


Impact of dysploidy and polyploidy on the diversification of high mountain *Artemisia* (Asteraceae) and allies

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Abstract Molecular cytogenetics and the study of genome size have been used to understand evolutionary and systematic relationships in many species. However, this approach has seldom been applied to alpine plants. A group of dysploid–polyploid high mountain *Artemisia* species, distributed from the European Sierra Nevada to Central Asian mountains, through the Pyrenees, the Alps and the Caucasus, is a good model to consider changes at the genome and chromosome levels. These small perennial *Artemisia*, found frequently in isolated populations, present highly disjunct distributions. Some are considered rare or even endangered. Here, we show results for nine species and 31 populations, including genome size (2C-values), fluorochrome banding and fluorescent in situ hybridisation of ribosomal RNA genes (rDNA). Significant intraspecific genome size variation is found in certain populations of *A. eriantha* and *A. umbelliformis*, but without taxonomic significance due to the absence of morphological or

ecological differentiation. The number and position of GC-rich DNA bands is mostly coincidental with rDNA although there is an expansion of GC-rich heterochromatin in centromeres in some taxa. Ancestral character states have been reconstructed and $x = 9$ is inferred as the likely ancestral base number, while the dysploid $x = 8$ has appeared repeatedly during the evolution of *Artemisia*. On the basis of cytological observations, Robertsonian translocations are proposed for the appearance of dysploidy in the genus. A remarkable presence of $x = 8$ -based species has been detected in the clade including high mountain species, which highlights the important role of dysploidy in the diversification of high mountain *Artemisia*. Conversely, polyploidy, though present in the alpine species, is more common in the rest of the genus, particularly in arctic species. Hypotheses on the mechanisms underpinning the relative abundance of dysploids and scarcity of polyploids in high mountain *Artemisia* are discussed.

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Introduction

High mountain communities are systems that endure harsh conditions, including extreme cold and fluctuating temperatures, exposure to snow, ice and strong winds, poor and rocky soils, high levels of UV radiation and little oxygen availability. Moreover, elevation gradients allow large environmental changes in short spatial distances (Nieto Feliner 2011). All these factors promote local adaptations which, coupled with processes such as long-term isolation

and secondary contacts, may lead to an increased biodiversity at high altitudes (Nagy and Grabherr 2009). Distribution areas of cold-adapted species were fragmented and restricted to higher elevations during interglacial periods, which created the current pattern of vicariant taxa between mountain tops in the Mediterranean Basin (Nieto Feliner 2014). In this regard, multiple glacial refugia have been identified in mountain ranges across Europe and North Africa, both in plants (Španiel et al. 2011) and animals (Canestrelli and Nascetti 2008).

Polyploidy and genome size studies in high mountain plants: state of the art

Several studies have hypothesised that high mountain plants undergo polyploidy more frequently (Löve and Löve 1949; Petit and Thompson 1999). It is thought that the ranges for polyploid species would include more extreme environments, such as high altitudes and latitudes, than their diploid relatives (Levin 2002; Weiss-Schneeweiss et al. 2013). However, the pattern stating the prevalence of polyploid taxa in alpine environments is still controversial. Besides, dysploidy (i.e. change in basic chromosome number, usually by fusion or fission, without a significant gain or loss of genetic material) has also contributed to the diversification of several alpine groups such as *Phyteuma* (Schneeweiss et al. 2013), *Saxifraga* (Mas de Xaxars et al. 2015) or *Leontopodium* (Russell et al. 2013) and, together with polyploidy, it is another important mechanism of karyotype evolution.

The C-value (i.e. genome size or the total amount of DNA in the unreplicated haploid or gametic nucleus) has been used in many taxonomic, systematic and evolutionary studies in a range of plant groups. Although genome size has been frequently correlated with environmental, phenotypic or ecological variables (see a review in Greilhuber and Leitch 2013), it has rarely been investigated in the context of high mountain communities. Albach and Greilhuber (2004) found significantly higher DNA amounts in alpine species from *Veronica*, while Mráz et al. (2009) found negative correlations with altitude and latitude in alpine populations of *Hieracium alpinum* L. Loureiro et al. (2013) explored genome size variation, beyond polyploidy incidence, in plants from two Spanish mountain ranges, finding no significant genome size differences or correlates. However, they stated that large genomes were difficult to find in high mountain environments.

***Artemisia* L. and its high mountain species**

Artemisia comprises about 500 species with a circumpolar and northern hemisphere distribution, with only a few species in South America and Africa (Tkach et al. 2008). The

fossil pollen record places the onset of differentiation of *Artemisia* back to the Early Miocene, about 19.8 Ma (Sanz et al. 2011). The main speciation and diversification centre of the genus is Central Asia, with secondary centres located in the Irano-Turanian and Mediterranean regions and in western North America (Vallès et al. 2011). *Artemisia* species are mostly perennial and particularly diverse in mountains, grasslands and semidesertic areas (Ling et al. 2006). The traditional classification of the genus, which relies on capitulum characters, is partially incongruent to lineages identified in molecular studies (Sanz et al. 2008). Nevertheless, the classical subgenera [*Artemisia*, *Ab-sinthium* (Mill.) Less., *Dracunculus* (Besser) Rydb., *Seriphidium* Besser ex Less., and *Tridentatae* (Rydb.) McArthur] are still widely used for the circumscription of the genus.

Within *Artemisia* two main basic chromosome numbers are recognised, $x = 9$ and $x = 8$, the former being the most common. Abundant karyological and cytogenetic information is available for the genus (for a review see Vallès et al. 2011). Polyploidy is common in the genus, ploidy levels have been found up to 16x and many species exist in polyploid series. Genome size variation has also been widely explored in *Artemisia*, which has become one of the best known genera in the family from this point of view.

A suitable model for studying the implications of genome size variation, dysploidy and polyploidy in the diversity and evolution of high mountain plants is found in a group of alpine *Artemisia*. This group is distributed from Sierra Nevada (Iberian Peninsula) to the Central Asian Mountains, through the Pyrenees, the Alps and the Caucasus. Some of the species associated with high elevations are currently considered as rare or endangered, such as *Artemisia granatensis*, endemic to Sierra Nevada. These small herbaceous perennials are usually found in isolated populations growing on siliceous rocky ledges or limestone substrates, often in crevices, at elevations between 1500 and 3400 m above sea level. Some taxa, such as *A. eriantha* and *A. umbelliformis*, show a highly disjunct distribution, reflecting the geographical complexity of the Pyrenees and the Alps (Sanz et al. 2014).

In the present study, we aim to explore genome size variation, ploidy and base chromosome number changes, and heterochromatin and rDNA loci evolution in alpine *Artemisia* and considering a phylogenetic context. Given the wealth of information currently available on the genus, we have the opportunity to address genome dynamics and evolutionary factors in this group of alpine plants. Our findings will contribute further evidence to shape the genomic/cytogenetic characteristics of alpine species. To fulfil these goals, two main questions have been specifically addressed: (1) is there any significant difference in basic chromosome number, genome size, heterochromatic or

rDNA distribution patterns in the high mountain *Artemisia* with respect to their lower altitude counterparts? and (2) are polyploidy and/or dysploidy more frequent in this group than in other non-high mountain *Artemisia*, and what is their biological significance? Additionally, and taking advantage of all available phylogenetic, cytogenetic and genome size information, we will infer the ancestral state of the studied features in this group and in the genus, and we will discuss the prevalence of ancestral states in the present-day high mountain *Artemisia*.

