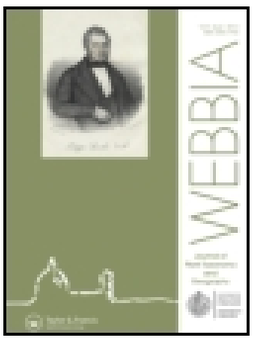

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Meiotic behaviour and B chromosomes in Argentine populations of *Chrysolaena verbascifolia* (Vernonieae: Asteraceae)

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ABSTRACT

Four populations of *Chrysolaena verbascifolia* (Vernonieae: Asteraceae) from localities in Argentina were cytologically analysed for meiotic behaviour and pollen viability. This is the first meiotic analysis in the species. All were diploids with $2n = 2x = 20$ and all showed quite regular meiotic behaviour. We also report the occurrence of accessory chromosomes in all the populations of the species. The majority of the populations displayed regular bivalent formation during late prophase stages and metaphase I, and chromosome segregation at anaphase. However, some chromosomal irregularities were recorded, mainly during meiosis I, including univalents, out-of-plate chromosomes and precocious segregation in metaphase, and laggards and chromatin bridges in anaphase. The B chromosomes and abnormal behaviours of the chromosomes during meiosis observed did not affect pollen development in these taxa, as viability was always high.

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KEYWORDS
Bivalents; meiotic abnormalities; pollen viability; univalents

Introduction

Chrysolaena H. Rob. (Vernonieae, Asteraceae) is a South American genus with 18 species geographically concentrated in southern Brazil and northern Argentina, with some species extending to Uruguay, Paraguay, Bolivia and Peru (Dematteis 2009). It is characterized by sericeous or velutine indumentum, style without basal node, and glandular anthers and cypselae (Robinson 1988). Other distinctive characteristics are the pollen morphology (pollen type 'C') and the basic chromosome number $x = 10$ (Dematteis 2009; Via do Pico and Dematteis 2013a, 2013b).

Chrysolaena is a cytogenetically very diverse genus; with significant interspecific and intraspecific chromosomal variation. Species with ploidy levels ranging from diploid ($2n = 2x = 10$) to octoploid ($2n = 8x = 80$) have been found, including odd chromosome numbers (Dematteis 2009; Via do Pico and Dematteis 2014). *Chrysolaena verbascifolia* (Less.) H. Rob. is one of the scarcely studied species of the genus. It is a perennial erect shrub that can reach up to 1 m in height, with well-developed xylopodia. It has very dense tomentose-velutine indumentum. The capitula are showy with violet or purple flowers. It grows on high fields, dry, sandy or rocky soil in northeast Argentina (mainly in the province of Misiones), southern Brazil and eastern Paraguay (Dematteis 2009; Via do Pico et al. 2016). The cytological background of this species indicates chromosome number $2n = 2x = 20$ and a karyotype formed by metacentric and submetacentric chromosomes (Angulo and Dematteis 2009b; Dematteis 2009; Via do Pico and Dematteis 2013a). The occurrence of polymorphism in a chromosome pair and the presence of accessory chromosomes or B chromosomes has also been reported (Via do Pico and Dematteis 2013a). However, there is no study of the meiotic behaviour.

The presence of B chromosomes has been documented in numerous groups of plants. It is estimated to occur in approximately 10–15% of flowering plants (Jones 1995). Jones (1975) defined the B chromosomes as extra chromosomes of a basic chromosomal set that clearly do not fit in the category of aneuploidy or polyploidy. It is a very common phenomenon among Asteraceae (Jones 1995). In Vernonieae there are numerous species with accessory chromosomes (Jones 1979; Galiano and Hunziker 1987; Dematteis 1998; Oliveira et al. 2007; Angulo and Dematteis 2009b, 2012, 2015). In *Chrysolaena*, besides *C. verbascifolia*, B chromosomes have been reported in populations of *Chrysolaena cognata* (Less.) Dematt., *Chrysolaena cristobaliana* Dematt., *Chrysolaena flexuosa* (Sims.) H. Rob., *Chrysolaena platensis* (Spreng.) H. Rob., *Chrysolaena propinqua* (Hieron.) H. Rob. and *Chrysolaena sceptrum* (Chodat.) Dematt. (Via do Pico and Dematteis 2013a, 2014).

