

EVOLUTIONARY DYNAMICS OF AUTOPOLYPLOIDS IN NATURAL POPULATIONS: THE CASE OF *TURNERA SIDOIDES* COMPLEX



DINÁMICA EVOLUTIVA DE AUTOPOLIPLOIDES EN POBLACIONES NATURALES: EL CASO DEL COMPLEJO *TURNERA SIDOIDES*

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ABSTRACT

Turnera sidoides ($x=7$) is one of the few well-studied South American autopolyploid complexes. Since polyploidy has played a prominent role within this complex, ongoing studies in *T. sidoides* focus on understanding the mechanisms involved in the origin and the establishment of polyploids using integrative approaches. This paper synthesises the results of more than 20 years of research on this topic. Cytogenetics analysis provided evidences for the production of unreduced male and female gametes, supporting the hypothesis of bilateral sexual polyploidization as the mechanism of origin of polyploids in *T. sidoides*. The finding of viable triploids suggested that unilateral sexual polyploidization could also be an important mechanism for the origin of tetraploids in *T. sidoides*. The occurrence of plants continuously forming many unreduced gametes would play a key role in the establishment of neopolyploids in natural populations. Also, the higher number of propagules that tetraploids contribute to subsequent generations, the ability to multiply asexually by rhizomes, and the occurrence of occasional cases of self-compatibility and successful illegitimate crosses in polyploids increase the likelihood that a low frequency of neopolyploids can be maintained in natural populations of *T. sidoides*. In addition, integration of cytogeographic and genetic divergence data together with past niche modelling provided further insights supporting the hypothesis that historical climatic and geomorphological events provided favourable conditions for the establishment of autopolyploids, with the wider distribution of tetraploids of *T. sidoides* being the result of their range expansion.

Key words: cytogeography, establishment, origin, polyploidy, unreduced gametes

RESUMEN

Turnera sidoides ($x=7$) es uno de los pocos complejos autopoliploides sudamericanos bien estudiados. Como la poliploidía ha tenido un papel destacado en el complejo, los estudios en curso en *T. sidoides* se centraron en la comprensión de los mecanismos implicados en el origen y el establecimiento de los poliploides mediante diferentes enfoques. En este trabajo se sintetizan los resultados de más de 20 años de investigación sobre este tema. El análisis citogenético proporcionó evidencias de la producción de gametos masculinos y femeninos no reducidos, sustentando la hipótesis de la poliploidización sexual bilateral como mecanismo de origen de los poliploides en *T. sidoides*. Sin embargo, el hallazgo de triploides fértiles sugirió que la poliploidización sexual unilateral también sería un mecanismo importante de origen de tetraploides en *T. sidoides*. La ocurrencia de plantas que forman continuamente gametos no reducidos desempeñaría un papel clave en el establecimiento de neopoliploides. Además, el mayor número de propágulos que los tetraploides aportan a las siguientes generaciones, la capacidad de multiplicación asexual por rizomas y los casos ocasionales de autocompatibilidad y cruzamientos ilegítimos exitosos aumentarían la probabilidad de que se mantenga una baja frecuencia de neopoliploides en las poblaciones naturales de *T. sidoides*. Asimismo, la integración de datos citogeográficos y de divergencia genética junto con el modelado de nicho en el pasado aportó información que sustenta la hipótesis de que los eventos climáticos y geomorfológicos históricos proporcionaron las condiciones favorables para el establecimiento y expansión de los tetraploides de *T. sidoides*.

Palabras clave: citogeografía, establecimiento, gametos no reducidos, origen, poliploidía

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INTRODUCTION

Polyploidy is a common phenomenon across numerous eukaryotic taxa (Soltis *et al.*, 2014; Marchant *et al.*, 2016) and is believed to play a significant role in the evolution of vascular plants (Otto and Whitton, 2000; Levin, 2002). As many as 70–80% of Angiosperms, including many crop species, and up to 95% of Pteridophytes have a polyploid origin (Bennet, 2004; Gregory and Mable, 2005; Otto, 2007). In addition, owing to the role in the origin of evolutionary novelties and the maintenance of diversity in plant populations, polyploidy was recognized as an integral component of the ecological and evolutionary dynamics of plant species populations. In this regard, different theoretical and experimental efforts to examine the evolutionary significance of polyploidy have focused on the processes responsible for the origin of polyploid plants and the conditions favouring their establishment and persistence (Thompson and Lumaret, 1992).

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 plant species (Karpechenko, 1927; Darlington, 1937) are involved in polyploid origin and, that sexual polyploidization is the driving force giving rise to polyploid plant species (Harlan and de Wet, 1975; Camadro, 1986; Thompson and Lumaret, 1992; Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998; Brownfield and Köhler, 2011). Particularly, autotetraploids may be formed in single steps through the union of two $2n$ gametes produced by diploids (bilateral polyploidization) or, alternatively, may arise in two steps from matings involving viable triploids (triploid bridge hypothesis), themselves the result of the union of n and $2n$ gametes produced by diploids (unilateral polyploidization) (Harlan and de Wet, 1975).

