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Extensive ribosomal DNA (18S-5.8S-26S and 5S) colocalization in the North American endemic sagebrushes (subgenus *Tridentatae*, *Artemisia*, Asteraceae) revealed by FISH

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Abstract. Chromomycin A_3 banding and fluorescent *in situ* hybridization (FISH) have been performed for six *Artemisia* species with special emphasis on subgenus *Tridentatae*. Morphometrical data on karyotype characters were calculated and idiograms with the position of GC-rich regions and 18S-5.8S-26S and 5S sites of ribosomal DNA were constructed. These sites were all colocalized. To our knowledge, this is the first time in the large family Asteraceae, indeed in angiosperms in general, that colocalization of the two rDNA regions studied is found at every single marked locus. In addition, transcriptionally active nucleolar organizer regions were detected after silver nitrate staining. *Tridentatae* is a cytogenetically homogeneous subgenus, which suggests that evolution of these species has not been coupled with important karyotypic reorganization. However, a few species are taxonomically difficult and show substantial differences. A loss of rDNA loci has been detected in a tetraploid taxon with respect to the diploids studied. These data provide clarifying insight into interspecific relationships between the studied taxa and overall evolutionary and systematic relationships of the *Tridentatae*.

Key words: Colocalization, Compositae, diploidization, fluorochrome banding, fluorescent *in situ* hybridization, genome organization, nucleolar organizing regions.

Introduction

The genus *Artemisia* L. (Asteraceae, Anthemideae, Artemisiinae) is one of the largest Asteraceae genera, comprising some 500 species, with many economically important uses (food, medicine or forage, among others). The genus is currently divided into five main groups [*Artemisia*, *Absinthium* (Mill.) Less., *Dracunculus* (Besser) Rydb., *Seriphidium* Besser and *Tridentatae* (Rydb.) McArthur] but subgeneric classification is subject to rearrangements in the light of recent molecular studies (Vallès et al. 2003). From a cytological perspective, *Artemisia* has been extensively investigated, with many recent chromosome records (Vallès et al. 2005, Garcia et al.

2006, Pellicer et al. 2007) and molecular cytogenetic studies (Torrell et al. 2001, 2003).

The subgenus *Tridentatae* is endemic to western North America. Broadly defined, it consists of 10 to 13 species (depending on the authors; see Shultz, 2005 for a recent summary) of perennial shrubs. These plants, known as sagebrushes, are landscape-dominant, and among the most common woody plants in North America. Polyploidy and hybridization are processes which have facilitated speciation in this group (McArthur et al. 1981, McArthur and Sanderson 1999). The subgenus is considered to be monophyletic and homogeneous but there remain some unresolved taxonomic problems (Kornkven et al. 1998, Vallès et al. 2003).

The sagebrushes have also been the subject of several comprehensive chromosome studies (Ward 1953, McArthur et al. 1981, McArthur and Sanderson 1999), which have established $x = 9$ as the base chromosome number. The current study is the first to focus on molecular cytogenetics of the *Tridentatae*. Fluorescent *in situ* hybridization (FISH) and fluorochrome banding data are useful analytical tools in elucidating systematic and evolutionary relationships within a group of closely related species (Zoldos et al. 1999). The sites of ribosomal genes constitute reliable landmarks for chromosome identification (Castilho and Heslop-Harrison 1995). The number of rDNA chromosomal loci, as well as the number of rDNA repeats within the genome, mostly vary between related species, thus revealing their relationships (Maluszynska and Heslop-Harrison 1991, Cerbah et al. 1998). Herein, we present karyotypes of five taxa of subgenus *Tridentatae* and of a species, *A. filifolia* Torr. (subgenus *Dracunculus*), which previous studies suggested may be related to the *Tridentatae* (McArthur and Pope 1979, Kornkven et al. 1999). The five *Tridentatae* taxa are *A. argillosa* Beetle, *A. cana* Pursh. ssp. *bolanderi* (Gray) G. H. Ward, *A. pygmaea* Gray, *A. rigida* (Nutt.) Gray, and *A. tripartita* Rydb. ssp. *rupicola* Beetle. The placement of *Artemisia pygmaea* and *A. rigida* has been questioned several times (Kornkven et al. 1999 and references therein); *A. argillosa* is a narrow endemic not always recognized as a distinct taxon (Shultz 2005). The main objectives of our study were: a) to characterize the patterns of distribution of GC-rich bands and of these ribosomal gene families; b) to detect any karyological difference taxonomically relevant between these taxa; c) to compare with previous results in *Artemisia* (Torrell et al. 2003, and references therein); d) to analyse these new data in view of their recently studied genome sizes (Garcia et al. unpubl. data); and finally, e) the elucidation of evolutionary shifts of rDNA organization in different ploidy levels.