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Table 1. Meiotic chromosome numbers (*n*), ploidy level and voucher information of the populations of *Chrysolaena verbascifolia* investigated.

Species	*n*	Ploidy level	Location and voucher specimens
C. verbascifolia	10	2*x*	Argentina. Misiones. Dep. San Javier. 20.4 km west of San Javier. Camino a Azara. *Via do Pico & al. 28* (CTES)
C. verbascifolia	10	2*x*	Argentina. Misiones. Dep. General Manuel Belgrano. Campina de Americo. Cementerio. *Via do Pico & al. 34* (CTES)
C. verbascifolia	10	2*x*	Argentina. Misiones. Dep. Concepción. 2 km northwest of Concepción de la Sierra. Camino a Apóstoles. *Via do Pico & al. 41* (CTES)
C. verbascifolia	10	2*x*	Argentina. Misiones. Dep. San Javier. 20.4 km west from San Javier. Camino a Azara. *Via do Pico & al. 58* (CTES)

Cytological information available on *Chrysolaena* comes primarily from analysis of mitotic chromosomes. The analysis of meiotic chromosomes in the genus is almost non-existent. Meiotic chromosome counts have been reported in only a few entities. Meiotic studies explain some reproductive phenomena of species, and help to improve the understanding of the mechanisms of heritability, evolutionary processes and genetic variability. Meiosis is one of the sources of genetic variation used by organisms to adapt themselves to their environment and perpetuate through their offspring (Caetano 2003). The knowledge of the events that occur during the meiotic divisions of *C. verbascifolia* is particularly interesting due to the high frequency of B chromosomes observed in some populations.

Therefore, the primary objective of this study was to provide for the first time a detailed cytogenetic analysis of the meiotic behaviour of *C. verbascifolia*, as well as to analyse the occurrence and effect of B chromosomes in this process.

Materials and methods

Material was obtained from natural populations of different localities of the province of Misiones (Argentina). Voucher specimens are deposited in the herbarium of Instituto de Botánica del Nordeste (CTES). The populations of *C. verbascifolia* studied and collections used are listed in Table 1. Figure 1 shows the geographic distribution of the populations studied.

Meiosis was studied in young inflorescences fixed in lactic acid : ethanol (1 : 5) and refrigerated until examined. Anthers were macerated and squashed using 2% lacto-propionic orcein. Permanent slides were prepared using Euparal.

The analysis included counting the chromosome number, determination of ploidy level and the analysis of meiotic behaviour of Pollen Mother Cells of each sample. All meiotic stages found were analysed and the frequency of each observed phenomenon was recorded. Photographs were taken through a Zeiss Axioplan microscope with a Canon Power Shot A640 camera.

Pollen fertility was estimated by staining with carmine : glycerine (1 : 1) (Pittenger and Frolik 1951). Uniformly stained pollen grains were considered fertile whereas the non-stained grains were scored as sterile. Approximately 300–500 pollen grains were analysed per population.

Results

The meiotic chromosome numbers, configurations, meiotic behaviour and pollen viability of the populations of *C. verbascifolia* analysed are detailed in Tables 2 and 3.

All populations studied showed basic chromosome number $x = 10$ and diploid cytotype ($2n = 2x = 20$). Analysis of the Pollen Mother Cells in diakinesis/metaphase I showed seven meiotic configurations, which included the formation of univalent (I) and bivalent (II) (Table 2). All populations showed 100% of bivalent formation in diakinesis/metaphase I, with the presence of zero to six B chromosomes per cell. These were seen as univalent during different phases of meiosis I and II. The meiotic behaviour showed a high percentage of out-of-plate chromosomes at metaphase I and lagging chromosomes in anaphase I.

Population no. 28 showed two configurations in diakinesis/metaphase I. Almost all of the cells with 10II (Figure. 3F) and a few cells with 10II + 1B. Besides, it presents the lowest percentage of irregularities during the two meiotic divisions; including out-of-plate chromosomes (Figure. 3G), laggards and bridges (Figure. 3H).