On the other hand, the process of neopolyploids establishment in diploid populations may be limited by the difficulty of mating with other plants of the same ploidy level, the unviability 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; Erilova *et al.*, 2009; Chrtek *et al.*, 2017). Self-pollinating plants and individuals with multiple breeding opportunities throughout life can more easily overcome the difficulties associated with intercytotypic mating (Rodriguez, 1996; Bever and Felber, 1998; Ramsey and Shemske, 1998; Husband, 2000). However, in outbreeding plants and if pollination is random, neopolyploids will be at a disadvantage, as most of their megaspores will be fertilised by n microspores of diploids forming triploids, whereas most of the relatively

abundant diploid megaspores will be conveniently fertilised. Consequently, a neopolyploid is likely to be excluded from a diploid population (Levin, 1975).

Besides, polyploidy can potentially contribute to the acquisition of new morphological, genetic and/or physiological traits, which may improve the competitive ability, fitness or ecological tolerance of polyploids compared to the diploid parents. These events, which have occurred on time scales ranging from ancient to contemporary, are assumed to be of fundamental importance for plant adaptation and range expansion (Levin, 1983; Udall and Wendel, 2006; Van de Peer *et al.*, 2021). Thus, polyploids may have a distinct or peripheral distribution compared to the parental diploids along climatic or environmental gradients (Levin, 2002). Such differences in geographical ranges between cytotypes may reflect historical patterns of colonisation or genetic differentiation that have occurred in association with or subsequent to polyploid formation (Levin, 1983). Alternatively, the spatial patterns of cytotypes may be explained through frequency-dependent production of hybrids with low frequency (Levin, 1975). 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; Castro *et al.*, 2018).

Turnera sidoides L. (Passifloraceae, Turneroideae) is one of the few well-studied autopolyploid complexes in South America (Fernández, 1987; Solís Neffa, 2000; Solís Neffa and Fernández, 2001, 2002; Elías *et al.*, 2011; Kovalsky and Solís Neffa, 2012, 2015, 2016; Kovalsky *et al.*, 2014, 2018; Roggero Luque *et al.*, 2015, 2017; Solís Neffa *et al.*, 2022). Because of the prominent role played by polyploidy within *T. sidoides*, in order to understand the evolutionary significance of autopolyploidy, an investigation is in progress in this complex using integrative approaches combining cytogenetics, together with genetic, morphological, biogeographic and environmental data. As a part of this study, we selected this autopolyploid complex as an ideal biological model system to investigate the evolutionary dynamics of polyploids in natural populations. In this paper we synthesise the results of more than 20 years of research on this topic.

TURNERA SIDOIDES

Turnera sidoides (Figure 1) is a complex of perennial, rhizomatous herbs that is naturally distributed in southern Bolivia and Brazil, southwestern Paraguay, Uruguay and Argentina, reaching up to 39° S (Arbo, 1985; Solís Neffa, 2000). It grows in a wide variety of habitats ranging from mountain regions (up to 2700



Figure 1. *Turnera sidoides* complex. **A)** *T. sidoides* subsp. *carnea*. **B)** *T. sidoides* subsp. *holosericea*. **C)** *T. sidoides* subsp. *integrifolia*. **D)–E)** *T. sidoides* subsp. *pinnatifida* **D)** Andino morphotype. **E)** Serrano morphotype. **F)** *T. sidoides* subsp. *sidoides*.

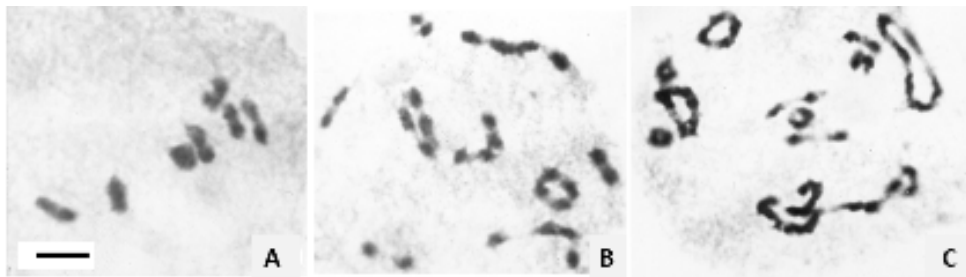


Figure 2. Meiotic chromosomes of *Turnera sidoides*. **A)** Diploid cytotype, 7 II. **B)** Tetraploid cytotype, 4 II + 5 IV. **C)** Hexaploid cytotype, 8 II + 5 IV + 1 VI. Bar = 5 μ m.

m a.s.l) to the sea level and encompassing different climatic regimes (Arbo, 1985; Solís Neffa, 2000, 2010).

