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SECCIÓ DE BOTÀNICA

UNA APROXIMACIÓ A L'ESTUDI DE L'EVOLUCIÓ I LA SISTEMÀTICA D'*ARTEMISIA* I GÈNERES AFINS en els àmbits de la citogenètica i filogènia moleculars

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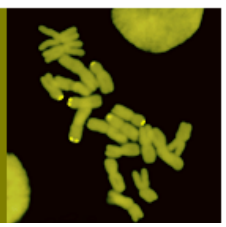

1. Variació de la quantitat de DNA en 47 poblacions de la subtribu *Artemisiinae* i tàxons relacionats (*Asteraceae*, *Anthemideae*): implicacions cariològiques, ecològiques i sistemàtiques.

Sònia GARCIA, María SANZ, Teresa GARNATJE, Agnieszka KREITSCHITZ, E. Durant MCARTHUR i Joan VALLÈS.
Genome 47: 1004-1014 (2004).

S'ha estimat la mida del genoma mitjançant citometria de flux en 47 poblacions de 40 espècies de la tribu *Anthemideae* (*Asteraceae*), principalment en espècies del gènere *Artemisia*, altres gèneres de la subtribu *Artemisiinae* i tàxons relacionats. S'ha trobat un rang de variació des de 3,54 fins a 21,22 pg. La quantitat de DNA per genoma haploide oscil·la entre 1,77 i 7,70 pg. Es donen les primeres dades sobre mida del genoma per a una subtribu, 10 gèneres, 32 espècies i dues subespècies. La quantitat de DNA es correlaciona amb alguns caràcters cariològics, fisiològics i ambientals i es revela una eina útil en la interpretació de relacions evolutives dins d'*Artemisia* i gèneres afins.

Variation of DNA amount in 47 populations of the subtribe Artemisiinae and related taxa (Asteraceae, Anthemideae): karyological, ecological, and systematic implications

Sònia Garcia, María Sanz, Teresa Garnatje, Agnieszka Kreitschitz, E. Durant McArthur, and Joan Vallès

Abstract: Genome size has been estimated by flow cytometry in 47 populations of 40 species of the tribe Anthemideae (Asteraceae), mainly from *Artemisia* and other genera of the subtribe Artemisiinae and related taxa. A range of 2C values from 3.54 to 21.22 pg was found. DNA amount per basic chromosome set ranged from 1.77 to 7.70 pg. First genome size estimates are provided for one subtribe, 10 genera, 32 species, and two subspecies. Nuclear DNA amount correlated well with some karyological, physiological and environmental characters, and has been demonstrated as a useful tool in the interpretation of evolutionary relationships within *Artemisia* and its close relatives.

Key words: Artemisia, C value, ecology, evolution, flow cytometry, genome size, nuclear DNA amount variation, phylogeny, polyploidy, systematics.

Résumé : La taille du génome de 47 populations de 40 espèces de la tribu Anthemideae (Asteraceae), principalement du genre *Artemisia* et d'autres représentants de la sous-tribu Artemisiinae ou de groupes proches à celle-ci, a été estimée par cytométrie en flux. Les valeurs 2C sont comprises entre 3,54 et 21,22 pg. La quantité d'ADN par dotation chromosomique de base est comprise entre 1,77 et 7,70 pg. La taille du génome a été déterminée pour la première fois dans une sous-tribu, 10 genres, 32 espèces et deux sous-espèces. La quantité d'ADN nucléaire est en très bonne corrélation avec des caractères caryologiques, physiologiques et écologiques ; elle s'est avérée aussi utile pour l'interprétation des relations évolutives chez *Artemisia* et ses taxons apparentés.

Mots clés : Artemisia, cytométrie en flux, écologie, évolution, phylogénie, polyploïdie, systématique, taille du génome, valeur C, variation de la quantité d'ADN nucléaire.

Introduction

The amount of nuclear DNA (C value) is a fundamental biodiversity character, directly or indirectly related to many phenotypic traits and other important factors such as reproductive biology, ecology, and plant distribution (Bennett 1998). More than 100 positive or negative correlations with nuclear DNA amount have been documented. Measurements of the amount of nuclear DNA, which initially focused on cytogenetics, physiology, and ecology, have recently become more important in systematic and phylogenetic research (Kellogg 1998; Leitch et al. 1998). With the growing recognition of its relevance, there is a need for additional DNA C-value assessments in plants (Bennett and Leitch 1995; Bennett 1998; Hanson et al. 2001*a*, 2001*b*). Bennett and colleagues have assembled six reference lists of nuclear DNA amounts since 1976; these data are available through an internet database (http://www.rbgkew.org.uk/cval/homepage.html; Bennett and Leitch 2003), which facilitates comparative studies and other data-based research. Nevertheless, the

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existing data scarcely represent the global angiosperm flora (Bennett and Leitch 1995): fewer than 2% of angiosperm species have a known C value and more than 50% of angiosperm families lack even a single estimate of nuclear DNA amount for any species.

Artemisia (Asteraceae, Anthemideae), the principal focus of the present study, is the largest of the tribe Anthemideae and among the largest genera in the family Asteraceae. It comprises from 200 to more than 500 taxa at the specific or subspecific level, according to various authors (see Vallès and McArthur 2001 and references therein). Five large groups (*Absinthium*, *Artemisia*, *Dracunculus*, *Seriphidium*, and *Tridentatae*) are classically considered within *Artemisia*, at sectional or subgeneric levels (Torrell et al. 1999). However, the present infrageneric classification does not represent natural groups (Persson 1974; McArthur et al. 1981; Vallès and McArthur 2001) and there is still no agreement about the global treatment of the genus. Several genera have been segregated from *Artemisia* (Vallès et al. 2003 and references therein): big ones, such as *Seriphidium*, and small, often monotypic ones, such as *Mausolea*. In addition, other genera, such as *Ajania*, are systematically close to *Artemisia* or in taxonomic conflict with it. They are the basis for the subtribe Artemisiinae (Bremer and Humphries 1993), and have close relationships with genera belonging to the subtribes Handeliinae, Tanacetinae, Leucantheminae and Chrysantheminae. Molecular biology can shed light on the real structure of this pool of genera and studies based on DNA sequences have been and are being carried out to clarify its systematics (Watson et al. 2002; Vallès et al. 2003), which indicate the need of rearrangements to achieve a phylogenetically based organization of the Artemisiinae.

