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Molecular cytogenetic characterization of some representatives of the subgenera *Artemisia* and *Absinthium* (genus *Artemisia*, Asteraceae)

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Abstract
A molecular cytogenetic study has been performed in three species of the genus *Artemisia*, complementing previous works on two subgenera that had been scarcely studied from this standpoint, *Artemisia* (*A. chamaemelifolia*, *A. vulgaris*) and *Absinthium* (*A. absinthium*). Chromomycin A_3 and 4',6-diamidino-2-phenylindole (DAPI) banding have been carried out, as well as fluorescent *in situ* hybridization (FISH) of 5S and 18S-5.8S-26S ribosomal DNA. Morphometrical data of karyotype characters were calculated and idiograms with the position of the AT- and GC-rich regions as well as rDNA loci were constructed. Colocalization of most of these regions has been observed, confirming previous findings in this genus. Both ribosomal DNA appear always colocalized, which is a distinct feature with respect to most angiosperms surveyed. Regarding the differential characteristics of each species, a symmetrical karyotype has been found in the species studied. *Artemisia absinthium* shows long chromosomes and absence of centromeric banding signals that, conversely, are absent in *A. vulgaris* and *A. chamaemelifolia*. The last species also presents B-chromosomes in which ribosomal DNA and heterochromatin have been detected. Despite these differences, karyotype morphology and signal pattern of the three species are quite coincidental. This might reflect a close phylogenetic relationship between both subgenera, which is consistent with the available molecular phylogenies presenting species of the subgenera *Artemisia* and *Absinthium* intermixed.

Keywords: *Artemisia*, Asteraceae, colocalization, fluorescent *in situ* hybridization, fluorochrome banding, karyotype analysis.

Introduction

The genus *Artemisia* L. (Asteraceae, Anthemideae) comprises more than 500 species, basically perennial and worldwide distributed (a variable number depending on the authors consulted: McArthur, 1979; Mabberley, 1990; Ling, 1991a, b, 1995a, b; Bremer & Humphries, 1993; Kubitzki, 2007). Many species of this genus are useful in different fields: food (*A. absinthium* L., *A. dracunculus* L., *A. genipi* G. Weber in Stechm.), medicine (*A. annua* L., *A. santonica* L.), forage (*A. herba-alba* Asso, *A. tridentata* Nutt.), ornamentals (*A. arborescens* L., *A. vulgaris* L.). After different rearrangements and systematic treatments, *Artemisia* has been divided into five large groups treated at sectional or subgeneric levels: *Absinthium*, *Artemisia* (= *Abrotanum*), *Dracunculus*, *Seriphidium* and *Tridentatae* (Torrell *et al*., 1999 and references therein). Nevertheless, this classification does not accurately represent natural groups. Molecular phylogenetic studies, based on the analysis of chloroplast (cpDNA) and nuclear ribosomal (nrDNA) sequences, have helped elucidating the

systematic relationships within *Artemisia* (KORNKVEN *et al*., 1998; TORRELL *et al*., 1999; WATSON *et al*., 2002; VALLÈS *et al*., 2003; SANZ *et al*., 2008), although important questions still remain unresolved.

The study of chromosomes has provided essential information for the systematics and the evolution of the genus, since the first studies (WEINEDEL-LIEBAU, 1928; EHRENDORFER, 1964; KOROBKOV, 1972) until present (VALLÈS & SILJAK-YAKOVLEV, 1997; TORRELL *et al*., 2003; VALLÈS & GARNATJE, 2005; GARCIA *et al*., 2007). Two different basic chromosome numbers have been described for the genus: $x = 9$, which is detected in all the subgenera, and the less frequent $x = 8$, only reported in the subgenera *Absinthium*, *Artemisia* and *Dracunculus* (SOLBRIG, 1977; OLIVA & VALLÈS, 1994; MCARTHUR & SANDERSON, 1999). Both basic chromosome numbers show polyploid series with known levels up to hexadecaploid for $x = 9$-based species and hexaploid for $x = 8$ (EHRENDORFER, 1964; PERSSON, 1974; VALLÈS *et al*., 2001; GARCIA *et al*., 2006; PELLICER *et al*., 2007).