Materials and methods

Plant material

As part of an ongoing project focusing on the biogeography of two of the most abundant alpine *Artemisia* species, *A. eriantha* and *A. umbelliformis* (Sanz et al. 2014, Sanz et al. unpublished), samples of these as well as closely related species were collected to perform cytogenetic and genome size assays. All specimens were gathered from natural sites except one population of *A. eriantha* obtained via Index Seminum from the Alpine Botanical Garden of Lautaret (Hautes-Alpes, France), which had been formerly collected near the Col du Lautaret at 2300 m above sea level. Achenes were germinated in pots and cultivated in the greenhouse at the Faculty of Pharmacy, Universitat de Barcelona (UB). Vouchers have been deposited in the herbarium BCN, of the Centre de Documentació de Biodiversitat Vegetal, UB. Table 1 shows the populations studied. Figure 1 indicates the geographical locations of the populations assessed across the high mountain ranges. Seeds of *Pisum sativum* ‘Express Long’ and *Petunia hybrida* ‘PxPC6’ used as internal standard for flow cytometry measurements, were obtained from the Institut des Sciences du Végétal (CNRS, Gif-sur-Yvette, France). Root tip meristems were obtained from seedlings produced by germinating achenes on wet filter paper in Petri dishes in the dark at room temperature.

DNA content assessments

DNA 2C-values of the selected species were estimated using flow cytometry. As outlined above, *P. sativum* and *P. hybrida* (2C = 8.37 and 2.85 pg, respectively; Marie and Brown 1993) were used as internal standards. We have analysed 29 populations of 8 species, with special emphasis on *A. eriantha* (12 populations) and *A. umbelliformis* (10 populations), because these are two of the most widely distributed species of this group. Young healthy leaf tissues from the species to be studied and the calibration standard were placed together in a plastic Petri dish and chopped in LB01 buffer (Doležel et al. 1989) with a razor blade. The suspension of nuclei was

filtered through a nylon mesh with a pore size of 70 µm and stained for 20 min with propidium iodide (60 µg/mL; Sigma-Aldrich, Madrid, Spain) and supplemented with 100 µg/mL ribonuclease A (Boehringer, Meylan, France). More details on the methodology can be found in Garcia et al. (2006). Mean values and standard deviations were calculated based on the results for five individuals per population. Results on genome size have been submitted to the GSAD database (<http://www.asteraceagenomesize.com>).

Chromosome preparations

Root tips were pre-treated with 0.05 % aqueous colchicine, at room temperature, for 2.5–4 h. The material was fixed in absolute ethanol and glacial acetic acid (3:1) and then stored at 4 °C. Protoplast preparations for fluorochrome banding and fluorescent in situ hybridisation (FISH) were carried out using the air-drying technique of Geber and Schweizer (1987), with some modifications as explained in Garcia et al. (2007).

Fluorochrome banding and fluorescent in situ hybridisation

In order to reveal GC-rich DNA bands, chromomycin A₃ was used, following the protocol in Garcia et al. (2007). DNA hybridisation was carried out also following Garcia et al. (2007) with minor changes: the 18S-5.8S-26S rDNA probe was obtained from the clone pTa71 (Gerlach and Bedbrook 1979) and was labelled with direct Cy3 -red- (GE Healthcare, Buckinghamshire, UK); the 5S rDNA probe was obtained from the clone pTa794 (Gerlach and Dyer 1980) and labelled with digoxigenin-11-dUTP-green-(Boehringer, Meylan, France). The preparations were counterstained with Vectashield (Vector Laboratories, Burlingame, CA, USA), a mounting medium containing DAPI. FISH preparations were observed with an epifluorescent Zeiss Axiophot microscope with different combinations of Zeiss excitation and emission filter sets (01, 07 and 15). Hybridisation signals were analysed and photographed using the highly sensitive CCD camera (Princeton Instruments, Trenton, NJ, USA), and an image analyser software (Metavue, version 4.6, Molecular Devices Corporation, Sunnyvale, CA, USA). Results on number and position of rDNA loci for each of the populations studied have been submitted to the Plant rDNA database (<http://www.plantrdnadatabase.com>).

Phylogenetic analyses and reconstruction of character evolution

Beyond the high mountain *Artemisia*, the sampling strategy for the phylogenetic analysis includes several complementary species to survey representative taxa across the different