Artemisia is a widely distributed genus in the northern hemisphere, mainly in temperate areas, (Bremer 1994) and is rather scarce in the southern hemisphere. It is better represented in Eurasia than in North America. Central Asia constitutes its main centre of speciation and diversification (McArthur and Plummer 1978; Vallès and McArthur 2001). The species of the genus can be found from sea level to high mountains, frequently colonizing semiarid environments. Some *Artemisia* species occur in isolation, but more commonly they form extensive, landscape-dominant populations. Most of the species are perennial, only around 15 are annual or biennial. Polyploidy is a frequent phenomenon in the genus, which also has dysploidy, with two basic chromosome numbers ($x = 8$ and $x = 9$). Many *Artemisia* species have a high economic value, in that thay have medical, food, forage, or ornamental uses; on the other hand, some taxa are invasive weeds that can adversely affect agronomic harvests (Vallès and McArthur 2001 and references therein; Wright 2002).

The present study also includes six additional Artemisiinae and four genera belonging to three other Anthemideae subtribes, as detailed in the Materials and methods. These taxa, particularly those belonging to the Artemisiinae, are phylogenetically close to *Artemisia*, up to the point that some of them had been previously classified as members of *Artemisia* (the proximity to this genus can be deduced from the complex synonymy of many of these species presented in Table 1), but alternatively these taxa are placed in other genera.

Materials and methods

Plant material

Table 1 shows the 47 populations studied, grouped by subtribe, genus, and subgenus, along with their site of origin and herbarium voucher information. The study material includes 27 species and four subspecies from the genus *Artemisia*, four from *Dendranthema* (including one subspecies and one variety), and one each from *Brachanthemum*, *Filifolium*, *Kaschgaria*, *Mausolea*, and *Neopallasia* (all from subtribe Artemisiinae). Other Anthemideae taxa represented in the study include one species each of *Lepidolopsis* (Handeliinae), *Nipponanthemum* (Leucantheminae), *Hippolytia*, and *Tanacetopsis* (both, Tanacetinae). The chosen populations represent, apart from different taxonomic groups, distinct geographic areas, life forms, ploidy levels, and chromosome numbers. Vouchers for most materials are deposited in the herbarium of the Centre de Documentació de Biodiversitat Vegetal de la Universitat de Barcelona (BCN). Other vouchers are in the herbaria of the Shrub Sciences Laboratory, Provo, Utah (SSLP), the Botanical Institute V.L. Komarov of the Russian Academy of Sciences, Saint Petersburg (LE), and A. Kreitschitz, Wrocław. Some species have been obtained from botanical gardens through Index Seminum (Sapporo Botanic Garden, Hokkaido University, Japan; and Vladivostok Botanical Institute, Russian Academy of Science, Russia), with known original location.

Young leaves used for flow cytometry assays were taken from plants cultivated in pots. The achenes or adult plants were collected in natural populations. Seeds of *Pisum sativum* L. 'Express Long' and an adult *Petunia hybrida* Vilm. 'PxPc6', both used as internal standards for flow cytometric measurements, were obtained from the Institut des Sciences du Végétal (CNRS, Gif-sur-Yvette, France).

Flow cytometry measurements

DNA 2C values of the tested species were estimated using flow cytometry. *Pisum sativum* L. 'Express Long' and *Petunia hybrida* Vilm. 'PxPc6' (2C = 8.37 and 2.85 pg, respectively; Marie and Brown 1993) were used as internal standards to cover the range of 2C values found. In one case, when the peak of the unknown *Artemisia absinthium* and the internal standard *Pisum sativum* overlapped, an *Artemisia* species (*A. abrotanum*, 2C = 11.41 pg) that had previously been assessed with *Pisum* for nuclear DNA amount estimation, was used as internal standard (Torrell and Vallès 2001); this exceptional case was due to the lack of *Petunia hybrida* at that moment. Young healthy leaf tissues from the species to be studied and a calibration standard were placed together in a plastic Petri dish and chopped with a razor blade in Galbraith's isolation buffer (Galbraith et al. 1983). The amount of target species leaf (about 25 mm^2) was approximately twice that of the internal standard. The suspension of nuclei in the isolation buffer was filtered through a nylon mesh with a pore size of 70 μm and stained for 20 min with propidium iodide (Sigma-Aldrich Química, Alcobendas, Madrid, 60 μg/mL), the chosen fluorochrome standard (Johnston et al. 1999); tubes were kept on ice during staining and then left at room temperature until measurement. For each population, five individuals were analyzed; two

samples of each individual were extracted and measured independently. Measurements were made at the Serveis Cientificotècnics generals de la Universitat de Barcelona using an Epics XL flow cytometer (Coulter Corporation, Hialeah, Fla.). The instrument was set up with the standard configuration: excitation of the sample was done using a standard 488-nm air-cooled argon-ion laser at 15 mW power. Forward scatter (FSC), side scatter (SSC), and red (620 nm) fluorescence for propidium iodide were acquired. Optical alignment was based on optimized signal from 10-nm fluorescent beads (Immunocheck, Epics Division, Coulter Corporation). Time was used as a control of the stability of the instrument. Red fluorescence was projected on 1024 monoparametrical histograms. Gating single cells by their area versus peak fluorescence signal excluded aggregates. Acquisition was automatically stopped at 8000 nuclei. The total nuclear DNA content was calculated by multiplying the known DNA content in *Pisum* or *Petunia* by the quotient between the 2C peak positions of the target species and the chosen internal standard in the histogram of fluorescence intensities for the 10 runs, based on the assumption that there is a linear correlation between the fluorescence signals from stained nuclei of the unknown specimen and the known internal standard and the DNA amount. Mean values and standard deviations were calculated based on the results for the five individuals.