Population no. 34 showed the highest number of meiotic configurations and B chromosomes (zero to six per cell); it being most usual to find four or five B chromosomes per cell (39.91% and 41.18% of cells, respectively) (Figures. 2A–D). At metaphase I a high percentage of cells with accessory out-of-plate chromosomes was observed (82.28%). Lagging chromosomes in anaphase/telophase I and II were observed in a 27.56% and 10.26%, respectively (Figure. 2E).

The diakinesis/metaphase I of population no. 41 show three meiotic configurations and zero to three B chromosomes per cell, 10II being the most common (Figure. 2F). Irregularities were observed during meiosis I and II. A high percentage of cells with B chromosomes out-of-plate at metaphase I (73.89%) (Figures. 2G, H, 3A) and metaphase II (55.88%) was observed. Precocious migration of chromosomes (5.09%) was also observed. In anaphase I and II a low frequency of laggard chromosomes (13.33% and 12.5%) (Figure 3B) and bridges and fragments (2.67% and 0.83%, respectively) were observed (Figure. 3C).

Figure 1. Geographic distribution of the populations of *Chrysolaena verbascifolia* studied.

Table 2. Meiotic configurations of the populations of *Chrysolaena verbascifolia* analysed.

Species and voucher	Configurations	N° de cells	%
C. verbascifolia 28	10II + 1B	3	17.65
	10II	14	82.35
	Total	17	100
C. verbascifolia 34	10II + 6B	3	4.41
	10II + 5B	28	41.18
	10II + 4B	27	39.71
	10II + 3B	4	5.88
	10II + 2B	4	5.88
	10II	2	2.94
	Total	68	100
C. verbascifolia 41	10II + 3B	1	4.76
	10II +1B	5	23.81
	10II	15	71.43
	Total	21	100
C. verbascifolia 58	10II	21	100

Population no. 58 showed a single meiotic configuration (10II), and few irregularities and at low frequency; mainly out-of-plate chromosomes in metaphase I (Figure 3D) and II, and bridges in anaphase I (Figure. 3E). Although no B chromosomes as univalents were observed in diakinesis, a few cells with out-of-plate chromosomes were observed in metaphase I and II.

Pollen viability was high in all populations, between 91.36% in population no. 41 and 96.28% in population no. 58.

Discussion

This work studied for the first time the meiotic behaviour of *C. verbascifolia* and reports the second case of B chromosomes in the species, but the first in meiosis.

The basic number and chromosome numbers found in the four populations analysed are consistent with previous counts made in the species (Dematteis 1996, 1997; Angulo and Dematteis 2009a; Via do Pico and Dematteis 2013a). Chromosome numbers are useful to distinguish the different South American genera of the tribe Vernonieae. Species belonging to *Lessingianthus*, for example, have basic number $x = 16$ (Angulo and Dematteis 2009a, 2009b, 2012), *Vernonanthura* presents basic number $x = 17$ (Vega and Dematteis 2016), whereas *Chrysolaena* presents basic number $x = 10$, commonly found in Old World members of the tribe (Dematteis 2009; Via do Pico and Dematteis 2012, 2013a). *Lepidaploa* is the only heterogeneous group

Table 3. Number of pollen mother cells (No.), percentage of meiotic irregularities (%) and pollen fertility of the populations of *Chrysolaena verbascifolia* analysed.

Phase	*C. verbascifolia 28*		*C. verbascifolia 34*		*C. verbascifolia 41*		*C. verbascifolia 58*	
MI	No.	%	No.	%	No.	%	No.	%
Regular	176	88.44	31	17.72	33	21.02	22	88
Precocious migration	–	–	–	–	8	5.09	–	–
Off-plate chromosomes	23	11.56	144	82.28	116	73.89	3	12
Total	199	100	175	100	157	100	25	100
AI/TI								
Regular	204	97.61	92	72.44	63	84	55	91.67
Laggard chromosomes	4	1.91	35	27.56	10	13.33	–	–
Bridges	1	0.48	–	–	2	2.67	5	8.33
Total	209	100	127	100	75	100	60	100
MII								
Regular	86	95.55	–	–	15	44.12	14	87.5
Off-plate chromosomes	4	4.45	–	–	19	55.88	2	12.5
Precocious migration	–	–	–	–	–	–	–	–
Total	90	100	–	–	34	100	16	100
AII/TII								
Regular	72	93.51	35	89.74	104	86.67	–	–
Laggard chromosomes	5	6.49	4	10.26	15	12.5	–	–
Bridges	–	–	–	–	1	0.83	–	–
Total	77	100	39	100	120	100	–	–
Pollen fertility								
Fertile	819	92.22	565	94.8	719	91.36	622	96.28
Sterile	69	7.78	31	5.2	68	8.64	24	3.72
Total	888	100	596	100	787	100	646	100

