

Ribosomal DNA, heterochromatin, and correlation with genome size in diploid and polyploid North American endemic sagebrushes (*Artemisia*, Asteraceae)

Sònia Garcia, Teresa Garnatje, Jaume Pellicer, E. Durant McArthur, Sonja Siljak-Yakovlev, and Joan Vallès

Abstract: Subgenus *Tridentatae* (*Artemisia*, Asteraceae) can be considered a polyploid complex. Both polyploidy and hybridization have been documented in the *Tridentatae*. Fluorescent in situ hybridization (FISH) and fluorochrome banding were used to detect and analyze ribosomal DNA changes linked to polyploidization in this group by studying four diploid-polyploid species pairs. In addition, genome sizes and heterochromatin patterns were compared between these populations. The linked 5S and 35S rRNA genes are confirmed as characteristic for *Artemisia*, and a pattern at the diploid level of three rDNA loci located at telomeric positions proved to be typical. Loss of rDNA loci was observed in some polyploids, whereas others showed additivity with respect to their diploid relatives. Genome downsizing was observed in all polyploids. Banding patterns differed depending on the pair of species analysed, but some polyploid populations showed an increased number of heterochromatic bands. FISH and fluorochrome banding were useful in determining the systematic position of *Artemisia bigelovii*, for which a differential pattern was found as compared with the rest of the group. Additionally, FISH was used to detect the presence of the *Arabidopsis*-type telomere repeat for the first time in *Artemisia*.

Key words: allopolyploidy, *Arabidopsis*-type telomere, autopolyploidy, Compositae, chromomycin, DAPI, FISH, fluorochrome banding, genome organization, *Tridentatae*.

Résumé : Le sous-genre *Tridentatae* (*Artemisia*, Asteraceae) peut être considéré un complexe polyploïde. L'autopolyploïdie et l'allopolyploïdie sont des facteurs bien documentés comme ayant joué un rôle dans l'évolution de ces espèces. L'hybridation in situ fluorescente (FISH) et la coloration différentielle aux fluorochromes ont été utilisées pour la détection et l'analyse des changements de l'ADN ribosomique qui sont rattachés à la polyploïdisation chez ce groupe par le biais de l'étude de quatre couples diploïde-polyploïde. La taille du génome et les modèles de répartition de l'hétérochromatine ont été aussi comparés entre ces populations. Les gènes liés 5S et 35S sont confirmés comme typiques d'*Artemisia* et un modèle constitué, au niveau diploïde, par trois loci de l'ADN ribosomique en position télomérique s'est avéré comme typique du sous-genre *Tridentatae*. Des pertes de loci de l'ADN ribosomique ont été observées chez quelques polyploïdes, tandis que d'autres ont montré de l'additivité par rapport à leurs diploïdes. Une diminution de la taille du génome haploïde a été observée chez tous les polyploïdes. Les distributions des bandes marquées aux fluorochromes sont différentes selon la paire d'espèces diploïde-polyploïde étudiée, mais quelques populations polyploïdes montrent un nombre plus élevé de bandes hétérochromatiques. L'utilité taxonomique de l'analyse FISH et de la coloration différentielle aux fluorochromes est exploitée pour élucider la position d'*Artemisia bigelovii*, où un modèle particulier a été trouvé en comparaison avec le reste du groupe. En plus, l'analyse FISH a servi à détecter la présence de la répétition télomérique du type *Arabidopsis* pour la première fois chez le genre.

Mots-clés : allopolyploïdie, *Arabidopsis*-type télomère, autopolyploïdie, Compositae, chromomycine, DAPI, FISH, coloration différentielle aux fluorochromes, organisation génomique, *Tridentatae*.

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Introduction

The subgenus *Tridentatae* of the genus *Artemisia* (Asteraceae) is a natural grouping of species commonly called sagebrushes, which are endemic to western North America (Beetle 1960; McArthur et al. 1981; Shultz 2006). The subgenus consists of about a dozen landscape-dominant and xerophytic taxa, probably the most common woody plants in terms of area occupied and number of individual plants in the western United States. Due to their ecological and economical importance, sagebrush species have been the subject of numerous studies in different disciplines. Karyological studies are particularly abundant (McArthur and Sanderson 1999; Garcia et al. 2007 and references therein). Polyploidy and interspecific hybridization have been important in the speciation and diversification of the central sagebrush species core (McArthur et al. 1981). *Tridentatae* species exhibit abundant polyploidy, a feature that is also widespread in *Artemisia* as a whole (Ehrendorfer 1964; Estes 1969; Persson 1974; Stahevitch and Wojtas 1988; Vallès and Siljak-Yakovlev 1997; Garcia et al. 2004; Pellicer et al. 2007). Some taxa are thought to be of hybrid origin (McArthur et al. 1988; Winward and McArthur 1995). The most abundant species (*A. arbuscula*, *A. cana*, *A. nova*, and *A. tridentata*) as well as many less common species (*A. bigelovii*, *A. longiloba*, *A. rigida*, *A. rothrockii*, and *A. tripartita*) exhibit broad polyploidy and hybridization phenomena, especially evident at ecotonal interfaces and within populations (McArthur and Sanderson 1999). Given the extensive and overlapping areas of distribution of its species, *Artemisia* subgenus *Tridentatae* fits the definition of a polyploid complex, in which taxonomically close and interbreeding plants with different ploidy levels allow genetic exchanges between species, creating a complex network of interrelated taxa (Babcock and Stebbins 1938).

Interspecific hybridization and polyploidy are widespread phenomena in flowering plants and are considered key elements in plant diversification (Stebbins 1950; Levin 1983; Soltis and Soltis 1999; Otto and Whitton 2000). Whole genome duplications have played a key role in the diversification of plants (Masterson 1994), and molecular analyses suggest that the genomes of more than 90% of extant angiosperms have undergone one or more ancient genome-wide duplications (Cui et al. 2006). Although it is not entirely clear why polyploids are so abundant among angiosperms, their prevalence and success imply that polyploidy can confer some evolutionary advantage. This is usually attributed to the genetic variability of polyploids, as they can harbour greater genetic diversity than their diploid relatives, especially in the case of allopolyploids (Doyle et al. 1999). It is known that polyploidy causes many genetic and epigenetic changes such as sequence rearrangements, activation of transposable elements, gene silencing, DNA methylation alteration, chromatin remodeling, and genome downsizing (Adams and Wendel 2005; Leitch and Leitch 2008), processes that can arise readily after the onset of polyploidy or later within several generations. Polyploidy can also have a buffering effect of gene redundancy on mutations (Comai 2005), although it is also possible that the additional DNA may not have any significant function but simply is not strongly selected against (Leitch et al. 2008).

