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Chromosome numbers, meiotic abnormalities, and 2n pollen formation in accessions of the wild species *Chrysolaena flexuosa* (Vernonieae, Compositae) from its distribution range in Argentina

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Summary: *Chrysolaena flexuosa* is a South American species of potential ornamental value (basic chromosome number of x=10). Diploid (n=10) and tetraploid (n=20) cytotypes have been reported for its distribution area, although one hexaploid (n=30-32ca.) cytotype has been reported for its most southern distribution. To investigate if ploidy and latitude are positively related in *Ch. flexuosa* natural populations and if sexual polyploidization could have had a role in the origin of the polyploid cytotypes, we determined chromosome numbers, DNA content, and pollen viability and size, and analyzed microsporogenesis in samples of seven Argentinian accessions. Two of the northeastern accessions were diploid and one was tetraploid, whereas the four southeastern accessions were hexaploid. Ploidy levels determined both by chromosome countings and flow cytometry coincided, although monoploid genome size significantly decreased with increasing ploidy. In all accessions, variability was observed for pollen viability and size, as well as for large (presumably 2n) pollen production. This variability was underlined by abnormal cytological events in meiosis and at the tetrad stage (lagging chromosomes, parallel spindles, triads). The results would indicate that there is, apparently, a positive relation between ploidy and latitude, and suggest a likely role of sexual polyploidization in the origin, establishment and expansion of *Ch. flexuosa* populations.

Key words: Polyploid cytotypes, DNA content, flow cytometry, meiotic abnormalities, abnormal spindle orientation, 2n pollen.

Resumen: Número de cromosomas, anomalías meióticas y formación de polen 2n en accesiones de la especie silvestre *Chrysolaena flexuosa* (Vernonieae, Compositae) de su rango de distribución en Argentina. *Chrysolaena flexuosa* es una especie sudamericana de potencial valor ornamental. Para el área principal de su distribución fueron reportados citotipos diploides (n=10) y tetraploides (n=20) mientras que para el área más austral sólo existe un registro correspondiente a un citotipo hexaploide (n=30-32ca.). Para investigar, en poblaciones naturales de *Ch. flexuosa*, una relación positiva entre la ploidía y la latitud y la posible participación de la poliploidización sexual en el origen de los citotipos poliploides, se determinó, en siete introducciones argentinas, el número cromosómico, contenido de ADN y tamaño y viabilidad de polen y se analizó la microesporogénesis. Las introducciones del noreste resultaron diploides y tetraploides mientras que las introducciones del sudeste resultaron hexaploides. El nivel de ploidía determinado a partir de conteos cromosómicos coincidió con lo obtenido mediante citometría de flujo; el tamaño genómico se redujo significativamente con la ploidía. Todas las introducciones presentaron variabilidad en viabilidad y tamaño de polen así como producción de granos grandes (presumiblemente polen 2n). Esta variabilidad fue acompañada por eventos citológicos anormales en meiosis y en el estadio de tétrada. Los resultados evidencian una potencial relación entre la ploidía y la latitud y la posibilidad de poliploidización sexual en el origen, establecimiento y expansión de las poblaciones de *Ch. flexuosa.*

Palabras clave: Citotipos poliploides, contenido de ADN, citometría de flujo, anormalidades meióticas, orientación anormal de los husos, polen 2n.

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Introduction

Increased world attention on biodiversity has promoted the study of wild species (Balmford *et al.* 2002) because they provide various ecosystem services. Thus, knowledge of basic aspects of their reproductive biology is relevant for both ecological and genetic studies aimed at their conservation and eventual utilization for applied purposes.

Vernonieae (Asteraceae) is one of the major tribes of the flowering plants. To this tribe belongs genus *Vernonia* Schreb., which includes species of agronomic and industrial value (e.g. *Vernonia galamensis* (Cass.) Less., *V. amygdalina* Delile) and others that could potentially be introduced into the market as ornamentals. The genus was originally composed of more than 1,000 species distributed in both hemispheres (Jones, 1979, 1981; Keeley *et al.* 2007). However, this number was reduced by Robinson (1987a, b, c; 1988a, b) who created new genera, among them *Chrysolaena* H. Rob. (*Vernonia* sect. *Lepidaploa* subsect. *Oligocephalae* Benth. & Hook).

Genus *Chrysolaena* H. Rob. contains eighteen South American species (Robinson, 1988b; Dematteis, 2014) but only eight of these grow in Argentina, mostly in the northeastern region of the country. One of these species, *Chrysolaena flexuosa* (Sims) H. Rob. (*Vernonia flexuosa* Sims), appears to have the farthest known southern geographical distribution, as natural populations of this species have been found from southern Brazil to central Argentina, being Buenos Aires province the southern limit of this species distribution (Cabrera, 1963; Dematteis, 2014).

Ch. flexuosa appears to be of potential ornamental value (Echeverría & Alonso, 2012). It is a perennial xylopodial wild herb, with an atypical inflorescence with a zig-zag branching pattern, protandrous flowers, and a palette of flower colours, from pure white to deep purple (Fig 1).

Many studies have been carried out to determine chromosome numbers in natural *Ch. flexuosa* populations, and diploid (2n=2x=20) and tetraploid (2n=4x=40) cytotypes have been recorded (Dematteis, 2009; Vía do Pico & Dematteis, 2012, 2013). However, those studies did not include populations from Buenos Aires province, an agricultural region in which the species has been found only in the Tandilia hill range, where no other species of the *Chrysolaena* genus or even the Vernonieae Tribe have been reported as growing in sympatry (Cabrera, 1963; Frangi, 1975; Alonso *et al.* 2009, Dematteis, 2014). For that range, there is only one record of a hexaploid cytotype, with the chromosome number determined in meiosis (n=30–32 *ca.*) (Hunziker *et al.* 1990).

Polyploidy is a widespread phenomenon in the plant kingdom (Ramsey & Schemske, 1998), with a relevant role in the origin, evolution and geographical distribution of higher plants. Polyploids and their diploid counterparts often occupy different habitats. Thus, it has been proposed that, being evolutionary novelties, polyploids are superior colonizers than diploids (Levin, 1983; Otto & Whitton, 2000; Soltis & Soltis, 2000). Polyploids can originate via either asexual polyploidization, by somatic chromosome doubling, or sexual polyploidization, by functioning of 2n gametes (numerically unreduced gametes or gametophytes with the sporophytic chromosome number) formed by pre- or post-meiotic chromosome doubling or by meiotic nuclear restitution mechanisms (Mok & Peloquin, 1975a; Veilleux *et al.* 1982; Camadro, 1986; Carputo *et al.* 2003).

Ploidy levels are usually determined by chromosome countings in either mitosis or meiosis. However, measurement of nuclear DNA content by flow cytometry has been suggested as a simple and fast alternative for large-scale ploidy screenings, particularly in plant groups with either small chromosomes or high chromosome numbers (Roux *et al.* 2002, Doležel *et al.* 2007; Suda *et al.* 2007). The basic assumption in the assignment of ploidy levels to individual samples using this technique is that increments in DNA content are the result of increments in the number of chromosomes. In that sense, it has been proposed that tetraploid and hexaploid taxa or populations are expected to have, respectively, twice and three-fold DNA content in relation to their diploid counterparts (Torrell & Vallés, 2001; Doležel *et al.* 2007; Suda *et al.* 2007).