Statistical analyses (analysis of variance, means comparison by least significant difference test) were carried out to evaluate the relationships between the studied variables (DNA content, DNA per basic chromosome set, altitude, and life cycle, among others). All the analyses were performed with the program Statgraphics Plus 5.0 (Statistical Graphics Corp., Rockville, Md.). In addition to the data obtained in the present study (Table 2), those from a previous paper on *Artemisia* genome size (Torrell and Vallès 2001) were also used for the statistical analyses of the present work.

Results and discussion

The results of flow cytometric assessment of the nuclear DNA content of 47 populations of 40 species belonging to the tribe Anthemideae are presented in Table 2, together with genome size data in megabase pairs (1 pg = 978 Mbp, Doležel et al. 2003), other karyological characters, and information on life cycle and on the internal standard used for each estimation. The analyses were of good quality (mean half peak coefficient of variation (HPCV) = 3.06%). This second study of *Artemisia* DNA by flow cytometry also includes some related genera. It expands the flow cytometry database by a factor of three — the earlier work reported 21 *Artemisia* species (Torrell and Vallès 2001). In addition to the flow cytometry work, nuclear DNA content had been estimated for only seven *Artemisia* species by cytodensitometry after Feulgen staining (Nagl and Ehrendorfer 1974; Geber and Hasibeder 1980; Greilhuber 1988; Bennett and Smith 1991; S.R. Band, personal communication; Dąbrowska 1992).

For the genera *Brachanthemum*, *Dendranthema*, *Filifolium*, *Hippolytia*, *Kaschgaria*, *Lepidolopsis*, *Mausolea*, *Neopallasia*, *Nipponanthemum*, and *Tanacetopsis* the DNA content values presented in this paper are the first estimates (Bennett and Leitch 2003). Likewise, the DNA content assessment reported here is the first record for the subtribe Handeliinae, 32 species (13 of the 10 above-cited genera and 19 of *Artemisia*) and three *Artemisia* subspecies (Table 2).

When all the *Artemisia* species with available genome size data — those from this paper, those reported by Torrell and Vallès 2001, and those from the papers cited in the first paragraph of this section, noted in the Bennett and Leitch (2003) database — are taken into account, variations are, respectively, 7.33- and 4.40-fold for DNA amount and DNA amount per basic chromosome set. The variation is 3.04 fold for 2C value and 3.53 fold for DNA per basic chromosome set in the other genera studied.

Relationships with karyological characters

As might be expected, 2C value means are significantly different ($p < 0.005$) for chromosome number and ploidy level. Both minor and major differences are even found between $2n = 16$ and $2n = 18$ taxa (Table 2 and Torrell and Vallès 2001). However, there are exceptions to this positive relationship; diploid *A. abrotanum* has only 5.78 pg of nuclear DNA amount with 18 chromosomes, whereas *A. leucodes* has 15.39 pg with the same chromosome number and, surprisingly, *A.* × *wurzellii*, with 34 chromosomes, has 8.60 pg. Similar results have been seen in other groups of Asteraceae e.g., *Siebera pungens* with a 2C value of 16.98 pg and 20 chromosomes and *Amphoricarpus neumayeri* with 1.73 pg and 24 chromosomes (Garnatje et al. 2004). Nevertheless, the general trend is an increase of nuclear DNA amount with the increase of chromosome number (*Papaver*, Srivastava and Lavania 1991; *Achillea*, Dąbrowska 1992).

Although genome size and ploidy level are highly correlated, nuclear DNA amount per basic chromosome set decreases with polyploidy. Analysis of variance (ANOVA) shows a significant difference ($p < 0.05$) in nuclear DNA amount mean values between diploids and tetraploids, the latter having less nuclear DNA amount per basic chromosome set than the former; we did not perform analyses with other ploidy levels, because we had only minimal representation of each one. This supports the Grant's (1969) hypothesis that there is a decrease in nuclear DNA amount in polyploids associated to an adaptive response for the stabilization of the higher polyploids (dodecaploids) in *Betula*. Nuclear DNA loss per basic chromosome set in polyploids has also been reported in many other taxa (Bennett 1972; Murray et al. 1992; Ohri 1996; Dimitrova and Greilhuber 2000; Friedlender et al. 2002).

Aneusomaty may be another source of genome size variation. Some of the highest standard deviation values in the plants studied, such as those of *Artemisia campestris* subsp. *sericea* (Table 2) or *A. dracunculus* (Torrell and Vallès 2001) correspond to aneusomatic populations (Kreitschitz 2003; Kreitschitz and Vallès 2003). Similar variations have been reported in aneusomatic *Helianthus annuus* (Cavallini and Cremonini 1985; Michaelson et al. 1991).

Systematic implications: intraspecific and interspecific variation

Nuclear DNA amount can be useful in the interpretation of evolutionary relationships. C value may increase or decrease with evolution and comparisons between the different

Table 1. Provenance of the populations of Anthemideae studied.