Research in plant genomes, polyploids included, has been enhanced by use of fluorescent in situ hybridization (FISH) methodology. The most commonly used probes in FISH are ribosomal DNAs, given their abundance and highly conserved sequence in vascular plants. Fluorochrome banding has accompanied FISH in many studies, contributing more data on heterochromatin distribution and composition in both diploids and polyploids. These techniques have been useful for karyotype characterization, enabling the discrimination of different chromosomes (Castilho and Heslop-Harrison 1995; Moscone et al. 1999; Hasterok et al. 2006; Garcia et al. 2007), and in understanding genomic rearrangements in polyploids (Heslop-Harrison 1991, 2000; Lim et al. 2007; Leitch et al. 2008). As the number of rDNA loci and banding patterns may be conserved or vary significantly between related taxa, these procedures have an application in plant systematics, revealing interspecific relationships. FISH has been useful in addressing chromosomal evolutionary questions and providing better information on genome organization (Lim et al. 2000, 2007; Pires et al. 2004; Kovářik et al. 2005; D'Hont 2005). Current application of the FISH technique allows mapping of species-specific repetitive DNAs or single-copy genes (Weiss-Schneeweiss et al. 2007). Even so, a substantial portion of plant cytogenetic research is still being done with the classical rDNA probes and fluorochromes.

The use of molecular cytogenetic tools has contributed interesting data in the study of karyotype structure and evolutionary relationships in the genus *Artemisia* (Mendelak and Schweizer 1986; Oliva and Vallès 1994; Vallès and Siljak-Yakovlev 1997; Torrell et al. 2001, 2003; Garcia et al. 2007; Pellicer et al. 2008). Data for some 30 *Artemisia* species of different ploidy levels are currently available, which facilitates summarizing the general characteristics of *Artemisia* karyotypes. Perhaps the most distinctive feature of *Artemisia* genomes is their unusual tandemly arranged ribosomal DNA unit (see Fig. 1A) that integrates the 5S rRNA gene in the intergenic spacer of the large 35S rRNA gene, as described recently by Garcia et al. (2009). In addition, typical *Artemisia* species have symmetrical karyotypes formed by mostly metacentric and submetacentric chromosomes, with 2 to 4 always telomeric ribosomal DNA loci at the diploid level. Banding patterns of GC- or AT-rich DNA are more variable among species (Vallès and Siljak-Yakovlev 1997; Torrell et al. 2001, 2003), with a usually telomeric position. However, telomere sequence composition, to our knowledge, has never been assessed in *Artemisia*.

We have conducted fluorochrome banding and in situ hybridization assays with the primary objective of describing and comparing ribosomal DNA and heterochromatin patterns in several diploid-polyploid species pairs within the subgenus *Tridentatae*. The study complements a previous report on this subgenus (Garcia et al. 2007) and aims to characterize *Artemisia bigelovii*, one of its systematically problematic species (Kornkven et al. 1998; Shultz 2006). We also discuss a possible relationship between the observed changes in genome size in polyploids (Garcia et al. 2008), the signal pattern differences of these species, and diploidization of the polyploid karyotypes. Finally, we describe the presence of the most usual plant telomeric repeat, (TTTAGGG)_n, in *Artemisia*.

Table 1. Provenance of the populations of *Artemisia* studied.

Taxon	Origin of materials	Ploidy level	Collection No.*
<i>A. bigelovii</i> Gray	Emery Co., Utah, USA; 1801 m	2x	2869
<i>A. bigelovii</i> Gray	15 km east of Fremont Junction, Emery Co., Utah, USA; 1777 m	4x	3050
<i>A. nova</i> A. Nels.	Birch Springs Road, Mount Borah, Custer Co., Idaho, USA; 2120 m	2x	3053
<i>A. nova</i> A. Nels.	Tunnel Spring, Desert Experimental Range, Millard Co., Utah, USA; 1703 m	4x	2876
<i>A. nova</i> A. Nels. var. <i>duchesnicola</i> Welsh & Goodrich	Tridell Road, Uintah Co., Utah, USA; 1702 m	6x	3029
<i>A. tridentata</i> Nutt. subsp. <i>tridentata</i>	Salt Cave Hollow, Salt Creek Canyon, Juab Co., Utah, USA; 1870 m	2x	2871
<i>A. tridentata</i> Nutt. subsp. <i>tridentata</i>	Dove Creek, Dolores Co., Colorado, USA; 2167 m	4x [†]	U74
<i>A. tripartita</i> Rydb. subsp. <i>tripartita</i>	Dubois Sheep Station, Clark Co., Idaho, USA; 1650 m	2x and 4x [†]	2845

*E.D. McArthur collection numbers; vouchers are deposited in the herbarium of the Shrub Sciences Laboratory, Rocky Mountain Research Station, Provo, Utah, USA (SSLP).

[†]These populations were originally described as diploids, but spontaneous tetraploid cytotypes have been found, as presented here.

Material and methods

Plant material

Ripe achenes from adult plants were collected from wild populations of each taxon. Root tip meristems from seedlings were obtained by germinating them on wet filter paper in Petri dishes in the dark at room temperature. At least 10 individuals were analyzed per population. The provenance of the species studied is shown in Table 1.

Chromosome preparations

Root tips were pretreated either with 0.05% aqueous colchicine or with a saturated aqueous solution of Gammexane (hexachlorocyclohexane; Sigma Aldrich) at room temperature for 2 h 30 min to 4 h and subsequently fixed in absolute ethanol and glacial acetic acid (3:1).