To investigate if ploidy and latitude are positively related in Argentinian *Chrysolaena flexuosa* populations and if sexual polyploidization could have had a role in the origin of the polyploid cytotypes, we determined chromosome numbers, DNA content, and pollen viability and size, and analyzed microsporogenesis in accessions from the northeastern-southeastern distribution range in

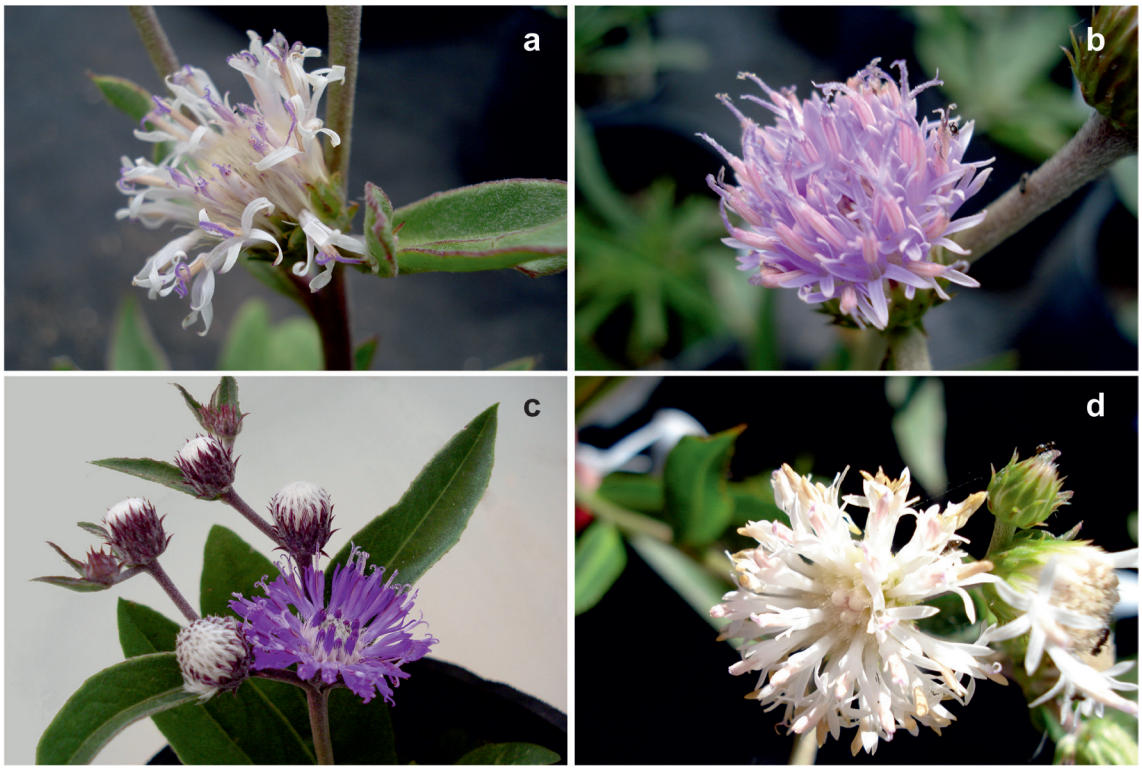

Fig 1. *Chrysolaena flexuosa* inflorescences with various flower colour: a) white corollas and lilac pistils, b) lilac corollas and pistils, c) deep purple corollas and lilac pistils, d) white corollas and pistils.

the country, following a transect of approximately 1,000 km. Moreover, we determined ploidy levels by both, root tip chromosome countings and flow cytometry for ascertaining the reliability of the latter technique for its use in further cytogeographical studies.

Material and methods

Plant material

Seven accessions of *Chrysolaena flexuosa* (Table 1) were used in the study. Accessions from the provinces of Misiones, Corrientes and Entre Ríos (Fig 2) were kindly provided by the Instituto de Botánica del Nordeste (IBONE), Argentina. Those from Buenos Aires province (Fig 2), were collected for this study and are deposited in Unidad Integrada Balcarce (UIB): Instituto Nacional de Tecnología Agropecuaria – Facultad de Ciencias Agrarias, Buenos Aires, Argentina. The accessions cover the distribution range of the species in Argentina, characterised by various temperature and rainfall regimes (Fig 3 a, b). The reference specimens are deposited in the herbarium of the Unidad Integrada Balcarce (BAL). Plants grown from seeds, and at the 2-3 leaf stage, seedlings were transplanted into individual pots with a mixture of sterilised soil, sphagnum moss and perlite (3:1:1, v/v/v), and cultivated in a greenhouse without light supplementation. In spring, the potted plants were placed in the open in a plot at the Unidad Integrada Balcarce (37°45'48" S; 58°17'60" W).

Chromosome number

Chromosome countings were carried out in root tips from at least five plants per accession, pre-treated with 8-hydroxyquinoline 0.002 M for 3 to 4 h, fixed in ethanol 96°: glacial acetic acid (v/v 3:1) for at least 24 hs, hydrolysed in HCl 1N at 62° C for 12 min, stained with basic fuchsine, squashed on a glass slide, and observed under a light microscope.

Table 1. Geographical origin of *Chrysolaena flexuosa* accessions from Argentina.

Accessions	Location	Geographic coordinates	Voucher specimens and herbarium collection No.
AloEch 1	Buenos Aires province: partido Gral. Puyrredón, Sierra de Los Padres	37° 34' 16" S; 57° 28' 04" W	Echeverría, M.L. 301; BAL No.: 8615
AloEch 2	Buenos Aires province: partido Balcarce, Sierra La Barrosa	37° 53' 40,7" S; 58° 16' 14" W	Echeverría, M.L. 302; BAL No.: 8616
AloEch 3	Buenos Aires province: partido Balcarce, Sierra La Chata	37° 52' 13,1" S; 58° 22' 40" W	Echeverría, M.L. 303; BAL No.: 8617
Nu 1	Buenos Aires province: partido Balcarce, Sierra La Bachicha	37° 47' 52" S; 58° 08' 36" W	Echeverría, M.L. 304; BAL No.: 8618
VdPDFV 26	Misiones province: Dpto. Capital	27° 26' 47" S; 55° 54' 11" W	Echeverría, M.L. 305; BAL No.: 8619
VdPDFV 45	Corrientes province: Dpto. Itá Ibaté	27° 26' 47" S; 57° 20' 1" W	Echeverría, M.L. 306; BAL No.: 8620
VdPDFV 14	Entre Ríos province: Dpto. Federación	30° 42' 12" S; 58° 00' 40" W	Echeverría, M.L. 307; BAL No.: 8621

Accession collectors: Alo= Alonso, S.I., Ech= Echeverria, M.L., Nu= Nuciari, M.C., VdP= Via do Pico, G., D= Dematteis, M., F= Farco, G., V= Vega, A. The reference specimens are deposited in the UIB Herbarium in Balcarce, Argentina (BAL).