Fluorochrome banding and fluorescent *in situ* hybridization (FISH) with rDNA are useful markers for chromosome identification (LEITCH & HESLOP-HARRISON, 1993) and have provided a valuable tool in order to determine genome organization in plants (RAINA *et al.*, 2001; SINGH *et al*., 2001) and the phylogenetic relationships between close taxa (ANSARI *et al*., 1999). The detection of 18S-5.8S-26S and 5S ribosomal DNA by fluorescent *in situ* hybridization and fluorochrome banding have become frequently used techniques for establishing the physical mapping of a certain type of repeated DNA, and in *Artemisia* previous researches have been carried out in this sense (TORRELL *et al*., 2001, 2003; HOSHI *et al*., 2006; GARCIA *et al*., 2007).

Within the framework of systematic and evolutionary studies in *Artemisia*, in particular those providing molecular cytogenetic data (TORRELL *et al*., 2007; GARCIA *et al*., 2007), the present work uses fluorochrome banding to characterize AT- and GC-rich chromosome regions, and fluorescent *in situ* hybridization (FISH) to obtain the distribution patterns of 18S-5.8S-26S and 5S rDNA loci in two subgenera scarcely studied from this point of view: two species from subgenus *Artemisia* (*A. chamaemelifolia* Vill., *A. vulgaris*) and one from subgenus *Absinthium* (*A. absinthium*). In previous works (TORRELL *et al*., 2001, 2003; GARCIA *et al*., 2007) we have studied from molecular cytogenetic viewpoint some representatives of subgenera *Seriphidium* and *Tridentatae* (plus one annual member of the subgenus *Artemisia*, related to *Seriphidium* in the molecular phylogeny (TORRELL *et al*., 1999; VALLÈS *et al*., 2001). Now we are presenting the results of the first investigation of two more subgenera, *Artemisia* and *Absinthium*.

MATERIALS AND METHODS

Plant material

Table 1 shows the species studied, grouped by subgenera, with their origins and vouchers, deposited at the herbarium of the Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona (BCN).

Root-tip meristems were obtained from wild-collected achenes germinated on wet filter paper in Petri dishes at room temperature in the dark.

Chromosome preparations

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

Chromosome preparation and staining for counts and karyotype elaboration

Root tips were hydrolysed in 1N HCl at 60ºC during 2 minutes. Subsequently they were washed in distilled water, stained in 2% aceto-orcein during 2-8 hours at room temperature, and squashed in a drop of 45% acetic acid and glycerol (9:1).

Fluorochrome banding

Chromosome preparations for fluorochrome banding and fluorescent *in situ* hybridization (FISH) were done using the air-drying technique of GEBER & 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

Table 1. Origin and herbarium vouchers of the populations studied.

Taxon	Location and herbarium voucher
Subgenus *Absinthium*	
Artemisia absinthium L.	Armenia, Ekhegnadzor, Vernashen: between the village of Vernashen and the church of Gladzor, 1,300 m, *G. Faivush, E. Gabrielian, N. Garcia-Jacas, M. Guara, M. Oganesian, A. Susanna S-1507, K. Tamanian, J. Vallès,* 20.viii.1995 (BCN 11601)
Subgenus *Artemisia*	
Artemisia chamaemelifolia Vill.	Andorra, Canillo: margins of the path from the church of Sant Joan de Caselles towards Vall de Riu, 1,600 m, *M. Torrell, J. Vallès*, 17.xi.1996 (BCN 13222)
Artemisia vulgaris L.	France, Paris: Bois de Boulogne, margins of a forest, 100 m, *M. Torrell, J. Vallès*, 8.ii.1997 (BCN 15297)

solution [4% cellulase Onozuka R10 (Yakult Honsha), 1% pectolyase Y23 (Sigma) and 4% hemicellulase (Sigma)] at 37ºC 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 100 µl fixative, at 4,000 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. In order to reveal GC-rich bands, chromomycin A_3 was used, following the protocols of SCHWEIZER (1976) and CERBAH *et al.* (1995).

Fluorescent in situ *hybridization (FISH)*

DNA hybridization was carried out according to 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). Preparations were mounted in a medium containing DAPI, which revealed AT-rich DNA bands.