showing four basic chromosome numbers, $x = 14$, $x = 15$, $x = 16$ and $x = 17$ (Dematteis 2002). Chromosomal backgrounds have shown that *Chrysolaena* is a cytologically very complex genus, and that polyploidy has played a very important role in the evolution of the group. Ninety per cent of the species studied so far are polyploid, and 81% of the species present intraspecific polyploidy (Angulo and Dematteis 2009a, 2009b; Dematteis 2009; Via do Pico and Dematteis 2012, 2013a, 2014). Despite this chromosomal variability, the mitotic studies carried out show that *C. verbascifolia* is exclusively diploid (Dematteis 1996, 1997; Angulo and Dematteis 2009a; Via do Pico and Dematteis 2013a). The results of this meiotic analysis continue to support this assumption.

All specimens showed quite regular meiotic behaviour. In all populations analysed, normal formation of bivalents during the final stages of prophase and metaphase I, and normal segregation of chromosomes in anaphase, have been visualized. However, some meiotic abnormalities that included the formation of univalent, out-of-plate chromosomes and precocious migration in metaphase, laggards and bridges in anaphase, both meiosis I and meiosis II, were recorded. The occurrence of univalents was observed in all populations, mainly in late prophase and metaphase I. Less frequently they were seen as laggard chromosomes. Bivalent formation is caused by the lack of pairing of homologous chromosomes and this can occur by synaptic mutations caused by mating failures in zygotene (asynapsis), anomalous behaviour of the chiasma, absence of crossover, or early resolution of chiasmata (desynapsis) (Lacadena 1996). An important factor that determines the formation of chiasmata between homologies is the size and morphology of the chromosomes. Small chromosomes tend not to form chiasmata and remain like univalents, and chromosomes with centromeres in the middle position tend to have lower chiasmata frequency than those with a subterminal centromere (Darlington 1965). The univalents formed may exhibit erratic behaviour. Generally, they migrate randomly to either pole or may fail to reach the pole and become laggards, or can also be divided into their sister chromatids as in mitosis (Swanson et al. 1981). In this study, the formation of univalents is closely linked to the high frequency of accessory chromosomes observed in all entities. These univalents are the B chromosomes, as all cells found in diakinesis/metaphase I showed 10II of the basic chromosome complement. Another irregularity was the early chromosome segregation in metaphase, particularly in population no. 41. It is considered that this asynchrony in segregation depends on the number and location of the chiasmata. When chiasmata are terminal, separation of the bivalent is faster and simpler than when they are in an interstitial position (Darlington 1965). Generally, the behaviour of chromosomes that migrate early in metaphase and those that become laggards in anaphase determines the formation of micronuclei (Koduru and Rao 1981). In *C. verbascifolia*, the percentage of cells with meiotic abnormalities was greater in metaphase I and decreased to telophase II, indicating that some chromosomes were included in the main nuclei. This seems to be the regular

