

Evidence of the production of $2n$ eggs in diploid plants of the autopolyploid complex *Turnera sidoides* L. (Passifloraceae)

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Received: 29 July 2015 / Accepted: 1 December 2015
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Abstract *Turnera sidoides* is a complex of distylous perennial rhizomatous herbs with five subspecies in which diploid ($2n = 2x = 14$) to octoploid ($2n = 8x = 56$) cytotypes were found. Previous studies have suggested an autopolyploid origin of the complex, and provided evidence for the production of $2n$ pollen in experimental conditions as well as in natural populations. However, only the production of $2n$ pollen has been demonstrated so far, while the production of $2n$ eggs on experimental crosses or in natural populations of *T. sidoides* remains unknown. In this paper we investigate the production of $2n$ eggs in diploid individuals to understand how they may have contributed to the origin and establishment of polyploids in diploid populations of *T. sidoides*. Our progeny test and flow cytometric analysis of seeds collected in natural populations of this species complex, show that the triploid embryos originated from $2n$ eggs, suggesting that $2n$ eggs can contribute to the origin of neopolyploids by sexual unilateral polyploidization as well as by sexual bilateral polyploidization. The occurrence of plants that continuously form many $2n$ eggs and pollen would play a key role in the establishment of neopolyploids in natural diploid populations.

Keywords $2n$ eggs · Polyploidy · Sexual polyploidization · *Turnera sidoides*

Introduction

Polyploidy is an important process of adaptation and speciation in flowering plants (Otto and Whitton 2000; Mable 2003; Adams and Wendel 2005; Otto 2007; Soltis et al. 2009, 2014; Brownfield and Köhler 2011). Despite the evolutionary significance of polyploidy, many important questions about the mechanisms by which polyploids are formed and become established in natural populations remain unanswered (Soltis et al. 2010).

Spontaneous doubling of somatic chromosomes (zygotic or meristematic) was long considered the predominant mode of polyploid origin in flowering plants (Winge 1917). However, it became soon clear that $2n$ gametes detected in many plants species (Karpechenko 1927; Darlington 1937) are involved in polyploid origin and, that the union of $2n$ gametes (i.e., sexual polyploidization) is the driving force giving rise to polyploid plant species (Harlan and De Wet 1975; Thompson and Lumaret 1992; Bretagnolle and Thompson 1995; Ramsey and Schemske 1998; Brownfield and Köhler 2011).

$2n$ gametes have been detected in hybrid and non-hybrid cultivars as well as in wild species (Clausen and Goodspeed 1925; Karpechenko 1927; Satina and Blakeslee 1935; Storey 1956; Mok and Peloquín 1975; Rhoades and Dempsey 1966; Parrot and Smith 1985; Tavoletti et al. 1991, Werner and Peloquín 1991; De Haan et al. 1992; Bretagnolle 2001; Ramsey 2007). Due to their easy detection because of the differences in size (Tyagi 1988; Orjeda et al. 1990, Jansen and Den Nijs 1993; De Storme et al. 2013) and DNA content (Pan et al. 2004; Dewitte

Handling editor: Martin Lysak.

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et al. 2009) between n and $2n$ pollen grains, the formation of $2n$ male gametes has received much more attention than $2n$ eggs.

Unreduced female gametes were identified by their size (Stelly and Peloquín 1986) but mostly indirectly, by estimating the ploidy level of the progeny of $2x \times 4x$ experimental crosses. In such crosses, a tetraploid descendant was considered a strong evidence of the fertilization of a $2n$ gamete of the diploid female parent by an n gamete of the tetraploid male parent (Marks 1966; Tavoletti et al. 1991; Calderini and Mariani 1997; Ramsey 2007). In outcrossing species in which interploidy barriers are well established, all descendants are tetraploids, because the triploid block prevents the development of triploid embryos in $2x \times 4x$ crosses (Alexander and Beckett 1963; De Haan et al. 1992; Bretagnolle and Thompson 1995; Ramsey 2007; Köhler et al. 2010). Alternatively, $2n$ eggs can easily and precisely be detected in natural populations by analyzing the ploidy level of the embryo and endosperm in mature seeds through the use of screening techniques such as flow cytometry (Matzk et al. 2000; Mable 2003). Different ploidy levels can occur depending on whether the embryo sacs are n or $2n$, and if the egg cell and/or central cells are fertilized by n or $2n$ male gametes. Diploid sexual plants with Polygonum-type of embryo sacs form a diploid embryo and a triploid endosperm (generated by the fusion of the nuclei of central cells with one generative nucleus from the pollen). Instead, seeds with tetraploid endosperm have triploid embryos resulting from $2n$ male gametes, while seeds with pentaploid endosperm have embryos that resulted from $2n$ female gametes (Table 1, Matzk et al. 2000).