Materials 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. An indication of the provenance of the species studied is shown in Table 1.

Chromosome preparations. Root tips were pretreated with 0.05% aqueous colchicine, at room temperature, for 2 hours 30 minutes to 4 hours. The material was fixed in absolute ethanol and glacial acetic acid (3:1) and then stored at 4°C for 48 hours. Subsequently, the materials were transferred to 70% ethanol and stored at 4°C.

In order to obtain the karyotypes, samples were hydrolyzed in 1 N HCl for 2–6 minutes at 60°C, stained in 1% aqueous aceto-orcein for 1–2 hours at room temperature, squashed and mounted in a drop of 45% acetic acid-glycerol (9:1).

The chromosome preparations for fluorochrome banding, silver staining 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 M citric acid – sodium citrate, pH = 4.6) for 15 minutes, 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 minutes, depending on the species and meristematic thickness. The lysate of 8–10 root-tips was centrifuged twice in 100 µl buffer and once in

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

Taxon	Origin of materials	Collection number[1]
A. argillosa	Coalmont, Jackson Co., Colorado. 2,489 m.	McArthur and Sanderson 3034. November 5, 2004.
A. cana ssp. *bolanderi*	17 km northwest of Bridgeport, Mono Co., California. 2,270 m.	McArthur 3047. November 20, 2004.
A. filifolia	Moccasin, Mohave Co. Arizona. 1,585 m.	McArthur 2868. December 28, 2003.
A. pygmaea	Yuba Dam Road, Juab Co. Utah. 1,535 m.	McArthur 2870. December 28, 2003.
A. rigida	Malheur Reservoir, Malheur Co., Oregon. 1,035 m.	McArthur and Sanderson 2859. November 11, 2003.
A. tripartita ssp. *rupicola*	Pole Mountain, Albany Co., Wyoming. 2,647 m.	McArthur and Sanderson 3033. November 5, 2004.

The superscript indicates:[1]E. D. McArthur collection numbers (with collector and date of collection); vouchers are deposited in the herbarium of the Rocky Mountain Research Station, Provo, USA (SSLP)

100 µl fixative, at 4000 rpm for 5 minutes for each centrifugation, and removing the supernatant each time. The final pellet was resuspended in 50 µl of fixative, about 10 µl were dropped onto a clean slide, and air-dried. This technique has provided good metaphase spreads with low background due to the minimum presence of cytoplasm, which has resulted in a high reproducibility of FISH signals.

Fluorochrome banding. In order to reveal GC-rich DNA bands, chromomycin A_3 was used, following the protocol in Vallès and Siljak-Yakovlev (1997). To detect the presence of AT-rich chromosome regions, we used bisbenzimide Hoechst 33258 but this method did not reveal such zones in any of the analyzed species.

Fluorescent in situ hybridization. DNA hybridization was carried out following Torrell et al. (2003), with minor changes: the 18S-5.8S-26S rDNA probe was labelled with direct Cy3 -red- (Amersham) and the 5S rDNA probe with digoxigenin-11-dUTP -green- (Boehringer Mannheim). The preparations were counterstained with Vectashield (Vector Laboratories), a mounting medium containing DAPI.

Silver staining. To determine the transcriptional activity of the 18S-5.8S-26S ribosomal genes (nucleolar organizer regions, NORs), the silver nitrate staining was performed, as two or more pairs of these sites were detected in all species. The protocol followed was that of Kavalco and Pazza (2004) with slight modifications. Two drops of 1% aqueous gelatine solution with 0.25% formic acid and four drops of silver nitrate at 25% were placed on test slides which were then incubated for 5–10 minutes at 65°C. After incubation, the coverslips were removed and the slides were washed under tap water, stained with 5% Giemsa for 30 seconds and air dried.

Karyological analyses. Several micromorphic measurements and subsequent analyses were performed (see Table 2 for details). Data of the total karyotype length were also calculated, and nuclear DNA content (Garcia et al., unpubl. data) is included for comparative purposes. These data were used to construct idiograms (mean values were obtained from at least five metaphase plates corresponding to five different individuals for each taxa, Fig. 1).

The best plates were photographed with a digital camera (AxioCam MRc5 Zeiss) coupled on a Zeiss Axioplan microscope and images were analyzed with Axio Vision Ac software version 4.2. FISH preparations were observed with an epifluorescent Zeiss Axiophot microscope with different combinations of Zeiss excitation and emission filter sets (01, 07 and 15). Hybridization signals were analyzed and photographed using the highly sensitive CCD camera (Princeton Instruments), and an image analyser software (Metavue, version 4.6, Molecular Devices Corporation). Morphometric karyotypic parameters were calculated with MicroMeasure 3.3 (Colorado State University). Graphics of the haploid idiograms were performed with PowerPoint (Microsoft Office XP Professional v. SP3).