Turnera sidoides is outbreeder because of distyly and genetic self-incompatibility, being legitimate crossings those between short-styled (S) and long-styled (L) morphs, S x L or L x S (Arbo, 1985; Solís Neffa, 2000). Pollination is carried out by butterflies, small wasps and bees, and seeds are dispersed by gravity and ants. Discrete populations, ranging from less than ten to hundreds of plants, are separated from each other by a few to several kilometres (Solís Neffa, 2000).

Five subspecies were recognized on the basis of geographical distribution and the variability of some morphological features (Arbo, 1985): *T. sidoides* subsp. *carnea* (Cambess.) Arbo, *T. sidoides* subsp. *holosericea* (Urb.) Arbo, *T. sidoides* subsp. *integrifolia* (Griseb.) Arbo, *T. sidoides* subsp. *pinnatifida* (Juss. ex Poir.) Arbo, and *T. sidoides* subsp. *sidoides*. Besides, populations of *T. sidoides* subsp. *carnea* were grouped into two morphotypes (*grandense* and *mercedeno*) according to the leaf consistency, the colour of the flowers and geographical distribution (Moreno *et al.*, 2021); while in

T. sidoides subsp. *pinnatifida* five morphotypes (*andino*, *chaqueño*, *mesopotamico*, *pampeano* and *serrano*) have been distinguished based on the degree of incision of the leaf blade, the colour of the flowers and geographical distribution (Solís Neffa, 2010).

In addition to the morphological variability, polyploidy is the most outstanding feature of *T. sidoides* complex, with ploidy levels ranging from diploid ($2n=2x=14$) to octoploid ($2n=8x=56$), all based on $x=7$. Polyploid series within each subspecies/morphotype were reported (Fernández, 1987; Solís Neffa, 2000; Solís Neffa and Fernández, 2001; Solís Neffa *et al.*, 2004; Speranza *et al.*, 2007; Elías *et al.*, 2011; Moreno *et al.*, 2015, 2021; Solís Neffa *et al.*, 2022). From the study of meiosis (Figure 2) and pollen viability it was suggested that polyploids of *T. sidoides* would have an autopolyploid origin (Fernández, 1987; Solís Neffa, 2000). These observations were supported by the results obtained from the analysis of the effect of polyploidy in some morphological, cytological, and biochemical traits (Solís Neffa, 2000; Solís Neffa and Fernández, 2002; Roggero Luque *et al.*, 2022; Solís Neffa *et al.*, 2003).

MECHANISMS OF POLYPLOID ORIGIN IN *TURNERA SIDOIDES*

Evidence of $2n$ microspore production in natural diploid populations of *T. sidoides*

Aiming to contribute to the understanding of the mechanisms involved in the origin and the establishment of polyploids, we firstly investigate the occurrence and frequency of unreduced microspores in diploids of *T. sidoides*. Early experimental studies provided the first evidences for the production of $2n$ and $4n$ microspores by analysing the size range of pollen and the constitution of the sporads in diploid plants (Panseri *et al.*, 2008). Different facts supported that the giant pollen grains observed in the complex correspond to unreduced gametes. First, the volume of the giant grains relative to the mean pollen size of the population studied was comprised in the range proposed by different authors as typical of unreduced gametes (Darlington, 1965; Orjeda *et al.*, 1990; Ramsey and Schemske, 1998). The existence of unreduced microspores in some plants was further confirmed by a particular bimodal distribution of pollen volume. Additionally, the presence of triads and dyads in plants with giant pollen indicates that $2n$ gametes certainly occurred and that the formation of $2n$ gametes would be expected. Moreover, the finding of monads suggested that “jumbo” ($4n$) gametes may also be formed. The mean frequency of unreduced gametes observed in such populations was in agreement with the estimations of the production of unreduced gametes for non-hybrid plants (Ramsey and Schemske, 1998; Ramsey, 2007).

Then, to further contribute to the understanding of the mode of polyploid formation in *T. sidoides*, we selected natural diploid populations that had been experimentally identified for its high production of $2n$ gametes (Panseri *et al.*, 2008). In these natural populations we studied the occurrence of unreduced microspores and estimated the frequency of their production by analysing the pollen size distribution and the constitution of the sporads (Figure 3) from samples obtained in the field (Kovalsky, 2012; Kovalsky and Solís Neffa, 2012). Some of these plants were then grown under the same soil and irrigation conditions at the greenhouse of the Instituto de Botánica del Nordeste for, at least, one year. The exhaustive study on such natural populations of *T. sidoides* (Kovalsky and Solís Neffa, 2012) revealed that some plants were more capable of inducing unreduced microspores production than others. The percentage of $2n$ gamete producer plants detected (~26%) evidenced that the production of unreduced microspores was not uncommon in diploid populations of *T. sidoides*. These studies also showed that, within populations, S and L plants differed in their ability to produce $2n$ microspores and, that the frequency of $2n$ gametes production was highly variable. Such variation in the frequencies of unreduced microspores