Turnera sidoides L. (*Passifloraceae*) represents an ideal study system to investigate the mechanism of polyploid formation involving $2n$ gametes in natural populations. This complex of perennial, rhizomatous herbs is widely distributed from the southern regions of Bolivia and Brazil, through Paraguay to Uruguay and Argentina, reaching 39°S (Arbo 1987; Solís Neffa 2000). Five subspecies and five morphotypes are recognized on the basis of the geographical distribution and the great variability of some morphological features (Arbo 1985; Solís Neffa 2010). *Turnera sidoides* is an obligate outbreeder due to dystily

(Arbo 1985) and genetic self-incompatibility. Seeds are dispersed by gravity and by ants, and tend to concentrate in localized areas, generating a distribution pattern in which discrete populations are frequently observed (Solís Neffa 2000).

The cytogenetics of *T. sidoides* are well documented. The subspecies of the complex are known to exhibit different ploidy levels based on $x = 7$, ranging from diploid ($2n = 2x = 14$) to octoploid ($2n = 8x = 56$) (Fernández 1987; Solís Neffa and Fernández 2001; Solís Neffa et al. 2004; Elías et al. 2011; Kovalsky and Solís Neffa 2012). Furthermore, cytogenetic analysis has revealed the autopolyploid origin of polyploid cytotypes (Fernández 1987; Solís Neffa 2000; Solís Neffa and Fernández 2002). The different cytotypes mostly occur in single areas (i.e. areas with only one cytotype). Diploids of *T. sidoides* have restricted and disjunct distributions, and among polyploids, tetraploids are the most widespread, while populations with higher ploidy levels and odd polyploids are rare and usually located at marginal areas (Solís Neffa 2000; Solís Neffa and Fernández 2001; Solís Neffa et al. 2004; Elías et al. 2011). Diploids and tetraploids are often spatially segregated also at a microgeographical scale, although different cytotypes can occur in close proximity in diploid–tetraploid contact zones where diploid, triploid and tetraploid plants frequently grow in mixed patches (Elías et al. 2011; Kovalsky and Solís Neffa 2012).

Previous studies in experimental diploid populations of *T. sidoides*, provided evidence for the production of $2n$ pollen (Panseri et al. 2008). Furthermore, an exhaustive study on natural populations of *T. sidoides* subsp. *carnea* (Cambess.) Arbo (Kovalsky and Solís Neffa 2012) revealed a large variation in the frequency of $2n$ and $4n$ pollen. This study also showed that, within populations, short-styled (S) and long-styled (L) plants differ in their ability to produce $2n$ pollen and, that the production of $2n$ pollen may increase up to 22 % under certain environmental conditions during the flowering period. The analysis of the progeny of $2n$ pollen producers and non-producers evidenced that, in *T. sidoides*, the capacity of $2n$ gametes production is a heritable trait (Kovalsky and Solís Neffa 2015). Nuclear restitution at both the first and the second meiotic division are involved in the production of $2n$ male gametes in this species (Kovalsky et al. 2014). Production of $2n$ gametes also appears to enhance the probability of establishment and persistence of newly formed polyploids in natural diploid populations of *T. sidoides* (Kovalsky and Solís Neffa 2012, 2015). Moreover, it plays a role in the dynamic of the diploid–tetraploid contact zones of this species complex (Elías et al. 2011; Kovalsky and Solís Neffa 2012). However, only the production of $2n$ pollen has so far been reported in this species, whereas it remains

Table 1 Ploidy level ratios expected according to the type of gametes involved in fertilization

Gametes	Male n	Male $2n$
Female n	Embryo $2x$	Embryo $3x$
	Endosperm $3x$	Endosperm $4x$
Female $2n$	Embryo $3x$	Embryo $4x$
	Endosperm $5x$	Endosperm $6x$

unclear whether female $2n$ eggs are also produced in *T. sidoides*.

To investigate the occurrence of $2n$ eggs in diploid populations of *T. sidoides* and their contribution to the origin of polyploids, in this paper we analyze the production of $2n$ eggs in diploid individuals by progeny test and by flow cytometric analysis of seeds collected in natural populations of this species complex.

Materials and methods

Plant material

We investigated diploid plants from natural populations of *Turnera sidoides* subsp. *carnea* and from a diploid - tetraploid contact zone of *T. sidoides* subsp. *pinnatifida* (Juss. Ex Poir.) Arbo (Table 2). These populations were selected because a high production of $2n$ pollen was detected in previous studies (Panseri et al. 2008; Kovalsky 2012; Kovalsky and Solís Neffa 2012). Voucher specimens were deposited in the Herbarium of the Instituto de Botánica del Nordeste (CTES), Corrientes, Argentina. Some individual plants of each site were also transported to Corrientes where they were grown under homogeneous greenhouse conditions.

Ploidy levels of all individuals here analyzed were determined in previous studies (Elías et al. 2011; Kovalsky 2012; Kovalsky and Solís Neffa 2012). Moreover, our analyses include 33 individuals in which $2n$ pollen were previously detected (Kovalsky 2012; Kovalsky and Solís Neffa 2012).

