Running title: rDNA loci changes in polyploid *Artemisia*

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Do polyploids require proportionally less rDNA loci than their corresponding diploids? Examples from *Artemisia* subgenera *Absinthium* and *Artemisia* (Asteraceae, Anthemideae)

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Abstract

Fluorescent *in situ* hybridization (FISH) of 5S and 18S-5.8S-26S ribosomal DNA has been carried out in two species of the genus *Artemisia*, belonging to the subgenera *Artemisia* (*A. medioxima*) and *Absinthium* (*A. lagocephala*) each one showing both low and high ploidy levels (2x and 16x, and 2x, 4x and 6x, respectively). Both species have a base chromosome number of $x = 9$. Linkage of both rDNA genes has been observed confirming previous researches. Diploid *A. lagocephala* ($2n = 18$) shows three rDNA loci and the hexaploid six. Also in *A. medioxima*, the number of rDNA loci does not increase in the proportion given by the ploidy level, and a relative loss is found. In this species, the diploid population shows two rDNA loci, the tetraploid four, and the hexadecaploid has around 20. The results evidence a relative loss of rDNA loci and heterochromatin, a phenomenon more pronounced at higher ploidy levels. Nevertheless, the DAPI banding pattern of *A. lagocephala* does not follow this trend, as it shows a spectacular increase of heterochromatic bands at the hexaploid level. These results are discussed in the light of possible chromosome restructurations and gene silencing mechanisms that take place during polyploidy, and more especially the allopolyploids formation.

Keywords: *Absinthium*, *Artemisia*, Asteraceae, constitutive heterochromatin, fluorescent *in situ* hybridization, rDNA.

Introduction

The genus *Artemisia* L. (Anthemideae, Asteraceae) comprises ca. 500 species and constitutes one of the larger genera in this family (Vallès & Garnatje 2005). This genus is very abundant in the Northern hemisphere and dominates many landscapes, indicating the ability of these plants to colonize different habitats. The plasticity of *Artemisia* genome is revealed especially from the genome size point of view, as evidenced in previous studies carried out by several authors (Vallès & Torrell 2001; Garcia et al. 2004, 2006b, 2008; Pellicer et al. 2008). Polyploidy, a phenomenon with high incidence in plant evolution (Otto & Whitton 2000), has been particularly relevant in *Artemisia* (Pellicer et al. 2007b and references therein). Recently, Soltis (2005) pointed out that all angiosperms are ancient or recent polyploids.

Artemisia is currently divided into five subgenera (Vallès & Garnatje 2005). Two of them, *Absinthium* and *Artemisia*, appear mixed in all molecular phylogenetic reconstructions obtained (Torrell et al. 1999; Vallès et al. 2003; Watson et al. 2002; Tkach et al. 2007; Sanz et al. 2008). This work focuses on two representatives of those subgenera with different ploidy levels, *A. lagocephala* (Bess.) DC. (subgenus *Absinthium*, 2x and 6x ploidy levels) and *A. medioxima* Krasch. ex Poljak. (subgenus *Artemisia*, 2x, 4x and 16x ploidy levels from), with the purpose to compare the rDNA loci number and localization throughout the polyploid series. *Artemisia lagocephala* is a perennial undershrub growing in stony and rocky places, endemic to the Russian Arctic, Siberia and Far East regions (Poljakov 1961). *Artemisia medioxima* is also a perennial species with long rhizomes occupying the forestry zones, leaf-bearing forests and meadows in the Russian Far East and Northern China (Poljakov 1961). The phylogenetic position of these species is not yet solved. In a more complete phylogenetic framework than the above cited (Pellicer et al. unpublished results), *A.*

lagocephala is completely merged into a clade constituted by species belonging to subgenus *Absinthium*, along with *A. krushiana* Besser and *A. litophila* Turcz. ex DC., whereas *A. medioxima* appears closely related to *A. atrata* Lam., *A. laciniata* Willd. and *A. tanacetifolia* L., both taxa belonging to the subgenus *Artemisia*.

Chromosome banding and fluorescent *in situ* hybridization (FISH) have demonstrated to be a valuable tool for studies on genome organization and evolution in plants (Sumner 1990; Jiang & Gill 1994). In particular, the detection of sites 18S-5.8S-26S and 5S ribosomal DNA is useful for chromosome identification and for evolutionary studies of *Artemisia* species (Vallès & Siljak-Yakovlev 1997; Torrell et al. 2001, 2003; Garcia et al. 2008, 2009; Pellicer et al. 2008). In these works, the FISH pattern was not always clearly related with the phylogenetic position of the species but in some cases, it was more likely associated to specific features as the life cycle.