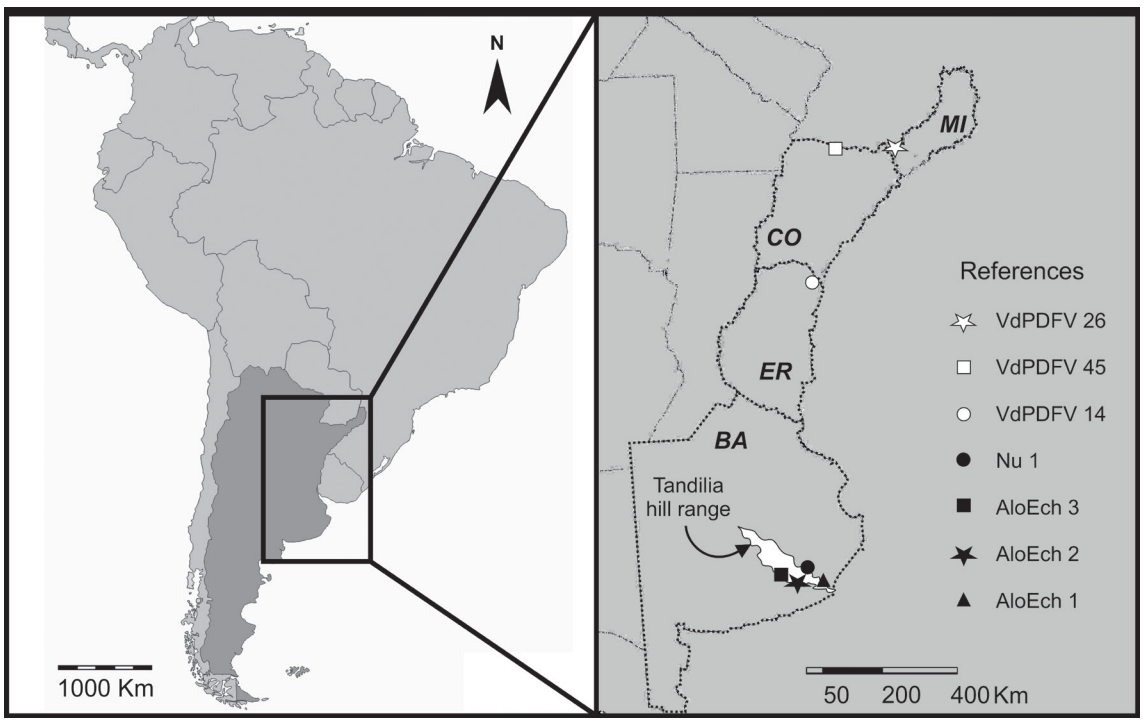

Fig 2. Geographic distribution of the seven *Chrysolaena flexuosa* accessions used in this study. The provinces of Misiones (*MI*), Corrientes (*CO*), Entre Ríos (*ER*) and Buenos Aires (*BA*) are delimited by dotted lines.

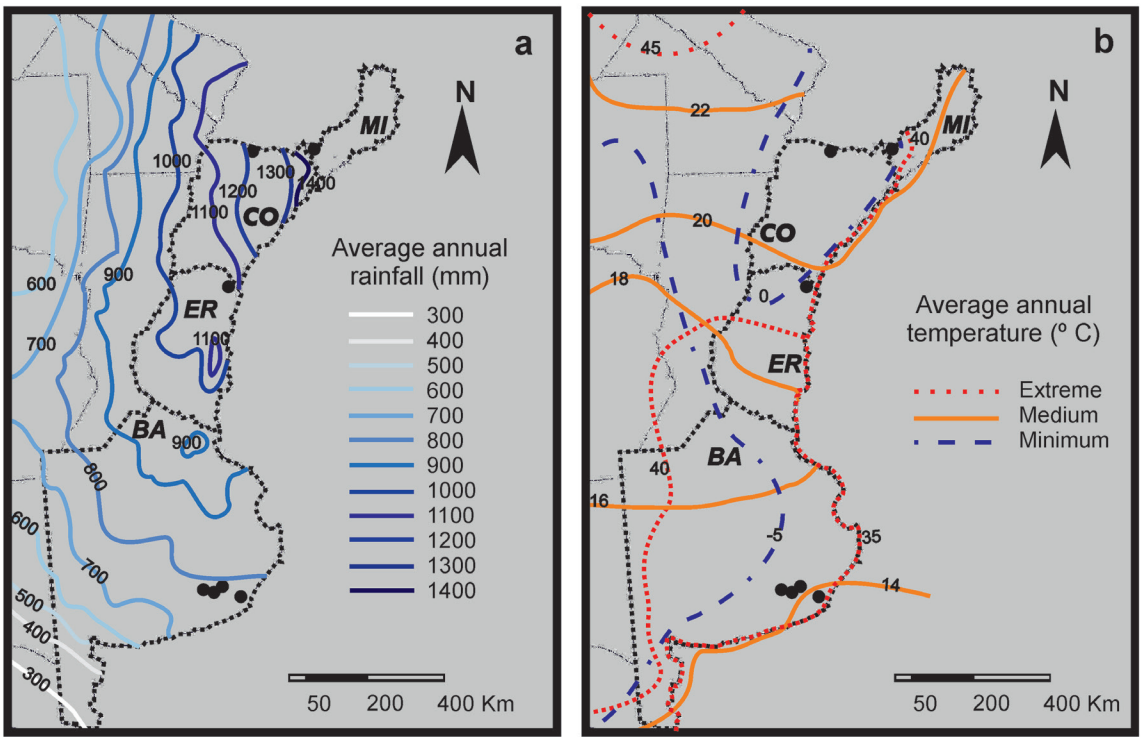

Fig 3. Average annual rainfall (a) and temperature (b) of *Chrysolaena flexuosa* distribution range and adjacent geographic areas in Argentina (adapted from Mozo, 2016).

Estimation of ploidy level by flow cytometry

Fresh leaves were used to estimate DNA content in a Partec flow cytometer according to Doležel and Göhde´s (1995) proposal. Cell nuclei were stained with propidium iodide (IP) and *Secale cereale* L. cv. 'Daňkovské', 2C DNA=16.19 pg (Doležel and Bartos 2005), was used as an internal standard. For each individual plant, 5 cm^2 leaf samples were co-chopped with 5 cm^2 leaf sample of the standard using a razor blade, in a Petri dish with 0.5 mL of Otto I buffer (citric acid 0.1 M and 0.5 % v/v of Tween 20). Every sample was filtered through a nylon mesh (50 µm) and then 0.5 mL of buffer Otto II buffer (0.4 M Na2HPO4 .12H2O) supplemented with PI (50 mg mL21 final concentration) and RNase (50 mg mL21 final concentration) were added. Samples were incubated in the dark for 45 min (15 min at room temperature and 30 min at 4º C).

Nine individual plants were analysed per accession, and three estimations of DNA content were made per each of them (2,000-6,000 nuclei per estimation). DNA content was estimated from gated fluorescence histograms (Fig 4). Data analysis was performed using Flowing 2.5.1 software (www.flowingsoftware.com). Total 2C DNA content was determined according to Doležel *et al.* (2007) as follows:

$$\text{Sample 2C value (DNA pg)} = \text{Standard 2C value (pg)} \times \frac{\text{Sample 2C mean peak position}}{\text{Standard 2C mean peak position}}$$

One plant of each *Ch. flexuosa* accession -whose chromosome number had been previously determined in mitosis- was included in the analysis as a control. In order to detect individuals potentially differing in ploidy level from others of the same accession, the 2C DNA content mean of each individual plant was compared with the 2C DNA content mean of its corresponding control by Dunnett test (α=5%).