Progeny test

Twelve diploid plants from population S₂₁₅ which are growing under greenhouse conditions were tested for $2n$ eggs production by using them in experimental crosses. Four tetraploid plants from populations S₄₂₉ and S₄₃₀ were

used as a pollen source (Table 2). Pollen viability of diploid ($\approx 95\%$) and tetraploid plants ($\approx 85\%$) used in this study were previously determined (Kovalsky 2012; Kovalsky et al. 2014; Kovalsky pers. com.). All crosses consisted of legitimate combinations between L and S plants: $2x$ (L) \times $4x$ (S) and $2x$ (S) \times $4x$ (L), for a total of 30 crosses (Table 3). Crossings were carried out in greenhouse conditions to exclude pollinators and followed protocols by Fernández and Arbo (1989). Open flowers used as females were emasculated before pollinating them with pollen from anthers of plants selected as males. The number of crosses for each parental combination varied according to the availability of plants and on the occurrence of simultaneous flowering (Table 3). Maturing capsules were wrapped in small tulle bags to prevent loss of seeds during dehiscence. Seed capsules fully developed in approximately 20 days, after which mature seeds were collected. Viable seeds of each cross were sown in individual pots, and the resulting individuals were transplanted after having developed the first pair of leaves.

The ploidy level of the progeny was determined by estimating the relative DNA content by flow cytometry. The analyses were performed using leaf tissue and following the recommendations with the Partec kit CyStain UV Precise P (05-5002), which was used to prepare the samples. For each individual, 0.5 cm² of leaf was placed in a petri dish with a comparable amount of tissue from an internal standard (*T. sidoides* subsp. *carnea*, individual S₂₁₅₋₄₈), the ploidy level of which was inferred previously from chromosome counts in meiosis (Kovalsky and Solís Neffa 2012). After adding 0.5 ml of extraction buffer, the leaf tissue was chopped with a razor blade. Following a 2-min incubation, samples were filtered through a 50 μ m nylon mesh into the sample tube with 1.5 ml of DAPI (4,6-diamidino-2-phenylindole) staining solution. The mixture was incubated for 2 min at room temperature and then analyzed. The fluorescence intensity of DAPI-stained nuclei was determined using a Partec PA II flow cytometer (Partec GmbH, Münster, Germany) with the wavelength

Table 2 Geographical location of populations of *Turnera sidoides* analyzed in this study

<i>T. sidoides</i>	Population	Locality (voucher references)	Ploidy level
subsp. <i>carnea</i>	S ₂₁₅	Argentina, Corrientes, Dpt. Mercedes. 29°33'44"S, 57°30'40"W, 66 m a. s. l. (SN and S 960)	2x
	S ₂₁₆	Argentina, Corrientes, Dpt. Mercedes. 29°33'2"S, 57°30'14.2"W, 41 m a. s. l. (SN 981)	2x, 3x
	S ₄₂₉	Argentina, Corrientes, Dpt. Mercedes. 29°31'52.8"S, 57°32'53.7"W, 68 m a. s. l. (SN et al. 2221)	4x
	S ₄₃₀	Argentina, Corrientes, Dpt. Mercedes. 29°33'00"S, 57°32'15.5"W, 69 m a. s. l. (SN et al. 2222)	4x
subsp. <i>pinnatifida</i>	S ₂₃₅	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°51'57.8"S, 64°29'29.3"W, 1082 m a. s. l. (SN and S 967)	2x
	S ₃₁₉	Argentina, Córdoba, Dpt. Punilla, Capilla del Monte. 30°51'47.7"S, 64°29'51.3"W, 1070 m a. s. l. (Elías s.n.)	2x

S: JG Seijo; SN: VG Solís Neffa

Table 3 Results of the $2x \times 4x$ experimental crosses performed in *Turnera sidoides*