Table 1 Provenance of the populations of *Artemisia* studied

Species	Origin	LatN long	PL ¹	2n ²	2C ³ (pg)	SD ⁴	2C ⁵ (Mbp)	1Cx ⁶ (pg)	Standard ⁷
<i>A. assoana</i> Balb.	Spain, Teruel, Puerto del Esquinazo	40°53'N 0°53'W	2	18	7.97	(0.11)	7475.86	3.98	<i>Petunia</i>
<i>A. eriantha</i> Ten.	Andorra, Ordino, Pic de Casamanya	42°34'N 01°33'E	2	18	6.17	(0.07)	6034.26	3.09	<i>Petunia</i>
	Bulgaria, Rila, Jostifika Mountain	42°00'N 23°30'E	2	18	6.24	(0.08)	6102.72	3.12	<i>Petunia</i>
	France, Alpes-Maritimes, Pas des Ladres	44°05'N 07°24'E	2	18	6.36	(0.08)	6220.08	3.18	<i>Petunia</i>
	France, Hautes-Alpes, Col du Galibier	45°03'N 06°24'E	2	18	5.72	(0.05)	5594.16	2.86	<i>Petunia</i>
	France, Hautes-Alpes, Col du Laurichard	45°06'N 06°24'E	2	18	6.33	(0.09)	6152.76	3.17	<i>Petunia</i>
	Greece, Epirus, Epiro Ploskos	39°58'N 20°46'E	2	18	5.93	(0.22)	5799.54	2.97	<i>Petunia</i>
	Greece, Tharsis, Mount Olympus	40°05'N 22°22'E	2	18	5.83	(0.24)	5701.74	2.92	<i>Petunia</i>
	Italy, Alpi Maritime, lake Ischiator	44°18'N 07°02'E	2	18	6.11	(0.15)	5975.58	3.06	<i>Petunia</i>
	Poland, Tatra Mountains, Liliowe Turnie	49°13'N 19°54'E	2	18	5.94	(0.22)	5809.32	2.97	<i>Petunia</i>
	Rumania, Dâmbovița, Bucegi-Caraiman	45°26'N 25°27'E	2	18	6.26	(0.21)	6122.28	3.13	<i>Petunia</i>
	Spain, Huesca, Ibón de Urdiceto	42°40'N 00°15'E	2	18	6.18	(0.14)	6044.04	3.09	<i>Petunia</i>
	Rumania, Argeș, Făgăraș	45°35'N 25°39'E	2	18	6.44	(0.35)	6259.68	3.22	<i>Petunia</i>
<i>A. genipi</i> Weber ex. Stechm.	Italy, Valle d'Aosta, Cogne de Scaletta	45°36'N 07°21'E	2	18	5.60	(0.09)	5476.80	2.80	<i>Petunia</i>
<i>A. glacialis</i> L.	Italy, Valle d'Aosta, Valnontey	45°35'N 07°20'E	2	16	8.52 ^a	(0.15)	8332.56	4.26	<i>Petunia</i>
	France, Alpes-Maritimes, Col de Tortisse	44°19'N 06°56'E	2	16	8.92	(0.41)	8723.76	4.46	<i>Petunia</i>
<i>A. granatensis</i> Boiss.	Spain, Granada, Sierra Nevada 1	37°05'N 03°23'W	2	16	7.43	(0.11)	7266.54	3.72	<i>Petunia</i>
	Spain, Granada, Sierra Nevada 2	37°80'N 03°24'W	2	16	7.61	(0.15)	7442.58	3.81	<i>Petunia</i>
<i>A. melanolepis</i> Boiss.	Iran, Māzandarān, Elburz Mountains	36°04'N 51°44'E	2	16	8.04	(0.18)	7863.12	4.02	<i>Petunia</i>
<i>A. nitida</i> Bertol.	Italy, Alpi Apuane, Monte Macina	44°04'N 10°14'E	6	54	18.40	(0.14)	17259.2	3.06	<i>Pisum</i>
<i>A. splendens</i> Willd.	Iran, Āzarbāijān-e Gharbī, Doughron mountains	38°44'N 46°40'E	4	32	13.59 ^b	(0.21)	13291.02	3.40	<i>Pisum</i>
<i>A. umbelliformis</i> Lam.	France, Alpes-Maritimes, Col de la Bonette	44°20'N 06°48'E	4	34	13.18	(0.19)	12899.82	3.30	<i>Pisum</i>
	France, Alpes-Maritimes, Col de Tortisse	44°18'N 06°57'E	4	34	13.03	(0.22)	12743.34	3.26	<i>Pisum</i>
	France, Hautes-Alpes, Col du Galibier	45°02'N 06°14'E	4	34	13.21	(0.08)	12919.38	3.30	<i>Pisum</i>
	France, Pyrénées-Orientales, Canigó	42°31'N 02°28'E	4	34	13.10	(0.18)	12811.8	3.28	<i>Pisum</i>
	Italy, Valle d'Aosta, Valnontey	45°35'N 07°20'E	4	34	13.40	(0.10)	13105.20	3.35	<i>Pisum</i>
	Spain, Catalonia, Girona, Noucreus	42°25'N 02°09'E	4	34	12.94	(0.42)	12650.49	3.23	<i>Pisum</i>
	Spain, Granada, Sierra Nevada 3	37°48'N 03°14'W	4	34	13.18	(0.12)	12890.04	3.30	<i>Pisum</i>
	Spain, Huesca, Collaradeta	42°44'N 00°31'W	4	34	12.40 ^b	(0.25)	12127.20	3.10	<i>Pisum</i>
	Spain, Palencia, Aguja de Cardaño	43°01'N 4°43'W	4	34	13.27	(0.06)	12978.06	3.32	<i>Pisum</i>
	Switzerland, Valais, Riffelberg	45°59'N 6°45'E	4	34	13.44	(0.05)	13144.32	3.36	<i>Pisum</i>

(1) Ploidy level; (2) chromosome number; (3) genome size in pg; (4) standard deviation; (5) genome size in Mbp (1 pg = 978 Mbp; Doležel et al. 2003); (6) monoploid genome size; (7) internal standard: *Petunia hybrida* 'PXP6' (2C = 2.85 pg), *Pisum sativum* 'Express Long' (2C = 8.37 pg). Genome size data from: ^a Garcia et al. (2004); ^b Torrell and Vallès (2001). Chromosome numbers confirmed and/or extracted from the Index to Chromosome numbers in Asteraceae (http://www.lib.kobe-u.ac.jp/infolib/meta_pub/G0000003asteraceae_e) except for *Artemisia melanolepis* (new count)

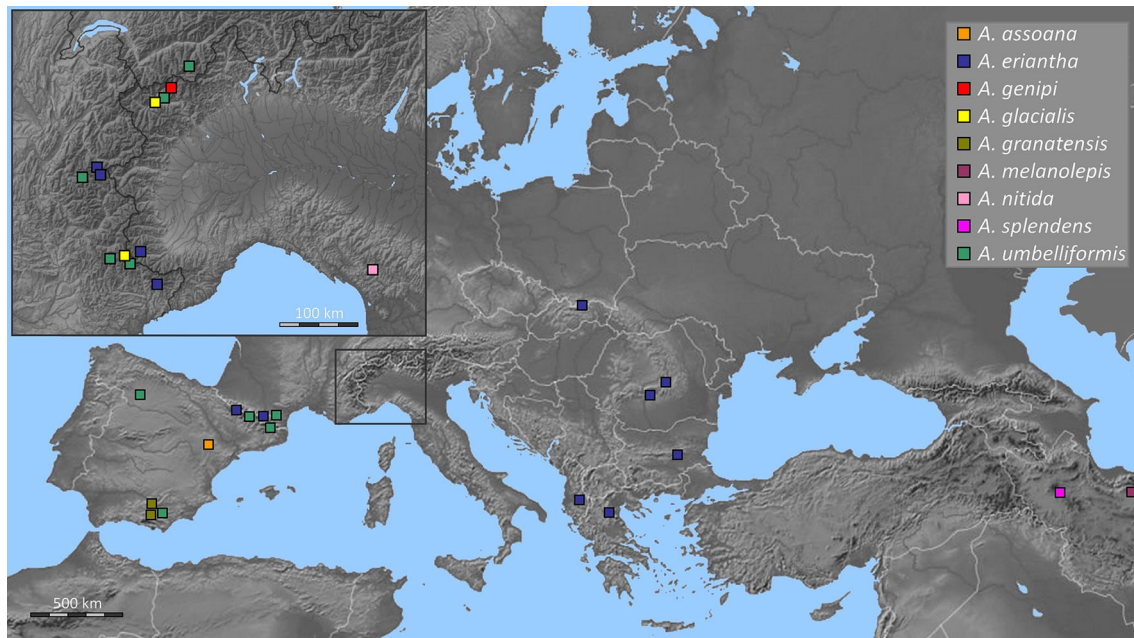


Fig. 1 Map indicating the provenance of the populations studied. Zoomed area in the *upper left corner* corresponds to the Alps, where many of the populations studied are present

recognised subgenera in *Artemisia*, bearing in mind availability of previous cytogenetic and genome size (2C-values) data. Species of *Argyranthemum*, *Nipponanthemum* and *Tanacetum* were selected as outgroups based on phylogenetic results of Sanz et al. (2008) and Zhao et al. (2010), and the availability of complete cytogenetic and genome size information. The nuclear ITS1 + ITS2 (470 bp) and the ETS sequences (374 bp), downloaded from GenBank (Supplementary Materials S1 and S2), and the newly generated ETS sequence for *A. genipi* (KT954132), were manually edited and concatenated using BioEdit v. 7.1.3.0 (Hall 1999). For phylogenetic analyses, only the 3' end of the ETS region was used due to the existence of subrepeats and indels causing alignment problems. Bayesian phylogenetic analysis was performed in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003) using the SYM + G model previously determined from jModeltest v. 2.1.3 (Darriba et al. 2012) under the Akaike information criterion (Akaike 1979). The Markov chain Monte Carlo (MCMC) sampling approach was used to calculate posterior probabilities (PPs). Four consecutive MCMC computations were run for 2,000,000 generations, with tree sampling every 100 generations. The first 1000 samples were discarded as the burn-in period. PPs were estimated through the construction of a 50 % majority-rule consensus tree.