Taxa	Origin of materials	Herbarium voucher
Subtribe Artemisiinae		
Genus *Artemisia*		
Subgenus *Absinthium*		
A. absinthium L.	Czeszów, Lower Silesia, Poland	Hb. A. Kreitschitz
A. aschurbajewii C. Winkler	Asku-Zhabagli nature reserve, Zhambul district, Kazakhstan	BCN 11693
A. austriaca Jacq.	Swieta Katarzyna, Lower Silesia, Poland	Hb. A. Kreitschitz
A. frigida Willd.	Almond, Colo.	BCN 11568
A. glacialis L.	Valmontey, Aosta valley, Italy	BCN 11566
A. lagocephala (Fischer ex Besser) DC.	Snezhnaya mountain,USSR	I. S. Vladivostok N53, BCN S-805
A. persica Boiss.	Asku-Zhablagli nature reserve, Zhambul district, Kazakhstan	BCN 11696
A. sieversiana Ehrh. in Willd.	Khanatalap, Almaty district, Kazakhstan	BCN 11692
Subgenus *Artemisia*		
A. abrotanum L.	Wrocław (Tarnogaj), Lower Silesia, Poland	Hb. A. Kreitschitz
A. abrotanum L.	Wrocław (Kozanów), Lower Silesia, Poland	Hb. A. Kreitschitz
A. afra Jacq.	Transwaal, Makapan, South Africa	BCN 11570
A. princeps Pampan.	Nopporo forest park, Ebetsu, Sapporo, Japan	BCN S-812
A. santolinifolia Turcz ex H. Kraschen.	Asku-Zhabagli nature reserve, Zhambul district, Kazakhstan	BCN 11694
A. stelleriana Besser	Glazkovka, USSR	I. S. Vladivostok N55, BCN S-813
A. tournefortiana Reichenb.	Karapalpakstan, Uzbekistan	BCN 11630
A. vulgaris L.	Chrząstawa Mała, Lower Silesia, Poland	Hb. A. Kreitschitz
A. vulgaris L.	Staniszów, Lower Silesia, Poland	Hb. A. Kreitschitz.
A. vulgaris L.	Lhasa, Tibet, People's Republic of China	BCN 11590
A. × *wurzellii* C. M. James & Stace in C. M. James, Wurzell & Stace	Northumberland park, London, UK	BCN 11670
Subgenus *Dracunculus*		
A. arenaria DC.	Volgograd, USSR	LE (Korobkov)
A. campestris L.	Wayne County, Utah	SSLP (McArthur 2777)
A. campestris L.	Konotop, Wielkopolska Region, Poland	Hb. A. Kreitschitz
A. campestris L.	Zagan, Ziemia Lubuska Region, Poland	Hb. A. Kreitschitz
A. campestris L. subsp. *sericea* (Fr.) Leuwke & Rothm.	Hel, Helska sandbank, Poland	Hb. A. Kreitschitz
A. filifolia Torrey	Mohave County, Ariz.	SSLP (McArthur 2784)
A. scoparia Waldst. & Kit.	Sultanuizdag Mountains, Karkalpakstan, Uzbekistan	BCN 11628
Subgenus *Seriphidium*		
A. leucodes Schrenk	Dgizak, Uzbekistan	BCN 11631
Subgenus *Tridentatae*		
A. arbuscula Nutt.	Millard County, Utah	SSLP (McArthur 2779)
A. bigelovii A. Gray	Emery County, Utah	SSLP (McArthur 2778)
A. cana Pursh. subsp. *viscidula* (Osterhout) Beetle	Wasatch County, Utah	SSLP (McArthur 2775)
A. nova Nelson	Nye County, Nev.	SSLP (McArthur 2739)
A. pygmaea A. Gray	Emery County, Utah	SSLP (McArthur 2780)
A. tridentata Nutt. subsp. *tridentata*	Juab County, Utah	SSLP (McArthur U-79)
A. tridentata Nutt. subsp. *vaseyana* (Rydb.) Beetle	Juab County, Utah	SSLP (McArthur 2507)
Genus *Brachanthemum*		
Brachanthemum titovii H. Kraschen.	Aktogai, Almaty district, Kazakhstan	BCN 11690

Table 1 (*concluded*).

Taxa	Origin of materials	Herbarium voucher
Genus *Dendranthema*		
D. arcticum Tzvelev subsp. *maekawanum* (Kitam.) H. Koyama	Sapporo, Japan	I.S. Sapporo, BCN S-815
D. indica Des Moul. var. *coreanum* Levl. & Van.	Sapporo, Japan	I.S. Sapporo, BCN S-809
D. maximowiczii (Komarov) Tzvelev	Glazkovka, USSR	I.S. Vladivostok N70, BCN S-810
D. zawadskii (Herbich) Tzvelev	Sapporo, Japan	I.S. Sapporo, BCN S-814
Genus *Filifolium*		
F. sibiricum (L.) Kitam. (*Artemisia sibirica*(L.) Maxim., *Tanacetum sibiricum* L.)	Oktyabrsky, USSR	I.S. Vladivostok N76, BCN S-806
Genus *Kaschgaria*		
K. brachanthemoides (C. Winkl.) Poljakov, *Artemisia brachanthemoides* C. Winkl., *Tanacetum brachanthemoides* (C. Winkl.) H. Kraschen.)	Kurtagai canyon, Almaty district, Kazakhstan	BCN 11691
Genus *Mausolea*		
M. eriocarpa (Bunge) Poljakov (*Artemisia eriocarpa* Bunge)	Gazli, Bukhara, Uzbekista.	BCN 11629
Genus *Neopallasia*		
N. pectinata (Pall.) Poljakov (*Artemisia pectinata* Pall.)	Southern slope of eastern Tien-Shan, Republic of Xingjan-Uigur, People's Republic of China	LE
Subtribe Handeliinae		
Genus *Lepidolopsis*		
L. turkestanica (Regel & Schmalh.) Poljakov (*Crossostephium turkestanicum* Regel & Schmahl., *Artemisia turkestanica* (Regel & Schmalh.) Franch., *Tanacetum turkestanicum* (Regel & Schmalh) Poljakov)	Sostube, Chimkent district, Kazakhstan	BCN S-807
Subtribe Leucantheminae		
Genus *Nipponanthemum*		
N. nipponicum (Franchet ex Maxim.) S. Kitamura (*Chrysanthemum nipponicum* (Franchet ex Maxim.) Sprenger, *Ch. nipponicum* Matsum., *Leucanthemum nipponicum* Franchet ex Maxim.)	Higashi-Hiroshima, Japan	BCN S-811
Subtribe Tanacetinae		
Genus *Tanacetopsis*		
T. goloskokovii (Poljakov) Karmysch.	Sogeti Mountains, Almaty district, Kazakhstan	BCN S-808
Genus *Hippolytia*		
H. megacephala (Rupr.) Poljakov (*Artemisia megacephala* Rupr.)	Asku-Zhabagli nature reserve, Zhambul district, Kazakhstan	BCN 11695