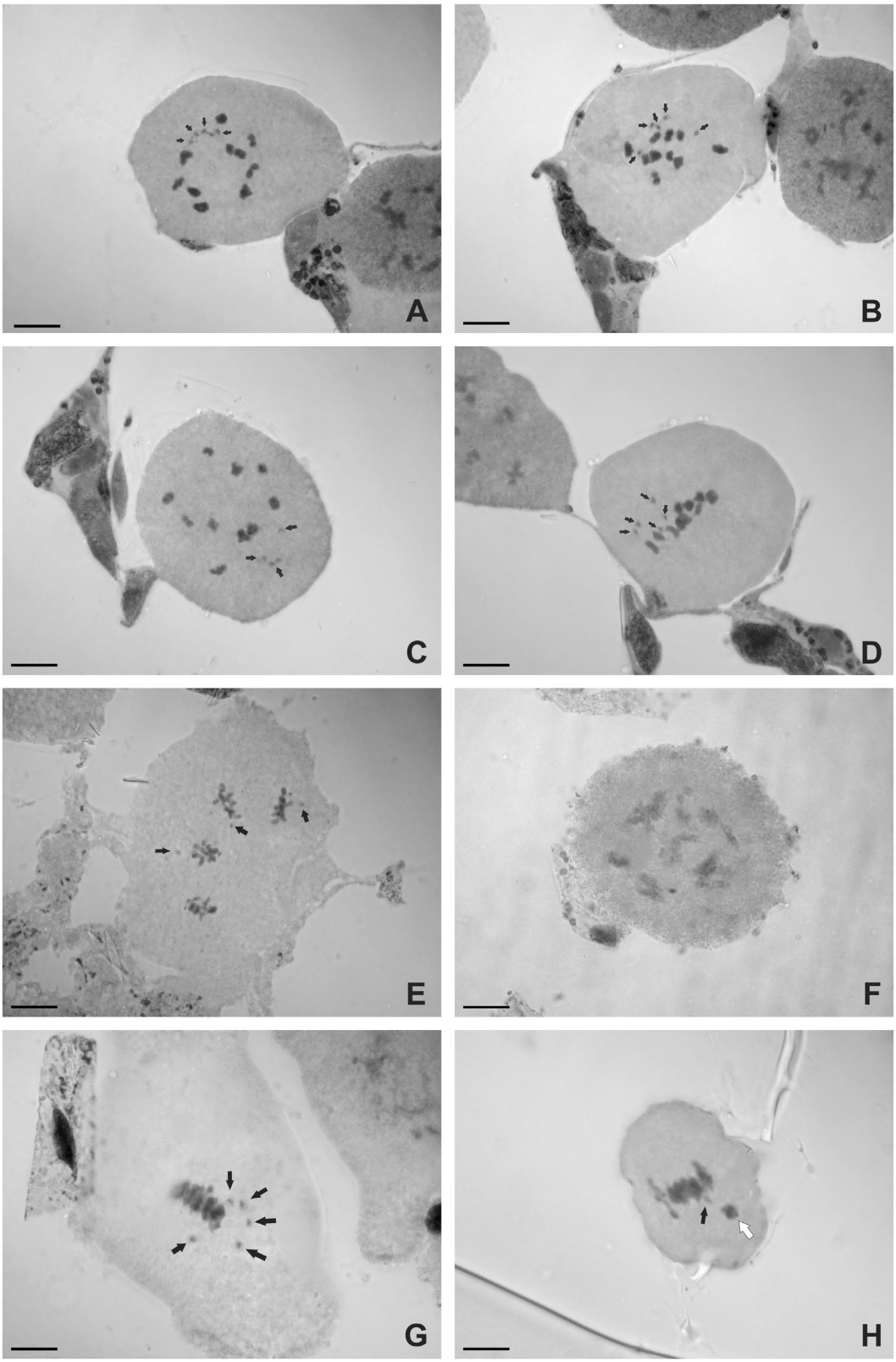

Figure 2. Meiotic behaviour of populations of *Chrysolaena verbascifolia*. (A–E) *C. verbascifolia* population no. 34: (A) Diakinesis with 10II + 4 B chromosomes; (B) Diakinesis with 10II + 5 B chromosomes; (C) Diakinesis with 10II + 3 B chromosomes; (D) Diakinesis with 10II + 5 B chromosomes; (E) Anaphase II with laggards B chromosomes. (F–H) *C. verbascifolia* population no. 41: (F) Diakinesis with 10II; (G) Metaphase I with B chromosomes out-of-plate; (H) Metaphase I with B chromosomes and bivalent out-of-plate. Black arrows show B chromosomes. White arrows show bivalents out-of-plate. Scale bar: 10 µm.

behaviour of many species of plants (Koduru and Rao 1981).