The ancestral character reconstructions were performed with Mesquite v. 3.02 software (Maddison and Maddison 2015). All taxa included in these analyses were diploid in order to avoid the bias of polyploidy in the estimated nuclear DNA contents and number of rDNA sites. The 50 %

majority-rule consensus tree resulting from Bayesian Inference analysis was used as the input tree file (after pruning polyploid taxa). Basic chromosome numbers ($x = 8$ and $x = 9$) and the number of rDNA sites were transformed into categorical data (discrete and ordered). Ancestral states using the “trace character history” function were then inferred using maximum likelihood under the Mk1 model, in which all changes are equally probable, following the same approach as Vaio et al. (2013). For reconstructing the ancestral holoploid genome sizes (2C), we selected squared-change maximum parsimony as implemented for continuous characters in Mesquite. This approach has been widely used to reconstruct ancestral genome size states in many plant groups (e.g. Burleigh et al. 2012).

Statistical analyses

Analyses of regression and one-way ANOVA (or alternatively the Kruskal–Wallis test), Shapiro–Wilk test for normality and Barlett’s test for equality of variances were performed with RStudio, v.0.98.1078, a user interface for R (<http://www.rstudio.com>). Additionally, to analyse variation of the basic chromosome number, holoploid genome sizes, number of chromomycin A₃ positive (CMA+) bands and of rDNA sites in a phylogenetic context, the phylogenetically based generalised least squares (PGLS) algorithm, as implemented in the *nlme* package for R (Version 3.1-118), was used (Pinheiro et al. 2015). Data on holoploid genome size and ribosomal DNA loci for the complementary and outgroup species were extracted from the Genome

Size in the Asteraceae Database (<http://www.asteraceagenomesize.com>) and the Plant rDNA database (<http://www.plantrdnadatabase.com>).

Results

Genus *Artemisia* is well known from the molecular cytogenetic perspective, yet the large subgenus *Absinthium* and, in particular, the group of taxa inhabiting high mountains, had not been the focus of any cytogenetic research so far. With the present study, known genome size and molecular cytogenetic profiles of *Artemisia* span up to 32 and 10.4 % of the accepted taxa, respectively (531 taxa according to The Plant List Version 1.1 <http://www.theplantlist.org>, accessed March 13th, 2015).

Phylogenetic placement of high mountain *Artemisia*

The nuclear ribosomal DNA original dataset included 73 *Artemisia* species selected to provide a good representation of all subgenera (Vallès et al. 2011). The concatenated aligned dataset consisted of 844 characters (bp), excluding the 5.8S gene. According to our phylogenetic reconstruction (Fig. 2 and Supplementary Material S2), all high mountain taxa, except *A. atrata*, are grouped in a strongly supported clade (PP = 100 %) that also includes other members of subgenera *Absinthium* and *Artemisia*. For simplicity, we have named this group ‘high mountain and allies’ (HMA clade hereafter). When polyploid species are pruned from the tree, 52 species are retained and the HMA clade still retains statistical support (PP = 99 %).

Basic chromosome numbers

Our sample contains representatives of the main basic numbers of the genus ($x = 8$ and $x = 9$) and from the diploid to the hexaploid level. For both the genus and the HMA clade $x = 9$ has been reconstructed as the ancestral chromosome number, while $x = 8$ has appeared independently in the three main clades of the phylogeny (Fig. 2). In particular, $x = 8$ has arisen repeatedly in the HMA clade, with 50 % of its members having this basic chromosome number. Overall, comparing the number of $x = 8$ species with the number of $x = 9$ species, the incidence of the former is significantly higher in this clade than elsewhere in the genus ($F_{2,59} = 5.397$, $p = 0.007$).

Genome size variation and relationship with dysploid karyotypes

Table 1 provides holoploid genome size results (2C-values) obtained in the species studied, and a plot illustrating mean

genome sizes of $x = 8$ and $x = 9$ based *Artemisia* is presented in Supplementary Material S3. Results range from 5.6 pg for the diploid *A. genipi* to 18.4 pg for the hexaploid *A. nitida*, which means a global 3.28-fold range, with a 1.59-fold at the diploid level. The low HPCV (half peak coefficient of variation corresponding to ten samples of five different individuals) mean value (3.22 %) indicates good quality of the flow cytometric assessments. Intraspecific (interpopulation) differences have been detected in cases in which several populations were assessed, reaching up to 11.26 % in diploid *A. eriantha* and 8.39 % in the tetraploid *A. umbelliformis*. Within these species, several populations (those usually with values at the limits of the range) had significantly different genome sizes ($F_{11,40} = 5.882$, $p < 0.0001$ for the populations of *A. eriantha* and $F_{9,40} = 10.963$, $p < 0.0001$ for the populations of *A. umbelliformis*). Two populations of each for *A. glacialis* and *A. granatensis* were also measured, but differences were small, 1.48 % and 2.42 %, respectively.

Using the phylogenetically generalised least squares method (PGLS) we have observed that holoploid genome size (2C) is significantly and positively correlated with ploidy level ($t_{2,47} = 8.345$, $p = 0.0039$) and chromosome number ($t_{2,47} = 8.358$, $p = 0.023$), as expected, while monoploid genome size (1Cx) decreases significantly with both ($t_{2,47} = -1.680$, $p = 0.0000$; $t_{2,47} = -1.631$, $p = 0.0000$, respectively), indicating a certain degree of genome down-sizing. There is an approximate genome size reduction of 13 and 20 % at the 4x and 6x levels, respectively.

Fluorochrome banding (GC-rich heterochromatin) and rDNA loci

Results of chromomycin A₃ (CMA+) banding, indicative of GC-rich bands, and rDNA FISH are presented in Figs. 3 and 4. The number of CMA+ signals in one population of *A. eriantha* and *A. genipi* is four, while in another population of *A. eriantha* it is eight, all located at terminal position and overlapping with rDNA sites. *Artemisia umbelliformis* presents six terminal sites, although one faint centromeric signal is also visible. *Artemisia melanolepis* chromosomes bear five to six terminal sites, while the closely related tetraploid *A. splendens* displays eight CMA+ signals. The remaining species display much more CMA+ signals, some of them being particularly abundant in pericentromeric position. This is the case for *A. glacialis*, showing up to 20 CMA+ signals out of which 18 are pericentromeric, and *A. assoana* with 16–18 visible CMA+ bands, out of which at least 12 are pericentromeric. *Artemisia nitida* shows c. 50 CMA+ bands although few are pericentromeric in this case. DAPI after FISH has resulted in signals at both chromosome ends in most chromosomes of *A. glacialis* and *A. assoana*, and in some of *A. nitida*.