Until now, the works dealing with rDNA loci mapping in *Artemisia* have been carried out mostly in low ploidy levels (mainly $2x$ and $4x$, and only in one case in $8x$; Garcia et al. 2008 and references therein). The objective of the present paper is to compare the rDNA loci distribution within a species showing different ploidy levels, including the one in which the highest ploidy level has been detected in the genus ($16x$, *A. medioxima*, Pellicer et al. 2007b).

Material and methods

Plant material

The populations of the species studied are listed in Table 1, classified by subgenera, with the indication of their origins, vouchers (deposited in the herbarium BCN, Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona) and collectors. Root tips were obtained from achenes germinating in wet paper in Petri dishes and

subsequently pretreated in 0.05% aqueous colchicine solution at room temperature during 2 h 15 min. After this first step, the root tips were fixed in 3:1 absolute ethanol and glacial acetic acid, stored at 4°C and transferred in a solution of ethanol 70% after a few days in the fixative.

Chromosome (protoplast) preparation

Root tips were washed in distilled water during 10 min and prepared following the method described in Leitch & Heslop-Harrison (1993), with minor modifications. The tips were incubated in a microcentrifuge tube containing 200 µl of an enzymatic solution [3% Cellulase Onozuka-RS (Yakult Honsha) and 0.5% Pectolyase Y-23 (Kikkoman)] during 40-50 min, depending on species, at 37°C. They were washed in distilled water for a 5 min, placed in a clean slide, crushed into a drop of fixative and air-dried. Staining of the slides with 1% acetic orcein was carried out to find the metaphase plates. The slides were frozen overnight at – 80°C to make easier the cover slides removal, and for acetic orcein destaining, they were washed with 45% acetic acid for 1 h and subsequently two times with distilled water during 10 min and air-dried.

Probe preparation and fluorescent in situ hybridization

The 18S-5.8S-26S rRNA gene of wheat, pTa71, which contained the intergenic spacer region, was used as 45S rDNA probe (Gerlarch and Bedbrook, 1979). Total genomic DNA of *Artemisia princeps* Pamp. was extracted from young leaves following the method of Doyle & Doyle (1987) modified by Cullings (1992). The 5S rDNA probe was amplified by PCR using the primers described in Hoshi et al. (2006) and with the following PCR conditions: one cycle of 5 min at 94.2°C, 35 cycles of (30 sec at 94.2°C, 30 sec at 55.5°C and 30 sec at 72.2°C) and one cycle for final extension at 72.2°C during

7 min. The PCR reaction mixture (final volume of 50 μ l) contained 60 ng of DNA template, 200 pmol of each primer, 0.1 mM of each dNTP, 10 mM of Tris-HCl pH 8.3 buffer, 50 mM of KCl, 1.5 mM of MgCl₂ and 1 unit of Taq polymerase (Tanaka). The 5S and 18S probes were labelled with random primed DNA labelling with digoxigenin-dUTP (Roche Diagnostics, Mannheim, Germany) and with Avidin-FITC BioNick labelling system (Invitrogen, USA), respectively, following the manufacturer's instructions. Hybridization mixtures contained 50% formamide, 10% dextran sulfate, and each probe (concentration of 4 ng/ μ l in 2xSSC) were subsequently denatured during 10 min at 95°C.

Slide preparations were incubated in 100 μ g/ml DNase-free RNase in 2xSSC for 1 h at 37°C in a wet chamber, washed once in 2xSSC (pH 7) for 10 min with slow shaking and then 10 min in 1xPBS (pH 7), treated with 4% paraformaldehyde in 1xPBS during 10 min., denatured at 72°C with 70% deionized formamide in 2xSSC 1.5 min., and dehydrated through an ethanol series (70°, 90° and 100°) and air dried. After denaturation of probe mixtures, approximately 15-20 μ l of probe were loaded on the slide and covered with coverslips. The preparations were then denatured during 5 min at 75°C, and transferred down to 37°C overnight for hybridization in a wet chamber. Posthybridization stringency washes were done with agitation as follows: two washes in 4xSSC at 42°C for 10 min followed by a wash in 2xSSC (with 0.2% Triton-100) at room temperature. For 5S signal detection, the slides were treated with 1% (w/v) bovine serum albumine (BSA) in 2xSSC with 0.2% Triton-100 for 45 min at 37°C, and then incubated for 1.5 h at 37°C in 20 μ g/ml Anti-digoxigenin-rhodamine Fab fragments (Roche Diagnostics, Mannheim, Germany) in the same buffer. Slides were washed two times for 10 min in 2xSSC with 0.2% Triton-100 at 42°C, once in 2xSSC at room temperature 5 min, once in distilled water at room temperature for 5 min, and finally

