Ann. Bot. Fennici 47: 321–329 ISSN 0003-3847 (print) ISSN 1797-2442 (online)
Helsinki 29 October 2010

Evolutionary relationships between the diploid *Turnera grandiflora* and the octoploid *T. fernandezii* (Turneraceae)

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Received 6 Mar. 2009, revised version received 19 May 2009, accepted 22 May 2009

Fernández, A., Rey, H. & Solís Neffa, V. G. 2010: Evolutionary relationships between the diploid *Turnera grandiflora* and the octoploid *T. fernandezii* (Turneraceae). — *Ann. Bot. Fennici* 47: 321–329.

Aiming to analyze the evolutionary relationships between the diploid species *Turnera grandiflora* (2n = 2x = 10) and the octoploid *T. fernandezii* (2n = 8x = 40), interspecific hybrids were recovered by *in vitro* embryo rescue methods. The full-grown plants obtained were all pentaploids (2n = 5x = 25) confirming their hybrid nature. The chromosome associations observed in the hybrids during meiosis were indicative for an autopentaploid, suggesting that *T. fernandezii* carries the genome of *T. grandiflora* (C^gC^g) but at the octoploid level (C^gC^g C^gC^g C^gC^g C^gC^g). This fact confirms a close evolutionary relationship between the species and supports the hypothesis that *T. grandiflora* is the progenitor of *T. fernandezii*. A tentative hypothesis regarding the autopolyploid origin of *T. fernandezii* is finally formulated.

Key words: autopolyploidy, chromosome pairing, embryo rescue, genetic relationships, karyology

Introduction

The genus *Turnera* (Turneraceae) comprises about 100 species classified into nine series (Urban 1883), which are distributed from the southern United States to central Argentina with two species occurring also in Africa. Chromosome numbers are known for 35 *Turnera* species (Raman & Kesavan 1964, Hamel 1965, Barrett 1978, Arbo & Fernández 1983, Barrett & Shore 1987, Fernández 1987, Solís Neffa & Fernández 1993, 2001, Solís Neffa *et al*. 2004). The basic chromosome number x = 7 is found in the *Salicifolieae*, *Stenodictyae*, *Mycrophyllae* and *Leiocarpae* series, x = 5 is found in the *Turnera* series and x = 13 in series *Papilliferae* (Fernández 1987). Cytological investigations documented the occurrence of diploid to decaploid populations. Polyploids may be autopolyploids as well as allopolyploids (Raman & Kesavan 1964, Barrett 1978, Arbo & Fernández 1983, Shore & Barrett 1985, Fernández 1987).

Cytogenetic studies are particularly detailed in the *Turnera* series and focus on the taxon-

omy and evolutionary relationships. This series presents the most complex floral structure in the family. The flowers are epiphyllous, solitary and the floral tube shows five nectar pockets formed by marginal adnation of each staminal filament to the petal-claws up to the throat (Arbo 2005). The series comprises about 22 species, which are divided into the subseries *Turnera* and *Umbilicatae* based on seminal features (Arbo 2005). Subseries *Turnera* includes two groups of species, one with yellow flowers and one with blue ones (Fernández & Arbo 1989, Arbo 2005). Distyly and the associated dimorphic self-incompatibility system is widespread in the genus (Barrett & Shore 1987) although self-compatible homostyly arose independently at least three times in the genus (Truyens *et al*. 2005).

To clarify the evolutionary relationship among species of *Turnera* series, a controlled crossing program has been carried out since 1982. Several interspecific hybrids were obtained and the genomic relationships of several yellow-flowered species and of the diploid blue-flowered species were analyzed (Arbo & Fernández 1987, Fernández & Arbo 1989, 1990, 1993a, 1993b, 1996, 2000a, 2000b, Fernández & Solís Neffa 2004).