Note: Most of the vouchers are deposited in the herbarium of the Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona (BCN). Some others are in the herbarium of the Rocky Mountain Research Station, Provo, Utah (SSLP), in the herbarium of the Botanical Institute V.L. Komarov of the Russian Academy of Sciences, Saint Petersburg (LE), or in the herbarium of A. Kreitschitz (Wroclaw). I.S. indicates that the achenes have been obtained through an Index Seminum.

Table 2. Nuclear DNA content and other karyological characters of the populations studied.

Taxa	Life cycle[a]	2C ± SD (pg)[b]	2C (Mbp)[c]	$2n$[d]	Ploidy level	DNA per basic chromosome set	Standard[e]
Subtribe Artemisiinae							
Genus *Artemisia*							
Subgenus *Absinthium*							
A. absinthium	P	9.06±0.07	8860.7	18(1)	2*x*	4.53	*A. abrotanum*
*A. aschurbajewii**	P	10.36±0.29	10132.1	36(2)	4*x*	2.59	*Petunia*
*A. austriaca**	P	5.95±0.15	5819.1	16(3)	2*x*	2.98	*Pisum*
*A. frigida**	P	5.25±0.06	5134.5	18(4)	2*x*	2.63	*Pisum*
*A. glacialis**	P	8.52±0.15	8332.6	16(4)	2*x*	4.26	*Petunia*
*A. lagocephala**	P	6.75±0.06	6601.5	18(4)	2*x*	3.38	*Petunia*
*A. persica**	P	6.55±0.02	6405.9	18(2)	2*x*	3.28	*Pisum*
A. sieversiana	A	6.17±0.07	6034.3	18(2)	2*x*	3.09	*Petunia*
Subgenus *Artemisia*							
*A. abrotanum** (Tarnogaj)	P	11.41±0.11	11159.0	36(1)	4*x*	2.85	*Pisum*
*A. abrotanum** (Kozánow)	P	5.78±0.07	5652.8	18(1)	2*x*	2.89	*Pisum*
*A. afra**	P	6.31±0.34	6171.2	18(4)	2*x*	3.16	*Pisum*
*A. princeps**	P	14.60±0.24	14278.8	52(4)	6*x*	2.43	*Pisum*
*A. santolinifolia**	P	4.62±0.07	4518.4	18(4)	2*x*	2.31	*Pisum*
*A. stelleriana**	P	6.10±0.07	5965.8	18(4)	2*x*	3.05	*Petunia*
A. tournefortiana	A/B	7.06±0.07	6904.7	18(5)	2*x*	3.53	*Pisum*
A. vulgaris (Tibet)	P	12.15±0.52	11882.7	36(4)	4*x*	3.04	*Pisum*
A. vulgaris (Mala)	P	6.23±0.04	6092.9	16(3)	2*x*	3.12	*Pisum*
A. vulgaris (Staniszów)	P	6.49±0.32	6347.2	16(3)	2*x*	3.25	*Pisum*
A. × *wurzellii**	P	8.60±0.22	8410.8	34(4)	4*x*	2.15	*Petunia*
Subgenus *Dracunculus*							
*A. arenaria**	P	10.29±0.15	10063.6	36(4)	4*x*	2.57	*Petunia*
A. campestris (Utah)	P	6.38±0.05	6239.6	18(4)	2*x*	3.19	*Petunia*
A. campestris (Konotop)	P	9.78±0.13	9564.8	36(3)	4*x*	2.45	*Pisum*
A. campestris (Zagan)	P	9.92±0.18	9701.8	36(3)	4*x*	2.48	*Pisum*
A. campestris ssp. *sericea**	P	10.61±0.45	10376.6	36(1)	4*x*	2.65	*Pisum*
*A. filifolia**	P	7.14±0.18	6982.9	18(4)	2*x*	3.57	*Petunia*
*A. scoparia**	A	3.54±0.05	3462.1	16(5)	2*x*	1.77	*Petunia*
Subgenus *Seriphidium*							
*A. leucodes**	A	15.39±0.43	15051.4	18(5)	2*x*	7.70	*Pisum*
Subgenus *Tridentatae*							
*A. arbuscula**	P	9.22±0.11	9017.2	18(5)	2*x*	4.61	*Petunia*
*A. bigelovii**	P	15.49±0.10	15149.2	36(6)	4*x*	3.87	*Pisum*
A. cana ssp. *viscidula**	P	8.54±0.09	8352.1	18(6)	2*x*	4.27	*Petunia*
*A. nova**	P	6.37±0.14	6229.9	18(6)	2*x*	3.19	*Petunia*
*A. pygmaea**	P	11.54±0.18	11286.1	18(6)	2*x*	5.77	*Pisum*
A. tridentata ssp. *tridentata**	P	8.17±0.08	7990.3	18(6)	2*x*	4.09	*Petunia*
A. tridentata ssp. *vaseyana**	P	8.66±0.07	8469.5	18(6)	2*x*	4.33	*Petunia*
Genus *Brachanthemum**							
*B. titovii**	P	6.98±0.08	6826.4	18(2)	2*x*	3.49	*Pisum*
Genus *Dendranthema**							
D. arcticum ssp. *maekawanum**	P	20.03±1.30	19589.3	72(4)	8*x*	2.50	*Petunia*
D. indica var. *coreanum**	P	12.14±0.11	11872.9	36(4)	4*x*	3.04	*Pisum*
*D. maximowiczii**	P	15.78±0.17	15432.8	54(4)	6*x*	2.63	*Pisum*
*D. zawadskii**	P	21.22±0.52	20753.2	72(4)	8*x*	2.65	*Pisum*
Genus *Filifolium**							
*F. sibiricum**	P	9.44±0.31	9232.3	18(4)	2*x*	4.72	*Petunia*
Genus *Kaschgaria**							
*K. brachanthemoides**	P	14.09±0.31	13780.0	18(2)	2*x*	7.05	*Pisum*
Genus *Mausolea**							
*M. eriocarpa**	P	13.79±0.13	13486.6	36(5)	2*x*	3.45	*Pisum*
Genus *Neopallasia**							
*N. pectinata**	A	10.56±0.21	10327.7	36(4)	2*x*	2.64	*Pisum*