FISH preparations were observed with an epifluorescent Zeiss Axiophot microscope with different combinations of Zeiss excitation and emission filter sets (01, 07 and 15). The best metaphase plates were photographed with a digital camera (AxioCam MRc5 Zeiss) mounted on a Zeiss Axioplan microscope, and images were analysed with Axio Vision Ac software version 4.2. Hybridization signals were analysed and photographed using the highly sensitive CCD camera (Princeton Instruments), and an image analyser software (Metavue, version 4.6, Molecular Devices Corporation).

Karyological analyses

Chromosome counts and measurements were done and subsequent analyses were performed (see Table 2 for details). The total karyotype length was also calculated, and compared with nuclear DNA content provided in previous works (TORRELL *et al*., 2001; GARCIA *et al*., 2004) 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 taxon (Fig. 1).

RESULTS

Results of the karyological and molecular cytogenetic assays are presented in Fig. 1 and Table 2. The chromosome counts carried out in the species studied revealed the presence of both basic chromosome numbers commonly reported in the genus, $x = 9$ (*A. absinthium*, Fig. 1A; *A. chamaemelifolia*, Fig. 1E) and $x = 8$ (*A. vulgaris*, Fig. 1I). All the species studied are diploid, *A. absinthium* and *A. chamaemelifolia* having $2n = 18$ chromosomes

Table 2. Karyological data. The superscripts indicate: [1]Nuclear DNA content (from TORRELL *et al.*, 2001; GARCIA *et al.,* 2004); [2]chromosomal formula according to LEVAN *et al.* (1964); [3]mean chromosome length; [4]chromosome length range; [5]symmetry class according to STEBBINS (1971); [5]ROMERO (1986) indexes; A1: intrachromosomal asymmetry index, A2: interchromosomal asymmetry index.

Taxon	2*n*	Ploidy level	Mean 2C (pg)[1]	Chromosomal formula[2]
A. absinthium	18	2*x*	8.75	12m + 2m^{sat} + 4sm
A. chamaemelifolia	18 + (1-5)B	2*x*	6.04	10m + 2m^{sat} + 4sm + 2sm^{sat}
A. vulgaris	16	2*x*	6.26	14m + 2sm^{sat}

				Romero indexes[6]	
Taxon	MCL[3]±(SD) (µm)	CLR[4] (µm)	Stebbins class[5]	A1	A2
A. absinthium	4.27 ± 0.23	38.46	2A	0.30	0.15
A. chamaemelifolia	3.08 ± 0.10	27.76	2A	0.09	0.09
A. vulgaris	3.69 ± 0.25	29.51	2A	0.24	0.18

(with the presence of one to three B-chromosomes in the case of *A. chamaemelifolia*) and *A. vulgaris* 2*n* = 16.

Artemisia absinthium presents long chromosomes (MCL: 4.27 ± 0.23 µm) and the values of the asymmetry indexes (STEBBINS, 1971) reveal a very symmetric karyotype (Table 2). We have detected a secondary constriction in pair VII, constituting a satellite in its short arm, which is positively stained both with chromomycin and the two FISH probes (Fig. 1A-D). The same pattern is found in the short arm of pair III, although no satellite is visible in this chromosome. Two loci with both rDNA (18S-5.8S-26S and 5S) colocalized were detected for this specimen. Several AT-rich bands, stained with DAPI, have also been detected at chromosome ends, as shown in Fig. 1D.

Artemisia chamaemelifolia displays again a very symmetric karyotype. The presence of CMA+ and DAPI+ heterochromatin signals has been detected at centromeric and telomeric position (Figs. 1F; 1G). As found in *A. absinthium*, four GC-rich DNA bands located at satellites coincide with the two rDNA loci, with both 5S and 18S-5.8S-26S rDNA colocalized. The presence of up to three B-chromosomes has been also observed in the studied population but these kind of chromosomes are normally variable in number depending on the specimen studied. Some of these B's show heterochromatic bands and, occasionally, ribosomal DNA loci have been detected in some of these supernumerary chromosomes (data not shown).

Artemisia vulgaris also presents a symmetrical karyotype, with abundant GC-rich bands, mainly at telomeric position, but also near the centromeres (Figs. 1J; 1K). As observed in the other taxa considered, both kinds of rDNA are colocalized (also in this case, two rDNA loci are found), and coincidental with some CMA-rich regions. Additionally, as in the other species studied, some AT-rich bands are detected.