In this context, our objective here is to investigate the relationships between two blue-flowered species, diploid *Turnera grandiflora* (2n = 2x = 10) and octoploid *T. fernandezii* (2n = 8x = 40). The species are morphologically similar and *T. fernandezii* is included within the geographic distribution range of *T. grandiflora*. Hence, the latter is more widely distributed, occurring in Brazil (Mato Grosso do Sul), Bolivia (Santa Cruz), Paraguay and northern Argentina; while *T. fernandezii* is endemic to Mato Grosso do Sul (Brazil) and NE Paraguay. In the overlapping area, both species often grow at the same sites (Arbo 2005).

However, despite that 237 interspecific crossings were made involving two accessions of *T. grandiflora* and one accession of *T. fernandezii* (Arbo & Fernández 1987), all crossings completely failed to yield F_1 hybrids since the fruits obtained aborted after 12 days, probably due to embryo collapse in the early embryogenesis of the hybrid. In hybrid seeds, there often is a paucity of endosperm tissue or its development is abnormal, and the embryo dies at an early stage of development, although it is potentially capable of normal growth. In these cases, embryo rescue methods have proven very effective, and were utilized for diverse interploid, interspecific and intergeneric crosses (Watanabe 1977, Sharma *et al*. 1996, Momotaz *et al*. 1998, Kato *et al*. 2001, Fratini & Ruiz 2006).

In this study, we firstly conducted interspecific crosses in an attempt to recover hybrids between *T. grandiflora* and *T. fernandezii*. Moreover, we employed *in vitro* embryo rescue methods to overcome hybrid sterility and used cytological evidence to evaluate the relationships among both species. Finally, we formulated a tentative hypothesis regarding the autopolyploid origin of *T. fernandezii*.

Material and methods

Plant material

Two accessions of *T. grandiflora* and one accession of *T. fernandezii* were used in the present study (Table 1). Voucher specimens were deposited in the herbarium of the Instituto de Botánica del Nordeste (CTES), Corrientes, Argentina.

Table 1. Material studied.

Turnera	2n	Ploidy	Locality and Collector	Floral morph	Code
grandiflora	10	2x	Argentina, Formosa. *Arbo 2696*	S	G_4
	10*	2x	Argentina, Corrientes, Capital. *Fernández s/n*	S	G_{11}
fernandezii	40*	8x	Paraguay, Amambay. Arbo 8882	L	G_{10}
fernandezii × *grandiflora*	25*	5x	Argentina, Corrientes, Capital. *Fernández s/n*	S	

*Chromosome counts on additional accessions. L = long-styled, S = short-styled.

Crossing methods

Since both *T. grandiflora* and *T. fernandezii* are dystylous (Arbo 2005), the 30 crosses performed consisted of legitimate combinations between long-styled (L) and short-styled (S) plants. Crossings were made according to Fernández and Arbo (1989) under greenhouse conditions to avoid undesired insect pollination. Moreover, although both species are self-incompatible (Shore *et al*. 2006), open flowers used as females were emasculated prior to pollination with anthers of plants selected as males. Pollinated flowers were individually marked indicating the pollen donor. The number of flowers that were pollinated was different for each parental combination used, depending on the availability of plants and on the chance of simultaneous flowering (Table 2).

Establishment of seedlings *in vitro*

Pollination was followed by embryo rescue, i.e. cultivation of immature seeds on Murashighe and Skoog's (1962) medium (MS). Immature fruits were collected ten days after pollination. The fruits were washed 1 min in 70% ethanol, surface-sterilized for 20 min with sodium hypochlorite solution (1.1% available chlorine) containing 1 ml of Tween 20, and then rinsed three times with sterile distilled water. After surface-sterilization, 85 immature seeds were placed on the surface of MS medium in glass tubes that were covered with Resinite AF50® and, incubated in a conditioned chamber at 27 ± 2 °C with a photoperiod of 14 hrs (116 μmol m^{-2} s^{-1}). To induce rooting, the germinated seeds were transferred to solid culture medium containing 1/2 strength MS supplemented with 3% sucrose and 1 g l^{-1} active charcoal (AC). Before the addition of 0.7% agar (Sigma A 1296), the pH was adjusted to 5.8 with KOH and/or HCl. The glass tubes were covered with aluminum foil and sterilized in an autoclave at 0.101 MPa for 20 min. The rooted shoots were cloned *in vitro* in order to increase the number of hybrid plants obtained.