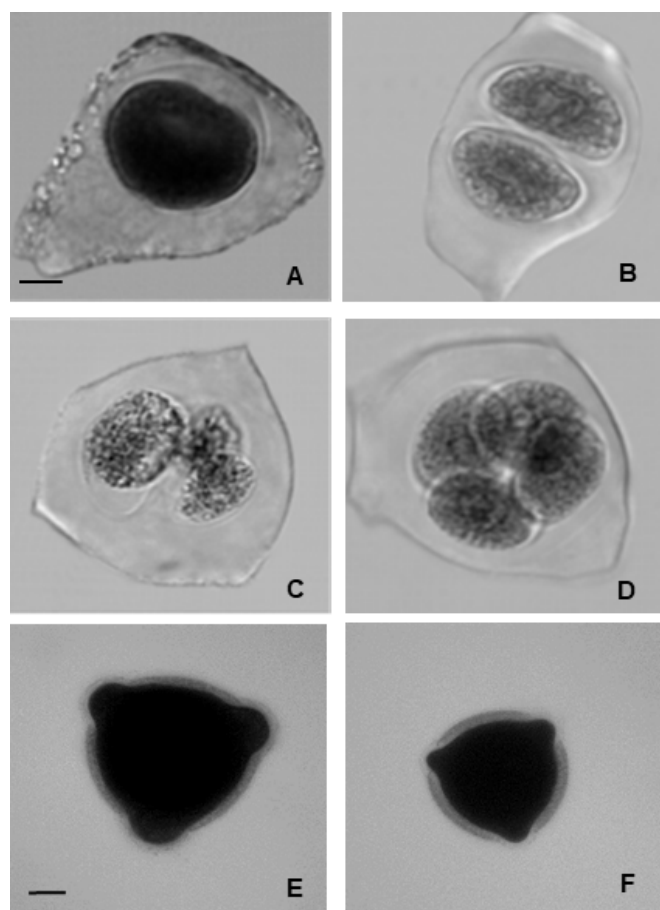


Figure 3. Sporads and pollen in *Turnera sidoides*. **A-D)** Sporads: **A)** Monad. **B)** Dyad. **C)** Tryad. **D)** Tetrad. **E-F)** Pollen grains. **E)** Unreduced pollen grain ($2n$). **F)** Reduced (n) pollen grain. Bar = 5 μ m.

production observed in *T. sidoides* was attributed to different factors. Considering that only some individuals produced unreduced microspores, the frequency of $2n$ microspores production varied among producer plants and that, producer plants identified in the field were consistently classified as producers in the greenhouse we concluded that production of $2n$ microspores in this species is under genetic control. However, the variation in the frequency of $2n$ microspores production among producer plants and also among anthers of the same flower suggested differences in gene expression. On the other hand, although the flowering period of *T. sidoides* is extended throughout spring and summer (Solís Neffa, 2000), the frequency of $2n$ microspores yield by each producer plant cultivated in the greenhouse increased up to 22% in summer. Therefore, it was suggested that the higher production of unreduced microspores might be caused by the environmental stress due to the high temperatures to which plants of *T. sidoides* were exposed (Kovalsky and Solís Neffa, 2012).

Finally, in order to contribute to the understanding of the cytological mechanisms involved in the production of unreduced male gametes, we analysed the meiotic behaviour and pollen viability of $2n$ and $4n$ gamete

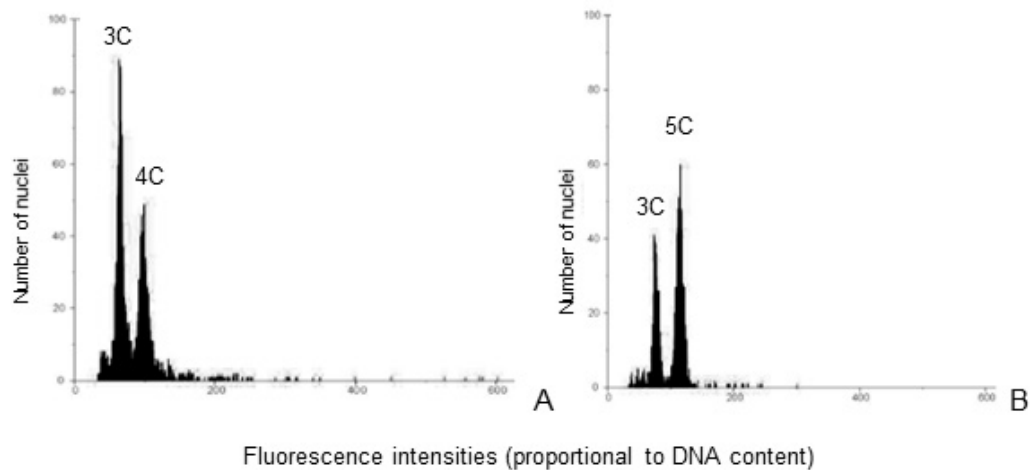


Figure 4. Flow cytometry histograms of triploid seeds of *Turnera sidoides*. **A)** Triploid seed originated from an $2n$ microspore showing an embryo with a peak at 3C and a peak at 4C corresponding to the endosperm. **B)** Flow cytometry histogram of a triploid seed originated from an $2n$ megaspore showing an embryo with a peak at 3C and a peak at 5C corresponding to the endosperm.