Table 2 (*concluded*).

Taxa	Life cycle[a]	2C ± SD (pg)[b]	2C (Mbp)[c]	$2n$[d]	Ploidy level	DNA per basic chromosome set	Standard[e]
Subtribe Handeliinae*							
Genus *Lepidolopsis**							
*L. turkestanica**	P	11.14±0.34	10894.9	18[2]	2*x*	5.57	*Petunia*
Subtribe Leucantheminae							
Genus *Nipponanthemum**							
*N. nipponicum**	P	11.87±0.17	11608.9	18[4]	2*x*	5.94	*Pisum*
Subtribe Tanacetinae							
Genus *Tanacetopsis**							
*T. goloskokovii**	P	9.73±0.27	9515.9	18[4]	2*x*	4.87	*Petunia*
Genus *Hippolytia**							
*H. megacephala**	P	12.47±0.19	12195.7	18[2]	2*x*	6.24	*Pisum*

Note: The taxa for which genome size has been estimated for the first time are marked with an asterisk (*).

[a]Life cycle: A, annual; B, biennial; P, perennial).

[b]2C nuclear DNA content (mean value ± standard deviation of 10 samples).

[c]1 pg = 978 Mbp (Doležel et al. 2003).

[d]Somatic chromosome number. (1) Kreitschitz and Vallès (2003); (2) Vallès et al. (2001*b*); (3) Kreitschitz (2003); (4) unpublished counts performed by the present authors; (5) Vallès et al. (2001*a*); (6) McArthur and Sanderson (1999). All counts have been carried out in the populations studied in the present paper.

[e]Internal standard used in each case (see text for details about *Pisum* and *Petunia*; for *A. absinthium*, the standard used was another *Artemisia*, *A. abrotanum*, previously measured (2C = 11.41 pg, Torrell and Vallès 2001) against *Pisum*).

genome sizes provide a natural explanation of phylogenetic relationships and systematics of many taxonomic groups (Ohri 1998). Our nuclear DNA results agree with the molecular phylogeny of *Artemisia* and other genera of Artemisiinae (Torrell and Vallès 2001; Vallès et al. 2003), as in other Asteraceae groups (Godelle et al. 1993; Zoldos et al. 1998; Cerbah et al. 1999).

Highly significant statistical differences ($p < 0.005$) have been detected in DNA amount per basic chromosome set in the five subgenera of *Artemisia*, particularly between *Seriphidium* and *Dracunculus*, and between *Tridentatae* on the one hand and *Artemisia* and *Dracunculus* on the other (Table 3). Moreover, subgenus *Tridentatae* is endemic to North America and also forms a well supported clade in the molecular phylogeny based on ITS analysis (Vallès et al. 2003). These data support standing of the subgenus *Tridentatae* as an independent group rather than its inclusion in *Serphidium*.

An important taxonomic character in subtribe Artemisiinae is pollen grain exine ornamentation. Genera belonging to subtribe Artemisiinae (Bremer and Humphries 1993) can be separated, on the basis of exine ornamentation, in two groups: one with *Artemisia* pollen type (with small spines) and another with *Anthemis* pollen type (with longer spines) (Martín et al. 2001, 2003). The genera *Brachanthemum* and *Dendranthema* and other phylogenetically close genera from other subtribes (*Hippolytia*, *Lepidolopsis*) present the *Anthemis* pollen type, while members of *Artemisia* and other Artemisiinae genera such as *Filifolium*, *Kaschgaria*, *Mausolea* and *Neopallasia* present the *Artemisia* pollen type. Pollen morphology is an indicator that the traditional classification of subtribe Artemisiinae is unnatural (Martín et al. 2001, 2003). Genome size data also support separation of the groups by pollen type: species with *Artemisia* pollen type have significantly ($p < 0.01$) less nuclear DNA than species with *Anthemis* pollen type. Genome size variation supports the established correlation between pollen grain ornamentation and the ITS phylogeny (Vallès et al. 2003).