The best regenerated plantlets were also acclimatized to conditions *ex vitro* in a mixture of soil, sand and pearlite (1:1:1) and maintained during 15 days in a conditioned chamber at 27 ± 2 °C, at a luminance intensity of 336 μmol m^2 s^{-1} and 14 hrs photoperiod. The plants were then transferred to the greenhouse.

Cytological studies

Chromosome numbers of the accessions G_{10} and G_{11} used as parents were obtained from meiosis, while the chromosome number of accession G_4 was previously determined (Fernández 1987). Meiotic chromosomes were examined in pollen mother cells (PMC) of young buds, fixed in 5:1 absolute ethanol:lactic acid (Fernández 1973) for 12 hrs at 4 °C and stored in 70% ethanol at 4 °C. Anthers were stained using the Feulgen technique and squashed in 3% aceto-orcein. Slides were made permanent in Euparal using Bowen's (1956) method. In the diploid parents, microspores at tetrad stage were also examined to explore the production of unreduced gametes. For this analysis, young floral buds were fixed as described above and the anthers were stained with carmine:glycerin. Expected n, 2n and 4n pollen percentages were calculated from tetrad, triad, dyad and monad frequencies, taking into account that tetrads form four n gametes, dyads two 2n gametes, while each monad originates one 4n gamete only. In order to estimate pollen viability, pollen stainability was also estimated using carmine-glycerin 1:1. At least 300 grains per flower were scored.

The hybrid character of the plants raised was checked by counting their chromosome number. Furthermore, the analysis of meiotic behavior and pollen stainability of the progeny were also performed according to the methods described for the parents.

Table 2. Crosses carried out between *Turnera grandiflora* and *T. fernandezii*.

Parents	*N*	Ploidy	Morphotypes
$G_{10} \times G_4$	3	8x × 2x	L × S
$G_{10} \times G_{11}$	21	8x × 2x	L × S
$G_{11} \times G_{10}$	6	2x × 8x	S × L

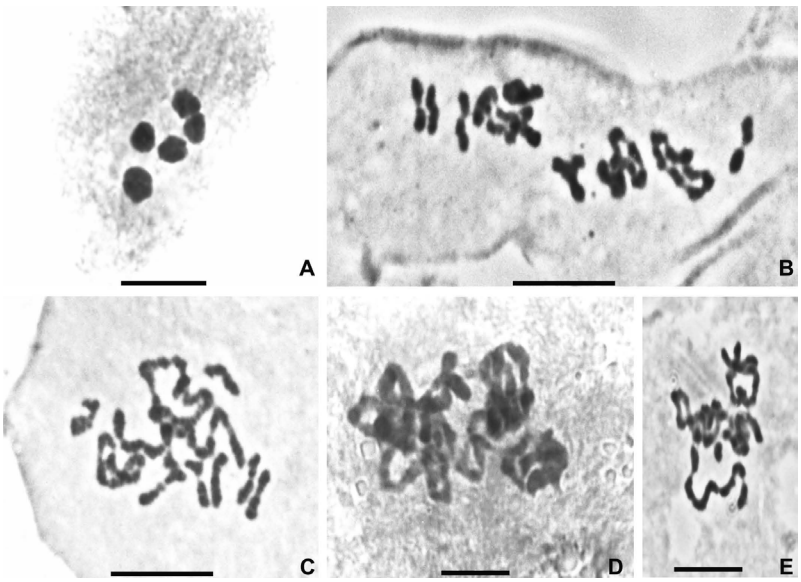

Fig. 1. Chromosome pairing at MI of the parental species. — **A**: *Turnera grandiflora* (2n = 2x = 10) with 5II. — **B**: *T. fernandezii* (2n = 8x = 40) with 4II + 2IV + 3VIII. — **C**: *T. fernandezii* with 6II + 2IV + 1VI + 2VIII. — **D** and **E**: *T. fernandezii* with 5VIII. Bar = 5 μm.