In each individual plant, genome size (1Cx) was also estimated by dividing the 2C DNA content by its ploidy level (Leitch & Bennett 2004). 2C DNA and 1Cx content data were subjected to an analysis of variance using a completely randomized design with accessions as treatments and individual plants as replicates. Tukey's multiple comparison test (α=5%) was performed to detect statistical differences between accessions.

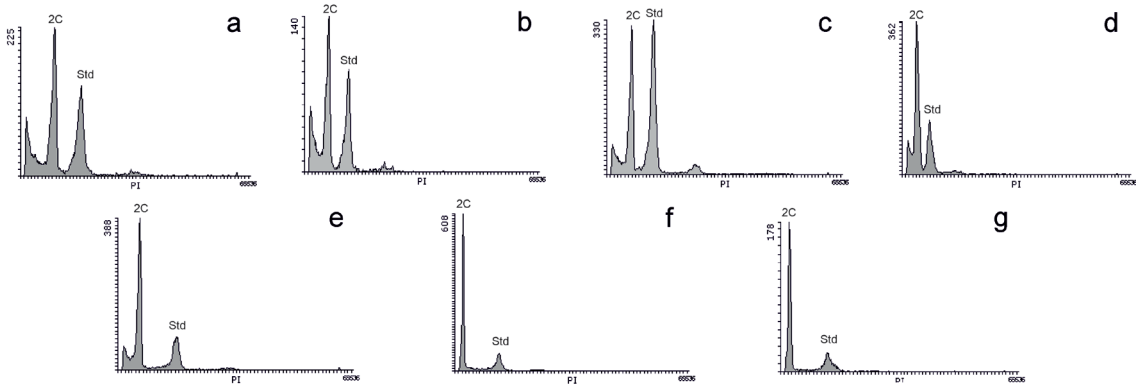

Fig 4. Fluorescence histograms of PI-stained nuclei isolated from individuals of seven *Chrysolaena flexuosa* accessions and an internal standard. a) AloEch 1, b) AloEch 2, c) AloEche 3, d) Nu 1, e) VdPDFV 26, f) VdPDFV 14, g) VdPDFV 45. 2C indicates G0/G1 peaks from *Ch. flexuosa* samples and Std indicates G0/G1 peaks from the standard *Hordeum vulgare* L. cv. 'Daňkovské'.

Pollen viability and size

At flowering, three to five flowers of each of 15 -21 plants per accession were removed to estimate pollen viability and size. In at least five microscope fields per sample, 250 pollen grains, stained with acetocarmine (3%), were scored. Well-stained, turgid and well-shaped grains were considered viable whereas those with shrunken protoplasm or either faintly or not stained were considered non-viable. In pollen samples of heterogeneous size, individual grains were assigned to one of the following categories: n (mean pollen grain size in the sample), >n (1.26 the diameter of n pollen and larger) and <n (Larrosa *et al.* 2012).

Meiotic analyses

At flowering, immature buds at various developmental stages were fixed in a 96% alcohol: glacial acetic acid solution (3:1, v/v) for 24 h and then transferred to 70% ethanol until use. Buds were rinsed with 45% glacial acetic acid and anthers from every floral primordium were removed under a drop of acetocarmine (0.5%) on a glass slide, to release the meiocytes with the help of a needle. Observations were made in at least one individual plant per accession under a light microscope, starting from the more advanced phases and working backwards to earlier ones if abnormalities in the meiotic process were detected.

Results

Chromosome numbers

Root-tip chromosome counts revealed that the analysed plants from VdPDFV 26 and VdPDFV 45 were 2n= 2x= 20, the ones from VdPDFV 14 were 2n= 4x = 40, and those from AloEch 1, AloEch 2, AloEch 3 and Nu 1 were 2n= 6x=60 (Fig 5).

Estimation of ploidy level by flow cytometry

Chromosome numbers, mean 2C DNA content and 1Cx mean of each accession are given in Table 2, along with the number of plants in which chromosome numbers were determined. Mean 2C DNA content per accession varied between 2.95 and 8.65. The coefficient of variation (CV) for the histogram peaks varied between 5% and 8%.

In analysing the 2C DNA content, non-significant differences were detected with Dunnett test (α=5%) between controls and the tested plants of each accession. However, significant differences (p= <0.0001) were detected among accessions, which could be separated by Tukey test into three main groups: VdPDFV 45 and VdPDFV 26, with a mean 2C DNA content of 2.95 pg, VdPDFV 14 with a mean 2C DNA content of 5.83 pg and finally AloEch 1, AloEch 2, AloEch 3 and Nu 1 with a mean 2C DNA content between 8.62 and 8.65 pg.

Regarding 1Cx, significant differences (p= <0.0001) were also detected among accessions, which could be separated by Tukey test into three

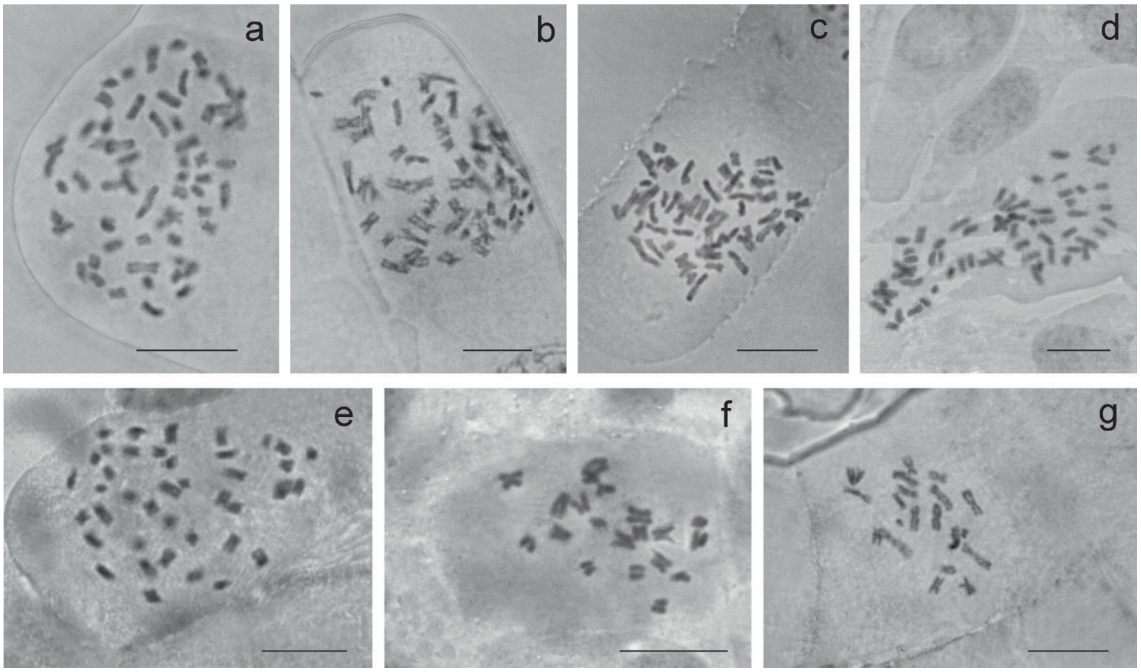

Fig 5. Mitotic metaphases in plants of seven accessions of *Chrysolaena flexuosa*. a) AloEch 1 (2n= 6x= 60); b) AloEch 2 (2n= 6x= 60); c) AloEch 3 (2n= 6x= 60); d) Nu 1 (2n= 6x= 60); e) VdPDFV 14 (2n=4x=40); f) VdPDFV 45 (2n=2x=20); g) VdPDFV 26 (2n=2x=20). Scale bar, 10 µm.

groups that matched the ploidy level of their corresponding internal controls. A significant decrement in 1Cx was observed with increasing ploidy: 1.47 pg for VdPDFV 45 and VdPDFV 26, 1.46 pg for VdPDFV 14 and 1.44 pg for AloEch 1, AloEch 2, AloEch 3 and Nu 1.