The formation of dicentric bridges and fragments in anaphase are other irregularities observed in most of the analysed populations. This type of irregularities can be explained by the occurrence of U-type reciprocal exchanges between sister chromatids (John 1990). Lewis and John (1966) suggested that U-type exchanges

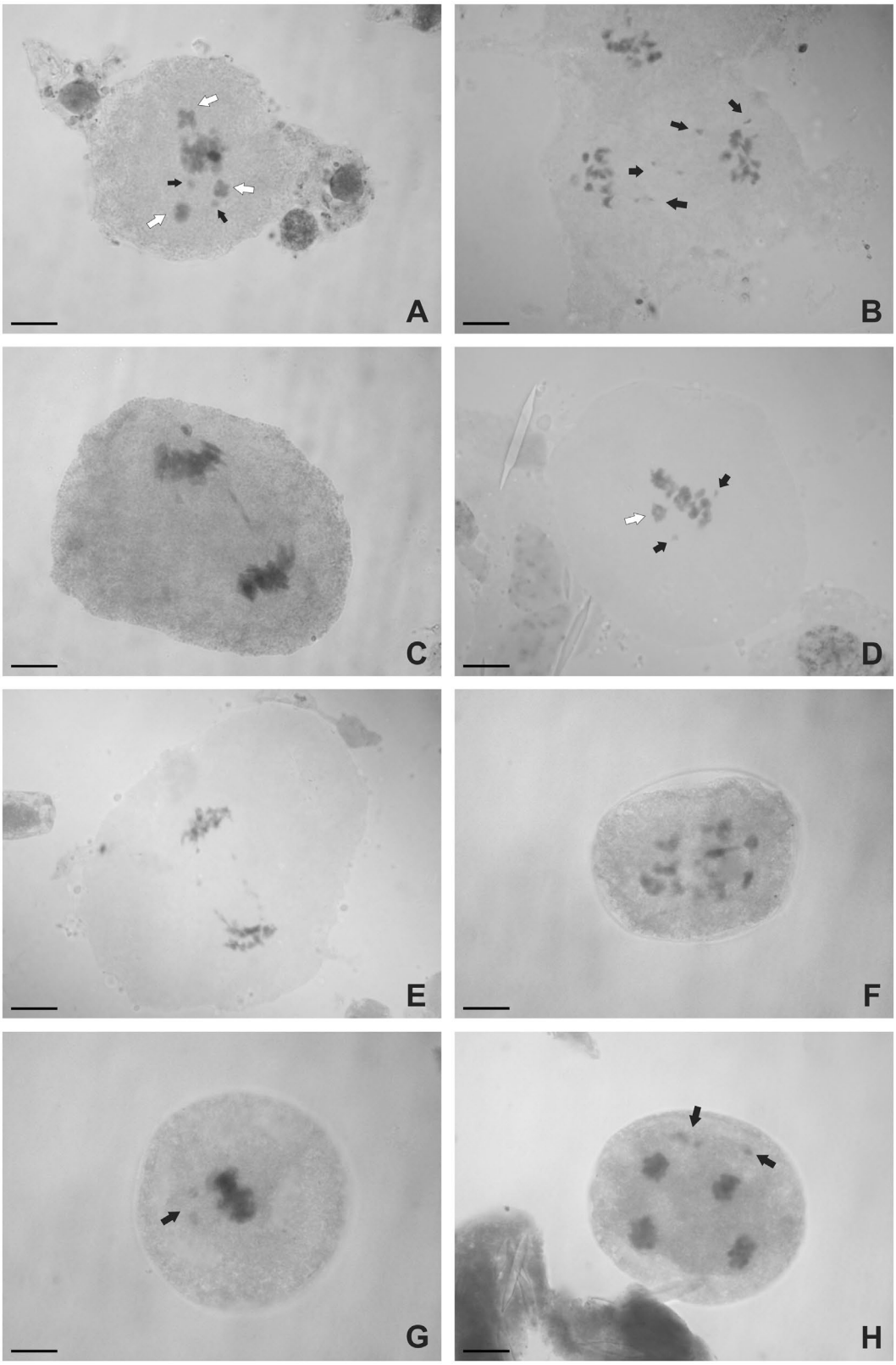

Figure 3. Meiotic behaviour of populations of *Chrysolaena verbascifolia.* (A–C) *C. verbascifolia* population no. 41: (A) Metaphase I with B chromosomes and bivalents out-of-plate; (B) Anaphase I with laggards B chromosomes; (C) Anaphase I with bridge and fragment. (D, E) *C. verbascifolia* population no. 58: (D) Metaphase I with B chromosomes and bivalent out-of-plate; (E) Anaphase I with bridges. (F–H) *C. verbascifolia* population no. 28: (F) Diakinesis with 10II; (G) Metaphase I with B chromosomes out-of-plate; (H) Anaphase I with laggards B chromosomes. Black arrows show B chromosomes. White arrows show bivalents out-of-plate. Scale bar: 10 µm.

occur by errors in chiasma formation, due to involving the chromatids and show strong correlation with the frequency and distribution of chiasmata. Most of the meiotic irregularities observed in *C. verbascifolia* are directly related to the formation of chiasmata in meiotic pachytene. The way in which this is resolved determines

the occurrence of abnormal events observed in the different phases of division.