Results

Crossings

Twenty-nine (96.67%) of the crosses between *T. grandiflora* and *T. fernandezii*, yielded no fruit, while the only crossing that gave fruits was *T. fernandezii* (G_{10}) L × *T. grandiflora* (G_{11}) S.

Establishment of seedlings *in vitro*

Seventy percent of the seeds germinated in culture after 15 days, 45 days later the plantlets gave several shoots. Eighty percent of the shoots cultivated in 1/2 MS + AC 1 g l^{-1} produced roots. From these shoots, eight plants were obtained and cloned *in vitro*, obtaining a total of 70 plants. Of these, only 20 gave full-grown plants. All flowering hybrid plants obtained so far were morphologically intermediates of their parents.

Cytological studies

Chromosome numbers of the parental plants and the F_1 hybrids are shown in Table 1. In *T. grandiflora*, all the PMCs analyzed had 5II (bivalents; Fig. 1A). However, the analysis of 7174 sporads showed, beside tetrads (75.30%, Fig. 2A), the production of dyads (0.04%, Fig. 2B), monads (0.03%, Fig. 2C) and other abnormal sporads (i.e. pentads, hexads and sporads with one or more micronuclei, 24.63%, Fig. 2D). The expected n, 2n and 4n pollen were 99.96%, 0.03% and 0.01%, respectively.

In *T. fernandezii,* up to five VIII (octovalents) were observed in metaphase I (Fig. 2B–D). All 20 plants that were obtained from interspecific crosses proved to be pentaploid hybrids (2n = 5x = 25; Fig. 3). A meiotic analysis of the hybrids revealed 16 different configurations in metaphase I (Table 3), with 5V (34.51%, Fig. 3A and B), 1II + 1III + 4V (19.02%) and 1I + 1IV + 4V (15.49%, Fig. 3F) being the most frequent. Chromosome associations at MI of the F_1 hybrids are summarized in Table 4. In anaphase I, laggard chromosomes (Fig. 4A and C) and bridges (Fig. 4B and C) were observed.

Pollen fertility of the parents was nearly 95% in *T. grandiflora* and 93.71% (98.68%–99.80%) in *T. fernandezii*; while the fertility of the pentaploid hybrid was 59.23% (55.56%–64.40%).