Chromosome preparations for fluorochrome banding and in situ hybridization were done using the air-drying technique of Geber and Schweizer (1987), with some modifications: root tips were washed with agitation in citrate buffer (0.01 mol/L citric acid – sodium citrate, pH 4.6) for 15 min, excised, and incubated in an enzyme solution (4% cellulase Onozuka R10 [Yakult Honsha], 1% pectolyase Y23 [Sigma], and 4% hemicellulase [Sigma]) at 37 °C for 20 to 25 min, depending on the species and meristematic thickness. Chromosome squashes were prepared following the enzymatic softening of material, as described in Leitch et al. (2001).

Fluorochrome banding

To reveal GC-rich DNA bands, chromomycin A₃ (CMA₃) was used, following the techniques of Schweizer (1976), Kondo and Hizume (1982), Cerbah et al. (1995), and Coulaud et al. (1995) with minor modifications: the slides were incubated in McIlvaine buffer, pH 7; treated with distamycin A (0.1 g/L in McIlvaine buffer, pH 7) for 10 min; stained with CMA₃ (0.1 g/L in McIlvaine buffer, pH 7, plus 5 mmol/L MgSO₄) for 60 min; rinsed in the same buffer;

counterstained with methyl green (0.1% in McIlvaine buffer, pH 5.5) for 10 min; rinsed in McIlvaine buffer, pH 5.5; and mounted in 1:1 glycerol – McIlvaine buffer, pH 7.

Fluorescent in situ hybridization

The same slides used for fluorochrome banding with chromomycin were faded with fixative, dehydrated through an ethanol series (70%, 90%, and 100%), and dried overnight (except in the case of *A. nova* var. *duchesnicola*, for which we only obtained FISH data). DNA hybridization was carried out according to the protocol described in Torrell et al. (2003), with minor changes. The probe used for 18S–5.8S–26S rDNA localization was a clone of a 9 kb *Eco*RI fragment of 18S–5.8S–26S rDNA and the intergenic sequence cloned from *Triticum aestivum* (Gerlach and Bedbrook 1979). This probe was directly labelled with Cy3 red (Amersham) by PCR. The probe used for 5S rDNA localization was a clone of pTa794, a 410 kb *Bam*HI fragment of 5S rDNA isolated from wheat and cloned in pBR322 (Gerlach and Dyer 1980). It contains the 5S rRNA gene (120 bp) and the noncoding intergenic spacer (290 bp). This probe was labelled with digoxigenin-11-dUTP-green (Boehringer Mannheim) by PCR. The plant (*Arabidopsis*-type) telomeric repeat probe (TTAGGG)_n was also labelled with digoxigenin-11-dUTP by PCR. Preparations were incubated in 100 µg/mL DNase-free RNase in 2× SSC (salt sodium citrate: 0.03 mol/L sodium citrate and 0.3 mol/L sodium chloride) for 1 h at 37 °C in a wet chamber, washed twice in 2× SSC for 5 min with slow shaking, incubated in pepsin (0.1 mg/mL in 0.01 N HCl), washed 3 times in 2× SSC for 5 min, dehydrated through an ethanol series (70%, 90%, and 100%), and air-dried. The rDNA probes were mixed for simultaneous hybridization, each at a concentration of 25–100 ng/µL, with 50% (v/v) formamide, 10% (w/v) dextran sulfate, 0.1% (w/v) sodium dodecyl sulfate, 250 µg/mL salmon sperm, and 20× SSC. The probe mixture was denatured at 75–80 °C for 10 min and immediately chilled on ice for 5 min to inhibit renaturation. Approximately 50 µL

Table 2. Number of chromomycin (CMA₃)-positive bands, number of rDNA loci, and genome size (2C value) of the diploid-tetraploid pairs of species studied.

Species	2x populations			4x populations			
	CMA ₃	rDNA	2C (pg)	CMA ₃	rDNA	2C (pg)	Reduction (%)
<i>A. bigelovii</i>	4	4	8.00	8	8 (4 weak)	15.32	4.25
<i>A. nova</i>	6	6	9.09	16	12 (6 weak)	17.10	5.9
<i>A. tridentata</i> subsp. <i>tridentata</i>	5/6	5/6	8.24	18–20	11	15.87	3.7
<i>A. tripartita</i> subsp. <i>tripartita</i>	6	6	8.85	12	6–8	15.32	13.44

Note: Data on genome sizes correspond to the mean values of the populations assessed up to now (Torrell and Vallès 2001; Garcia et al. 2004, 2008). In 4x populations, “Reduction” is the percent diminution of genome size of tetraploids with respect to diploids.

aliquots of probe mixture were loaded on the slides and covered with plastic cover slips. The preparations were then denatured for 10 min at 75–80 °C and transferred for 5 min to 55 °C. Hybridization was carried out overnight at 37 °C in a wet chamber. Post-hybridization washes were done with agitation as follows: after a first wash in 2× SSC at room temperature for 3 min, several washes for 5 min were done at 42 °C (three in 2× SSC, two in 20% formamide, one in 0.1× SSC, three in 2× SSC, and one in 4× SSC with 0.2% Tween 20), and a final wash was performed for 5 min in 4× SSC with 0.2% Tween 20 at room temperature. For 5S rDNA and plant telomere signal detection, the slides were treated with 5% (w/v) BSA in 4× SSC with 0.2% Tween 20 for 5 min, incubated for 1 h at 37 °C in 20 µg/mL anti-digoxigenin–fluorescein (Boehringer Mannheim) in the same buffer, and washed three times for 5 min also with the same buffer at room temperature. Counterstaining was done with Vectashield, a mounting medium containing DAPI. Hence, DAPI bands detected in some cases (Fig. 1C, panel 4; Fig. 2A, panel 4; Fig. 2D) are the result of denaturation after hybridization, when DAPI stains any constitutive heterochromatin. FISH preparations were observed with a Zeiss Axiophot epifluorescence microscope with different combinations of Zeiss excitation and emission filter sets (01, 07, and 15). Hybridization signals were analysed and photographed using a highly sensitive CCD camera (Princeton Instruments) and image analyser software (MetaVue, version 4.6, Molecular Devices Corporation). In some cases chromomycin staining and FISH have been photographed on the same metaphases (Fig. 1C, panels 1 and 2; Fig. 2A, panels 1 and 2; Fig. 2B, panels 3 and 4), whereas in other cases we show different metaphases. This is mainly because some chromomycin metaphases were over-denatured after hybridization and chromosome morphology was partially lost, so we preferred showing more intact chromosomes from other metaphase plates. Correlation of chromomycin and FISH signals was based on at least 10 different metaphases from different individuals. Images were processed for colour balance, contrast, and brightness uniformity with Adobe Photoshop (version 7.0.1). Graphics of the haploid idiograms were performed with PowerPoint (Microsoft Office XP Professional version SP3) based on the results from Garcia et al. (2007).