Type of crossing	No. of crosses	No. of the progeny plants	Ploidy level of the progeny	
			3x	4x
S ₂₁₅ -58 L (2x) × S ₄₂₉ -216 S (4x)	2	12	12	0
S ₂₁₅ -54 L (2x) × S ₄₃₀ -226 S (4x)	1	6	6	0
S ₂₁₅ -243 L (2x) × S ₄₃₀ -226 S (4x)	1	6	6	0
S ₂₁₅ -133 L (2x) × S ₄₃₀ -226 S (4x)	2	10	10	0
S ₂₁₅ -5 L (2x) × S ₄₃₀ -226 S (4x)	2	6	6	0
S ₂₁₅ -29 L (2x) × S ₄₂₉ -216 S (4x)	2	13	13	0
S ₂₁₅ -133 L (2x) × S ₄₂₉ -216 S (4x)	1	6	6	0
S ₂₁₅ -54 L (2x) × S ₄₂₉ -216 S (4x)	1	5	5	0
S ₂₁₅ -243 L (2x) × S ₄₂₉ -216 S (4x)	1	7	7	0
S ₂₁₅ -29 L (2x) × S ₄₃₀ -226 S (4x)	1	5	5	0
S ₂₁₅ -48 S (2x) × S ₄₃₀ -222 L (4x)	3	19	19	0
S ₂₁₅ -186 S (2x) × S ₄₃₀ -222 L (4x)	1	5	3	2
S ₂₁₅ -42 S (2x) × S ₄₃₀ -222 L (4x)	1	5	5	0
S ₂₁₅ -186 S (2x) × S ₄₂₉ -218 L (4x)	2	12	10	2
S ₂₁₅ -42 S (2x) × S ₄₂₉ -218 L (4x)	2	11	11	0
S ₂₁₅ -129 S (2x) × S ₄₂₉ -218 L (4x)	2	15	15	0
S ₂₁₅ -199 S (2x) × S ₄₂₉ -218 L (4x)	1	5	5	0
S ₂₁₅ -135 S (2x) × S ₄₂₉ -218 L (4x)	1	5	5	0
S ₂₁₅ -129 S (2x) × S ₄₃₀ -222 L (4x)	2	11	11	0
S ₂₁₅ -199 S (2x) × S ₄₃₀ -222 L (4x)	1	F	F	0

Ploidy level of progenitors is between brackets

L long-styled, S short-styled, F failed

used for excitation operating at 355 nm. About 3000 nuclei were measured in each sample. Ploidy levels were estimated by comparing the DNA peak of the samples to the internal standard. The data was analyzed using PA II's Partec FloMax software. The ploidy level of the progeny was confirmed by chromosome counts in meiosis. Meiotic chromosomes were examined in pollen mother cells of young buds, fixed in 5:1 absolute ethanol:lactic acid (Fernández 1973) for 12 h at 4 °C and stored in 70 % ethanol at 4 °C. Pollen mother cells were extracted in a drop of 2 % aceto-orcein after the tip of pollen sacs was cut. Then, a slight squash was done. Chromosomes were viewed and photographed with a Zeiss Axioplan HBO 100 W/2 microscope equipped with a computer-assisted Cannon Powershot A-640 digital camera system, at a magnification of 1000×.

Detection of $2n$ eggs production in natural populations

Flow cytometry experiments were set up in wild populations S₂₁₅ and S₂₁₆ of *T. sidoides* subsp. *carnea* and S₂₃₅ and S₃₁₉ of *T. sidoides* subsp. *pinnatifida* to screen the ploidy distribution pattern of the seeds and identify those derived from $2n$ eggs. Populations S₂₃₅, S₃₁₉ and S₂₁₆

were composed of fewer than 50 individuals, while population S₂₁₅ was composed of ≈ 150 individuals. For flow cytometry experiments, 1376 seeds belonging to 273 individuals were collected. Of them, 686 viable seeds belonging to 140 individuals were selected and analyzed. The pericarp was extracted from seeds, and samples were then processed as described in the previous section. The homogenate obtained was filtered through a 30 μ m nylon mesh. Ploidy levels of both the embryo and the endosperm were compared with those expected from crosses involving n and $2n$ pollen and eggs (Table 1). A similar number of L and S plants were analyzed in each population.

Results

Progeny test

Fruits developed in 93 % of the crosses performed. The progeny was mostly triploid, although some tetraploid progeny (2.44 %) was recovered from crosses involving S plants as a female parent (Fig. 1). Only one of the diploid individuals used as maternal parents produce $2n$ eggs (Table 3).

Detection of $2n$ egg production in natural populations

Triploid embryos were detected in populations S₂₁₅, S₂₃₅ and, S₃₁₉. Only in some seeds of populations S₂₁₅ and S₃₁₉ we detected a pentaploid endosperm indicating an origin of triploids by $2n$ eggs (Fig. 2, Table 4). In both the diploid populations of *T. sidoides* subsp. *carnea* and the diploid-tetraploid contact zone of *T. sidoides* subsp. *pinnatifida*, most of the 140 individuals analyzed were not producing $2n$ eggs, whereas in nine individuals we detected one or two $2n$ eggs. In two plants of population S₂₁₅, triploid embryos were originated from $2n$ eggs or from $2n$ pollen, both produced in the same plant. We found no tetraploid embryos.

In subspecies *carnea*, both S and L plants produced $2n$ eggs (3 and 4 plants, respectively), whereas in subspecies *pinnatifida* only L plants produced $2n$ eggs. All plants producing $2n$ eggs were previously reported to produce $2n$ pollen (Kovalsky 2012; Kovalsky and Solís

Neffa 2012). However, not all plants producing $2n$ pollen produced $2n$ eggs.