Discussion

Crossings

The analysis performed showed that embryo

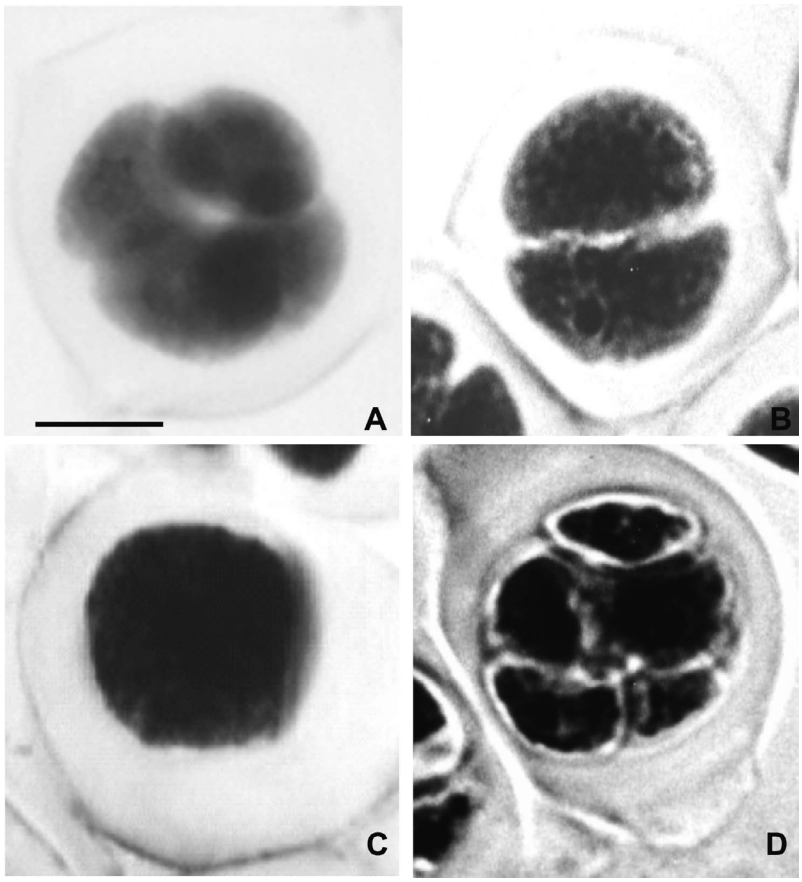

Fig. 2. Sporads of *Turnera grandiflora*. — **A**: Tetrad. — **B**: Dyad. — **C**: Monad. — **D**: Pentad. Bar = 5 μm.

rescue provided an effective means for the production of semi-fertile pentaploid hybrids between diploid *T. grandiflora* and octoploid *T. fernandezii*.

As a result of the 267 reciprocal experimental crosses between *T. fernandezii* and *T. grandiflora* presented in this paper and in a previous one (Arbo & Fernández 1987), fruits were recovered only once, when *T. fernandezii* was used as female parent. This result agrees with those obtained from interploidy crosses involving other *Turnera* species (Shore & Barret 1985, Arbo & Fernández 1987, Fernández & Solís Neffa 2004) and species of other genera (Stebbins 1958, Woodell & Valentine 1961, Ockendon 1968, Levin 1971). This could be explained by the ratio between the ploidy level of an embryo and its associated endosperm being a critical factor influencing seed development (Ramsey &

Table 3. Chromosome configurations at first metaphase of meiosis of the PMCs in F_1 hybrids between *T. fernandezii* × *T. grandiflora*.

Configuration	*N*	Percentage
5V	49	34.51
1II + 1III + 4V	27	19.02
1I + 1IV + 4V	22	15.49
1I + 2II + 4V	11	7.75
1I + 1II + 1III + 1IV + 3V	8	5.64
2II + 2III + 3V	7	4.94
1I + 3II + 1III + 3V	5	3.52
2I + 1III + 4V	4	2.82
2I + 4II + 2III +1IV +1V	2	1.41
2I + 1II + 2III + 3V	1	0.70
2I + 2IV + 3V	1	0.70
2I + 2II + 3III + 2V	1	0.70
4I + 3II + 3V	1	0.70
4II + 4III + 1V	1	0.70
5I + 3II + 1IV + 2V	1	0.70
5I + 4V	1	0.70