Results

Chromosome complements are $2n = 2x = 18$ for diploids, $2n = 4x = 36$ for tetraploids, and $2n = 6x = 54$ for the only

hexaploid studied (Table 2 summarizes results obtained for both 2x and 4x populations). All signals observed are located at telomeric regions. Karyotypes are rather symmetrical and most chromosomes are metacentric to submetacentric. As mentioned previously, all *Artemisia* species possess a characteristic ribosomal DNA type with both 5S and 35S rDNA unified to a single unit. Therefore, red (35S) and green (5S) probes hybridize to the same regions in these species and composed images show rDNA sites in an orange-red colour. Chromomycin (guanine-cytosine specific fluorochrome) stains only the same sites as rDNA probes in these diploids, but in some polyploids additional GC-rich bands are present (such as *A. nova* and *A. tridentata* subsp. *tridentata*).

Diploid *A. bigelovii* (Fig. 1B, panels 1 and 2) has 4 rDNA sites located at the short arms of one metacentric and one submetacentric chromosome pair. The remaining diploids show different numbers of signals: both *A. nova* (Fig. 1C, panels 1 and 2) and *A. tripartita* subsp. *tripartita* (Fig. 2B, panels 1 and 2) always present 6 GC-rich bands co-localized with rDNA sites; for *A. tridentata* subsp. *tridentata*, two cytotypes with either 5 or 6 rDNA sites have been found, belonging to different individuals of the same population (Fig. 2A). In some cases rDNA signals are clearly different in size, which allows distinguishing homologues at metaphase.

Tetraploid *A. bigelovii* shows 8 rDNA sites, 4 of which are weakly and the others more strongly stained (Fig. 1B, panel 4); all these rDNA sites co-localize with GC-rich DNA, and there are no additional chromomycin-positive bands. The tetraploid *A. nova* presents 16 GC-rich bands, with one chromosome pair stained at both ends; 12 of these sites also exhibit rDNA (Fig. 1C, panels 3 and 4). A non-reciprocal chromosomal translocation has also been detected for this population (marked with asterisks in Fig. 1C, panel 3). *Artemisia tridentata* subsp. *tridentata* shows 18 to 22 chromomycin-positive bands (depending on the individual observed), with one chromosome pair marked at both ends, and 11 sites presenting rDNA signals (Fig. 2A, panels 3 and 4) in all the individuals observed. The tetraploid *A. tripartita* subsp. *tripartita* (Fig. 2B, panels 3 and 4) has 12 to 14 GC-rich bands, 6–8 (depending on the individual) of which are co-localized with rDNAs. The hexaploid *A. nova* var. *duchensis* (Fig. 2D) presents 16 rDNA sites.

DAPI used as a counterstain after hybridization experiments revealed 8 bands in tetraploid *A. nova* (Fig. 1C, panel 4), 16 bands in tetraploid *A. tridentata* subsp. *tridentata* (Fig. 2A, panel 4), and 30 bands in the hexaploid *A. nova*

Fig. 1. (A) Schematic representation of 5S and 35S ribosomal RNA genes in most angiosperms (1) and in genus *Artemisia* (2), with both rRNA genes linked in the same unit. (B) Chromomycin (1, 3), FISH (2, 4), and a representation of the chromosomes carrying rDNA loci for diploid and tetraploid *Artemisia bigelovii*. Arrows in B1 and B3 indicate faint CMA₃-positive signals. Note weaker rDNA sites in B4. (C) Chromomycin (1, 3), FISH (2, 4), and a representation of the chromosome pairs carrying rDNA loci for diploid and tetraploid *Artemisia nova*. Open arrows in C3 mark a characteristic chromosome pair with CMA₃-positive bands at both ends; asterisks indicate a nonreciprocal chromosomal translocation. Scale bars = 10 μ m.

var. *duchesnicola* (Fig. 2D), always located at telomeric positions and in some cases coincident with CMA₃-positive bands. The Vectashield counterstaining after FISH procedure does not always allow detection of DAPI-positive bands (it depends strongly on the quality of materials and on the particularities of the methodology itself), so such bands may also be present but not visible in the other taxa.

Finally, the *Arabidopsis*-type telomere probe efficiently bonded to the ends of chromosomes in the tetraploid *A. nova* (Fig. 2C).

Discussion

The unique *Artemisia* rDNA and the *Tridentatae* karyotype

This study confirms previous reports that ribosomal DNA structure in *Artemisia* genomes has diverged radically from the expectation (Torrell et al. 2003; Garcia et al. 2007). As mentioned previously, contrary to the most typical separate organization of 5S and 35S rDNA in flowering plants (Fig. 1A, panel 1), in *Artemisia* both ribosomal RNA genes have become unified in a single unit (Fig. 1A, panel 2). This unusual organization of ribosomal DNA has been confirmed by PCR assays, Southern blot hybridization, and sequencing of this region (Garcia et al. 2009), which show that the 5S rRNA gene is embedded, in inverted orientation, in the intergenic spacer of the 35S rRNA gene unit. This discovery is the first clear evidence in angiosperms of an alternative organization of both ribosomal DNAs in a single tandemly repeated motif. A similar structure may also be common in the subfamily Asteroideae branch that includes the tribes Anthemideae and Gnaphalieae (sensu Bremer and Humphries 1993; Kadereit and Jeffrey 2007). Our ongoing studies will determine the precise distribution and structure of rDNA units and the extent of this trait or similar organizations in the Asteraceae.