Of the traditional subgeneric classification, the subgenus *Artemisia* is less supported by molecular phylogeny than are the subgenera *Dracunculus*, *Seriphidium* and *Tridentatae*. Its species are dispersed in the phylogenetic tree (Vallès et al. 2003). Furthermore, subgenus *Artemisia* is the most heterogeneous in terms of morphological, chemical, ecological, and karyological data (Ehrendorfer 1964; Torrell et al. 1999). Additionally, in the phylogenetic analysis of Vallès et al. (2003) 5 out of the 10 taxa that were not included in any clade belong to subgenus *Artemisia*, and members from this subgenus appear distributed in four of the eight clades, confirming again that the present infrageneric classification does not represent natural groups (Persson 1974; Vallès and McArthur 2001). Nuclear DNA amount analysis is thus quite useful in support of molecular phylogeny and pollen data. Further support of the heterogeneous nature of the subgenus *Artemisia* is that it has the highest ratio between maximum and minimum nuclear DNA amount per basic chromosome set (Table 4). Conversely, subgenus *Dracunculus*, the most homogeneous according to the molecular phylogeny (Vallès et al. 2003), is the one that presents the lowest genome size variability (the lowest ratio of all subgenera). Nuclear DNA amount per basic chromosome set of *Artemisia leucodes* (7.70 pg) is markedly different from the mean value of the subgenus to which this species belongs, *Seriphidium* (3.89 pg). Similarly, Torrell and Vallès (2001) found a nuclear DNA amount per basic chromosome set for *Artemisia judaica* of 5.76 pg, far different from the mean value of its subgenus, *Artemisia* (2.96 pg). In both cases, these taxa are placed out of their respective traditional subgenera by ITS phylogeny (Vallès et al. 2003). This confirms the value of nuclear DNA content as a systematic marker and agrees with the striking interspecific variation in genome size that occurs

Table 3. Comparison of means of DNA amount per basic chromosome set in the subgenera of *Artemisia*.

Subgenus	Mean (pg)	Homogeneous groups
Dracunculus	2.668	a
Artemisia	3.050	ab
Absinthium	3.563	bc
Seriphidium	3.892	bc
Tridentatae	4.088	c

Table 4. Maximum, minimum, and ratio (maximum/ minimum) of nuclear DNA amount per basic chromosome set (pg) in the subgenera of *Artemisia*.

Subgenus	Maximum	Minimum	Ratio max/min
Absinthium	4.53	2.59	1.75
Artemisia	5.76	1.44	3.27
Dracunculus	2.93	1.77	1.66
Seriphidium	7.69	2.67	2.88
Tridentatae	5.79	3.21	1.80

in many, though not all, major taxonomic groups (Hanson et al. 2001*a*, 2001*b*).

Amount of nuclear DNA per basic chromosome set statistically differs ($p < 0.005$) between *Artemisia* and its related genera from other subtribes (Anthemideae not Artemisiinae). Nuclear DNA amount per basic chromosome set of Artemisiinae (*Artemisia* excluded) also differs ($p < 0.05$) from those genera belonging to subtribes Tanacetinae, Leucantheminae and Handeliinae. On the other hand, there is no statistically significant difference in nuclear DNA amount per basic chromosome set between genus *Artemisia* and the other Artemisiinae analysed. In fact, many of the non-*Artemisia* Artemisiinae studied here had been previously included in *Artemisia*, and subsequently separated in different genera, often new and with only one or two species. DNA sequence analysis of these plants (Vallès et al. 2003) demonstrate most of these genera tightly embedded in the *Artemisia* clade; this could be interpreted to support the elimination of these new genera, and their species returned again to *Artemisia*. The absence of statistically significant difference in nuclear DNA amount per basic chromosome set between these groups also supports this hypothesis. In summary, all these results indicate that nuclear DNA amount is an important tool in the analysis of phylogenetic relationships.

Within the studied *Artemisia* taxa, in the present paper and an earlier one (Torrell and Vallès 2001), different populations have been analysed for some species. The differences detected in nuclear DNA amount give a low degree of variability in most of these species. This can be illustrated by the comparison between the very similar 2C values obtained in the present study and in Torrell and Vallès (2001) for *A. absinthium* (9.06 in the present study / 8.52 in Torrell and Vallès 2001), *A. vulgaris* (6.23, 6.49 / 6.08), *A. campestris* (diploid: 6.38 / 5.87; tetraploid: 9.78, 9.92 / 11.00), and different subspecies of *A. tridentata* (8.17, 8.86 / 8.18). *Artemisia abrotanum* also constitutes a case of nuclear DNA amount constancy: although the analysed populations have different ploidy levels (diploid and tetraploid), nuclear DNA amount per basic chromosome set of both species only differs in 1.40%. This fact is also interesting because polyploids ordinarily have significantly less nuclear DNA per basic chromosome set than corresponding diploids. However, in this case both specimens show quite a similar nuclear DNA amount per basic chromosome set and both *A. abrotanum* specimens came from the same geographic area (Wrocław, Poland), a circumstance that could partially explain this homogeneity. Another possibility could be an autopolyploid origin of the tetraploid population, which has almost exactly double DNA amount of the diploid. Further cytogenetic studies on *A. abrotanum* are necessary to confirm this hypothesis. Additionally, tetraploid *A. campestris* could have the same origin (2*x*, 5.87 pg; 4*x*, 11.0 pg; Torrell and Vallès 2001). Our results support that although nuclear DNA amount or C value is considered constant within a species, it is almost sure that a certain degree of genuine intraspecific variation exists; the processes or mechanisms that are usually able to cause it are duplications, deletions, chromosomal polymorphisms, the existence of B chromosomes, or the presence of transposable elements or repetitive sequences (Greilhuber 1998; Małuszyńska 1999).