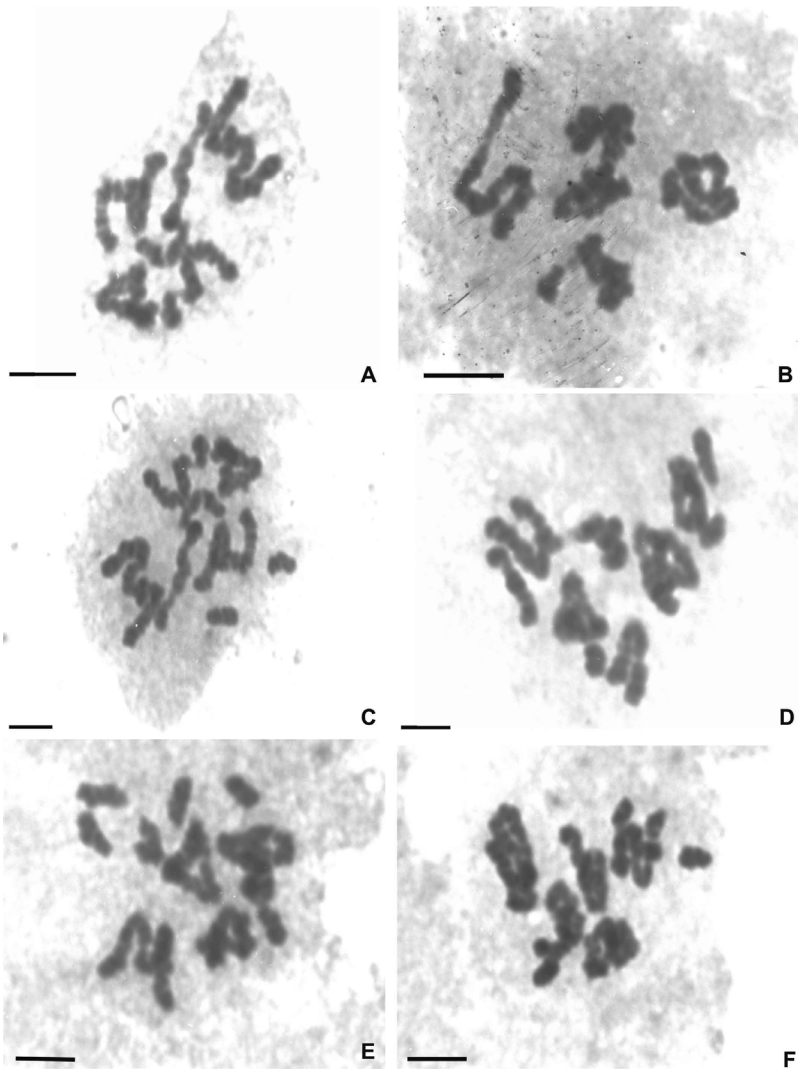

Fig. 3. Chromosome pairing at MI of the F_1 hybrid *Turnera fernandezii* × *T. grandiflora* (2n = 5x = 35). — A and B: 5V. — C: 2I + 2IV + 3V. — D: 3II + 3III + 2V. — E: 5I + 1II + 1III + 3V. — F: 1I + 1IV + 4V. Bar = 5 μm.

Schemske 1998). However, the embryo collapse due to unbalance of the embryo/endosperm rate would eventually be overcome when the ploidy level of maternal parents is the highest.

Cytological studies

Our chromosome counts confirm the numbers 2n = 2x = 10 and 2n = 8x = 40 previously reported for *T. grandiflora* and *T. fernandezii*, respectively (Fernández 1987). The analysis of hybrid chromosome complements of the full-grown plants confirms their hybrid nature, since all of them were pentaploids (2n = 5x = 25).

The analysis of chromosome pairing at metaphase I and pollen fertility of hybrids are useful for assessing the evolutionary relationship and genetic divergence between species (de Wet & Harlan 1972). Chromosome pairings and pollen fertility observed in the pentaploid *T. fernandezii* × *T. grandiflora* hybrid are indicative of an autopentaploid and suggest a close genetic relationship between *T. grandiflora* and *T. fernandezii*.