producers from diploid populations of *T. sidoides*. The results obtained showed that meiotic behaviour was mostly regular; however, some irregularities such as parallel spindles, fused spindles and nuclear restitution were also detected. Pollen viability varied from 44.75% to 95.82%. Overall, our results suggested that nuclear restitution at both the first and the second meiotic division were involved in the production of $2n$ male gametes in *T. sidoides* (Kovalsky *et al.*, 2014).

Evidence of the production of $2n$ eggs in diploid plants of *T. sidoides* complex

As a second step to understand how unreduced gametes may have contributed to the origin and establishment of polyploids in natural diploid populations of *T. sidoides*, we provide the first evidence of the production of $2n$ megaspores by progeny test and by flow cytometric analysis of seeds (Figure 4) collected in natural populations (Kovalsky and Solís Neffa, 2016). In spite of the low number of $2n$ megaspores detected, our results suggested that, in natural diploid populations of *T. sidoides*, some plants would be more likely to produce $2n$ megaspores than others. This data agrees with our previous findings in this species, which demonstrated that only some S and L plants (26%) were capable of producing $2n$ and $4n$ microspores and, that the capability of producing such unreduced gametes is under genetic control (Kovalsky and Solís Neffa, 2012, 2015). The finding of only some plants of *T. sidoides* that produced $2n$ megaspores suggests that their production could be under genetic control as well. Furthermore, although the frequency of plants producing $2n$ megaspores in *T. sidoides* was lower than that previously reported for plants producing $2n$ microspores (Kovalsky and Solís

Neffa, 2012), all plants that produced $2n$ megaspores also produced $2n$ microspores. However, the fact that plants producing $2n$ microspores do not always produce $2n$ megaspores it was proposed that their simultaneous production may be independent from each other. Our results also suggested differences in the relative contributions of $2n$ microspores and $2n$ megaspores to polyploid formation. In diploid–tetraploid crosses, $2n$ megaspores are supposed to be more likely to generate viable seeds than $2n$ microspores (Thompson and Lumaret, 1992; Ramsey and Schemske, 1998) and it was suggested that formation of neopolyploids in natural populations would proceed in a similar way to these interploidy crosses (Ramsey, 2007). However, this would not be the case of *T. sidoides*, since the major frequency of triploid embryos from $2n$ microspores in seeds collected in natural populations suggested that $2n$ microspores would contribute more than $2n$ megaspores to the origin of neopolyploids. Although, this would not imply that triploid embryos generated by fusion of $2n$ microspores and n megaspores result in plants which will effectively establish in populations of *T. sidoides*. In this species, plants which produced $2n$ megaspores develop more viable seeds per fruit, and seeds have a higher germination rate than those plants that exclusively produce $2n$ microspores (Kovalsky, 2012). This suggested that, although in *T. sidoides* $2n$ microspores 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 triploids originated from the fusion of $2n$ megaspores with n microspores an advantage during more advanced stages of their development and establishment, being effectively established in diploid populations (Kovalsky and Solís Neffa, 2016).

Unilateral vs bilateral sexual polyploidization

Both unilateral and bilateral sexual polyploidization may be involved in the origin of the polyploids of *T. sidoides* in natural populations. Because all polyploids of the complex so far analysed have an even ploidy level (Fernández, 1987; Solís Neffa and Fernández, 2001; Solís Neffa *et al.*, 2004), bilateral polyploidization was expected to be the most important mechanism of polyploid origin in the complex (Panseri *et al.*, 2008). However, taking into account that this species is dystilous and outbreeder (Solís Neffa, 2000), for bilateral sexual polyploidization to occur both, S and L individuals should produce $2n$ microspores and $2n$ megaspores. In this sense, our finding of $2n$ microspores (Kovalsky and Solís Neffa, 2012), together with the detection of $2n$ megaspores (Kovalsky and Solís Neffa, 2016) in non-hybrid diploid populations of *T. sidoides*, and the fact that both the L and S plants can produce both $2n$ microspores and $2n$ megaspores, suggested that bilateral sexual polyploidization can occur in natural populations of this species. However, owing to the limited chances of fertilization between simultaneously formed $2n$ microspores and $2n$ megaspores, bilateral polyploidization would occur less frequently than the unilateral sexual polyploidization. The finding of triploids in natural diploid populations of *T. sidoides* (Elías *et al.*, 2011; Kovalsky and Solís Neffa, 2012; Kovalsky *et al.*, 2018) may reflect triploid formation through the union of n and $2n$ gametes, suggesting that unilateral polyploidization by a triploid bridge may be an alternative mechanism of polyploid formation in this species complex.