Pollen viability and size

Variability for pollen viability and size was observed in all accessions (Fig 6 and Fig 7). The percentage of viable pollen, indirectly estimated by staining in 15 to 21 individual plants per accession, varied within and between accessions, from 18.8%

Table 2. Chromosome numbers and mean 2C DNA content of seven accessions of *Chrysolaena flexuosa* from Argentina. The number of plants analysed for chromosome number determination are indicated with parenthesis. Accessions with different letters have significantly different 2C DNA content means or 1Cx means ($p < 0.0001$ and $p < 0.0001$, respectively). S.D.: standard deviation.

Accession	Chromosome number (determined in mitosis)	2C DNA mean (pg) ± S.D.	1Cx mean (pg) ± S.D.
AloEch 1	2n=6x=60 (6)	8,63 ± 0,16 a	1.44 ± 0.03 a
AloEch 2	2n=6x=60 (7)	8,65 ± 0,08 a	1.44 ± 0.01 a
AloEch 3	2n=6x=60 (5)	8,62 ± 0,13 a	1.44 ±0.02 a
Nu 1	2n=6x=60 (5)	8,63 ± 0,09 a	1.44 ± 0.01 a
VdPDFV 14	2n=4x=40 (6)	5,83 ± 0,10 b	1.46 ± 0.02 b
VdPDFV 45	2n=2x=20 (6)	2,95 ± 0,10 c	1.47 ± 0.05 c
VdPDFV 26	2n=2x=20 (5)	2,95 ± 0,05 c	1.47 ± 0.03 c

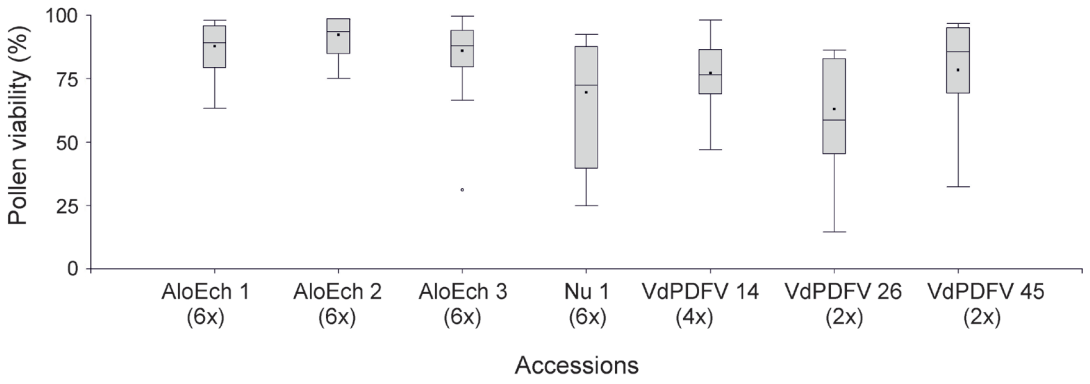

Fig 6. Pollen viability (%) in 15-21 individual plants of each of seven accessions of *Chrysolaena flexuosa*. Box= 50% of the central data; highlighted line= median; black dots= mean; white dot: outlier.

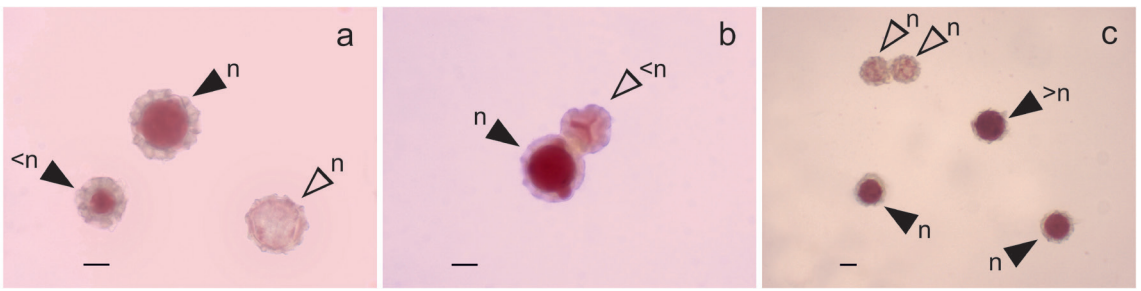

Fig 7. Examples of viable (filled arrows) and non-viable (empty arrows) pollen grains in *Chrysolaena flexuosa* according to size. a) Normal (n) and small (<n); b) normal (n) and small (<n); c) normal (n) and 2n (>n). Scale bar, 20 µm.

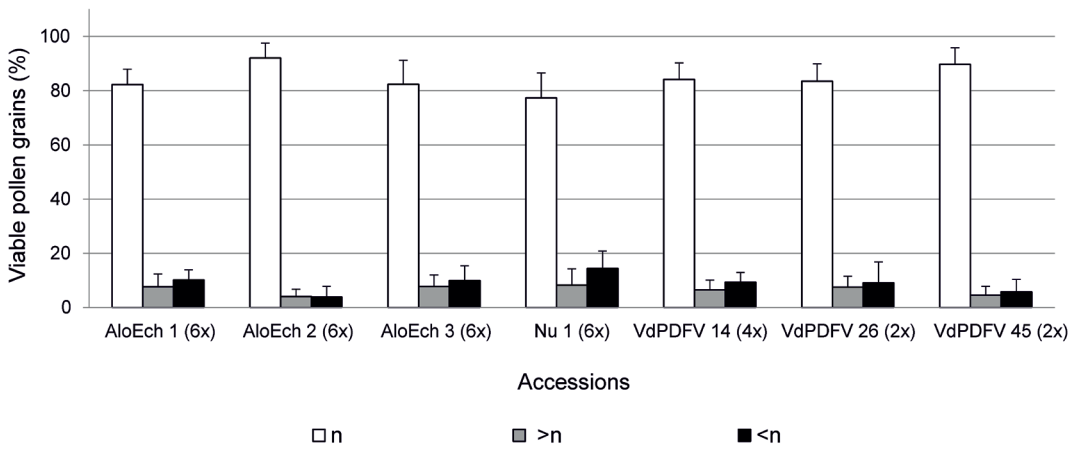

Fig 8. Mean values of viable pollen grains (%) in seven accessions of *Chrysolaena flexuosa* discriminated into three categories according to size: normal (n), >n (2n) and small (<n). Note: for each accession, the percentage of viable pollen grains in each category was calculated by averaging the percentages (for the same category) recorded in 15-21 individual plants.

in one plant of accession 2x VdPDFV 26 to 100% in two plants of accession 6x AloEch 2 and one of accession 6x AloEch 1. Accessions 2x VdPDFV 26 and 6x Nu 1 exhibited the lowest average percentage of viable pollen (65.0% and 71.6%, respectively) and the highest variability for that parameter (sd= 23.9% and 24.9%, respectively). On the other hand, accessions 6x AloEch 2 and 6x AloEch 1 had the highest average percentage of viable pollen (92.3% and 87.7%, respectively) and the lowest variability for that parameter (S.D.= 8.2% and 10.9%, respectively).