Discussion

The present study on *Turnera sidoides* provides the first evidence of the production of $2n$ eggs by diploid plants from natural populations in the genus *Turnera*. $2n$ eggs were previously detected only in interspecific experimental crosses between *T. caerulea* DC. ($2x$) \times *T. grandidentata* (Urban) Arbo ($4x$) and *T. krapovickasii* Arbo ($2x$) \times *T. grandidentata* ($4x$) (Fernández and Arbo 1990). Additionally, $2n$ eggs were mainly detected in diploid cultivars (Mendiburu and Peloquín 1977; Pfeiffer and Bingham 1983; Parrot and Smith 1985; Veronesi et al. 1986; Camadro and Espinillo 1990; De Haan et al. 1992; Veerle et al. 2002) and allopolyploid hybrids (Zhou et al. 2008; Koutecky et al. 2010), but rarely in natural autopolyploid populations. Our data provide further evidences of the

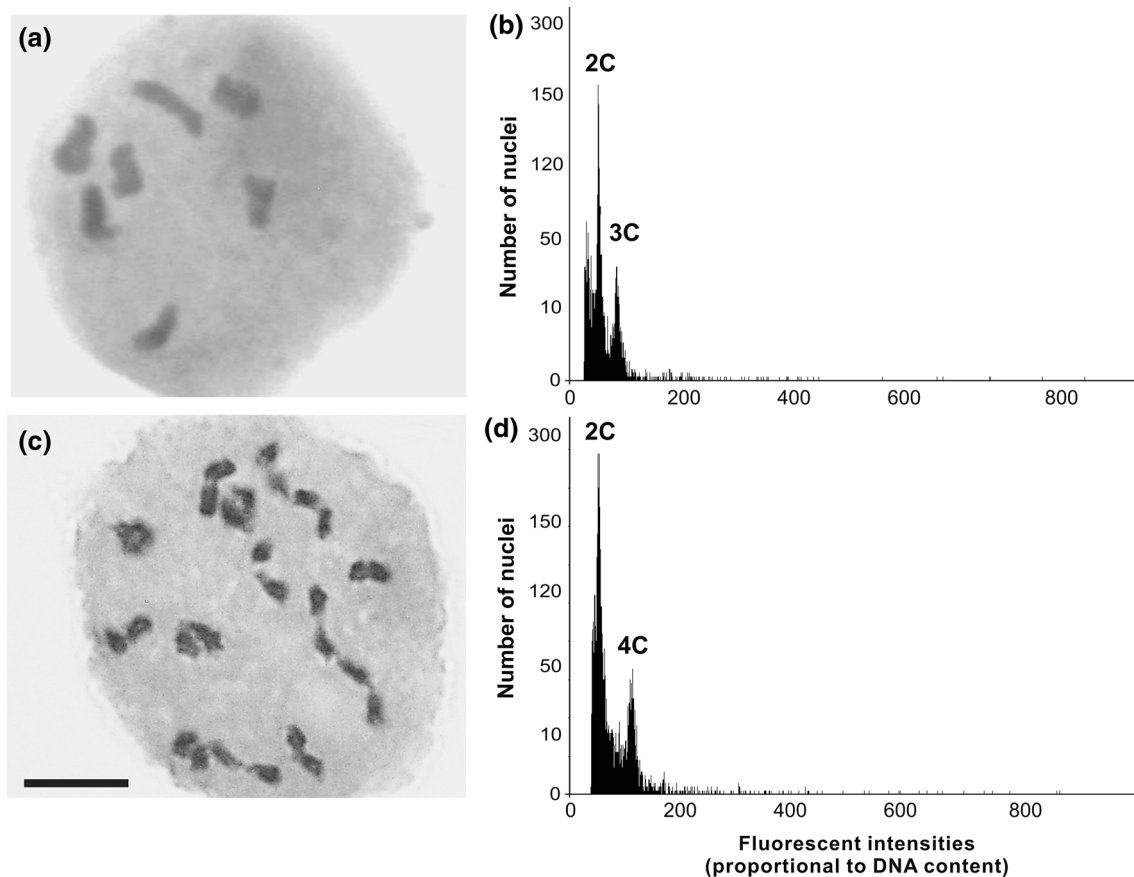


Fig. 1 Meiotic chromosomes and flow cytometric profiles of the progeny of experimental $2x \times 4x$ crosses of *Turnera sidoides*. **a**, **b** Triploid ($2n = 3x = 21$). **a** Diacinesis 7 III. **b** Flow cytometry histogram showing a peak at 3C and the standard (2C). **c**, **d** Tetraploid

($2n = 4x = 28$). **c** Diacinesis, 3VI + 8II. **d** Flow cytometry histogram of a tetraploid plant with a high peak at 4C and the diploid standard (2C). Bar 5 μm