The B chromosomes are dispensable components of genome that have originated from chromosomal fragments belonging to complement A, as a result of errors during meiotic divisions (Jones and Houben 2003). Usually these elements are small and heterochromatic with a large amount of repetitive DNA (Camacho 2005). They present irregular and non-Mendelian inheritance, which causes polymorphism in the number of B chromosomes within populations, or even within different lines of cells of a carrier individual (Houben et al. 2013). The presence of B chromosomes is a very common phenomenon in the Asteraceae (Jones 1995). In the tribe Vernonieae they have been reported in species of *Lessingianthus* H. Rob. (Jones 1979; Dematteis 1998; Angulo and Dematteis 2009b, 2012, 2015) and *Lepidaploa* (Cass.) Cass., two genera related to *Chrysolaena* (Galiano and Hunziker 1987; Oliveira et al. 2007; Angulo and Dematteis 2012). However, the presence of these accessory chromosomes has not been documented in *Vernonanthura* H. Rob. and *Vernonia* Schreb. (Dematteis 2002; Vega and Dematteis 2016). As previously mentioned, in *Chrysolaena* B chromosomes have been documented in seven species: *C. cognata*, *C. cristobaliana*, *C. flexuosa*, *C. platensis*, *C. propinqua*, *C. sceptrum* and *C. verbascifolia* (Via do Pico and Dematteis 2013a, 2014). In *C. verbascifolia* zero to seven metacentric B chromosomes per cell with an average length of 0.94 μm have been observed during mitotic metaphases, (Via do Pico and Dematteis 2013a).

During the meiotic analysis performed in this study accessory chromosomes were observed in all populations of *C. verbascifolia* analysed. The number of B chromosomes varied between individuals in a population and even among cells of the same plant. In meiosis, although B chromosomes do not pair with chromosomes of A complement, they can sometimes mate with each other according to some authors, and depending on their number can form univalent, bivalent or multivalent types (Houben et al. 2013). However, Jones and Rees (1982) argue that this does not occur in some species because they would not be homologous to each other or because they are too small to form pairs, as in *Allium cernuum* Roth (Grun 1959). The large amount of heterochromatin would also inhibit the formation of chiasmata. Most frequently, the B chromosomes form univalents that can be lost in the successive phases of meiosis (Mendelson and Zohary 1972; Jones and Rees 1982). In this study, B chromosomes were observed as univalents in different phases, mainly in diakinesis/metaphase I. No bivalent or multivalent associations of these elements were observed. B chromosomes were not observed in the later stages of meiosis, which suggests that they were removed in successive stages or have successfully migrated to the poles. On the other hand, the high frequency of B chromosomes reported in previous mitotic studies (Via do Pico and Dematteis 2013a) and that observed in this study suggest that there is a cumulative transmission mechanism and this would occur at higher rates than Mendelian inheritance mechanisms, ensuring its permanence in populations. In many species the B chromosomes seem to behave cytologically in such a way that their number increases in the progeny. The substance of this phenomenon comprises the lack of regular distribution of the B chromosomes, and their preferential migration towards a determined pole (Pole of accumulation). Preferential distribution can take place as much in mitosis as in meiosis. When meiotic preferential distribution takes place the B chromosomes appear as univalents; they do not divide at anaphase, and they distribute themselves towards a privileged pole (Kimura and Kayano 1961; Houben et al. 2013, 2014). Other evidence that an accumulation of B chromosomes would be happening in *C. verbascifolia* is that no micronuclei are observed in the final stages of meiotic division. Micronuclei are formed as a consequence of laggard B chromosomes during anaphases I or II and are very frequently left outside the four tetrad nuclei, becoming small micronuclei, which usually degenerate (Battaglia 1964).