All analysed plants, regardless of ploidy, produced pollen of heterogeneous size. The mean pollen grain size in the sample was considered to represent the n category, whereas the >n and <n sizes were taken as indications of the formation of gametophytes with chromosome numbers differing from the expected n=x in diploids, n=2x in tetraploids, and n=3x in hexaploids (Larrosa *et al*. 2012). The percentage of viable pollen represented by n grains varied from 77.3% in accession 6x Nu 1 to 92.0% in accession 6x AloEch 2 (Fig 8). Accession 6x AloEch 2 presented similar values of <n and >n pollen grains (3.9% and 4.1% respectively) whereas the rest of the accessions presented a slightly higher percentage of <n pollen grains in relation to >n (Fig 8). On the other hand, in all the accessions non-viable pollen grains were detected, which could be discriminated into four categories according to size and type of protoplasm, as follows: normal and shrunken, small and shrunken, normal and empty, and small and empty (Fig 9). Variability between and within accessions was observed for the different categories of non-viable pollen grains.

Meiotic analyses

Normal tetrads were observed in all analysed plants. However, with the exception of plant 16 from accession 6x Nu 1, abnormal tetrads with unequal-sized cells or a non-tetrahedral spatial disposition were also observed (Table 3; Fig 10 k, m, n and p). The highest percentage of abnormal tetrads was observed in the two plants analysed from accession 2x VdPDFV 26 (25.6% and 27.3%, respectively). Triads were also detected in 18% of the 473 meiocytes analysed in the eight plants

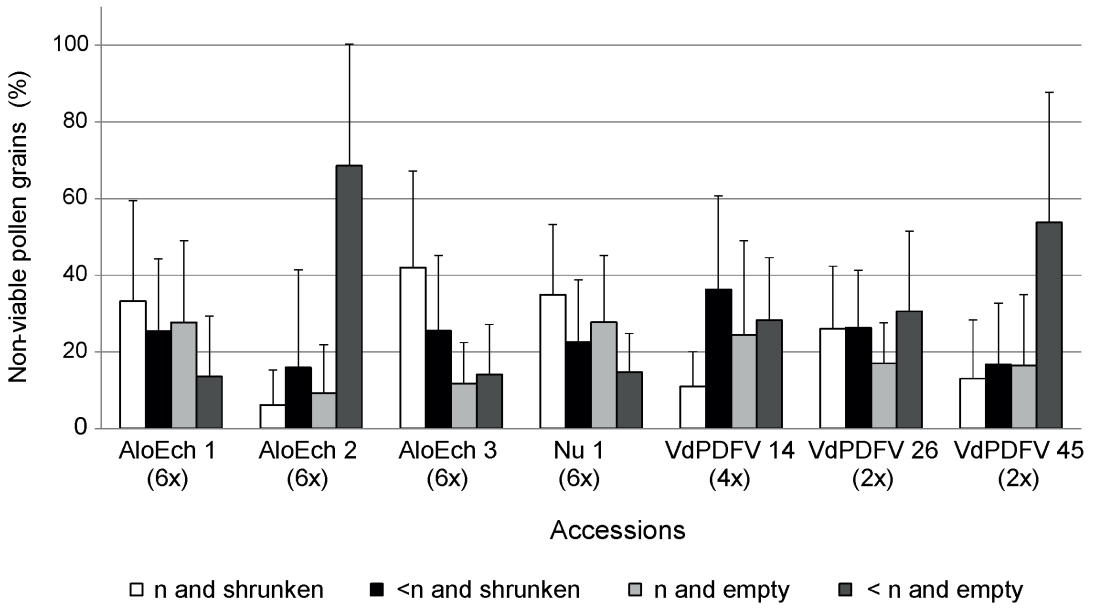

Fig 9. Mean values of non-viable pollen grains (%) in seven accessions of *Chrysolaena flexuosa* discriminated into four categories according to size and type of protoplasm: normal (n) and shrunken, small (<n) and shrunken, normal and empty, and small (<n) and empty. Note: for each accession, the percentage of non-viable pollen grains in each category was calculated by averaging the percentages (for the same category) recorded for 15-21 individual plants.

Table 3. Percentage of triads and tetrads at the tetrad stage of meiosis in individual plants (genotypes) of seven accessions of *Chrysolaena flexuosa*.

Accession	Ploidy	Plant denomination	Meiocytes (no.)	Triads (%)	Tetrads (%)	
					Normal	Abnormal
AloEch 1	Hexaploid	2	66	4.5	92.4	3.1
AloEch 2	Hexaploid	7	32	6.2	84.4	9.4
AloEch 3	Hexaploid	27	48	8.3	87.5	4.2
Nu 1	Hexaploid	16	26	7.7	92.3	0.0
VdPDFV 14	Tetraploid	20	120	10.8	88.3	0.9
VdPDFV 26	Diploid	1	55	12.7	60.0	27.3
	Diploid	4	74	2.7	71.7	25.6
VdPDFV 45	Diploid	23	52	15.4	80.8	3.8

(Table 3, Fig 10 n, p), with percentages varying from 2.7% in plant 4 from accession 2x VdPDFV 26 to 15.4% in plant 23 from accession 2x VdPDFV 45.

Other abnormalities detected were: lagging chromosomes and precocious chromosome migration in Metaphase I in all plants analysed (Fig 10 b); one chromosome bridge (Fig 10 d) and abnormal Metaphase II (Fig 10 f, h) in accession 6x AloEch 2. On the other hand, one plant of accession 2x VdPDFV 26 presented lagging chromosomes in Metaphase II and off-plate chromosomes in Telophase II. Moreover, in accessions 4x VdPDFV 14, 2x VdPDFV 26 and 6x AloEch 3, parallel spindle orientation was detected in Metaphase II (Fig 10 g).

Discussion

Chromosome numbers, ploidy levels and DNA content

Chromosome numbers determined in the three accessions from northeastern Argentina (2n=20 for VdPDFV 26 and VdPDFV 45, and 2n=40 for VdPDFV 15) were consistent with previous reports for other accessions from the same region and also from Uruguay, Bolivia and Brazil (Ruas *et al.* 1991; Dematteis, 2009; Via do Pico & Dematteis, 2012). On the other hand, those determined in the four accessions from the southeastern distribution were consistent with the only record, n=30-32 *ca.*, for a population from Tandil, in the Tandilia hill range (Hunziker *et al.* 1990), although the number of plants analysed in that study was not informed.

Regarding the flow cytometry results, the CV for the histogram peaks (5% and 8%) are considered tolerable for ploidy level estimations when replications within individuals are made and more than 1,000 nuclei per peak are acquired (Suda *et al.* 2007). Thus, according to the mean 2C DNA content, the following ploidy levels were assigned: diploid (VdPDFV 14 and VdPDFV 45), tetraploid (VdPDFV 26), and hexaploid (AloEch 1, AloEch 2, AloEch 3 and Nu 1).