Besides this peculiar rDNA organization, the data provided here also demonstrate a conserved karyotype structure for members of *Artemisia* subgenus *Tridentatae*; this structure is different from that of other subgenera and gives additional support to the distinctive placement of *Tridentatae* in molecular phylogenies (Kornkven et al. 1999; Sanz et al. 2008). The signal pattern of *A. bigelovii* (4 rDNA sites in the diploid and 8 in the tetraploid, Fig. 1B, panels 1 and 2) differs from the structure of the typical *Tridentatae* karyotype, which shows 6 rDNA sites at the diploid level (Table 2; Garcia et al. 2007). This contributes additional evidence of the possible misplacement of this species within the true sagebrushes (subgenus *Tridentatae*), as also suggested by floral morphology, essential oil composition, and molecular phylogeny (Holbo and Mzingo 1965; Geissman and Irwin 1973; Kornkven et al. 1998). *Artemisia bigelovii*, although generally treated as a species of *Tridentatae* on the basis of

other characters (wood anatomy, leaf form, karyotype morphology, RAPD genetic markers, and chloroplast DNA restriction site variation; McArthur et al. 1981, 1998; Kornkven et al. 1999), has also been considered to occupy an intermediate position between subgenus *Tridentatae* (the true sagebrushes) and subgenus *Artemisia*, particularly because the morphology of the flower heads is remarkably different. *Artemisia filifolia*, a species considered a member of subgenus *Dracunculus* but closely related to the sagebrushes, also shows the same floral morphology and only 4 rDNA sites (Garcia et al. 2007), and a molecular phylogeny based on chloroplast DNA restriction site variation placed *A. bigelovii* and *A. filifolia* together in the same clade (Kornkven et al. 1999). Similarly, *A. rigida*, another species classically included in the *Tridentatae* but, like *A. bigelovii*, of uncertain taxonomic placement, also revealed a differential signal pattern (Garcia et al. 2008). Molecular phylogenetic studies based on different nuclear and chloroplast DNA regions will shed light on the relationships between these species.

Heterogeneity in ribosomal DNA loci number

Apart from *A. bigelovii*, all the other diploid taxa of the study, *A. nova*, *A. tridentata*, and *A. tripartita*, present 6 rDNA sites. This pattern, together with their increased genome and chromosome size (Torrell et al. 2003; Garcia et al. 2007), distinguishes the *Tridentatae* from the remaining *Artemisia* subgenera. However, in *A. tridentata* subsp. *tridentata* (the only studied population of this species in the present work), we have found individuals, in similar proportions, with either 5 or 6 ribosomal DNA sites (Fig. 2A, panels 1 and 2 show the cytotype with only 5 sites). For another subspecies, the diploid *A. tridentata* subsp. *spiciformis* (Torrell et al. 2003), 6 ribosomal DNA sites were invariably detected in the population studied. We have also found individuals with either 6 or 8 ribosomal DNA sites in the tetraploid *A. tripartita* subsp. *tripartita*. A gene copy loss process could account for missing sites in these species.

Studies have shown that polymorphism in the number and chromosomal distribution of 5S and 35S rDNA loci can occur both among different subspecies and varieties of a given species and within a population (Hasterok et al. 2006; Pedrosa-Harand et al. 2006). A review of plant cytogenetic papers shows that differences in the number of rDNA sites within a species are not unusual; for instance, two studies report different numbers of 5S and 35S rDNA sites for *Brassica napus* (Schrader et al. 2000; Hasterok et al. 2001). Moscone et al. (1999) reported two different cultivars of *Phaseolus vulgaris* showing different numbers of rDNA loci, and later Pedrosa-Harand et al. (2006) demonstrated a high degree of variation in the number of 35S rDNA sites for this species, from 2 to 9 depending on the population studied, and also noted variation within some populations.