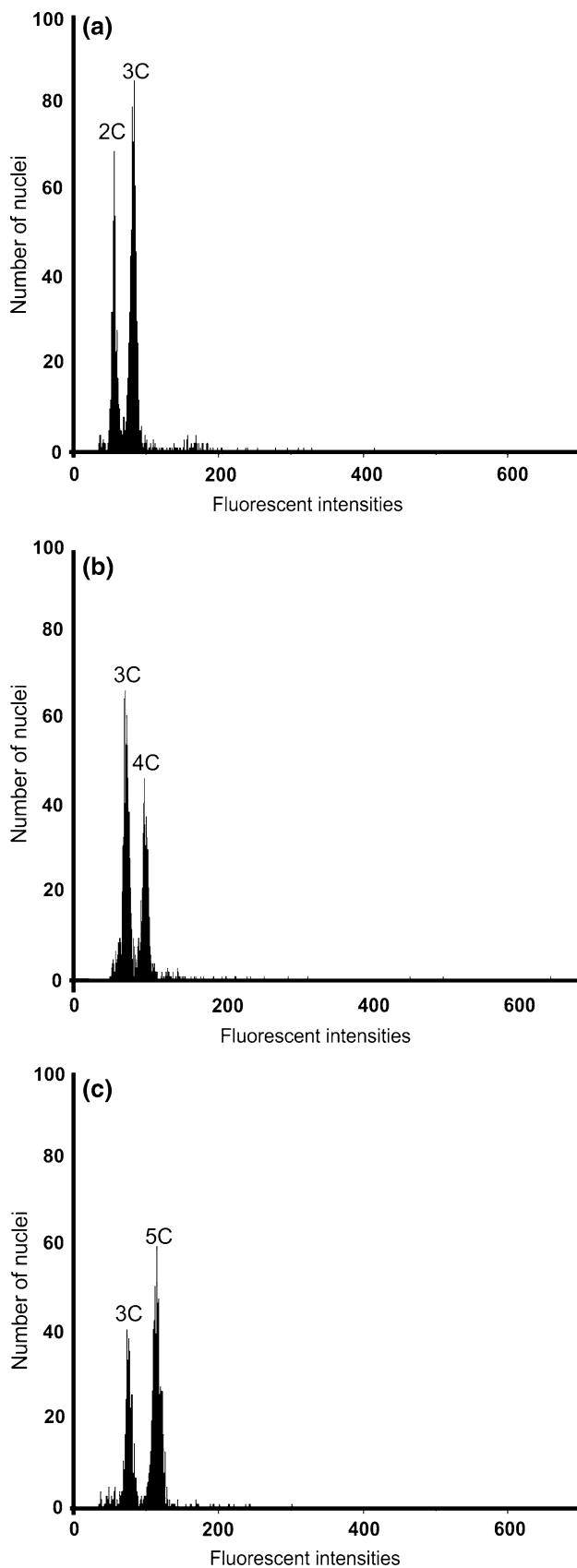


Fig. 2 Flow cytometry histograms of seeds of *Turnera sidoides*. **a** Flow cytometry histogram of a diploid seed showing an embryo with a high peak at 2C and a peak at 3C corresponding to the endosperm. **b** Flow cytometry histogram of a triploid seed originated from an $2n$ pollen showing an embryo with a high peak at 3C and a peak at 4C corresponding to the endosperm. **c** Flow cytometry histogram of a triploid seed originated from an $2n$ egg showing an embryo with a high peak at 3C and a peak at 5C corresponding to the endosperm

production $2n$ eggs in natural diploid populations of an autopolyploid complex.

In spite of the low number of $2n$ eggs detected, our results suggested that, in natural diploid populations of *T. sidoides*, some plants would be more likely to produce $2n$ eggs than others. These data agree with the previous findings in this species, which demonstrated that only some S and L plants (26 %) were capable of producing $2n$ and $4n$ pollen and, that the capability of producing such unreduced gametes is under genetic control (Kovalsky and Solís Neffa 2012, 2015). The finding in this paper of only some plants of *T. sidoides* that produced $2n$ eggs suggests that their production could be under genetic control as well. Furthermore, although the frequency of plants producing $2n$ eggs in *T. sidoides* was lower than that previously reported for plants producing $2n$ pollen (Kovalsky and Solís Neffa 2012), all plants that produced $2n$ eggs also produced $2n$ pollen. This pattern was also observed in hybrids of *T. grandidentata* (Fernández and Arbo 1990). A relation was proposed to exist in the capacity of plants to produce male and female $2n$ gametes (Ramsey and Schemske 1998), but it remains controversial (see Parrot and Smith 1985; Veilleux 1985; Veronesi et al. 1990; Tavoletti et al. 1991; De Haan et al. 1992). In *T. sidoides*, although some plants may produce both $2n$ pollen and $2n$ eggs, the fact that plants producing $2n$ pollen do not always produce $2n$ eggs suggests that their simultaneous production may be independent from each other.