Results

All the taxa studied have the same chromosome number, $2n = 18$, except *A. argillosa*, a tetraploid with $2n = 36$. In three species, the

Table 2. Karyological data

Taxon	2*n*	Ploidy level	Chromosomal formula[1]	MCL[2] (SD) (μm)	CLR[3] (μm)	TKL[4] (SD) (μm)	CI[5]	R[6]	A1[7]	A2[8]	Stebbins Class[9]	2C [10] (pg)	NORs[11]
A. argillosa	36	4*x*	30 m + 6 sm	6.11 (1.08)	2.76 – 8.36	226.76 (9.00)	43.07	1.16	0.24	0.17	1B	15.77	8(4)
A. cana ssp. *bolanderi*	18	2*x*	14 m + 4 sm	6.17 (0.77)	5.09 – 7.32	111.14 (2.50)	42.63	1.76	0.25	0.13	2A	9.01	6(1)
A. filifolia	18 + (0–2)B	2*x*	14 m + 2 m^{sat} + sm + sm^{sat}	4.76 (0.61)	3.88 – 5.58	85.75 (1.35)	44.97	1.22	0.18	0.13	1A	7.26	4(1)
A. pygmaea	18 + (0–1)B	2*x*	12 m + 2 m^{sat} + 2 sm + 2 sm^{sat}	7.08 (0.56)	6.13 – 8.04	127.53 (3.43)	44.43	1.70	0.19	0.08	1A	11.14	6(3)
A. rigida	18 + (0–4)B	2*x*	14 m + 2 m^{sat} + sm + sm^{sat}	5.57 (0.49)	4.95 – 6.34	100.25 (5.75)	44.29	1.13	0.19	0.09	2A	8.23	6(3)
A. tripartita ssp. *rupicola*	18	2*x*	14 m + 4 sm	5.75 (053)	4.96 – 6.40	103.61 (3.11)	42.31	1.18	0.26	0.09	1A	8.68	6(3)

The superscripts indicate: [1]chromosomal formula according to Levan et al. (1964); [2]mean chromosome length; [3]chromosome length range; [4]total karyotype length; [5]centromeric index (I index in Levan et al. 1964); [6]length ratio of long and short chromosome arms (Levan et al. 1964); [7]intrachromosomal asymmetry index (Romero 1986); [8]interchromosomal asymmetry index (Romero 1986); [9]symmetry class according to Stebbins (1971); [10]2C nuclear DNA content in pg (Garcia et al., unpubl. data); [11] Number of NORs detected with silver staining (the most frequent number is given, followed by the maximum number observed in brackets)