An ancient origin, probably during speciation, or during the stabilization of the species, has been assumed to explain the occurrence of B chromosomes. Occasionally, after the systematic differentiation of the species into individuals, chromosomic anomalies could occur for different reasons, leading to the formation of the B chromosomes. In such cases their origin is often easily understood. Independently from their ancient or recent origin, the mechanism responsible for the formation of B chromosomes is always an abnormal meiosis, which through translocations, inversions or deletions, produces supernumerary chromosomes morphologically rearranged, in fact B chromosomes (Battaglia 1964). As mentioned previously, karyotypic studies reported the occurrence of polymorphism in a chromosomal pair of a population of *C. verbascifolia*. We could not differentiate this heteromorphic pair from the other homologous pairs in the diakinesis/metaphase I of *C. verbascifolia* analysed in this study. Chromosomal heteromorphism is generally associated with the sex chromosomes of plants that have this genetic system, e.g. *Cannabis* L., *Humulus* L. (Cannabaceae), *Silene* L. (Caryophyllaceae) and *Coccinia* Wight & Arn. (Cucurbitaceae) (Vyskot and Hobza 2004; Weiss-Schneeweiss and Schneeweiss 2013). In these entities, accumulation of repetitive DNA and subsequent changes in the size and structure of one of the pairs of chromosomes would have occurred (Navajas-Pérez et al. 2006; Jamilena et al. 2008). However, there are cases of chromosomal heteromorphism that are not of this type and the authors explain this phenomenon by the occurrence of pericentric inversions, intrachromosomal

translocations and re-translocations (Walters 1952; Darlington 1965). There is probably a relationship between chromosomal heteromorphism and the presence of accessory chromosomes in *C. verbascifolia*, as both events are linked to the occurrence of chromosomal irregularities within the genome. However, other studies should be performed to better understand this issue.

The adaptive significance of B chromosomes in populations is largely unknown, despite many attempts to link them with variations in phenotypes and environment. In some cases, there is strong evidence to support the contention that they are simply 'selfish' and that they exist in populations as 'parasitic' chromosomes (Nur and Brett 1988; Jones 1991). Generally, in species of plants or animals carrying B chromosomes, the individuals in a population with or without B chromosomes cannot be distinguished phenotypically from each other. But there are cases in which the B chromosomes change certain morphological characteristics of individuals (Jones and Rees 1982; Jones and Houben 2003), or cause some selectively advantageous effect (Teoh and Rees 1977; Jones and Rees 1982). In low number, the B chromosomes show slight or null impact on their host individuals. However, higher numbers of B chromosomes cause phenotypic differences and reduce fertility (Jones and Rees 1982; Jones 1991, 1995; Camacho et al. 2000; Camacho 2005; Jones and Houben 2003; Jenkins and Jones 2004; Jones et al. 2008a, 2008b). Nonetheless, one of the great enigmas of B chromosomes is the near impossibility of showing any selective advantage that could explain their large polymorphism (Jones et al. 2008b). In the cytological analysis carried out here, it was not possible to distinguish the populations or individuals with B chromosomes from those who lack these for some particular feature. Therefore, apparently B chromosomes found in *C. verbascifolia* have no effect on phenotype or individual development.

In many cases a significant correlation between the B chromosomes and their geographical distribution exists. They are sometimes related to their origin, e.g. *Clarkia* Pursh (Lewis 1951, 1954) and rye (Müntzing 1958); and at other times to ecological factors such as in *Centaurea scabiosa* L. (Fröst 1958). Among the populations of *C. verbascifolia* analysed in this study, population 34 was the one with greater number and frequency of B chromosomes. Geographically, it is located farthest away, with respect to the others. Further investigations in specimens of *C. verbascifolia* from different localities are needed to determine whether B chromosomes are maintained in geographically distinct populations and their probable modes of origins.

With regard to the viability of the pollen grains, all meiotic anomalies found in *C. verbascifolia* have been recorded in the literature as possibly responsible for pollen sterility (Pagliarini 2000; Diao et al. 2009). However, the presence of these irregularities and the high frequency of B chromosomes does not seem to significantly affect the course of meiosis, as pollen fertility was high in all populations (> 90%).

Disclosure statement

No potential conflict of interest was reported by the authors.

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