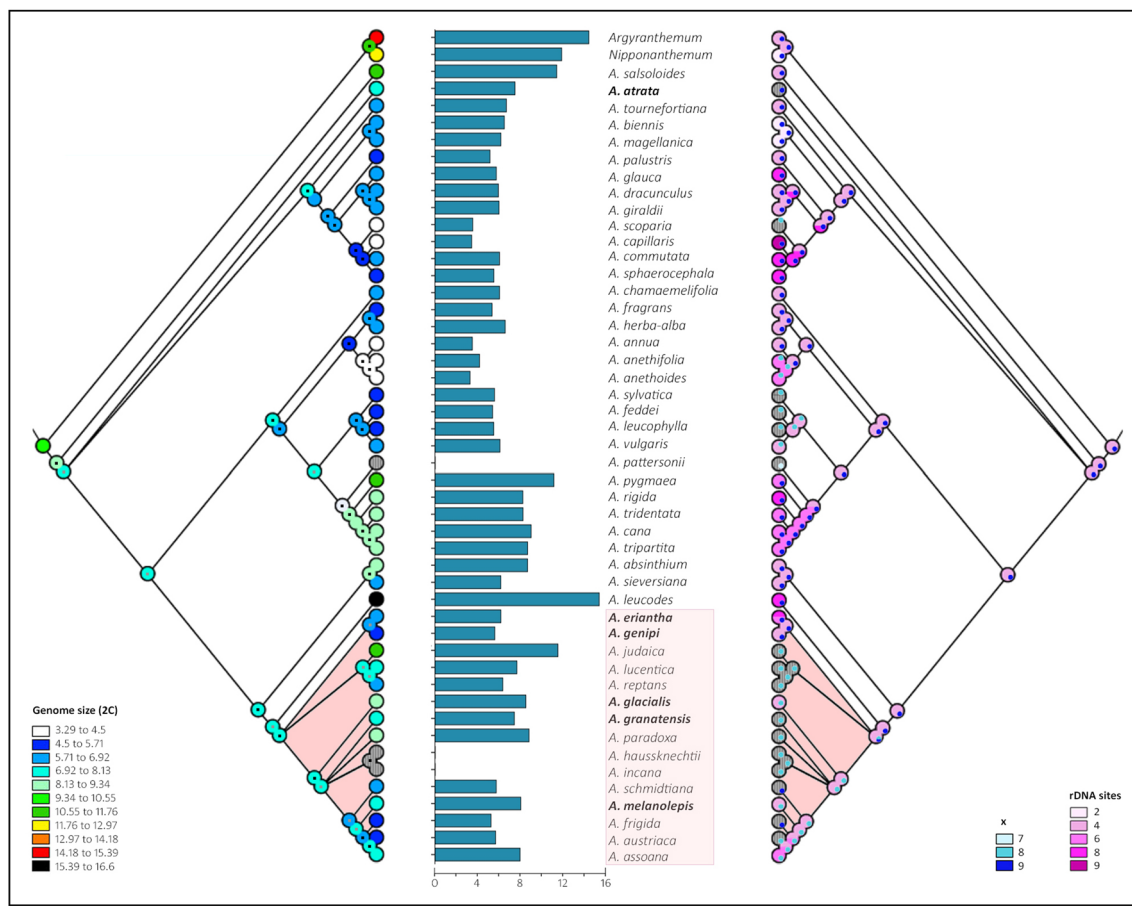


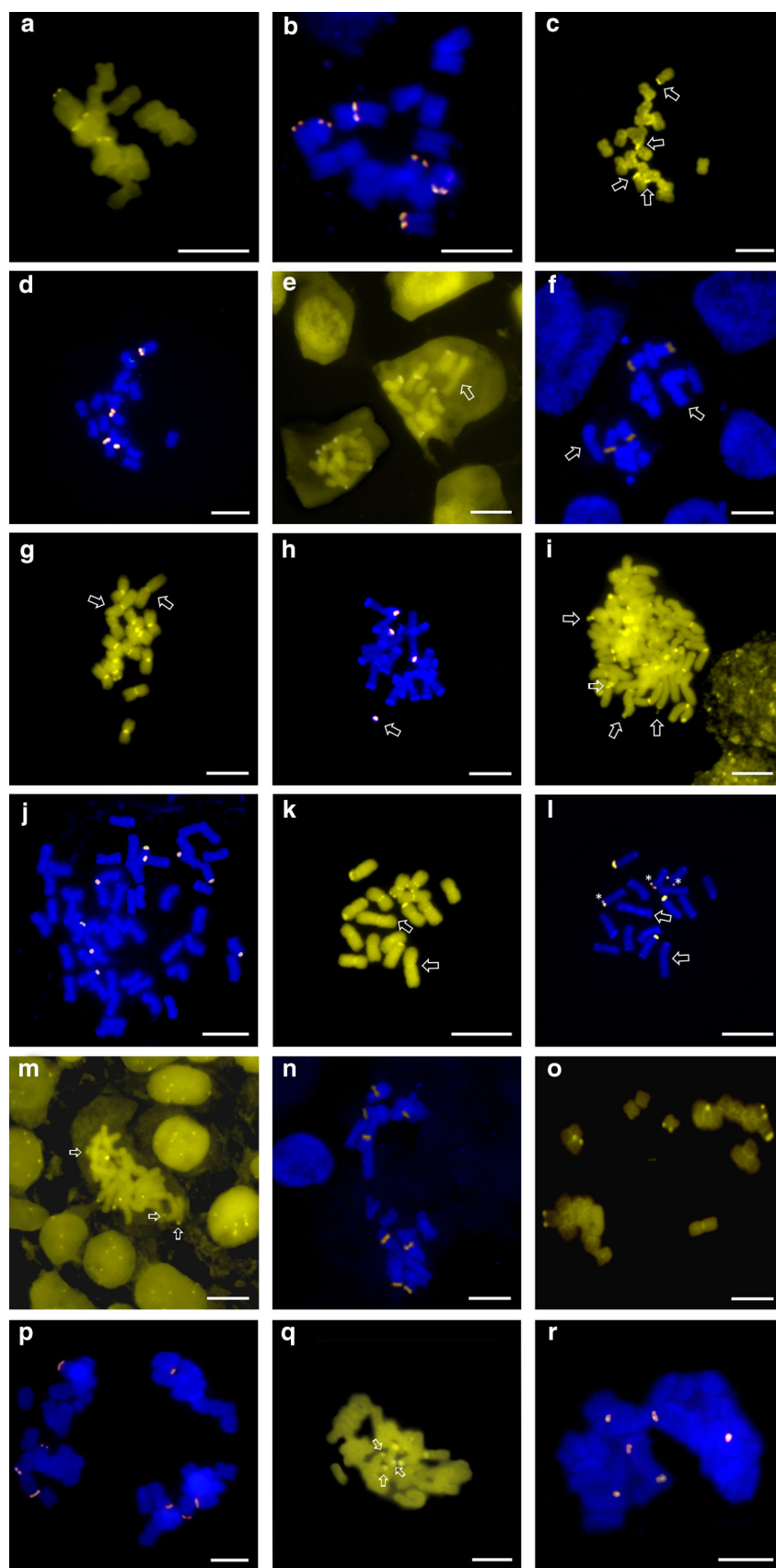
Fig. 2 Ancestral state reconstruction of genome size (*left*) and basic chromosome number and number of rDNA sites (*right*) for diploid *Artemisia* taxa. The HMA clade is highlighted in *pink* and the strictly high mountain species in *bold*. Species for which no data are available indicated in striped *grey*. Ancestral state reconstruction was estimated by Mesquite (v.3.02) using the 50 % majority-rule consensus