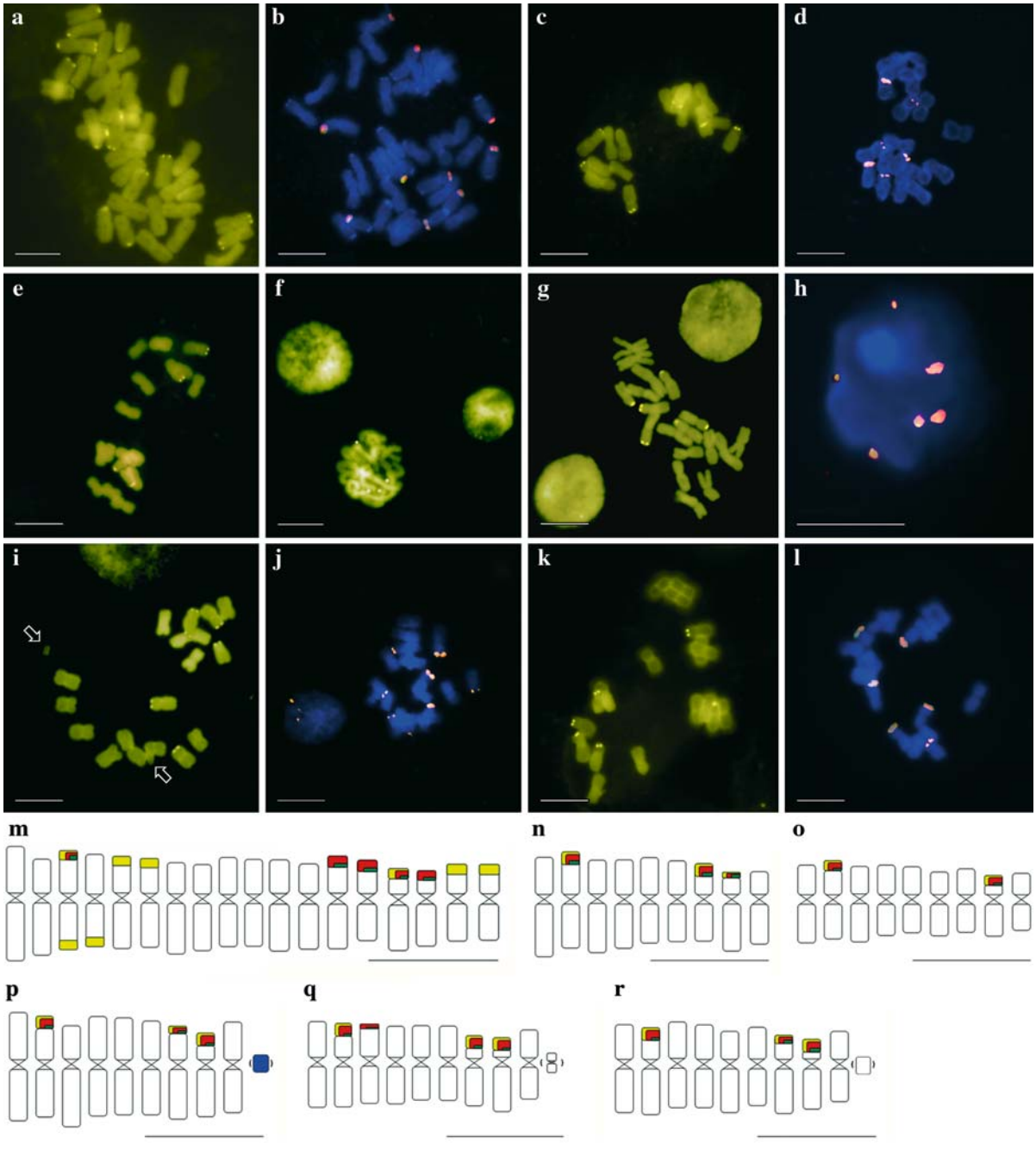

Fig. 1. Fluorochrome banding with chromomycin (**a**, **c**, **e**, **f**, **g**, **i**, **k**), fluorescent *in situ* hybridization (**b**, **d**, **h**, **j**, **l**) and haploid idiograms (**m-r**) of the different taxa studied. Scale bars = 10 μm for photographs and idiograms. (a, b, m) *A. argillosa*. (c, d, n) *A. cana* ssp. *bolanderi*. (e, f, o) *A. filifolia*. (g, h, p) *A. pygmaea*. (i, j, q) *A. rigida*; arrows in picture "i" indicate B chromosomes. (k, l, r) *A. tripartita* ssp. *rupicola*. ■ Chromomycin ■ DAPI ■ 18S-5.8S-26S rDNA loci ■ 5S rDNA loci

presence of B-chromosomes was detected. The chromosomes were all metacentric (m) or submetacentric (sm) by the Levan et al. (1964) classification system. According to Stebbins' classification (1971), these species belong to the most symmetrical types, 1A, 2A

and 1B (Table 2). Secondary constrictions were observed in all taxa, particularly in the 2nd and 8th chromosome pairs. The total karyotype length (TKL) was also calculated, ranging from 85.75 to 226.79 μm. A summary of all these morphometrical data is presented in Table 2 and Fig. 1.

With regard to the fluorochrome banding, GC-rich regions were detected in all species, from only four in *A. filifolia* (Fig. 1e, o), six in the remaining diploid *Artemisia*, and 16 in the tetraploid *A. argillosa* (Fig. 1a, m). *In situ* experiments revealed the presence of 18S-5.8S-26S and 5S rDNA loci (colocalized) in all species, and almost always colocalized with chromomycin positive bands. DAPI used as a counterstaining in hybridization experiments showed 26 bands in the tetraploid taxon, but as these bands were not visible in all the species they have not been considered for systematic purposes and these results are not shown in the idiograms. With respect to silver staining, in all cases excepting two (*A. argillosa* and *A. rigida*), the maximum number of stained nucleoli corresponded to the number of 18S-5.8S-26S regions. Banding, FISH and data concerning the NORs of the species studied are also presented in Fig. 1 and Table 2.