Multiple origin of polyploids

The formation of autopolyploids was considered a rare event, but it was later recognised that multiple origins typify polyploid plant species (Segraves *et al.*, 1999). In *T. sidoides*, the multiple diploid–polyploid transitions revealed in a phylogenetic analysis based on the sequences of the plastid DNA regions supported a multiple origin of autopolyploids in each subspecies and morphotypes (Solís Neffa *et al.*, 2022), as previously suggested for the complex (Solís Neffa and Fernández, 2001). Also, the finding of lineage-exclusive haplotypes in diploid and polyploid populations were interpreted as independent polyploidization events. The occurrence of polyploids with unique haplotypes, not directly related to any of the haplotypes detected within the diploids analysed, suggested an additional independent origin of polyploids (Solís Neffa *et al.*, 2022). Likewise, analyses of the genetic variability and structure of the diploid and polyploid populations of *T. sidoides* using nuclear molecular markers, showed that the greater genetic similarity of tetraploids to diploids from the same

geographic region than to diploids from other regions was consistent with the origin of polyploids in multiple polyploidization events from genetically differentiated diploid populations (Panseri, 2012; Dabrio *et al.*, 2020). Such multiple polyploidization events detected in *T. sidoides* complex were related to the capacity of many diploid populations to produce unreduced gametes (Panseri *et al.*, 2008; Kovalsky and Solís Neffa, 2012, 2016), but also to the ability of polyploids to survive and establish in nature.

POLYPLOID ESTABLISHMENT IN *T. SIDOIDES*

Owing to the lower rate of neopolyploid formation in *T. sidoides* complex, their establishment constitute a critical step. Thus, for a better understanding of the evolution of polyploidy in *T. sidoides* our next objective was to assess the possible factors influencing neotetraploids establishment in natural populations.

Patterns of cytotype variation of T. sidoides in a diploid-tetraploid contact zone

As a first step, we examined the cytotypes structure in a diploid-tetraploid contact zone of *T. sidoides* in the mountain ranges of central Argentina, aiming to analyse whether the frequency and distribution of cytotypes at microgeographical scale was explained by ecological sorting in heterogeneous environments or, alternatively, due to competitive cytotype exclusion (Elías, 2010; Elías *et al.*, 2011). The results evidenced that diploids and tetraploids were spatially segregated, although both cytotypes can occur in close proximity. Diploids grew in the piedmont of the hills, tetraploids in the adjacent valley, while patches of diploids and triploids plants were found, in the transition zone of both cytotypes. Since diploids and tetraploids occur under similar climatic conditions, it is unlikely that climate influences cytotype distribution in the contact zone. Also, the edaphic conditions would only partially contribute to the spatial segregation of cytotypes at local scale. The similar ecological preferences of both cytotypes and the lack of mixed diploid-tetraploid patches suggested that the separate distribution of cytotypes in the contact zone may be independent of the selective environment; diploids and tetraploids being unable to coexist as a result of reproductive exclusion (Elías, 2010; Elías *et al.*, 2011).

Additionally, to assess whether differences in the biological fitness between cytotypes would be influencing the cytotype distribution and frequency in the contact zone, we analysed the variation in reproductive and phenological traits of diploids and tetraploids (Elías, 2010; Panseri, 2012). From this study

it was evident that although the average number of seeds per fruit was similar in both cytotypes, the continuity of flowering and fruiting in tetraploids in relation to the discontinuity of diploids, the highest number of bloomed individuals per site as well as the greater number of fruits per individual in tetraploids result in an increase number of propagules that tetraploids contribute to the seed bank as well as to the next generation, increasing the frequency of tetraploids in each generation. This fact, together with the perennial condition of *T. sidoides* and its ability to multiply asexually by rhizomes (Solís Neffa, 2000) would favour the success and establishment of neotetraploids in natural populations. Also, the occurrence of occasional cases of self-compatibility and successful illegitimate crosses ($S \times S$ and $L \times L$) in polyploids of *T. sidoides* suggests that such polyploids may produce seeds by selfing or from crosses between plants of the same floral type, further increasing their chances of establishment and expansion (Solís Neffa, 2000; Solís *et al.*, 2020).