DISCUSSION

Chromosome number

For both *A. absinthium* and *A. chamaemelifolia* previous studies have detected diploid and tetraploid populations respectively. For *A. vulgaris*, either *x* = 8- or *x* = 9-based cytotypes have previously been reported, with 2*n* = 16 as a predominating chromosome number and, in addition, 2*n* = 18 and several unusual chromosome

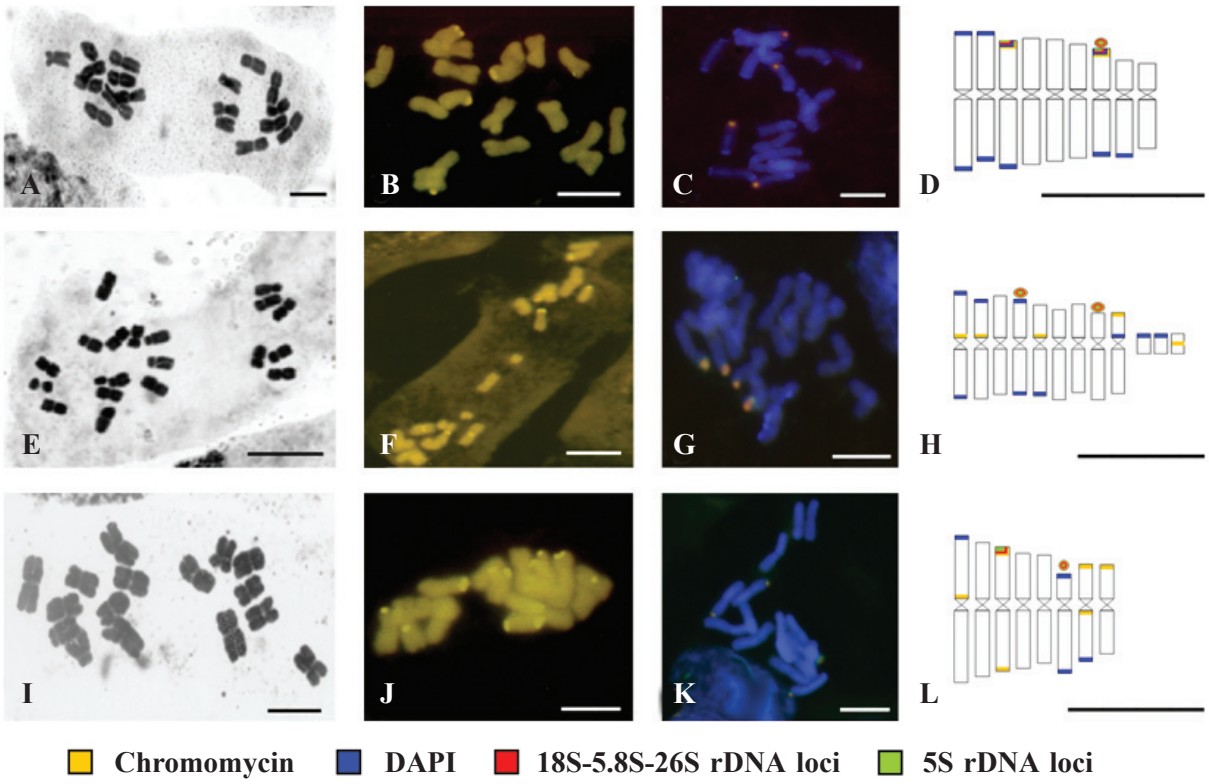

Figure 1. Metaphase plates stained with aceto-orcein (A, E, I), fluorochrome banding with chromomycin (B, F, J), fluorescent *in situ* hybridization (C, G, K) and haploid idiograms of the species studied (D, H, L). *Artemisia absinthium* (A, B, C, D), *A. chamaemelifolia* (E, F, G, H), *A. vulgaris* (I, J, K, L). Scale bars = 5 µm.

numbers ($2n$ = 24, 34, 40, 45), including the occasional presence of B-chromosomes (http://www-asteraceae.cla.kobe-u.ac.jp/search.html; 14/07/2007). In the latter species, Vallès & Siljak-Yakovlev (1997) reported the evidence of a chromosomal fusion and a certain centromeric fragility of the longest chromosome pair, which could explain the supposed $2n$ = 18 counts. The other numbers may belong to other species of the *A. vulgaris* complex (such as *A. princeps* Pamp. or *A.* x *wurzellii* C.M. James & Stace in C.M. James, Wurzell & Stace, James *et al.*, 2000, Vallès *et al.*, 2005), since many counts performed in genuine *A. vulgaris* populations have consistenly yielded $2n$ = 16 (Vallès *et al.*, unpubl. data).