There is only one report on DNA content of *Ch. flexuosa*, by Via do Pico and Dematteis (2013), who worked with one diploid accession from Northeastern Argentina and one tetraploid accession from Uruguay. The 2C DNA content estimated by these authors was, respectively, 11% and 17.5% higher than the 2C DNA content estimated for the diploid and tetraploid accessions analysed in the present study. They also found that the 1Cx value increased with the ploidy level. The discrepancies between the results of both studies could be due to genetic and/or methodological differences. In their paper, Via do Pico and Dematteis (2013) mentioned neither the number of analysed plants nor the CV of the histograms peaks; moreover, they used other reference standards, selected according to ploidy: *Paspalum intermedium* Munro "Sch 28857" for the diploid accession and *P. dilatatum* Poir. "Chirú" for the tetraploid one.

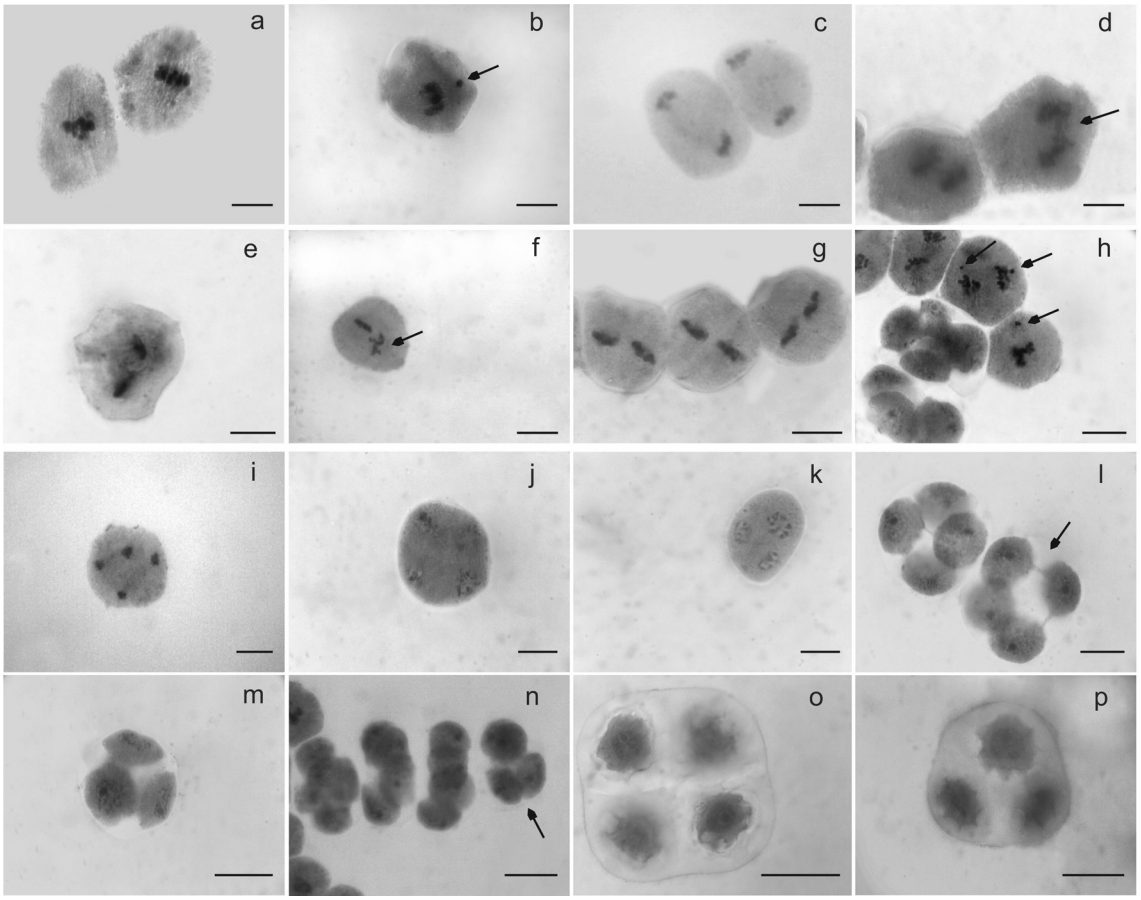

Fig 10. Examples of meiosis in accessions of *Chrysolaena flexuosa*. a) normal Metaphase I; b) lagging chromosome in Metaphase I (arrow); c) normal late Anaphase I; d) chromosome bridge in Anaphase I (arrow); e) normal Metaphase II; f) abnormal Metaphase II (arrow); g) parallel spindles in Metaphase II h) abnormal Metaphase II with lagging chromosomes (arrows); i) normal late Anaphase II; j) normal Telophase II; k) abnormal Telophase II with one large and two small groups of chromosomes; l) a normal tetrad and other tetrad with cytomixis (arrow) at the tetrad stage; m) triad at the tetrad stage; n) triad (arrow) and tetrads with abnormal disposition at the tetrad stage; o) tetrad at the tetrad stage; p) triad. Scale bar, 20 µm.

In the present study, the 2C DNA content of the tetraploid and hexaploid accessions was, respectively, 1.9 and 2.9 higher than the content of the diploid accession, instead of being the double and triple as expected according to the proposal of several authors for higher plants in general (Torrell & Vallés, 2001; Doležel *et al.* 2007; Suda *et al.* 2007). The observed differences between estimations and expectations can be better appreciated by considering the significant decrement in 1Cx value with increasing ploidy. Decrease in genome size is a widespread phenomenon among polyploids (Leitch & Bennett, 2004; Leitch & Leitch, 2013). In the Asteraceae family, reductions of 1Cx content with increasing ploidy have been documented in several genera, among them, *Artemisia* L., *Centaurea* L., *Colymbada, Cyanus, Hieracium* and *Lessingianthus* (Bancheva & Greilhuber, 2006; Pellicer *et al.* 2007; Chrtek *et al.* 2009; Angulo & Dematteis, 2013), and even in the species *Chrysolaena cognata* (Via do Pico & Dematteis, 2013).

Variation in DNA content after polyploidization could be caused by the loss or gain of genetic material

in response to a chromosomal rearrangement after the genomic shock caused by the hybrid genome combination (McClintock, 1984). Several authors have suggested that genome downsizing can occur by non-random deletion of coding and non-coding sequences, deletion of retroelements, gain or loss of chromosomes or entire genomes, altered patterns of gene expression and epigenetic modifications (Feldman & Levy, 2005; Ma & Gustafson, 2006; Jones & Langdom, 2013; Leitch & Leitch, 2013). The 1Cx values estimated in the present study combined with the chromosome countings would suggest that the observed differences between observations and expectations in DNA content are the result of chromosome rearrangements with loss of genetic material, not of aneuploidy. Non-coding DNA sequences constitute the major fraction of many eukaryotic genomes (Leitch & Bennett, 2004) and it has been demonstrated that this type of sequences can be eliminated in following polyploid formation in plants (e.g. Ozkan *et al.* 2001; Shaked *et al.* 2001). The loss of DNA material by polyploidization in *Ch. flexuosa* could be related to deletions of non-coding DNA sequences, because vital functions do not appear to have been affected in the polyploid cytotypes.

Pollen viability

Three of the hexaploid accessions -AloEch 2, AloEch 3 and AloEch 1- had the highest percentage of viable pollen (> 85%) whereas diploid VdPDFV 26 had the lowest (62.3%). Via do Pico and Dematteis (2012), working with another diploid population from the same geographical region, reported a pollen viability value of 92.1%. These differences in pollen viability could be due to the genetic composition of the populations, environmental growing conditions and/or methodological aspects of both studies. In fact, Via do Pico and Dematteis (2012) observed a total of 342 pollen grains but did not refer to the number of individual plants under study, whereas we analysed at least 15 plants/accession, that is a minimum of 3,750 pollen grains. The analysis of 250 pollen grains in each of several plants per accession revealed that pollen viability among plants, even within accessions, was variable, and that individual plant values are more meaningful than average accession values in this type of studies.