topology obtained by Bayesian inference phylogenetic analysis of the ITS1, ITS2 and ETS data sequence. The Bayesian clade-credibility values are indicated as *grey* and *black squares* (posterior probability >0.7 and >0.95, respectively). *Blue bars* depict genome sizes (2C values)

For rDNA loci, our results (Table 2) show a homogenised L-type organisation of ribosomal RNA genes, in which all rDNA sites, in all species, present overlapping 5S and 35S signals, thus both rRNA genes colocalise in the same loci. Perfect coincidence between the number of rDNA sites and the number of CMA+ bands is only seen, however, in *A. eriantha* (Fig. 4), *A. genipi* (Fig. 3c, d) and in *A. splendens* (Fig. 3m, n), while in another *A. eriantha* (Fig. 3a, b, corresponding to the Lautaret population) and in *A. umbelliformis* (Fig. 3o, p) not all rDNA sites are located in CMA+ regions, and in *A. melanolepis*, *A. glacialis*, *A. nitida* and *A. assoana* there are more CMA+ bands than rDNA sites—in the last three cases many more (Fig. 3g, i, k). The number of sites ranges from four to eight at the diploid, six to eight at the tetraploid, and nine at the hexaploid levels. The position of signals is invariably terminal and its number is constant within a population except for *A. eriantha* and *A. umbelliformis*, in which individuals presenting six and eight rDNA sites have been found; in one *A. eriantha* a small and

unpaired chromosome shows an rDNA site at each end (Fig. 4). As for genome size, the number of rDNA sites is positively correlated with chromosome number ($F_{1,62} = 82.06$, $p < 0.0001$) and ploidy level ($F_{1,62} = 79.18$, $p < 0.0001$), and both for the whole genus and for the HMA clade (the distribution of ploidy levels in *Artemisia* is presented in Fig. 5). Also, there is a significant ($F_{1,46} = 4.767$, $p = 0.0341$) reduction of the number of rDNA sites per chromosome complement with increasing ploidy. Considering data from all the diploid and tetraploid *Artemisia* known until present (mean $2C = 2.63$ vs. $2C = 1.93$ in $2x$ and $4x$, respectively), 26.6 % overall loci loss has been found. The number of sites per monopleid genome in *Artemisia* is gradually reduced at higher ploidy levels: hexaploids present a 33.5 % reduction in number of loci per monopleid genome with respect to the diploids, and the highest polyploids (decaploids) have 35.36 % less loci than the diploids. Finally, several species show considerable site size differences, such as *A. assoana* (Fig. 3l) and *A.*

Fig. 3 Chromomycin A₃-positive (CMA+) and FISH pictures of metaphases of *Artemisia* species from the HMA clade. **a, b** *A. eriantha*, $2n = 18$ (Urdiceto); **c, d** *A. genipi* $2n = 18$; **e, f** *A. melanolepis* $2n = 16$; arrows in **c** indicate CMA+ signals; arrow in **e** indicates one large chromosome; **g, h** *A. glacialis* $2n = 16$; arrows in **g** indicate a large chromosome pair and in **h** a detached ribosomal DNA locus; most CMA+ signals are pericentromeric in **g** (**i, j**) *A. nitida* $2n = 54$; arrow in **i** indicates probably decondensed rDNA loci; **k, l** *A. assoana* $2n = 16$; arrows in **k** and **l** indicate a large chromosome pair, and asterisks in **l** mark loci with low rDNA copy number; **m, n** *A. splendens* $2n = 32$; arrows in **m** mark faint CMA+ signals; **o, p, q, r** *A. umbelliformis* $2n = 34$; note 8 and 6 rDNA loci in pictures **p** and **r**, respectively; arrows in **q** indicate faint CMA+ signals



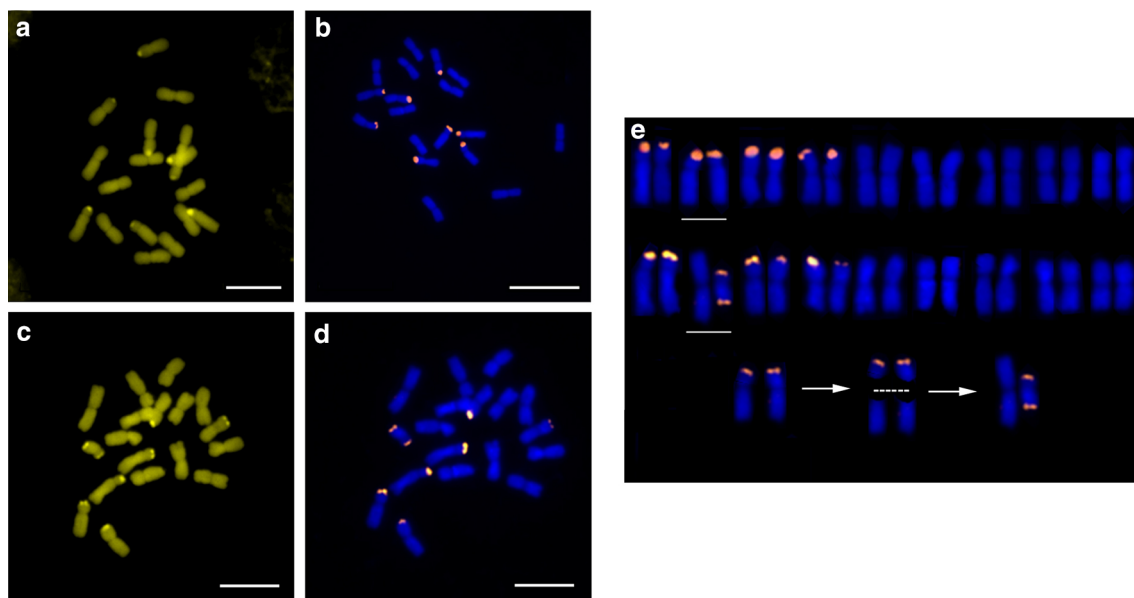


Fig. 4 Metaphases from *Artemisia eriantha*: regular metaphase (**a**, **b**) and metaphase with a likely Robertsonian translocation (**c**, **d**). Aligned chromosomes from **b** and **d** with the translocation pair underlined, and the possible mechanism of the translocation (**e**)