Kayotype features

Indexes of asymmetry

All three studied species present a very symmetrical karyotype, which is a common trait in the genus (Vallès & Garnatje, 2005, and references therein), and is consistent with the assumption of a symmetrical karyotype characterizing the species included in tribe Anthemideae (Schweizer & Ehrendorfer, 1983). Furthermore, *A. chamaemelifolia* shows a particulary low index of interchromosomal asymmetry, which is smaller than the average for the genus (Vallès & Garnatje, 2005, and references therein).

Karyotype morphology

Among the taxa studied, karyotype morphology is rather homogeneous. As a general pattern in these diploid species, we have detected the presence of six to eight metacentric (m) and one to three submetacentric (sm) chromosome pairs. Furthermore, the presence of secondary constrictions (SC) and satellites (SAT) has been observed. It is possible that more SC and SAT exist but they are not always detectable, depending on the degree of chromatin condensation, so they have not been used for chromosome identification (Vischi *et al.*, 2003).

These results are rather similar to those recorded in *Artemisia* in previous works (MCARTHUR & SANDERSON, 1999; TORRELL *et al*., 2003; GARCIA *et al.*, 2007). Karyotype homogeneity is another feature of the species included in the present study, as PERSSON (1974) stated for the whole genus. Only slight differences in size between chromosomes have been detected.

Karyotype size

Although MCL of the species studied are quite similar, some differences between each other are detected, which are mirrored in genome size differences. This illustrates once more the remarkable correlation between genome size and karyological data (BENNETT & LEITCH, 2005, and references therein). *Artemisia absinthium* is the species with the largest MCL (4.27 ± 0.23 μm) of the three, and also presents a higher nuclear DNA content (2C = 8.75 pg, Table 2). Contrarily, *A. chamaemelifolia* accounts for the smallest MCL (3.08 ± 0.10 μm), and its genome size (2C = 6.04 pg, Table 2) correlates negatively with the karyotype size. *Artemisia vulgaris*, with an intermediate karyotype length (3.69 ± 0.25 μm), presents a nuclear DNA amount (2C = 6.26 pg, Table 2) which ranges between those of the two other species.

Presence of B-chromosomes

B- or supernumerary chromosomes have been detected in one of the species studied, *A. chamaemelifolia* (Figs. 1E-H), as already pointed in TORRELL *et al*. (1999). The presence of B-chromosomes has been frequently observed in other subgenera of *Artemisia*, e. g. *Absinthium* (PELLICER *et al*., 2007) or *Tridentatae* (GARCIA *et al*., 2007). B's are extra chromosomes found in some, but no all individuals within a species, and have been described in many plants and animals (JONES & REES, 1982). Their function, composition and origins are not still well understood (TRIVERS *et al*., 2004). The number of B-chromosomes found is also variable, from 1 to 3, when present. The frequency of occurrence of this kind of chromosomes in *Artemisia* is also a subject of discussion (MCARTHUR & SANDERSON, 1999; GARCIA *et al*., 2007). Although different kind of B's might be found in plants linked to their origin (VALLÈS & SILJAK-YAKOVLEV, 1997), in *A. chamaemelifolia* we have only detected some compact, small chromatin bodies, in which the centromere is hardly seen. Most B-chromosomes are heterochromatic, composed of repetitive DNA sequences (CUADRADO & JOUVE, 1994), suggesting the idea that they are genetically inert. Nonetheless, some of them show the presence of ribosomal genes, as in *A. chamaemelifolia*, which might imply transcriptional activity (GREEN, 1998), although this point is still to be confirmed in this genus.

The three studied species present strongly homogenous karyotype traits. Although rather consistent at the genus level, karyotype resemblance generally does not allow identification and discrimination of *Artemisia* species (HOSHI *et al*., 2003). In this sense, fluorochrome banding and FISH of rDNA can provide useful data for karyotype characterization and distinction in closely related species.