Meiotic behaviour

In normal meiosis of Dicotyledonous plants, the first meiotic division is followed by a second meiotic division in which the Anaphase II spindles are orientated such as to form a 60° angle; then, simultaneous cleavage furrow formation occurs resulting in four equal-sized cells with the gametophyte chromosome number (n), which are contained in the meiocyte in a final tetrahedron disposition (tetrad stage) (Singh, 2003).

The variability observed in pollen grain size and viability in the present study can be accounted by the meiotic abnormalities detected in plants from all the accessions. Those abnormalities were of two types: in chromosome synapsis and/or segregation, and in spindle orientation. Regarding the first type, lagging and out of plate chromosomes could have resulted from asynapsis or desynapsis (Koduru & Rao, 1981) and/or irregular chromosome segregation, whereas the presence of a chromosome bridge in Anaphase I can be taken as an indication of crossing-over within the loop of an inversion heterozygote, which could have led to duplications and deletions of chromosome regions (Singh, 2003). Cells with unequal chromosome numbers were expected to be formed and, in fact, meiocytes with four unequal-sized cells at the tetrad stage, heterogeneity in pollen size in individual plant samples, and non-viable pollen grains were observed.

On the other hand, abnormalities in spindle orientation at Metaphase II can lead to the formation of linear or disorganized tetrads. However, if spindle orientation is parallel, fused or tripolar, either dyads can be formed at the tetrad stage with both cells with the sporophytic chromosome number (the first two orientations), or triads with two cells with the gametophytic chromosome number and one cell with the sporophytic number (Mok & Peloquin, 1975a). The observed parallel spindles at Metaphase II and meiocytes at the tetrad stage with two equal-sized cells and a larger third cell can explain the origin of the >n viable pollen grains with diameters approximately 1.26 larger that the diameter of the pollen grains of the most frequent size (n). Those pollen grains can be considered to be 2n (Camadro, 1986; Carputo *et al.* 2003; Kumar & Singhal, 2011).

Gene mutations and environmental conditions can explain the variability encountered in pollen viability and 2n gamete formation in higher plants

(Bretagnolle & Thompson, 1995; Peloquin *et al.* 1999). Heritable formation of 2n gametes has been attributed to the action of single recessive genes with incomplete penetrance and variable expressivity (Mok & Peloquin, 1975b; Bretagnolle & Thompson, 1995). In our study, plants of all the accessions were grown in the same environment; thus, it is highly likely that the cytological mechanism that led to the formation of parallel and tripolar spindles is under the same genetic control, as in other Dicotyledoneae (see Carputo *et al.* 2006).

Geographic distribution of polyploids

Crisci *et al.* (2001) suggested that the evolution of the Asteraceae in the Tandilia hill range was affected by geological events related to the uplift of the Andes and the Pleistocene glaciations. Dry and wet cycles caused fragmentation and differentiation of populations and semiarid vegetation extended into the continent (Simpson, 1975 and Prado & Gibbs, 1993 in Crisci *et al.* 2001). Fragmentation and differentiation may have resulted in an eventual separation of populations which would have become restricted to rocky and loose soils in more elevated regions (Crisci *et al.* 2001). Moreover, Stebbins (1947) proposed that polyploids might have a higher capacity to conquest new ecological niches than diploids. In this regard, Otto and Witton (2000) suggested that changes in metabolism, developmental rates, gene regulation and/or physiological tolerances, among other factors, could alter biotic interactions and ecological tolerances, playing critical roles in the establishment and diversification of newly formed polyploid lineages. Thus, the fact that only hexaploid cytotypes of *Ch. flexuosa* were found in the southern limit of its distribution is in accordance with Crisci´s et al. (2001) proposal and would give further support to Stebbins´s (1947) hypothesis. Morphological differences have been observed in flower color, size and number of inflorescences and leaves between plants from northeastern and southeastern populations of this species growing under the same environmental conditions (Echeverría, com. pers.). The southeastern region of the Buenos Aires province has significantly lower temperatures and incidence of rainfalls than the central area of the species distribution. Phenotypic changes resulting from polyploidization events in *Ch. flexuosa* could have played a role in the establishment of new cytotypes and their successful colonization of new geographical areas as southern Buenos Aires province. Although this hypothesis could explain the presence of hexaploid cytotypes in more moderate temperate climates, it does not explain why tetraploids have only been found in the northern distribution area along with the diploids. Anthropic interventions that modified the original landscapes over the last century could provide a possible explanation. In fact, the agroclimatic characteristics of the Buenos Aires province have given impulse to the advancement of agronomic practices and urbanization. These anthropic interventions have led to the almost complete disappearance of the original plant communities and many natural plant populations are no longer found in that area/region (Zalba & Villamil *et al.* 2002), although some native species, among them *Ch. flexuosa*, can still thrive in the Tandilia hills (Alonso *et al.* 2009).

Fitness and genetic flexibility of natural populations are related to the mode of reproduction and stability of the environment. Asexual reproduction of plants adapted to a given environment provides for high levels of fitness in stable conditions but low genetic flexibility under unstable ones, whereas the opposite holds true for sexual reproduction. Plants that have both types of reproduction available to them would be more successful over time that the ones that have either one or the other type. In this regard, *Ch. flexuosa* combines vegetative reproduction via the xylopodium and sexual reproduction by allogamy resulting from protandria (pers. observ.). Moreover, the observation of 2n pollen formation mediated by the same cytological mechanism in all accessions analysed can be taken as an indication that this mechanism is heritable. Thus, it can be hypothesized that sexual polyploidization was instrumental in the origin of the tetraploid and hexaploid cytotypes. The establishment and subsequent geographical expansion of *Ch. flexuosa* polyploid cytotypes could be explained by their greater capacity to store alleles than their diploid counterparts and, therefore, the occurrence of new intra and inter-locus interactions that could be exposed to selection, and which cannot occur at the lower ploidy level (see Mendiburu & Peloquin 1976).

Conclusions

Flow cytometry appears to be a reliable technique for ploidy estimations in *Chrysolaena flexuosa* populations because chromosome numbers and DNA content were in accordance. However, care must be taken in the application of the technique because significant changes in monoploid genome size with increasing ploidy were detected.

The high number of 2n pollen-producing plants by a cytological mechanism known to be genetically controlled in other plant groups and the irregularities observed in meiosis are indications that hybridization and sexual polyploidization could have played an important role in the origin, establishment and expansion of natural populations.

Regarding the distribution of the polyploid cytotypes in Argentina:

(1) in the area between the northeastern distribution and the southeastern Tandilia range, natural populations have not been apparently recorded, and there are no accessions in working collections. Thus, prospection and collection trips should be carried out. Particular emphasis should be placed in exploring southern Entre Ríos province, which limits with northern Buenos Aires province, to clarify the distribution of the polyploid cytotypes,

(2) there is also a need to perform cytogeographical studies with a representative number of plants per population to arrive at robust conclusions on the possible gene pools.

Sampling and ex situ multiplication strategies would be of utmost importance for conservation of the species natural genetic diversity and its effective use in breeding and other applied purposes (see Camadro 2012, for an example in wild potatoes).

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