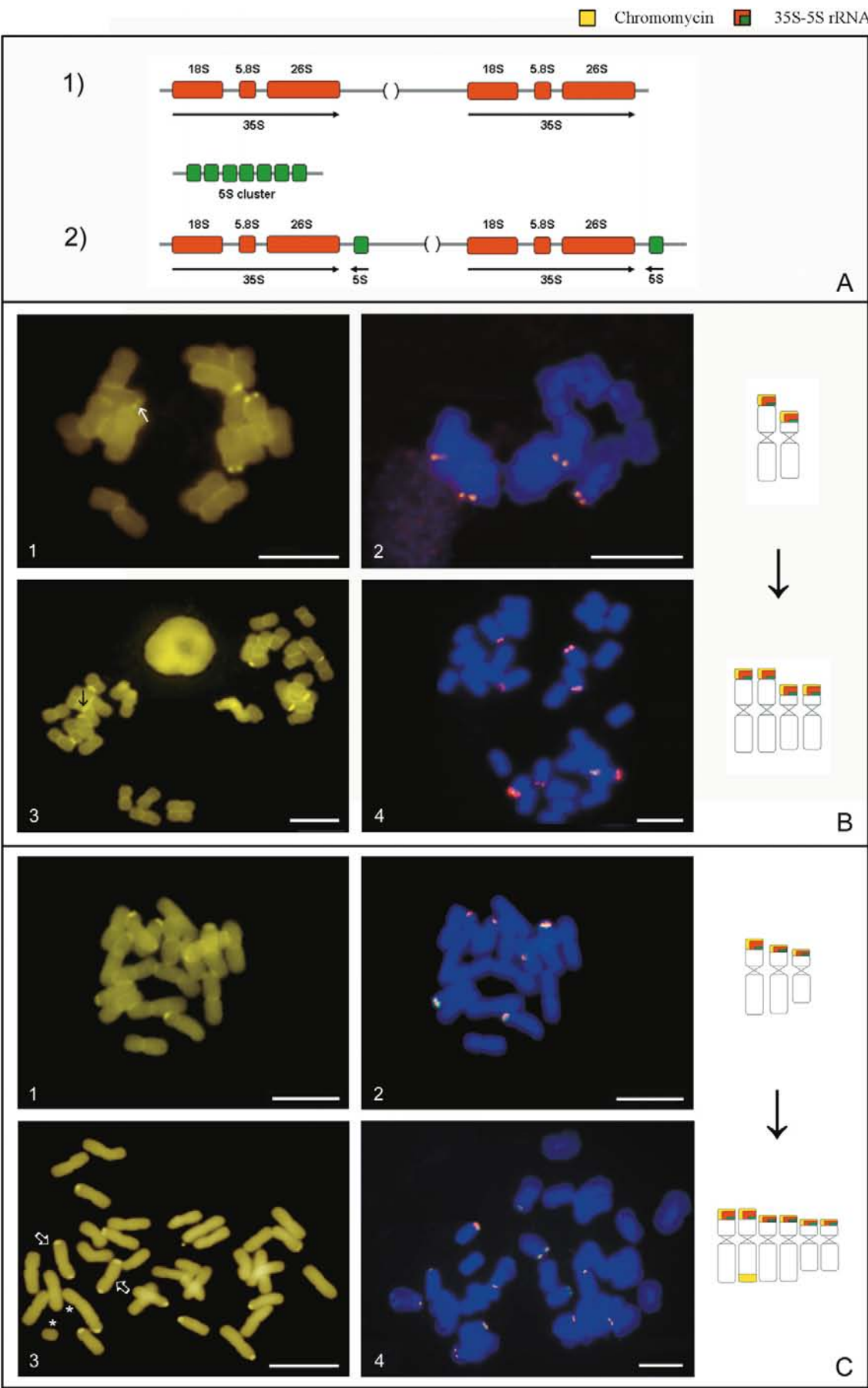


Fig. 2. (A) Chromomycin (1, 3), FISH (2, 4), and a representation of the chromosomes carrying rDNA loci for diploid and tetraploid *Artemisia tridentata* subsp. *tridentata*. Arrows in A3 indicate faint CMA₃-positive signals and open arrows mark a characteristic chromosome pair with CMA₃-positive bands at both ends; arrows in A4 point to DAPI-positive bands not coincident with CMA₃-positive bands. The chromosome marked with an asterisk does not represent a chromosome pair, but a single chromosome. (B) Chromomycin (1, 3), FISH (2, 4), and a representation of the chromosome pairs carrying rDNA loci for diploid and tetraploid *A. tripartita* subsp. *tripartita*. The asterisk indicates the occasional presence of one chromosome with CMA₃-positive bands at both ends. (C) *Arabidopsis*-type telomere probe hybridizing in a metaphase and prometaphase plate of tetraploid *A. nova*. (D) FISH of *A. nova* var. *duchesnicola*. Note the strong DAPI-positive bands at many chromosome ends. Scale bars = 10 μ m.

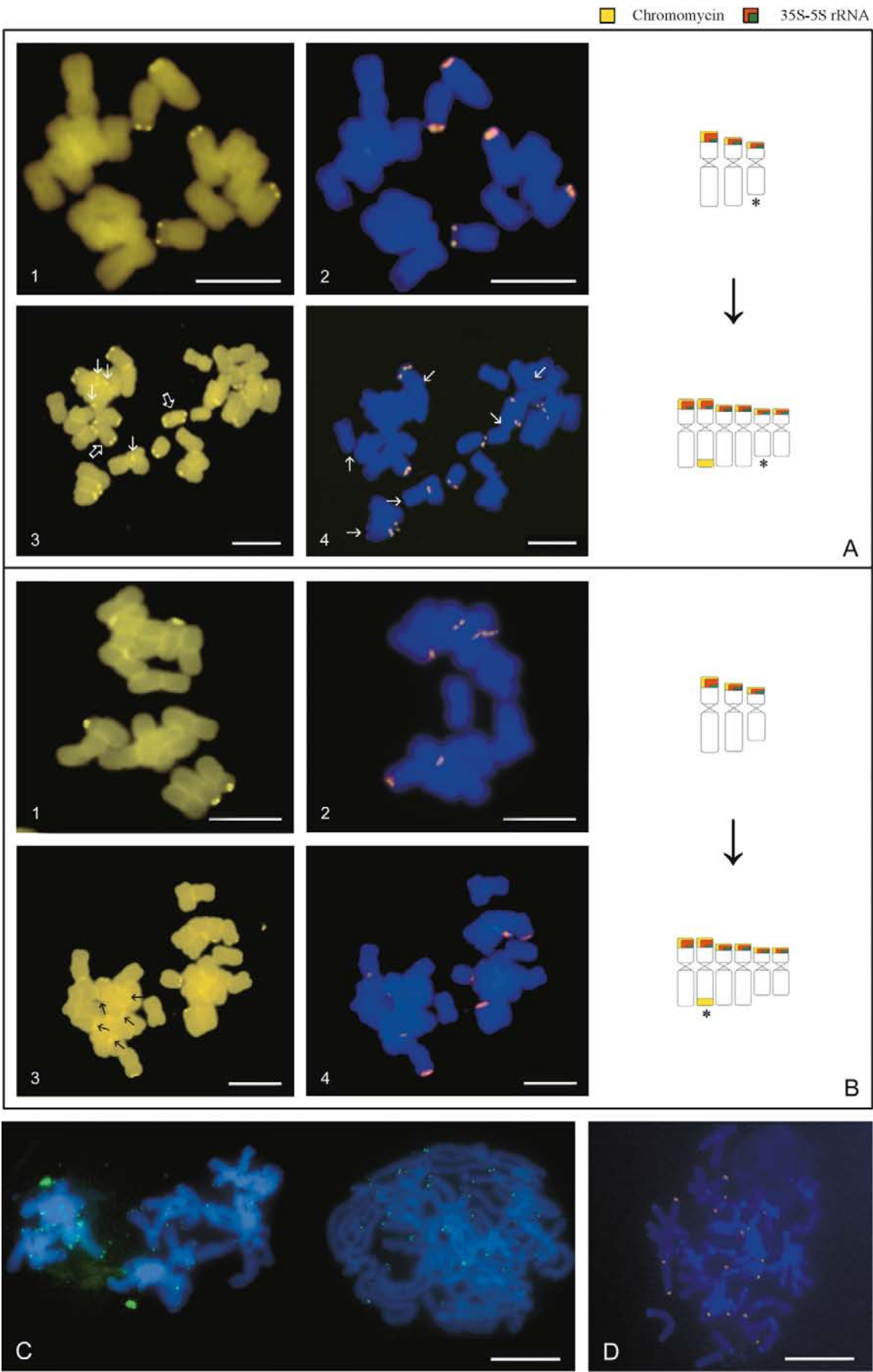
Youn-Kyu et al. (1999) described four 5S and eight 35S rDNA sites for *Capsicum anuum*, whereas Kwon and Kim (2009) reported two 5S and two and six 35S rDNA sites for different varieties of that species. Different variants of *Allium tuberosum* have also shown different numbers of 35S rDNA sites (Do et al. 1999). Pedrosa-Harand et al. (2006) explain that a mechanism responsible for this variation could be unequal interlocus crossing over: if recombination occurs between a chromosome end bearing a distal rDNA site and another end without rDNA, a new rDNA locus can be generated. In contrast, some genera show invariable rDNA loci number and karyotype structure; for example, all *Quercus* species studied by Zoldos et al. (1999) or all *Coffea* diploids investigated by Lombello and Pinto-Maglio (2004) display the same number and distribution of 5S and 35S rDNA loci.