Fluorochrome banding and fluorescent *in situ* hybridization (FISH)

Banding pattern

The banding and FISH patterns here shown are coincidental to a high degree with those established hitherto in different molecular cytogenetic researches centred in *Artemisia* (TORRELL *et al*., 2001, 2003; GARCIA *et al*., 2007). Fluorochrome banding with chromomycin revealed heterochromatin regions composed of GC-rich DNA (Figs. 1B; 1F; 1J), and in all cases these regions were located at distal ends, in some cases in satellites. Only in the species belonging to subgenus *Artemisia* (*A. chamaemelifolia*, *A. vulgaris*), centromeric GC-regions were detected, and sometimes the B-chromosomes of *A. chamaemelifolia* also revealed positive regions when treated with chromomycin. In a previous work (TORRELL *et al*., 2003), only four positive chromomycin bands were detected in diploid individuals of a species belonging to subgenus *Artemisia*, *A. annua*. In the present study we detected six or seven CMA+ signals. *Artemisia annua* being annual, the divergence observed with regard to CMA+ signal number of this taxa with respect to its perennial relative could be related to their difference in life cycle. Such a difference has been also found in other Asteraceae (e. g. HIDALGO *et al*., submitted).

Artemisia chamaemelifolia and *A. vulgaris* belong to the same subgenus, *Artemisia*, and both present positive CMA signals at centromeric position, which might reflect a closer phylogenetic relationship between these two taxa than with *A. absinthium*, as indicated by molecular phylogenetic studies (VALLÈS *et al*., 2003; SANZ *et al*., 2008). However,

phylogenetic inferences from cytogenetical data should be cautiously done because subgenus *Artemisia* may not to have a unique CMA signal pattern, and AT-rich (DAPI-positive) bands are presented much more variable among these three taxa. DAPI-positive signals are always telomeric except in the case of *A. chamaemelifolia*, in which a centromeric band has been detected in a single chromosome pair. In most cases, this kind of banding coincides or is located very close to GC-rich signals.

FISH pattern: colocalization of the 18S and 5S rDNA

Our FISH results show that all three *Artemisia* species present four signals (two loci) for 18S-5.8S-26S and 5S regions (Figs. 1C; 1G; 1K). In all cases these signals are colocalized, so 18S-5.8S-26S and 5S rDNA signals are always in the same number and in the same position. This peculiar distribution of the 45S (18S, 5.8S and 26S rRNA genes) and 5S rDNA, which in animals and plants are usually placed separately and transcribed by different RNA polymerases (SRIVASTAVA & SCHLESSINGER, 1991), has been previously reported in former investigations in *Artemisia* (TORRELL *et al*., 2003; HOSHI *et al*., 2006; GARCIA *et al*., 2007) and in other Anthemideae genera, e. g. *Chrysanthemum* L. (ABD EL-TWAB & KONDO, 2006), which is quite close to *Artemisia*. The overlapped position of these rDNA sites might be a frequent feature of chromosome evolution in *Artemisia*, and probably in some other Anthemideae (GARCIA *et al*., unpubl. res.). Hence, the results obtained in former studies and in the present one suggest that the existence of 18S-5.8S-26S rDNA linked to 5S rDNA is not an experimental artifact; previous works hypothesize a casual insertion of the 5S rDNA into the 45S repeat unit, (DROUIN & MONIZ DE SA, 1995), although a clear interpretation of this phenomenon is not still achieved.

CONCLUDING REMARKS

The present study reports a typical *Artemisia* and *Absinthium* signal pattern at the diploid level, with four rDNA loci located at telomeric positions, and sometimes in satellites of metacentric and submetacentric chromosomes. The different pattern of the distribution of GC-rich regions at telomeric and centromeric position between these two subgenera may reflect the different evolutionary histories of these species. All these results contribute to the cytogenetical knowledge of *Artemisia*, and, according to former investigations, we demonstrate that the patterns of distribution of the rDNA loci might be subject to the different evolutionary episodes that have taken place in the genus. The extensive colocalization of the 18S-5.8S-5S rDNA and 5S rDNA found in *Artemisia* should be considered as a particular trait of the evolution of ribosomal DNA in the genus, as well as in other Anthemideae genera. Further investigations would be needed to elucidate when and how these changes at genome level took place and what information they convey on the relationships at the species level within the *Artemisia* as well as at the tribal level.

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