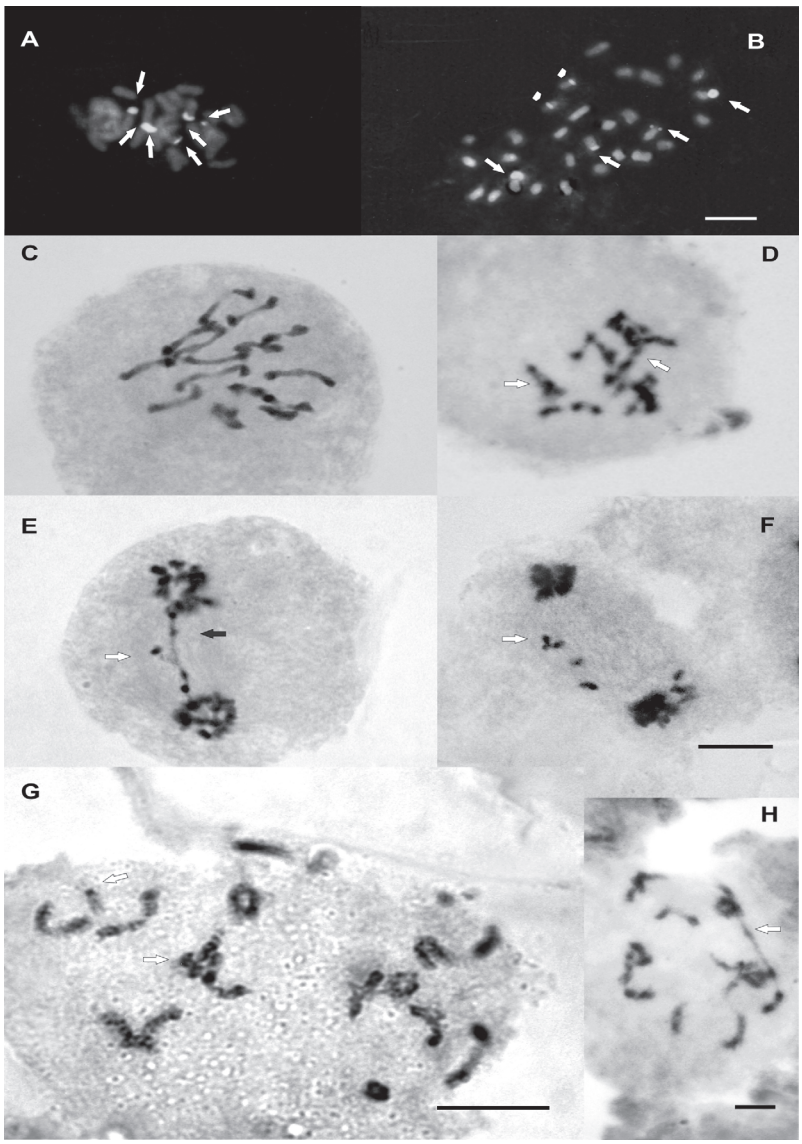

Fig. 2. — **A** and **B**: FISH using rDNA probe (pTa71) in mitotic metaphase of (**A**) *Turnera orientalis* and (**B**) *T. occidentalis*. Arrows indicate hybridization signal. Arrow tip indicates pericentromeric position of the signal. — **C–F**: *T. orientalis* × *T. occidentalis* hybrid. **C** and **D**: Metaphase I, 15II. **D**: Metaphase I, 12II + 2III (arrow shows III); **E** and **F**: Anaphase I. E: Fragment (white arrows) and bridge (black arrow); **F**: Laggard chromosomes. — **G** and **H**: Meiosis in *T. occidentalis* × *T. orientalis* hybrid. **G**: 2I + 14II (arrow shows I); **H**: 13II + 1IV (arrow shows IV). Bar = 5 μm, same bar for **A**, **B** and **C–F**.

reciprocal crosses. The mean number, variation range and standard deviation of univalents, bivalents, trivalents and tetravalents in the hybrids are shown in Table 2.

Table 2. Ploidy level, number of mother pollen cells (MPC) studied, mean ± SD (and range) of chromosome associations at MI and pollen viability of the F1 hybrids between *Turnera orientalis* and *T. occidentalis*.

	Ploidy level	MPC	I	II	III	IV	Pollen viability
T. orientalis × *T. occidentalis*	6x	24	0.083 ± 0.08 (0–2)	14.83 ± 0.13 (12–15)	0.083 ± 0.08 (0–2)	–	36.21%
T. occidentalis × *T. orientalis*	6x	48	0.875 ± 0.21 (0–6)	14.27 ± 0.14 (12–15)	0.083 ± 0.04 (0–1)	0.083 ± 0.04 (0–1)	53.08%

Discussion

Results of the cytological and molecular cytogenetic studies presented here support the division of *T. orientalis* into two species, as previously described by Arbo (2005). The species have different karyotypes, in *T. occidentalis* 22m + 8sm, while in *Turnera orientalis* it is 26m + 4sm (Solís Neffa & Fernández 1993), and is also more symmetric. Both species differ in the position of the secondary constriction and the satellite. In *T. orientalis* it is located on the submetacentric chromosome pair 14, while in *T. occidentalis* it is located on the metacentric chromosome pair 5.

The FISH analysis revealed that both species have six rDNA signals. One pair of signals of higher intensity is co-located with the NOR region detectable with conventional techniques. The other two pairs, albeit of lower intensity, are always present. These three pairs may correspond to the three genomes in the polyploids.

Numerous interspecific hybrids were produced from controlled crosses of *T. orientalis* × *T. occidentalis* and from the reciprocal crosses. The frequent presence of II indicates high homology between the genomes of the species since 15II was the most frequent configuration in all hybrids. The low frequency of multivalents (III and IV) suggests some degree of homeology among the genomes of these hexaploids. In addition, laggards were detected at low frequency possibly due to asynapsis or desynapsis, and the presence of bridges and fragments at anaphase I would indicate heterozygosity for a paracentric inversion. These results suggest the occurrence of a postzygotic reproductive isolation mechanism between the taxa.

Pollen viability was high but shows differences in reciprocal crosses, suggesting a nucleus-cytoplasm interaction decreasing viability when *T. orientalis* was used as a maternal progenitor.

Our results, coupled with differences in morphology, phenology and geographic distribution support the status of these taxa as separate species as proposed by Arbo (2005).

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