Table 2 Summary of the results (in number of signals) of the fluorochrome banding with chromomycin (CMA+) and of the fluorescent in situ hybridisation of rDNA

Species	Chromosome number	CMA+	rDNA
<i>A. melanolepis</i>	$2n = 2x = 16$	5 and 6	4
<i>A. glacialis</i> (Col de Tortisse)	$2n = 2x = 16$	20	4
<i>A. assoana</i>	$2n = 2x = 16$	18	6
<i>A. eriantha</i> (Urdiceto)	$2n = 2x = 18$	4	6
<i>A. eriantha</i> (Col du Lautaret)	$2n = 2x = 18$	8	8
<i>A. genipi</i>	$2n = 2x = 18$	4	4
<i>A. splendens</i>	$2n = 4x = 32$	8	8
<i>A. umbelliformis</i> (Noucreus)	$2n = 4x = 34$	6	6 and 8
<i>A. nitida</i>	$2n = 6x = 54$	c. 50	9

The position of signals is always terminal, except for CMA+ bands in *A. glacialis* and *A. assoana*, where most sites are pericentromeric. All taxa are high mountain species with the exception of *A. assoana*

nitida (Fig. 4j). Besides, a detached rDNA site is observed in *A. glacialis* (Fig. 3h).

Reconstruction of ancestral characters

The reconstruction of ancestral holoploid genome size onto the phylogeny (Fig. 2), based on diploid taxa, infers a holoploid genome size between 6.92 pg and 8.13 pg as the likely ancestral estimates both for the genus and for the HMA clade, in which ups and downs with respect to their putative common ancestors have occurred during the evolution of the genus. There has been an increase in the number of rDNA sites in 14 cases (45.16 %, three of them within the HMA clade, two within the high mountain) and a decrease in

only two cases (6.45 %); the remaining 15 (48.39 %) retain the alleged ancestral number of signals.

Discussion

Intraspecific genome size variation

Research on genome size variation within the species level has a long tradition, from the first reports in flax (Evans et al. 1966). Nevertheless, the extent of true intraspecific variation is still a source of debate. Critical reassessments of such variations have proved them to be artefacts in some cases, having identified technical problems, methodological errors

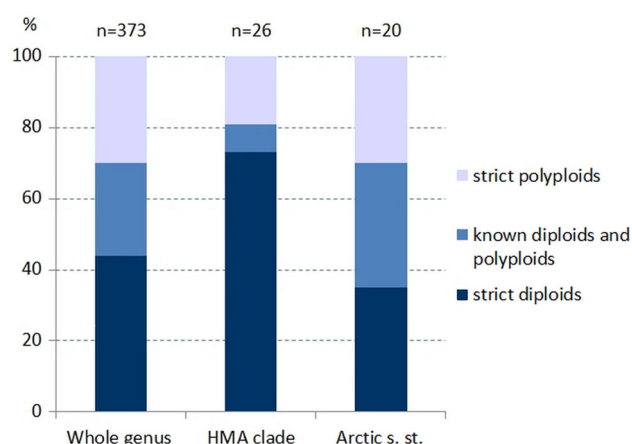


Fig. 5 Distribution of ploidy levels among different *Artemisia* groups. Data for the whole genus are taken from Vallès et al. (2011). Arctic *s. str.* species are selected from Tkach et al. (2008) and chromosome numbers are extracted from the index to chromosome numbers in Asteraceae. “n” indicates sample size

or presence of endogenous staining inhibitors as likely causes (Greilhuber 2005). However, many well-documented examples, based on accurate assessments, do suggest the existence of genuine intraspecific variation in nuclear DNA content (e.g. Suda et al. 2007; Trávníček et al. 2013).

With regards to the high mountain *Artemisia*, intraspecific genome size variation has been evaluated in *A. eriantha* (11.26 %) and *A. umbelliformis* (8.39 %), the most widely distributed species through the European mountains. The percentages here reported reflect a certain degree of variation, and interpopulational differences are significant in some cases in both species. Other studies on intraspecific variation across a distribution range performed in *Artemisia* have detected similar percentages: 8.8 % for 17 populations in the diploid *A. arborescens* (Garcia et al. 2006) and 9.22 % for 45 populations in the hexaploid *A. crithmifolia* (Pellicer et al. 2009). Yet it is difficult to set a cut-off point from which a given percentage of variation should be considered important. Intraspecific genome size variations have been related to microevolutionary differentiations and could be taxonomically significant, especially if there is some degree of morphological or ecological differentiation (Murray 2005). Neither *A. eriantha* nor *A. umbelliformis* show a clear morphological, geographical or ecological pattern to correlate with this variation; however, the detected differences are probably genuine. Possible sources of true intraspecific genome size variation that could explain the detected differences include the presence of aneuploidy or B-chromosomes, introgression or hybridisation processes (Morgan-Richards et al. 2004) or changes in repetitive DNA content (Trávníček et al. 2013).

Polyploidy is associated with genome downsizing and loci loss, but not necessarily with loss of GC-rich heterochromatin

Most studies have found genome downsizing on polyploids in multiple occasions, as it is the case with the alpine *Artemisia*. Recently, Meudt et al. (2015) found not only significant genome downsizing across *Veronica* but also a link with the diversification success of polyploid lineages. However, in some cases as in *Nicotiana* (Leitch et al. 2008, Renny-Byfield et al. 2011), despite the most common response to polyploidy is still genome downsizing, unchanged genome sizes and even genome upsizing have also been found linked to polyploidy processes in certain species.

Besides, the loci loss detected in these high mountain polyploids fits well with previous findings, particularly at the highest ploidies. Globally, most diploid *Artemisia* have four rDNA sites (58 %) while some have six (26 %), eight (13 %), and only in two cases nine and ten sites (<http://www.plantrdnadatabase.com>, accessed March 20th 2015). Regarding tetraploids, *Artemisia* species mostly show eight sites, consistent with our new findings for *A. splendens* and one population of *A. umbelliformis*. As with genome downsizing, loci loss is commonly observed across ploidy levels and seems to be a global response to polyploidy, however, others have found directly proportional increase (Bareka et al. 2012) or even loci gain (Hasterok et al. 2006). Factors such as qualitative and quantitative differences in transposable element (TE) composition of genomes, possible hybrid origins, and the amount of time elapsed after the polyploidisation event, may play a role on the extent of genome size loss after polyploidisation.

The amplification of certain GC-rich repetitive DNA families specific to certain species could explain the differential pattern observed in those species showing considerably more CMA+ signals, such as *A. nitida*. A similar finding was reported for the tetraploid *A. argilosa* (Garcia et al. 2007) and more recently, Olanj et al. (2015) found a striking number of CMA+ signals in *Tanacetum*, a genus closely related to *Artemisia* (reaching up to 66 at the diploid level, $2n = 18$). The observed abundance of pericentromeric CMA+ signals in certain species (*A. glacialis* and *A. assoana*) while most are (sub)terminal, would also point to other kinds of repetitive GC-rich DNA amplifying differently in these taxa. Contrasting to genome downsizing and loss of rDNA loci, there is no clear pattern with respect to the pattern of change of CMA+ bands with increasing ploidy: a given species can have very different profiles, and in some cases tetraploids have less CMA+ bands than diploids. As shown previously (Garcia et al. 2007, Olanj

et al. 2015), the underpinning evolutionary dynamics of GC-rich DNA is particularly elusive.