dehydrated (ethanol 70°, 90° and 100°). Counterstaining was done with Vectashield (Vector Laboratories, Burlingame CA, USA), a mounting medium containing 500 ng/ml DAPI (4',6-diamidino-2-phenylindole). FISH preparations were observed with an epifluorescence Nikon Eclipse E600 microscope using the following filters: UV-1A (365/410), B-2A (450/490), G-2A (510/560), Dia-ill. Hybridization signals were analysed and photographed using a CCD camera (Pixera, penguin 600CL), and an image analyser software (Metavue, version 4.6, Molecular Devices Corporation).

Results

The chromosome number, DNA amount and number of bands visualised with DAPI (after FISH denaturation), as well as rDNA FISH signals for each studied population, are summarized in Table 2 and presented in Figs. 1 and 2. All populations show a very symmetric karyotype, typical in *Artemisia* (Torrell & Vallès, 2001 and references therein) and, in general, in the tribe Anthemideae (Schweizer & Ehrendorfer 1983). Data on constitutive heterochromatin and rDNA loci of previously studied *Artemisia* species belonging to subgenera *Absinthium* and *Artemisia* has been used for comparative purposes.

Discussion

FISH pattern

The 18S-5.8S-26S and 5S rRNA genes are co-localized in both studied taxa, confirming the reports of all previous works on this subject in the genus *Artemisia* (Torrell et al. 2003; Garcia et al. 2007, 2009; Pellicer et al. 2008), in which both ribosomal RNA genes are linked in a single unit, a structure recently described for the first time in angiosperms (Garcia et al. 2009).

There is no correlation between the number of rDNA loci and ploidy level. It seems that the number of FISH marks does not increase proportionally to the ploidy level and moreover in the highest polyploids the number of FISH signals is much lower than expected (Fig. 3b,d). In *A. lagocephala*, six signals are detected in the diploid populations, whereas the hexaploid one presents only 12, some of them weak, instead of the expected 18 rDNA sites (Fig. 3b). The situation is similar in *A. medioxima*, where the proportionality, although maintained between the diploid and the tetraploid levels (with four and eight signals, respectively), is broken in the hexadecaploid population, which only shows ca. 20 marks (instead of the 32 expected, Fig. 3d).

A variation of the number of rDNA loci or copy number variation have been related to the polyploid formation, as well as genome downsizing, in which nuclear DNA amount of the polyploid is less than the sum of genome sizes of the parental diploids (Lim et al. 2000; Leitch et al., 2008; Leitch and Leitch 2008). On the one hand, the presence of some weaker ribosomal signals in the case of *A. lagocephala* could indicate that loss of gene copies might be taking place at these loci, as was documented in polyploid *Nicotiana* by (Leitch et al. 2008), even with an additive loci pattern. On the other hand, the hypothesis of gene loss may be also contemplated at the same level as other different consequences of polyploidy, such as evolution in function in duplicate genes or gene silencing (Adams & Wendel 2004). When considering that, the exact cause and the mechanisms of the rDNA loci loss in polyploids remain unclear, the simple excess of dispensable genetic material being one of the most plausible explanations for sequence elimination. In this respect, according to Reeder (1999), the total number of rDNA copies present in a genome is much beyond the necessary to supply the requirements of ribosomes, as many copies remain transcriptionally inactive, so the loss of rDNA loci (or copy number) in polyploids might not represent a problem

of transcriptional requirements to these species. The present results corroborate this fact, suggesting that some rRNA gene copies may not be strictly necessary in polyploids, so that they can be eliminated in the frame of DNA loss associated with polyploid formation and stabilization.

The polyploid populations of both studied species exhibit a relevant ratio of vegetative multiplication and an aptitude to occupy new habitats; particularly, the 16x *A. medioxima* population colonized a large area in a territory after being affected by a fire (A. A. Korobkov, pers. comm.). Vegetative reproduction, which gives the plants the ability to colonize new environments, is often linked to polyploidy (Urbanska 1986; Lumaret et al. 1997). Additionally This would be consistent with findings in *Nicotiana* polyploids, in which, although the number of rDNA loci was additive, considerable sequence elimination of individual copies had taken place (Leitch et al. 2008), cytogenetic instability has been described in plants with vegetative reproduction (Duncan 1945; Lewis 1970; Persson 1974; Couderc et al. 1985). In particular, aneusomaty has been reported in the hexaploid *Artemisia verlotiorum* Lamotte (Martinoli & Ogliotti 1970; Vallès 1987), a plant that usually reproduces vegetatively. The vegetative-spreading plants can better stand cytogenetic abnormalities, because meiosis is not so significant for them. Although a high amount of rDNA may be necessary for a higher protein synthesis in these plants that quickly develop in new habitats, a certain rate of rDNA loci or gene copy loss (in the context of the chromosomal irregularities proper to asexually-reproducing plants) should not be problematic.