Discussion

Morphometrical data. Karyotype morphology is rather homogeneous in the taxa studied. As a general rule in the diploids, there are 7 to 8 metacentric (m) and 1 to 2 submetacentric (sm) chromosome pairs. These results are similar to those reported by McArthur et al. (1981) for the *Tridentatae*: 5 metacentric, 3 submetacentric and 1 subtelocentric chromosomes, using a different analytic technique. As previously pointed, secondary constrictions (SC) and satellites (SAT) can be viewed. It is possible, however, that more SC are present but not always visible due to a high degree of chromatin condensation, so they have not been used as for chromosome identification (Vischi et al. 2003). Morphometrical data of the tetraploid species correspond to an almost doubled diploid *Tridentatae* karyotype. These findings rather agree with the statements of McArthur and Sanderson (1999) about the fairly homogeneous *Tridentatae* karyotype. Persson (1974) reported that the whole genus *Artemisia* has a relatively similar karyotypic morphology. Moreover, the karyotypes are both symmetrical and very similar for all species included in this study, which is also consistent with the high morphological similarity reported for the *Tridentatae* as a whole (McArthur et al. 1981, McArthur and Sanderson 1999). Karyotypic symmetry is a common feature in the tribe *Anthemideae* (Schweizer and Ehrendorfer 1983), and our current data confirm previous findings in these genus and subgenus. Although Stebbins (1971) considered that the evolution leads to an increase of karyotypic asymmetry, he and other authors have also suggested another interpretation, i.e. a derived secondary karyotypic symmetry caused by chromosomal rearrangements produced, for example, by fusions between acrocentric chromosomes (Vallès 1987, Garnatje et al. 2004). This is more consistent with the systematic placement of *Artemisia* in one of the most advanced angiosperm families, and in particular with the hypothesis that subgenus *Tridentatae* also occupies a derived position within this genus.

Morphometrical data also support the observation of McArthur and Pope (1979) that the karyotype of *A. filifolia* is fairly similar to the *Tridentatae* karyotype pattern (Table 2, Fig. 1e,o). However, the total karyotype length of *A. filifolia* is less than those of the diploid *Tridentatae* species analyzed, consistently with its lower genome size (Garcia et al., unpubl. data), and with a different signal pattern (this point will be commented afterwards).

The single tetraploid taxon analyzed in this study, *A. argillosa*, displays the less symmetrical karyotype (1B). As illustrated in Fig. 1a, b, m, *A. argillosa* chromosome pairs are more different in length between each other than in other species in this study. This is particularly true for the first and second

chromosome pairs, which are expected to be rather similar as they belong to the same quartet of the tetraploid. Such a difference had been previously observed in other *Artemisia* polyploids (Vallès 1987, Vallès and Siljak-Yakovlev 1997). Two possibilities may explain these observations. It could be interpreted as a sign of diploidization of the tetraploid karytoype, possibly as a strategy to maintain its reproductive isolation, which might also confirm a trend towards less chromosomal symmetry in tetraploids as compared to the diploids (McArthur et al. 1981). However, it can also be a consequence of the likely allopolyploid origin of this taxon, as it is thought that it is a hybrid (Beetle 1959). If this were the case, homeologous chromosomes might have properly formed quadrivalents despite their size differences. Indeed, both hypotheses are non-exclusive.

Additionally, nuclear DNA content is well correlated with total karyotype length (TKL). As expected, a highly significant positive correlation ($r = 0.99$, $P = 0.0003$) has been found between both parameters (Table 2). A higher TKL for *A. pygmaea*, visible in its bigger chromosomes (Fig. 1g), is also reflected in a much larger genome size than the other diploids studied.

Presence of B chromosomes. B or supernumerary chromosomes are extra chromosomes found in some, but not all, individuals within a species, and they have been described in many plants and animals; their function, composition, and origin are not completely known (Trivers et al. 2004). In our case study, *A. pygmaea*, *A. rigida* and *A. tripartita* ssp. *rupicola*, present B chromosomes (Fig. 1i, p, q, r). The number of B's in these species is also variable, from one to four per cell, when present. Supernumerary chromosomes had been previously detected in the *Tridentatae* by McArthur et al. (1981). A later cytogenetic study (McArthur and Sanderson 1999) considered that B's were present but in a low frequency; however, data of this study might point to a higher frequency of B chromosomes in the *Tridentatae*.