The role of 2n gametes in polyploid establishment of the T. sidoides

Theoretical models of polyploid evolution suggest 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 or to be maintained at a low frequency within diploid populations when they are formed recurrently through the union of $2n$ gametes (Levin, 1975; Felber and Bever, 1997). *Turnera sidoides* is an outbreeder, therefore, the occurrence of plants that continuously form many $2n$ microspores and $2n$ megaspores would play a key role in the establishment of neopolyploids in natural diploid populations. 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 polyploid dynamics in diploid populations. Moreover, since the capability to produce $2n$ microspores is a heritable trait in this species complex and, the frequency of production of $2n$ microspores was higher in the progeny of $2n$ microspores producers (Kovalsky and Solís Neffa, 2015), the frequency of $2n$ microspores and $2n$ megaspores and, consequently, the likelihood of origin of neopolyploids by sexual polyploidization would increase after successive generations. Additionally, considering that *T. sidoides* is a perennial species and, that its seeds are mostly dispersed by gravity 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, increasing the likelihood of crosses between $2n$ microspores and $2n$ megaspores producers. The continuous formation of neopolyploids as a consequence

of successive backcrosses between $2n$ gametes producers and their progeny would favour the establishment and persistence of such neopolyploids in diploid populations of *T. sidoides*. Furthermore, although diploid individuals produced low levels of $2n$ gametes but, under certain environmental conditions, such production may be increased, enhancing the likelihood that a low frequency of polyploids can be originated and maintained in this population (Kovalsky and Solís Neffa, 2015).

The role of triploids in the origin and evolution of polyploids of T. sidoides complex

Simulation models have demonstrated that the evolution of tetraploids in a diploid population depends on the reproductive efficiency of triploids and the ploidy level of functional gametes ($n=x$, $n=2x$ and $n=3x$) they produce, contributing to the formation of neopolyploids in each generation (Husband, 2004). Thus, aiming to account for the role of triploids in the origin and demographic establishment of tetraploids of *T. sidoides* in natural populations, we analysed the microsporogenesis of triploids as well as the crossability among diploid, triploid and tetraploid plants of the complex (Kovalsky *et al.*, 2018). Triploids exhibit irregular meiotic behaviour that results in unbalanced and/or non-viable gametes and, low pollen viability. Although, in spite of abnormalities in chromosome pairing and unequal chromosome segregation, triploids are not completely sterile and form viable gametes (pollen viability up to 67%). Triploids originated by the fusion of $2n \times n$ gametes showed more regular meiotic behaviour and higher fertility than triploids from the contact zone among diploids and tetraploids (Elías, 2010) and, triploids with hybrid origin (Moreno *et al.*, 2015). Also, our results evidenced that reproductive isolation among cytotypes of *T. sidoides* was not so strong. In this species complex most populations are constituted by a single cytotype, even though some mixed diploid – triploid – tetraploid populations were also detected (Elías *et al.*, 2011; Kovalsky and Solís Neffa, 2012; Moreno *et al.*, 2015). This fact together with our finding of triploids in the progeny of $2x \times 4x$ experimental crosses, evidence that triploid block may be frequently overcome, being not enough to prevent the regular occurrence of triploids in natural populations of *T. sidoides* (Kovalsky *et al.*, 2018). Moreover, although in this species the success of crosses involving triploids is generally low, their gametes can participate in fertilization, producing progeny of variable ploidy level. The diploid plants used for experimental crossings produced reduced gametes ($n=x$), therefore, the frequency of diploid and triploid progeny obtained from crosses $2x \times 3x$ constituted an indirect measure of the frequency of n and $2n$ gamete production by triploids. The results of our studies evidenced that triploids of *T. sidoides* produce n and $2n$

gametes, although n gametes were the most frequently produced. This finding was supported by the fact that the progeny of $3x \times 4x$ crosses was 100% triploid, resulted of the fusion of n gametes of triploid and tetraploid parents. Besides, the experimental crosses performed evidenced that triploids produce diploid and, triploid progeny, suggesting that new generations of triploids and tetraploids would originate by crossings between triploids or by backcrosses with diploid progenitors that produce $2n$ microspores and $2n$ megaspores. Therefore, triploids of *T. sidoides* would contribute both to the origin of new tetraploids (Kovalsky *et al.*, 2018) and to gene flow among diploids and tetraploids in the contact zones (Moreno *et al.*, 2015).

POLYPLOIDY AND RANGE EXPANSION

Since the analysis of geographical differentiation of cytotypes provides useful insights into the evolutionary dynamics and ecological differentiation of polyploids, we employed a cytogeographical approach for *T. sidoides*. The results so far have revealed that, at a geographical scale and considering the complex as a whole, diploids have restricted and disjunct distributions, while tetraploids are the most widespread, being the cytotype that gives continuity to the complex throughout the distribution area. Likewise, populations with higher ploidy levels are rare and were found in disjunct, and usually marginal, areas of the complex. These patterns of cytotypes frequencies and distribution support the classification of *T. sidoides* as a mature polyploid complex as was initially proposed (Solís Neffa, 2000) following the criteria of Stebbins (1971).