Ribosomal DNA loci number, genome downsizing, and polyploidy

Ribosomal DNA loci or copy number variation and genome downsizing, in which the nuclear DNA amount of the polyploid is less than the sum of genome sizes of the parental diploids, have been related to polyploidization (Volkov et al. 1999; Lim et al. 2000; Leitch et al. 2008; Leitch and Leitch 2008). Therefore, one of the main goals of the present study was to compare rDNA and genome size data of diploid-polyploid species pairs. In all cases, monoploid genome size loss was detected in tetraploids with respect to diploids (Table 2), and for the hexaploid *A. nova* var. *duchesnicola* (mean genome size 22, 66 pg; Garcia et al. 2008), the loss is almost 17% compared with the diploid *A. nova*. In these species, the higher the ploidy level attained, the larger the loss. We had already reported genome downsizing in *Artemisia* (Torrell and Vallès 2001; Garcia et al. 2004; Pellicer et al. 2007) and particularly in the *Tridentatae* polyploids (Garcia et al. 2008). However, in some other Asteraceae genera, genome sizes of polyploid taxa present additivity with respect to the genome sizes of their presumed diploid parents or their most closely related diploid ancestors (Price and Bachmann 1975; Marchi et al. 1983; Cerbah et al. 1999; Garnatje et al. 2006). In addition, there are also some limited data pointing to genome upsizing in older polyploids (Leitch et al. 2008). Nevertheless, genome downsizing is a widespread biological response to polyploidization, leading to diploidization of the polyploid genome (Leitch and Bennett 2004). Mechanisms resulting in loss of DNA can be an increased rate of illegitimate recombination (Grover et al. 2007), unequal homologous recombination, or the elimination of specific genes or DNA sequences (Ozkan et al. 2001). Loss of genes, including ribosomal DNA copies, has been reported in several polyploid groups, among them some intensively studied systems, e.g., *Nicotiana*, *Brassica*, and *Glycine* (Leitch and Bennett 2004).

Concerning the number of rDNA loci of the polyploids with respect to the diploids, two tendencies are present in the studied species. The first tendency is additivity, i.e., the number of rDNA sites in the tetraploids is twice the number found in diploids. This is the case for *A. bigelovii* and *A. nova*. An additive pattern of rDNA loci has been previously reported for the allopolyploid *Nicotiana rustica*, albeit with gene conversion to the parental rDNA type (Matyásek et al. 2003; Kovařík et al. 2004). In *Tragopogon*, an Asteraceae genus like *Artemisia*, the number of rDNA loci in some polyploids was also additive, and the distribution similar, to those observed in the diploids (Pires et al. 2004). Nevertheless, many weaker signals were detected in polyploid *A. bigelovii* and *A. nova* (well visible in the tetraploid *A. bigelovii*, Fig. 1B, panel 4), so it is possible that loss of gene copies might be taking place at these loci. This would be consistent with findings in *Nicotiana* polyploids, in which the number of rDNA loci was additive even though considerable sequence elimination of individual copies had taken place (Leitch et al. 2008). The second tendency is loci loss, i.e., less than the expected number of rDNA sites in the polyploid, as observed in the tetraploid *A. tripartita* subsp. *tripartita*, which shows individuals from the same population with either 6 or 8 rDNA loci (most individuals presenting 8 loci rather than 6) where 12 were expected (Fig. 2B, panel 4). Another case of rDNA site loss after polyploidization might have occurred in *A. tridentata* subsp. *tridentata*, where only 11 sites are present instead of the 12 expected, although this could also have been a consequence of crossing between the diploid cytotype carrying 5 sites (see above) and the cytotype with 6 sites (Fig. 2A, panel 4). Finally, the single hexaploid studied, *A. nova* var. *duchesnicola*, shows 16 rDNA sites instead of the 18 expected from the diploid *A. nova* (Fig. 2D). Ribosomal DNA loci loss after polyploidization has also been reported in other Asteraceae genera such as *Xeranthemum* (Garnatje et al. 2004) and in members of other plant families, i.e., *Sanguisorba* (Mishima et al. 2002) and *Zingiberia* (Kotseruba et al. 2003), among others.

There might be a relationship between the accentuated genome downsizing and the low number of rDNA loci for the diploid-polyploid pair of *A. tripartita* subsp. *tripartita* (see Table 2). The whole genome downsizing cannot be directly attributable to a diminution in rDNA loci or copy number; however, it is possible that ribosomal DNA, as a highly repetitive gene family, while itself making up a small part of the genome may bear some association with overall genome size (Prokopowich et al. 2003), and therefore part of this genome size loss may actually correspond to ribosomal DNA loci (or copy number) loss. As the total number of rDNA copies present in a genome is much beyond that necessary to supply the requirements of ribosomes (Reeder 1999), the loss of rDNA loci or copies in polyploids would not repre-



sent a problem of transcriptional requirements to these species.