This rDNA loci decreasing could be, in part, considered in the framework of the above-mentioned frequent occurrence of genome downsizing in polyploids (Leitch & Bennett 2004). This phenomenon has been repeatedly reported in *Artemisia* (Torrell & Vallès 2001; Garcia et al 2004, 2008; Pellicer et al. 2007b). It is also confirmed for the species studied in the present paper, with a 1Cx-value downsizing around 15% in the hexaploid *A. lagocephala* and around 47% in the hexaidecaploid *A. medioxima* with respect to their diploid relatives (Pellicer et al. submitted). Restructuration during homoeologous chromosome pairing in allopolyploids, leading sometimes to deletions and the elimination of specific DNA sequences after polyploid formation are two of the possible explanations for this non-proportional DNA content increase in polyploids (Leitch & Bennett 2004, and references therein). Furthermore, evidences have been reported in several genera of gene loss in polyploids, including rDNA loci (Wendel 2000; Ozkan et al. 2001; Leitch & Bennett 2004). Reduction of rDNA loci in polyploids has also been documented in *Artemisia*. Diploid *A. herba alba* Asso subsp. *valentina* (Lam.) Mascl. and tetraploid subsp. *herba-alba* present the same number (four) of rDNA loci (Torrell et al. 2003), and tetraploid *A. umbelliformis* exhibits eight rDNA loci instead of the 12 expected considering the six present in the related diploid *A. eriantha* (Garcia et al. unpubl. res.).

In the studied *Artemisia* species, the basic pattern of the variation of rDNA loci number with polyploidy is consistent with the findings in other genera, as it corresponds either to the exact proportionality (e.g. tetraploid *A. medioxima*, Fig. 3d; in *Tragopogon*, Pires et al. 2004; in *Nicotiana*, Leitch et al. 2008) or to a relative loss of sites (e.g. highest polyploids of *A. lagocephala* and *A. medioxima*; loss of a 5S sites in *Sanguisorba*, Mishima et al. 2002; loss of 18S-5.8S-26S loci in *Thinopyrum*, Li et al. 2004).

DAPI banding pattern

Since DAPI was used after chromosome denaturation, the regions detected with this fluorochrome correspond to constitutive heterochromatin and are equivalent to those obtained with Giemsa C-banding (Bogunic et al. 2006). The pattern of DAPI banding observed in the studied species is less homogeneous than for rDNA loci, as the number of heterochromatin marks in polyploids can be either more (*A. lagocephala*; Fig. 3b) or less (*A. medioxima*; Fig. 3c) than expected with respect to the diploids .

A dramatic increase of DAPI bands from the diploid (two telomeric marks) to the hexaploid (76 telomeric plus two subtelomeric marks) levels occurs in *A. lagocephala* (Fig. 1c,d; Fig. 3a). The appearance of new heterochromatic blocks with polyploidy is rather unfrequent in the genus, and apart from *A. lagocephala*, it has been documented only in some representative of the subgenus *Tridentatae* (Garcia et al. 2007). Chromatin remodelling mechanisms, and especially the heterochromatinization of euchromatin, are known to play an important role in plant epigenetics (Meyer 2000). When euchromatin becomes condensed in heterochromatin, the genes are not accessible to the transcription machinery, providing a gene silencing mechanism fast, effective, and potentially reversible (Chen 2007). Epigenetic regulation of gene expression is particularly active in the early stages of polyploid formation (because of its fastness), and in the case of allopolyploids it enables the match of different genomes by solving the regulatory incompatibilities and gene dosage problems (Chen 2007). Other hypothesis to explain the emerging heterochromatin blocks might be that these correspond to some novel satellite, as Lim et al. (2007) found in *Nicotiana*, maybe also to species-specific repeats, that have appeared during the polyploidization.