Different kinds of B chromosomes seem to appear in these taxa, which is consistent with the hypotheses stated by Vallès and Siljak-Yakovlev (1997) and Camacho et al. (2000) that B's do not have a single mode of origin, but can arise in a variety of ways. On the one hand, there are some more or less spherical compact chromatin bodies, sized approximately a fourth part of a regular chromosome, similarly wide, and in which the centromer is hardly seen. In our case, they appear sometimes markedly poorly (*A. tripartita* ssp. *rupicola*) or richly (*A. pygmaea*) stained with DAPI. Indeed, an intense blue spot (DAPI-rich) is usually seen at the interphase nucleus in *A. pygmaea* (Fig. 1h, p), which is probably related to the habitual presence of this AT-rich B chromosome, and may also be linked with the higher genome size of this species by an increase in highly repetitive DNA. Most B chromosomes are heterochromatic, and basically composed of repetitive sequences (Cuadrado and Jouve 1994) promoting the general idea that these elements are genetically inert, although some of them have shown transcriptional activity or presence of ribosomal genes (Green 1988). In fact, one metaphase plate of *A. pygmaea* presented a B chromosome with ribosomal DNA at one end, but this has only been seen once (data not shown); presence of ribosomal genes dispersed throughout B chromosomes was also detected by Donald et al. (1995) and Stitou et al. (2000). However, detached satellites, usually not connected with any visible extension with the chromosomes, can lead to misidentification (Ohri and Ahuja 1990, Hidalgo et al., 2007), a possibility that should also be taken into account in the consideration of supernumerary chromosomes, but particularly in those stained more or less similarly to the rest of the chromosome complement. On the other hand, another kind of B chromosomes can only be seen in *A. rigida*: they have a similar shape to the A chromosomes although narrower, they measure only one fourth the length of a standard chromosome and with a clearly visible centromer (Fig. 1i, q).

Signal pattern. For the studied species, both chromomycin and FISH signals are always telomeric. This coincides with Schweizer and Ehrendorfer's (1983) statement of a general landmark pattern shared by all the *Anthemideae*; other studies focused on *Artemisia* also report similar telomeric signals (Torrell et al. 2001, 2003).

Fluorochrome banding. Fluorochrome banding with chromomycin revealed heterochromatin composed of GC-rich DNA. All the species studied show such regions, which are located at distal ends, most usually in the short arms, and in some cases, they clearly appear in satellites. The diploids, excepting *A. filifolia*, show the same organization of GC-rich DNA, namely, six chromomycin bands (Fig. 1n, p, q, r). Our findings are consistent with a previous study (Torrell et al. 2003), where six GC-rich regions were also found in the only *Tridentatae* species investigated. For *A. filifolia* only four GC-rich bands were detected (Fig. 1e, o), in this case clearly located at the satellites of the marked chromosomes. This result is confirmed with the observation of four chromomycin-positive spots at the interphase nuclei of this species (Fig. 1f). This difference in banding pattern supports standing of this species in a different subgenus than *Tridentatae*. *Artemisia filifolia* has traditionally been placed in the subgenus *Dracunculus* but some karyotypic (McArthur and Pope 1979) and cpDNA evidences (Kornkven et al. 1999), had suggested a relationship with the *Tridentatae*. A preliminary study centred in a small group of species from subgenus *Dracunculus*, detected however, six chromomycin-positive bands in a diploid (Torrell et al. 2001), whereas four GC-rich regions were also observed in different diploid species of other subgenera, *Artemisia* and *Seriphidium* (Torrell et al. 2003). As the genus *Artemisia* is currently circumscribed, these data show that there is not a specific banding pattern for each subgenus; however, this might either reveal that the extant classification is not natural or that there are different lineages within each subgenus that account for different banding patterns.

The single tetraploid *Artemisia* studied shows 16 chromomycin-positive bands (Fig. 1a), with a pair of chromosomes marked in both ends. Compared with the number of bands previously found in other *Artemisia* and even in other Asteraceae genera (Vallès and Siljak-Yakovlev 1997; Cerbah et al. 1998; Torrell et al. 2001, 2003; Garnatje et al. 2004) the number of GC-rich bands of this species is surprisingly high. As it is considered that polyploid species tend to reduce its monoploid genome size, usually by means of reducing repetitive DNA, of which GC-rich regions are an important part (Sharma and Sen 2002), this high number of chromomycin-positive bands is even more exceptional. When a diploid and a tetraploid of the same species have been observed (this has not been possible for *A. argillosa*, as this species is thought to be of allopolyploid origin), the number of GC-rich bands detected in the tetraploid was never more than double of that of the diploid, and in some cases equal number of bands were found in both ploidy levels (Vallès and Siljak-Yakovlev 1997, Torrell et al. 2001). As previously mentioned, 26 bright DAPI bands were found in the karyotype of this species, many of them colocalized with chromomycin-positive bands and always telomeric. This is a species of putative hybrid origin: Beetle (1959) described *A. argillosa* and performed a detailed study of its morphological characters, which indicate that it is an intermediate between *A. cana* Pursh ssp. *viscidula* (Osterhout) Beetle and *A. longiloba* (Osterhout) Beetle, both of which grow in its vicinity. *Artemisia argillosa* does not occur intermixed with its putative parents, so current active hybridization and introgression between these species is not apparent. This particular abundance of both kinds of repetitive DNA sometimes at the same loci, could then be explained as a mechanism of speciation. It might constitute a way of creating more genomic reproductive barriers which may prevent hybridization between this plant and relatives. This taxon is a narrow endemic of high elevations (ca. 2,500 m); the distinct

banding pattern of *A. argillosa* could also reflect an adaptation to the environmental conditions of high mountain areas, such as ultraviolet (UV) radiation. As telomeres are sensitive to DNA damaging agents, particularly UV radiation (Lansdorp 2005), the abundance of both kinds of constitutive heterochromatin at chromosome ends could have a protective function of these structures (Siljak-Yakovlev and Cartier 1986, Susanna, pers. comm. *in* Hidalgo, 2006).