Taking into account that *T. fernandezii* showed a maximum of three octovalents in MI (Fernandez 1987) and that it shares the same karyotype with 4 metacentric and 1 submetacentric with diploid *T. grandiflora* for all eight chromosome sets (Solís Neffa & Fernández 1993),

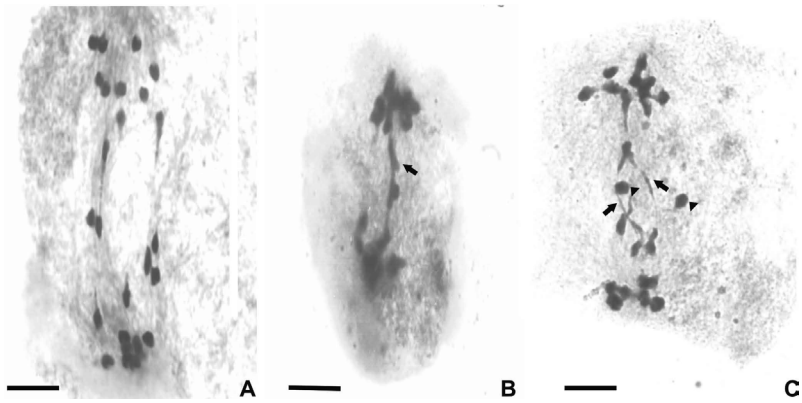

Fig. 4. Anaphase I of the F_1 hybrid *Turnera fernandezii* × *T. grandiflora* (2n = 5x = 35). — **A**: Laggard chromosomes. — **B**: Bridge (arrow). — **C**: Bridge (arrow) and laggard chromosomes (arrow head). Bar = 5 μm.

T. fernandezii was proposed to be an octoploid cytotype of *T. grandiflora*. Based on this cytological evidence as well as on the morphological similarity between the cytotypes, an autopolyploid origin from diploids was hypothesized to explain the origin of the octoploid cytotype (Fernández 1987). However, considering that no hybrids among the cytotypes had been obtained so far and that the octoploid can be distinguished from diploid *T. grandiflora* by its height and the leaf-indumentum, Arbo (2005) elevated the auto-octoploid cytotype to a species of its own, *T. fernandezii*. Therefore, the meiotic analysis of the pentaploid hybrids presented in this paper suggests that *T. fernandezii* (C^gC^g C^gC^g C^gC^g C^gC^g) inherited the genome C^gC^g of *T. grandiflora* and supports the hypothesis that *T. fernandezii* originated by autopolyploidy from *T. grandiflora*.

Our results also provide evidence that sexual polyploidization may have been involved in the origin of the auto-octoploid *T. fernandezii*. Sexual polyploidization involves the fusion of 2n gametes and was considered as the main mechanism of origin and evolution of polyploid plant species (Karpechenko 1927, Darlington 1937, 1956, Harlan & De Wet 1975, De Wet 1980, Bretagnolle & Thompson 1995). The sporad analysis performed showed the presence of dyads indicating that 2n gametes occurred in *T. grandiflora* and that the formation of 2n pollen has to be expected. Moreover, the presence of monads suggests that 4n pollen may also be formed. However, considering that in the diploid hybrid *T. subulata* × *T. scabra* secondary roots with duplicate (2n = 4x = 20) or quadruplicate (2n = 8x = 40) chromosome number were found (Fernández 1987) and that plants of *Turnera* may often grow from secondary roots, somatic chromosome doubling cannot be discarded as an alternative mechanism of polyploid formation in the genus *Turnera*. Consequently, octoploid plants of *T. fernandezii* may have also been formed from secondary roots of the diploid *T. grandiflora* with quadruplicate chromosome number.

Acknowledgments

The authors are grateful to Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Secretaría General de Ciencia y Técnica of the Universidad Nacional del Nordeste (UNNE) for financial support. The authors are members of the Carrera del Investigador Científico of CONICET.

Table 4. Mean, standard error and range of the number of observed chromosome associations at MI of the F_1 hybrids between *Turnera fernandezii* × *T. grandiflora*.

	I	II	III	IV	V
Mean ± SE	0.55 ± 0.07	0.73 ± 0.08	0.49 ± 0.06	0.25 ± 0.04	4.10 ± 0.07
Range	0–5	0–4	0–4	0–2	1–5

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