Proximal and historical nonexclusive hypotheses were proposed to explain the spatial patterns of the cytotypes in this species (Solís Neffa, 2000; Solís Neffa and Fernández, 2001; Solís Neffa *et al.*, 2004). Given that the widespread latitudinal and longitudinal range of *T. sidoides* encompasses a wide diversity of climates and ecological regions with contrasting precipitation regimes, it was first hypothesised that the geographical separation of the cytotypes was due to differences in habitat requirements. However, taking into account the relative frequency and geographical distribution of the cytotypes, it was also hypothesised that the distribution of extant cytotypes might reflect the Quaternary geomorphologic and climatic changes that affected most of the current geographical range of *T. sidoides*. Consequently, restricted diploids may be relictual populations that have survived these periods of change, while such historical events could provide favourable conditions for the establishment of polyploids, with the wider distribution of tetraploids being the result of range expansion (Solís Neffa and Fernández, 2001; Solís Neffa *et al.*, 2004; Elías *et al.*, 2011).

Additionally, the integration of cytogeographic and genetic divergence data together with past niche modelling provided further insights into the geographic context of the processes driving divergence and range expansion in *T. sidoides* complex (Solís Neffa *et al.*, 2022). The projected model for the mid-Pliocene (~3.3–3.0 Myr), revealed four areas of highest climatic suitability for a possible ancestor of *T. sidoides*. The main one, in the NW of the current species range (following the direction of main orographic systems) were coincident with the proposed refugial centres for *T. sidoides* complex based on plastid genome diversity (Speranza *et al.*, 2007, Moreno *et al.*, 2018). This area was also proposed as the ancestral geographic area for *T. sidoides* (Moreno *et al.*, 2018). Additionally, three smaller suitable areas were located in the Pampean ranges and in NE Argentina as well as in the SE of the current range, along the Serranías del Este (Uruguay) (Solís Neffa *et al.*, 2022). The spreading of large areas of semi-arid conditions in the Chaquean Domain concomitantly with the final uplift of the Andes (Gregory-Wodzicki, 2000) would have acted as a barrier to gene flow among populations of *T. sidoides*, promoting the divergence between the two main lineages by the time of the Great Patagonian Glaciations (~1.17 Myr). Further diversification of lineages within the two main clades of *T. sidoides* complex mostly coincides with colder periods, suggesting that cooling and drying during Pleistocene (Villagrán and Hinojosa, 1997; Rabassa *et al.*, 2011) may have caused the isolation and divergence of ancestral *T. sidoides* complex populations (Moreno *et al.*, 2018).

The predicted distribution of diploids during interglacial periods suggests a more continuous area of diploids than at present, probably along the most elevated terrains (Moreno *et al.*, 2018; Solís Neffa *et al.*, 2022). The evolutionary history of the complex from the Last Glacial Maximum up to present day mostly involved the range expansion of the allopatrically differentiated populations to their current limits. During the Mid-Holocene, the warmer and wetter climate would have promoted the westward reduction of the area of diploids and the range expansion of tetraploids. Semiarid conditions during the Late Holocene (Iriondo and García, 1993) may have determined the progressive retraction of the suitable area for diploids, while the subhumid climate period would have favoured the persistence of diploids in the inter-Andean valleys (Solís Neffa *et al.*, 2022).

EVOLUTIONARY SIGNIFICANCE OF AUTOPOLYPLOIDY IN *T. SIDOIDES*

Cytogenetic data together with past niche modelling as well as cytogeographic and genetic divergence analyses provided strong evidence that *T. sidoides* complex is in an active process of intraspecific allopatric diversification

at diploid level since the early Pleistocene. Further diversification of the complex involved the emergence of independent polyploid series in each morphologically divergent lineage.

Polyploidy did not produce extensive morphological changes in *T. sidoides*, so diploids and polyploids within each subspecies and morphotypes can only be distinguished from each other on the basis of detailed analysis of microcharacters (Panseri, 2012) and quantitative traits (Solís Neffa, 2000). These results together with cytogenetic (Solís Neffa, 2000; Roggero Luque *et al.*, 2015) and molecular data (Moreno *et al.*, 2015) suggested that autopolyploidy did not contribute significantly to the diversification of *T. sidoides*. Instead, changes associated with genome size variation and the gigas effect were recorded in the polyploid series of the complex (Solís Neffa, 2000). All these chromosome doubling-induced changes may have had an adaptive significance that may promote an increase range of subspecies and morphotypes through the expansion of polyploids arising from multiple diversified diploid populations of *T. sidoides* (Solís Neffa *et al.*, 2022). In this sense, despite the fact that polyploid series of each subspecies and morphotype of *T. sidoides* inhabits the same ecoregion, the cytotypes tend to be spatially segregated at a more local scale (Solís Neffa and Fernández, 2001; Solís Neffa *et al.*, 2004; Elías *et al.*, 2011; Solís Neffa *et al.*, 2022). A clear example was demonstrated in the *serrano* morphotype of subspecies *pinnatifida* (Elías *et al.*, 2011), in which diploids and polyploids, although inhabiting the same ecoregion, are segregated along altitudinal and bioclimatic gradients at regional/local scales. The wider distribution of tetraploids compared to their related diploids in each polyploid series of *T. sidoides* complex supports the hypothesis that autopolyploids may have been more effective colonisers of new ecological niches.

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