Fluorescent *in situ* hybridization. One of the most interesting outcomes of this study is that repeated units of the two different rDNA multicopy families analyzed were overlapped (detectable as red and green signals in hybridization experiments), almost always colocalized with chromomycin-positive regions (with the exception of three loci in *A. argillosa* and one in *A. rigida*), and therefore always located at telomeric position or in satellites. In most plants and animals the 45S rDNA (18S, 5.8S and 26S rRNA genes) is clustered in tandem and transcribed as one cistron by RNA polymerase I whereas the 5S rDNA is normally located separately and transcribed by RNA polymerase III (Srivastava and Schlessinger 1991). Sone et al. (1999) and Vitturi et al. (2002) also reported such an extensive colocalization in a bryophyte and in an earthworm, respectively. However, this is the first time to our knowledge in the large family Asteraceae and in the angiosperms as a whole that this feature appears in every marked position. Overlapping at some but not all marked loci has been recently described in one species of a genus closely related with *Artemisia*, *Chrysanthemum* (Abd El-Twab and Kondo 2006), in some other plant genera (*Silene*, Siroky et al. 2001, *Vicia*; Raina et al. 2001; *Linum*, Muravenko et al. 2004) and in a fish species (Rossi and Gornung 2005). Also in a previous study of *Artemisia* some but not all loci had showed this overlapping (Torrell et al. 2003). A very low copy number of the 5S units at specific positions might explain that visual detection at each locus has not always been evident.

Hence, the existence of the 5S rDNA without the 18S-5.8S-26S rDNA is not apparent in *Artemisia*. The significance of such an unusual association involving repeated units of different multigene families is still unclear and needs interpretation. An accidental insertion of the 5S rDNA into the 45S rDNA repeat unit by transposon-like DNA-mediated or retrotransposon-like RNA-mediated transposition may be a hypothesis for this phenomenon (Drouin and Moniz de Sa 1995).

Contrariwise, the interspersion of 18S rDNA sites with chromomycin-positive zones is easily explained as GC-rich DNA is linked to NORs, which are coded by 18S-5.8S-26S rDNA (Siljak-Yakovlev et al. 2002, and references therein). Additionally, most 18S-5.8S-26S rDNA loci are transcriptionally active in these species, as the maximum number of nucleoli stained with silver nitrate (Table 2) usually coincides with the number of 18S-5.8S-26S signals found (excepting in *A. argillosa* and *A. rigida*). However, the most frequent number of stained nucleoli is always lower than the maximum, as it is thought that they habitually fuse between themselves (Siljak-Yakovlev et al. 2002).

There is a similar pattern of organization of the two families of ribosomal genes in three of the studied diploid species, *A. cana* ssp. *bolanderi*, *A. pygmaea* and *A. tripartita* ssp. *rupicola*, which is quite coincidental with the only *Tridentatae* previously studied, *A. tridentata* Nutt. ssp. *spiciformis* (Osterhout) Kartesz & Gandhi (Torrell et al. 2003). As both *A. cana* Pursh and *A. tripartita* Rydb. had been formerly included in the *A. cana* lineage within the *Tridentatae* (Ward 1953, Beetle 1960, Shultz 1983), according to their leaf morphology, habitat preference and tendency to root sprout and layer, their cytogenetic similarity is not unexpected. The case of *A. pygmaea* is different. Its position within the *Tridentatae* has been questioned several times. As previously mentioned, the bigger chromosomes and the presence of an AT-rich B-chromosome account for the difference of this taxon. However, it shows the same signal distribution as the other *Tridentatae* diploids.