Our results also suggest differences in the relative contributions of $2n$ pollen and $2n$ eggs to polyploid formation. In diploid–tetraploid crosses, $2n$ eggs are supposed to be more likely to generate viable seeds, than $2n$ pollen (Thompson and Lumaret 1992; Ramsey and Schemske 1998). Formation of neopolyploids in natural populations would proceed in a similar way to these interploidy crosses (Ramsey 2007). This would not be the case of *T. sidoides*, since the major frequency of triploid embryos from $2n$ pollen in seeds collected in natural populations, suggests that, in this complex, $2n$ pollen would contribute more than $2n$ eggs to the origin of neopolyploids. However, this would not necessarily imply that triploid embryos generated by fusion of $2n$ pollen and n eggs result in plants

Table 4 Results of the flow cytometry analysis of the ploidy level of the embryo and endosperm in mature seeds from natural populations of *Turnera sidoides*

Population	Number of plants	Number of seeds	Number of $2x-3x$ seeds	Number of $3x-4x$ seeds	Number of $3x-5x$ seeds
S ₂₁₅	70	342	325	10	7
S ₂₁₆	30	146	146	0	0
S ₂₃₅	20	98	96	2	0
S ₃₁₉	20	100	97	0	3

which will effectively establish in populations of *T. sidoides*. Ploidy ratios among embryo, endosperm, and maternal tissue affect the development and viability of seeds generated by interploidy crosses (Ramsey and Schemske 1998). The embryo collapse due to unbalances of the embryo/endosperm rate would be eventually overcome by mean of the highest ploidy level of maternal parent, as it was observed in interploidy crosses involving other *Turnera* species (Shore and Barrett 1985; Arbo and Fernández 1987; Fernández and Solís Neffa 2004; Fernández et al. 2010) and species of other genera (Stebbins 1958; Woodell and Valentine 1961; Ockendon 1968; Levin 1971). Consequently, endosperm ploidy may also determine the efficiency of $2n$ gametes in unilateral sexual polyploidization (Ramsey and Schemske 1998). Moreover, studies performed in *Arabidopsis thaliana* revealed that parent-of-origin-specific expression (imprinting) of the regulatory genes of the polycomb group (PcG) gene MEDEA is one of the underlying cause responsible for abnormalities in growth and structure of the endosperm in *Arabidopsis* seeds with increased paternal genome contributions (Luo et al. 2000; Osborn et al. 2003; Erilova et al. 2009). In *T. sidoides*, plants which produced $2n$ eggs develop more viable seeds per fruit (100 %), and seeds have a higher germination rate (66 %) than those plants that exclusively produce $2n$ pollen (89 and 41 %, respectively, Kovalsky 2012). This suggest that, although in *T. sidoides* $2n$ pollen were involved in the origin of most triploid embryos found in natural populations, the ploidy ratios among embryo and endosperm and/or epigenetic processes might confer such triploids an advantage during more advanced stages of their development and establishment than those triploids originated from the fusion of $2n$ eggs with an n pollen, that would be effectively established in diploid populations of *T. sidoides*.

Evolutionary implications

Autotetraploids can arise in two steps from matings involving triploids (unilateral sexual polyploidization), that serves as an intermediate step in the production of a new tetraploid (triploid bridge hypothesis). Moreover, autotetraploids may arise within a single step (bilateral sexual polyploidization) by the fusion of two $2n$ gametes (Ramsey

and Schemske 1998). Consequently, so that sexual bilateral polyploidization can occur, it is necessary that both male and female $2n$ gametes are formed (Bretagnolle and Thompson 1995; Ramsey and Schemske 1998). Bilateral sexual polyploidization was proposed to be the most probable mechanism of polyploid formation in the *T. sidoides* complex (Panseri et al. 2008; Kovalsky and Solís Neffa 2012). Taking into account that this species is dystilous and outbreeder (Solís Neffa 2000), to bilateral sexual polyploidization occurs both, S and L individuals should produce $2n$ pollen and $2n$ eggs. Our finding of $2n$ eggs, together with the detection of $2n$ pollen in previous studies (Panseri et al. 2008; Kovalsky and Solís Neffa 2012) in non-hybrid diploid populations of *T. sidoides*, and the fact that both the L and S plants can produce $2n$ pollen and $2n$ eggs, suggest that bilateral sexual polyploidization can occur in natural populations of this species. Furthermore, the recent finding of some tetraploid plants growing in a diploid population of *T. sidoides* (Mola Moringa et al. 2015) may reflect the pathway of tetraploid formation through the fusion of two $2n$ gametes. However, owing to the limited chances of fertilization between simultaneously formed $2n$ pollen and $2n$ eggs, bilateral sexual polyploidization would occur less frequently than the unilateral sexual polyploidization as was also proposed in other species (Ramsey and Schemske 1998; Ramsey 2007). The presence of some triploids (2–3 %) in natural diploid populations of *T. sidoides* detected in this paper and in previous ones (Elías et al. 2011; Kovalsky and Solís Neffa 2012) suggest that unilateral polyploidization by a triploid bridge could be an important mechanism for the origin of tetraploids in this species complex.