Reconstruction of ancestral characters and *Artemisia* cytogenetic features

Studies that address the evolution of cytogenetic traits considering a phylogenetic and temporal perspective are not common and there is no clear pattern in the direction of their changes (Leitch et al. 2008). During *Artemisia*'s evolution, both genome size and number of rDNA loci have undergone ups and downs, as others have previously found (Burleigh et al. 2012), this showing the dynamic nature of plant genomes. The ancestral-like condition of four rDNA sites in *Artemisia* is consistent with the most frequently found numbers of rDNA sites in angiosperms (<http://www.plantrdnadatabase.com>). The (sub) terminal position is also the most common, both in *Artemisia* and in angiosperms as a whole, consistently with evolutionary constraints favouring the terminal position for rDNA (Roa and Guerra 2012). Holoploid genome size has mostly remained stable or increased during *Artemisia*'s evolution: the reconstruction of ancestral holoploid genome size onto the phylogeny (Fig. 2) infers a value between 6.92 and 8.13 as the likely ancestral estimates both for the genus and for the HMA clade. This is considered intermediate ($7 \leq 2C < 28$ pg) according to the genome size categories of plants (Leitch et al. 2005). In angiosperms, the ancestral genome size has been reconstructed as very small ($2C \leq 2.8$ pg) with several independent increases and decreases taking place (Bennett and Leitch 2005).

Low incidence of polyploid taxa in the high mountain *Artemisia*

Polyploidy in the HMA clade is not particularly abundant when compared to the whole genus. In *Artemisia*, 44 % of species are only diploid, 30 % only exclusively polyploid, while 26 % are known both at diploid and polyploid levels (Vallès et al. 2011). However, in the HMA clade, while 73.08 % are only diploid, 19.23 % are exclusively polyploid and 7.69 % are known both at diploid and polyploid levels (Fig. 5). Other few high mountain species not belonging to this clade are also diploid, such as *A. atrata*. Nie et al. (2005) only detected 22 % of polyploid taxa across the flora of the Hengduan Mountains (Qing-Hai Tibet Plateau). More recently, Loureiro et al. (2013) found 23 % of polyploids across two Spanish high mountain ranges, showing that polyploidisation is probably not essential in determining species adaptation to such environments. Conversely, Löve and Löve (1967) and Morton (1993) found a high rate of polyploids in the alpine zone of Mt. Washington and in the Cameroon Mountains, respectively. Likewise, Vamosi and

McEwen (2013) detected an increase of polyploids in high elevations of British Columbia. However, it has been suggested that a high genome size (product of polyploidisation) would not be selected in alpine environments, in principle (Baack 2004). This could have promoted a lower frequency of alpine polyploids in medium-large holoploid genome size genera such as *Artemisia*. Interestingly, as Tkach et al. (2008) pointed out, we confirmed a higher frequency of polyploids in arctic *Artemisia* species (Fig. 5). This suggests that polyploidy ranges, and their increase with latitude, could be more related with their ability to colonise new habitats, such as newly deglaciated environments (Brochmann et al. 2004), rather than with a greater cold-hardiness or higher altitudes (Stebbins 1971; te Beest et al. 2012).

Descending dysploidy by recurrent Robertsonian translocations

A pioneer study by Kawatani and Ohno (1964) suggested descending dysploidy in *Artemisia* on the grounds of the much larger proportion of $x = 9$ -based taxa. Dysploidy has contributed to species differentiation in many genera, occurring once or repeatedly (Blösch et al. 2009; Schneeweiss et al. 2013). As pointed out previously, within the HMA clade there is a significant number of $x = 8$ (dysploid) species (12 out of 26 species) in a mostly $x = 9$ based genus, tribe and family (Schweizer and Ehrendorfer 1983). Within *Artemisia*, $x = 8$ -based species are around 14 % of the total whose chromosome number is known (Vallès et al. 2011). Yet in the HMA clade this grows to 46 %. Our ancestral state reconstruction points to the fact that $x = 9$ is the ancestral condition in the genus, as in family Asteraceae, although Semple and Watanabe (2009) hypothesised $x = 10$ was the ancestral base number in tribe *Anthemideae* (to which *Artemisia* belongs). This would support a hypothesis of downwards dysploidy within the tribe. Descending dysploidy has been related to plants adapted to extreme habitats in some Asteraceae genera (Garnatje et al. 2004) and this might be linked to $x = 8$ being over-represented in the HMA clade.

Few works have shown in detail the specific process leading to a decrease in basic chromosome number. However, a likely case of Robertsonian (Rb) translocation that could account for the descending dysploidy has been found in *A. eriantha* (Fig. 4). In fact, similar processes had already been suggested for *Artemisia* on the basis of observed centromeric fragility in a large chromosome pair (Vallès and Siljak-Yakovlev 1997). Likewise, a Rb translocation could have been involved in the appearance of $x = 17$ in species such as *A. umbelliformis*. Given the distribution of $x = 8$ taxa across *Artemisia*'s phylogeny, Rb translocations may have occurred independently several times in the genus (notwithstanding any other mechanisms promoting

descending dysploidy). It is possible, however, that the chromosomes involved in the translocation are not the same in all instances where $x = 8$ appears (i.e. different translocations affecting different chromosomes). We hypothesise that this may be the case in *Artemisia* since, (1) a notably larger chromosome pair is seen in several $x = 8$ species from the HMA clade (Fig. 4, e, f, g, h, k, l, arrows) while this is rarely observed in the other $x = 8$ species outside this clade and (2) holoploid genome size of $x = 8$ species from the HMA clade is significantly higher than that of the other $x = 8$ species outside this clade (Supplementary Material S3) which could be related to their specific translocation process (involving different chromosomes, which would explain the observed size differences, and perhaps different genomic processes which somehow could be related with the genome size differences detected). Therefore, the fact that only species from the HMA clade share these features (i.e. large chromosome pair and increased genome size) indicates that the event leading to $x = 8$ in this group would be common and may differ from other emergences of the dysploid karyotype in other groups, leading to size differences in the translocation chromosomes and genome size accumulation.

Conclusions

This work shows that *Artemisia* species from high mountain habitats do not present, at first sight, particular karyological features as compared with other alpine plant groups or other non-high mountain *Artemisia*. However, we do detect a high frequency of dysploids in the clade containing alpine *Artemisia*. Besides, polyploids are remarkably less abundant in the high mountains than in the rest of the genus. Thus, it seems that evolutionary processes in high mountain *Artemisia* have been less influenced by polyploidy and more affected by dysploidy and other mechanisms involving genome size increase, such as, for example, mobilisation of transposable elements or amplification of repetitive DNA, which still remain to be proved in these taxa. As previously reported for high mountain species from genus *Phyteuma* (Schneeweiss et al. 2013), descending dysploidy could be associated with lineage diversification in alpine *Artemisia*.

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