Artemisia rigida presents the same number of GC-rich bands but with an additional colocalized 5S and 18S-5.8S-26S signal in the 3rd chromosome pair. A notable difference is observed in the intensity of this extra hybridization signal, which is weaker than the rest. The strength of hybridization signal is generally considered related to the copy number of genes (Maluszysnka and Heslop-Harrison 1991). *Artemisia rigida*, a low, spreading shrub, has also been considered as problematic from the systematic point of view, as it is uniquely adapted to poor soil and extreme xerophytic conditions (Kornkven et al. 1999). It had also been included by Beetle (1960) in the *A. cana* lineage with *A. tripartita* and *A. cana*, and it somewhat resembles *A. tripartita* in size, silverly pubescence, and the deeply, narrowly lobed leaves, but is distinguishable by the spike-like inflorescence, large leafy bracts that subtend the heads and deciduous leaves (Cronquist 1994). The difference in the signal pattern between *A. cana* and *A. tripartita* on the one hand and *A. rigida* on the other hand, could also be added to this difference in morphology. Moreover, preliminary results of a molecular phylogeny based on the analysis of ITS and ETS nuclear ribosomal DNA regions place this species apart from the *Tridentatae* clade (Garcia et al., unpubl. data).

FISH signals of *A. filifolia* are consistent with GC-rich bands, as it also presents two 5S and two 18S-5.8S-28S loci colocalized with these chromomycin-positive regions (Fig. 1o). As previously mentioned for fluorochrome banding results, the difference detected would support the non-inclusion of this species in the *Tridentatae;* its genome size (Garcia et al. 2004) and an ITS molecular phylogeny (Kornkven et al. 1998) are also non-supportive. *Artemisia filifolia* has been traditionally assigned to the subgenus *Dracunculus*, with which it shares morphological characters, but it has no evidence of close relatives in that subgenus. The available cytogenetic information does not support inclusion of *A. filifolia* in *Dracunculus* (Torrell et al. 2001). In common with *A. rigida*, a preliminary molecular phylogeny places *A. filifolia* independent from both the *Tridentatae* and *Dracunculus* clades, a result consistent with banding and *in situ* hybridization data of this study.

Finally, physical mapping of the ribosomal DNA of *A. argillosa* shows that the number of sites is not duplicated in this tetraploid species, with respect to the diploids of this study. This finding is in contrast to Torrell et al. (2003), wherein the tetraploid *Artemisia* studied corresponded fairly well to a duplication of a diploid karyotype of the same subgenus. Research focused in the genetic consequences of allopolyploidy in *Tragopogon*, another Asteraceae, showed that the number and distribution of the rDNA loci in some of the polyploids studied were additive of those observed in the diploids (Pires et al. 2004), and similar results have been found in most tetraploid cultivars of *Nicotiana tabacum* L. (Lim et al. 2000).

The examination of the signals and their precise location in *A. argillosa* karyotype reveals that the signal pattern corresponds to almost a doubled karyotype of a diploid *Tridentatae*, but with the loss of one site, as there are only ten loci instead of the twelve expected for a tetraploid. Similar findings have been reported in other Asteraceae genera as *Xeranthemum* (Garnatje et al. 2004), where it was hypothesized that the activity of this lacking rDNA locus was probably silenced (amphyplasty) during the genome duplication process. In other allopolyploids such as *Zingeria* (Kotseruba et al. 2003) and *Sanguisorba* (Mishima et al. 2002) some rDNA loci were lost after polyploidization. It is likely that diploidization, in the case of *A. argillosa* associated with locus loss, might have occurred after polyploidization. The signal pattern and karyotype morphology of *A. argillosa* might reflect whether it is a recent allopolyploid still showing both parent's karyotype differences and signal pattern, or an older autopolyploid which has started differentiation and diploidization. Together with its morphological distinctiveness and the preliminary results of a molecular phylogeny (Garcia et al. unpubl. data), the maintenance of specific status for

this taxon as proposed by Beetle (1959) is supported.

Conclusion

This study demonstrates a unique constituency of the *Tridentatae* from the perspective of molecular cytogenetics. This research supports the hypothesis that the evolution of the sagebrushes in North America is not accompanied by karyotypic rearrangements, but in all likelihood, speciation in this group has been facilitated by polyploidization and hybridization. This may be related with a recent evolutionary history of these plants, which has not been reflected in enough karyological, morphological, genic or chemical differences. However, the one non-*Tridentatae* included in this study was meaningfully distinctive, and the species with questionable taxonomic status also showed significant differences. The extensive colocalization found can be considered as a particular feature present in the genus *Artemisia*, a finding that can shed light on the evolution of ribosomal DNA. It would also be of great interest to detect when in the evolutionary history of the genus (or at higher taxonomic levels) this trait appeared for the first time, the objective of current research (Garcia et al. unpubl. data). For *A. argillosa*, knowing the signal pattern of its presumed parental species would be interesting to elucidate its possible hybrid origin; moreover, research focused on changes of the organization of ribosomal DNA during polyploidization within the *Tridentatae* is also underway (Garcia et al., unpubl. data).

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