Moreover, the production of $2n$ eggs and $2n$ pollen would also have important implications in the establishment of *T. sidoides* polyploids. Polyploid populations of Angiosperms are supposed to have originated from single polyploid individuals, themselves the products of $2n$ gametes within a diploid population (Harlan and de Wet 1975). The establishment of such polyploids may be limited by the difficulty of encountering a mate of the same ploidy level, the inviability of triploid hybrids, the viability and fertility of polyploids relative to diploids, and the potential for genetic swamping of the more frequent cytotype (minority cytotype exclusion) (Levin 1975;

Fowler and Levin 1984; Bever and Felber 1998). Autogamous plants and individuals with multiple lifetime opportunities for reproduction may overcome difficulties associated with intercytotype mating most readily (Rodríguez 1996; Bever and Felber 1998; Ramsey and Schemske 1998; Husband 2000). However, in outbreeder plants and if pollination is at random, new, and thus rare, polyploids will be at a disadvantage, since most of their eggs will be fertilized by n pollen of diploids forming triploids, while most eggs of the relative abundant diploid will be suitably fertilized. Consequently, a new or rare polyploid is likely to be excluded from a diploid population (Levin 1975). In addition, models of polyploid evolution showed that $2n$ gamete production by diploids is an essential factor in the dynamics of mixed diploid-tetraploid populations since tetraploids are more likely to establish within diploid populations (or to be maintained at a low frequency) when they are formed recurrently through the union of $2n$ gametes or depending on frequency-dependent selection (Levin 1975; Felber and Bever 1997).

Turnera sidoides is an outbreeder; therefore, the occurrence of plants that continuously form many $2n$ eggs and $2n$ pollen would play a key role in the establishment of neopolyploids in natural diploid populations. Although the number of plants producing $2n$ eggs found in *T. sidoides* is relatively low, the proportion of $2n$ eggs here detected is in agreement with the estimations of the production of $2n$ gametes (0.03–2 %) for non-hybrid plants (Ramsey and Schemske 1998; Ramsey 2007). Additionally, taking into account that *T. sidoides* grow in discrete populations (mostly with fewer than 100 individuals, Solís Neffa 2000), the occurrence of a low number of plants producing $2n$ gametes may be significant to polyploids dynamics in diploid populations. Moreover, since the capability to produce $2n$ pollen (and probably to produce $2n$ eggs) is a heritable trait in *T. sidoides* and, the frequency of production of $2n$ pollen was demonstrated to be higher in the progeny of $2n$ pollen producers (Kovalsky and Solís Neffa 2015), the frequency of $2n$ pollen and $2n$ eggs and, consequently, the likelihood of origin of neopolyploids by sexual polyploidization would increase after successive generations. Furthermore, considering that *T. sidoides* is a perennial species and, that its seeds are dispersed in such a way that individuals concentrate in localized areas (Solís Neffa 2000), the progeny of plants that produce $2n$ gametes would concentrate near the mother plant and, thus, would increase the likelihood of occurrence of crosses between $2n$ pollen and $2n$ eggs producers. The continuous formation of neopolyploids as a consequence of successive backcrosses between $2n$ gametes producers and their progeny would favor the establishment and persistence of such neopolyploids in diploid populations of *T. sidoides*. In addition, since triploids of this species are not completely

sterile, produce n and $2n$ viable gametes as well as diploid, triploid and tetraploid progeny in experimental crosses (Elías 2010; Kovalsky 2012) suggest that new generations of polyploids (triploids and tetraploids) would originate by crossings between triploids or by backcrosses with diploid progenitors that produce $2n$ pollen and $2n$ eggs. These facts together with the capacity of *T. sidoides* to multiply asexually by rhizomes (Kovalsky and Solís Neffa 2015), are all aspects that would enhance the likelihood that a low frequency of neopolyploids can be originated and maintained in natural diploid populations of *T. sidoides*.

Conclusions and prospects

Our progeny test and flow cytometric analysis of seeds collected in natural populations of *Turnera sidoides* showed that $2n$ eggs can contribute to the origin of neopolyploids of this species complex by sexual polyploidization and that they would play an important role in the establishment of such neopolyploids in natural diploid populations. Current studies of the frequency of spontaneous polyploidization, the spatial distributions of cytotypes and $2n$ gamete producers and the effective contribution of $2n$ pollen and $2n$ eggs to neopolyploid origin in natural diploid populations of *T. sidoides* allow us to understand, more accurately, the role of $2n$ gametes in the origin and establishment of autopolyploids.

Acknowledgments We are deeply indebted to anonymous referees for their helpful comments on earlier version of this manuscript. This research was partially supported by Grants of the Agencia Nacional de Promoción Científica, Tecnológica y de Innovación (ANPCyT-FONCyT, PICT 07-1329 and PICT 12-1812), the National Research Council of Argentina (CONICET, PIP 11220120100192CO), and the Secretaría General de Ciencia y Técnica de la Universidad Nacional del Nordeste (SGCyT-UNNE, PI-A003/10) to V. G. Solís Neffa. I. E. Kovalsky is a Post Doctoral Fellow of CONICET and the North-eastern National University (UNNE). V. G. Solís Neffa is a member of the Carrera del Investigador Científico of CONICET.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest

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