Artemisia medioxima shows less DAPI marks (than expected from the diploid population) at tetraploid and hexadecaploid levels (Fig. 3c). The diploid population ($2n = 18$) presents 12 bands, about 18 are observed in the tetraploid ($2n = 36$), and around 34 in the hexadecaploid ($2n = 144$; Fig. 2d, e, f; Table 2). The same trend is found in the subgenus *Absinthium*, between the diploid *A. eriantha* Ten., which shows 36 C-bands, and its related tetraploid *A. umbelliformis* Lam., which has 56 (Garcia et al. unpubl. res.). In another subgenus, *Dracunculus*, a tetraploid population of *A. campestris* L. has 34 C-bands, the same number of its related hexaploid *A. crithmifolia* L. (Torrell et al. 2001). This relative loss of heterochromatic bands follows the same tendency than rDNA loci or 1Cx genome size, and may respond to the same pressures acting toward a genome diploidization

Conclusions

The study of two diploid-polyploid pairs in two closely related *Artemisia* subgenera (*Absinthium* and *Artemisia*) has basically shown that polyploidy is associated with a proportional decrease of rDNA loci, and either a decrease or increase of constitutive heterochromatin regions. These results agree with previous reports on other subgenera of *Artemisia* (Torrell et al. 2001, 2003; Garcia et al. 2008, unpubl. res.), particularly *Seriphidium* and *Tridentatae* (and *Dracunculus* only for the heterochromatin).

Ongoing research aiming to complement the knowledge of the subgenera *Absinthium* and *Tridentatae* and especially an extensive review of subgenus *Dracunculus* (Pellicer et al. unpubl. res.) will allow us to depict precisely the evolution of heterochromatin and rRNA genes in the genus.

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Table 1. Origin, collectors and vouchers of the *Artemisia* species and populations studied.

Species	Origin, collectors and voucher
Subg. <i>Absinthium</i> (Mill.) Less.	
<i>Artemisia lagocephala</i> (Fischer ex Besser) DC.	Russia, Snezhnaya mountain. Index Seminum Vladivostok n° 53. (BCN S-805). $2n = 18$
<i>Artemisia lagocephala</i> (Fischer ex Besser) DC.	Russia, Sokha Republic (Yakutya), Aldan raion: Ugoyan, near the mouth of Tommozha river, forest, V.N. Zakharova, 26.viii.2005. (LE-Korobkov 06-29). $2n = 54$
Subg. <i>Artemisia</i>	
<i>Artemisia medioxima</i> Krasch. ex Poljakov	Mongolia, Tuv (Central) aimag: Mungunmort sum, 10 km north-west of the sum, path margins, steppe, Sh. Dariimaa, Sh. Tsooj, J. Vallès & E. Yatamsuren, 7.ix.2004 (BCN 23792). $2n = 18, 36$
<i>Artemisia medioxima</i> Krasch. ex Poljakov	Russia, Khabarovskii krai: Baninskii raion, Tumnin river basin, upper Akur river valley, base of rocky slopes, Larix and Betula forest, A.A. Korobkov, 21.x.2004 (LE-Korobkov). $2n = 144$

Table 2. Karyological and cytogenetic features of the studied species and populations.

Species	Chromosome number (2n)	DAPI (constitutive heterochromatin)	18S-5.8S-26S	5S
Subg. <i>Absinthium</i>				
<i>A. lagocephala</i>	18	2	6	6
<i>A. lagocephala</i>	54	78	12	12
Subg. <i>Artemisia</i>				
<i>A. medioxima</i>	18	12	4	4
<i>A. medioxima</i>	36	ca. 18	8	8
<i>A. medioxima</i>	144	≥ 34	ca. 20	ca. 20

Figure 1: Physical mapping of 5S and 18S-5.8S-26S rDNA loci revealed by FISH and DAPI signals in diploid (a, c) and tetraploid (b, d) populations of *Artemisia lagocephala* (subgenus *Absinthium*).

Figure 2: Physical mapping of 5S and 18S-5.8S-26S rDNA loci revealed by FISH in diploid (a, d), tetraploid (b, e), and hexaidecaploid (c, f) populations of *Artemisia medioxima* (subgenus *Artemisia*).

Figure 3: Graphical representation of the variation of DAPI marks and rDNA sites with increasing ploidy levels. **a-b** *Artemisia lagocephala*, DAPI marks ft. chromosome number (a), rDNA sites ft. chromosome number (b). **c-d** *Artemisia medioxima*, DAPI marks ft. chromosome number (c), rDNA sites ft. chromosome number (d). Full lines represent the observed values and dotted lines the expected ones.

Figure 1

Figure 2

Figure 3