The case of *A. tripartita* subsp. *tripartita* is also interesting because both diploid and tetraploid cytotypes have been found in the same population. Similarly, the tetraploid population of *A. tridentata* subsp. *tridentata* studied here comes from an originally diploid population (McArthur et al. 1981, 1988). Among the sagebrushes, spontaneous tetraploids in diploid populations have previously been detected (McArthur et al. 1981; McArthur and Sanderson 1999). The possibility of de novo production of $4x$ plants in $2x$ populations was verified in *A. tridentata* subsp. *vaseyana*, in which the recent origin of the polyploid cytotype was supported by RAPD analysis (McArthur et al. 1998). The formation of unreduced gametes in such populations is one of the most common mechanisms for the production of polyploids (Lewis 1980; Ramsey 2007). Previous work had suggested the autopolyploid nature of many of the *Tridentatae* (McArthur et al. 1981) and other *Artemisia* species (Estes 1969), although the wide occurrence of hybridization processes suggests that allopolyploidy might be a major force in the evolution of this group.

Heterochromatin bands in diploids and polyploids

Chromomycin stains not only ribosomal DNA but also other GC-rich heterochromatin regions, and DAPI is a specific fluorochrome that preferentially marks AT-rich heterochromatic regions. However, counterstaining with DAPI after FISH (and therefore after DNA denaturation and renaturation) does not reveal specifically AT-rich regions but mainly any constitutive heterochromatin, resulting in a C-banding-like pattern (Moscone et al. 1999). Considering this, we have found significant differences in the heterochromatin staining behaviour of the plants analyzed. On the one hand, diploid taxa show only GC-rich DNA bands co-localizing with ribosomal DNA sites, without any DAPI-positive band. As mentioned previously, DAPI staining depends on the quality of material and the methodology, so it is possible that some bands are actually present in these species but not visible. On the other hand, different patterns in the distribution of heterochromatin seem to occur in the tetraploids: *A. bigelovii* and *A. tripartita* subsp. *tripartita*, with double the number of GC-rich DNA bands with respect to the diploids (Table 2 and Figs. 1B and 2B), contrast with both *A. nova* and *A. tridentata* subsp. *tridentata*, which show more than twice the number of bands with respect to the diploids and have a particular chromosome pair with GC-rich DNA bands at both ends (Table 2 and Figs. 1C and 2A). The DAPI banding pattern is even more heterogeneous in these polyploids: no bands are observed for both *A. bigelovii* and *A. tripartita*, whereas 8, 16, and 30 are seen in *A. nova*, *A. tridentata*, and *A. nova* var. *duchesnicola*, respectively. Most DAPI bands coincide with chromomycin signals and additional DAPI signals are also detected in *A. tripartita* (Fig. 2A, panel 4, arrows), as Bogunic et al. (2006) reported for some *Pinus* species. Similar results were described for *A. argillosa*, another *Tridentatae* tetraploid showing 16 chromomycin-positive and 26 DAPI-positive bands (Garcia et al. 2007), and in the tetraploid *A. alba*, where many AT- and GC-rich bands were seen (Vallès and Siljak-Yakovlev

1997). The same applies to the hexaploid *A. nitida*, with many chromomycin- and DAPI-positive bands as well (Garcia et al., unpublished data). These observations suggest that in at least some *Artemisia* polyploids, new blocks of heterochromatin are emerging. It might be possible that some novel satellites, maybe species-specific repeats, have appeared in *Artemisia* polyploids. In this context, Lim et al. (2007) found new satellite repeats in the older *Nicotiana* allopolyploids, located at subtelomeric regions. A massive amplification of transposable elements as a consequence of the “genomic shock” caused by polyploidization (McClintock 1984) could also give this pattern; it is known, for example, that in wheat several tandemly arranged centromeric sequences have originated from retroelements (Cheng and Murata 2003). And finally, it is also possible that this increased number of heterochromatic signals reflects epigenetic changes associated with inactivation of chromosomal domains, promoting a higher degree of chromatin condensation (Aleš Kovařík, personal communication). All possibilities considered, the new heterochromatic blocks found in some of these polyploids together with genome downsizing and (or) rDNA loss are here interpreted as another step towards diploidization of their genomes.

Detection of the plant-telomere repeat

Telomeres are repeated highly conserved sequences that stabilize and protect chromosome ends, and most plant groups possess the *Arabidopsis*-type telomere repeat (TTTAGGG)_n, although this sequence is not ubiquitous to all flowering plants. This is the case for some groups in the order Asparagales (genus *Allium* among others) and for the genus *Cestrum* of the family Solanaceae (Fajkus et al. 2005), which lack the typical plant telomere. To our knowledge, telomere composition had never been assessed in *Artemisia*, although the most usual *Arabidopsis*-type telomere sequence was reported for the closely related genus *Chrysanthemum* (Abd El-Twab and Kondo 2006). As in *Chrysanthemum*, the *Arabidopsis*-type telomere probe hybridized at chromosome ends of *A. nova* (Fig. 2C).

Conclusions

The present study supports a typical *Tridentatae* signal pattern at the diploid level, with mostly 3 rDNA loci located at telomeric positions of metacentric and submetacentric chromosomes. Nevertheless, the analysis of more populations of each species would be desirable to definitely confirm such a pattern, since intrapopulation variation has been found in some cases. Similarities with previous research at the tetraploid level (Garcia et al. 2007) have been reported, and processes involving ribosomal DNA loci (or copy number) loss, amplification of heterochromatic DNA, and genome downsizing are found to be linked to polyploid formation in some cases; it is also likely that new tandemly arranged satellite repeats appear with polyploidy. More studies would be necessary to characterize these apparently novel repeats in *Artemisia* polyploids. From a systematic point of view, FISH data support the hypothesis of an improper placement of *A. bigelovii* in the *Tridentatae* core (as morphological, chemical, and phylogenetic data do), given its differential signal pattern. We have also shown for the

first time that *Artemisia* presents the *Arabidopsis*-type telomere repeat. Beyond these specific data, the present study is an example of the plasticity of angiosperm genomes to tolerate variation at the karyotype level, as intrapopulational variation in rDNA loci (and probably in copy number), heterochromatin differences, and even occasional translocations have been reported here. Echoing Leitch and Leitch (2008), such tolerance may enable the polyploids to form, establish, and occur so widely.

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