Ecology and life cycle

No statistically significant relationship exists between life cycle and C value among the species studied. However, the taxon with the lowest nuclear DNA amount, *A. scoparia*, is annual, and those with the highest C values are perennial, as was the case in a previous report in other *Artemisia* species (Torrell and Vallès 2001) or in other genera, including some Anthemideae (Bennett 1972; Nagl and Ehrendrofer 1974; Rees and Narayan 1981). It is generally assumed that a low C-value correlates with a high rate of development; in other words: if less nuclear DNA is duplicated, the cell cycle is faster, and the developmental rhythm, consequently, is more intense. This is specially useful for annual or ephemeral plants, which have only limited time to carry out their life cycle. The studied *A. scoparia* population inhabited an intermittently dry river bed, and its low C value (3.54 pg, the lowest of the present study) promotes a fast life cycle that is rapidly completed before the seasonal summer or fall floods. This case supports the premise that annual species have a smaller amount of nuclear DNA than perennials. In contrast, however, *Artemisia leucodes*, another annual species, has one of the biggest genomes (2C = 15.39 pg) of all the diploid species studied; its karyotype is made up of large chromosomes (Vallès et al. 2001*a*) and its high C value, despite its annual life cycle, is supported by the Nagl and Ehrendrofer (1974) explanation that large chromosomes could have a higher metabolic rate that facilitates an increase in RNA synthesis. This would increase the synthesis of the necessary proteins to permit a faster life cycle. Although many authors have found a positive correlation between genome size and life cycle duration, numerous exceptions suggest that it is not so clear as initially thought. Some authors have reported even a negative relationship between those parameters (e.g., *Pennisetum*, Martel et al. 1997), whereas others have found no relationship (Grime and Mowforth 1982).

The statistical analysis carried out on the species of this study did not reveal any significant difference between the

studied populations of higher or lower altitudes. The tetraploid *A. vulgaris* studied in this paper has a 24.7% difference with the one studied by Torrell and Vallès (2001) even though both populations are tetraploid. These populations grow in geographically and ecologically distinct conditions. The population with the higher nuclear DNA amount is a Tibetan population growing at 3650 m. An adaptation to altitude could at least partly explain the difference. Many studies on this subject have reported that species inhabiting arctic or high mountain areas tend to present larger genomes, and are most frequently polyploids (Gregory and Hebert 1999, and references therein). Some authors have concluded that natural selection favours the modulation of nuclear DNA content under certain weather conditions, mainly linked with altitude or latitude (Bennett 1976). The high taxonomic complexity of the *A. vulgaris* group may also contribute to an explanation of this difference. Similarly, *A. glacialis*, found in Italy at an altitude of 2300 m has a larger C value than the mean of the analysed diploid species of the genus, conforming to what other authors have stated about the positive correlation between DNA amount and altitude (Caceres et al. 1998). Nevertheless, similar studies have found a negative correlation or even no relationship between altitude and C value, (Creber et al. 1994; Reeves et al. 1998; Vilhar et al. 2002), similar to the relationship between life cycle and genome size, suggesting again that the link between the two is not clear.

It seems likely then that genome size variation in *Artemisia* species does not depend on altitude. Nonetheless, it appears to be a response to other kinds of selective pressures, such as adaptation to arid environments. Sanderson et al. (1989) and McArthur and Sanderson (1999) found a better adaptation to arid habitats in polyploid rather than in diploid *Artemisia* and *Atriplex* in North American semi-desert habitats, and Vallès et al. (2001*a*, 2001*b*) detected that tetraploid species of *Artemisia* were more widely distributed in arid lands than the related diploids. Both studies support the hypothesis that nuclear DNA amount increases — especially by means of polyploidy — in plants adapted to extreme environments. Our results agree with this idea, and show that there can be a DNA amount increase even in diploids. *Artemisia leucodes* and *A. pygmaea*, the two diploid taxa with the highest DNA amount per basic chromosome set of the *Artemisia* species analysed, inhabit desert or semi-desert regions of central Asia and North America, and are well adapted to the extreme conditions of high temperature and drought that characterize these environments. Moreover, diploid *A. filifolia*, another colonizing plant of sandy North American deserts, shows the highest nuclear DNA amount per basic chromosome set of its subgenus, *Dracunculus*.

Artemisia absinthium is a nitrophilous species usually grown in ruderal zones. As the presence of high concentrations of nitrogen in the soil can also be considered as a difficult, if not extreme, environmental condition, it is interesting to observe that our *A. absinthium* population shows a higher nuclear DNA amount per basic chromosome set than the mean for the genus. Torrell and Vallès (2001) reported the same relationship in another ruderal *Artemisia* species, *A. thuscula*, taxonomically related to *A. absinthium*. In both cases, the high nuclear DNA amount could be interpreted as a response to the presence of nitrogen; this would support Evans (1968), who detected a 10% increase in nuclear DNA amount in varieties of *Linum usitatissimum* growing in strongly nitrogenated soils and at high temperatures.

Concluding statement

C values in the subtribe Artemisiinae, including the large genus *Artemisia* and related taxa, are a useful adjunct in parallel or in correlation to other kinds of data, e.g., chromosome number, life form, pollen grain exine patterns, systematic placement, and ecology, in determining evolutionary relationships within this group of plants.

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2. Variació de la mida del genoma en alguns representants del gènere *Tripleurospermum*.

Sònia GARCIA, Huseyin INCEER, Teresa GARNATJE i Joan VALLÈS.
Biologia Plantarum 49: 381-387 (2005).

S'ha determinat mitjançant citometria de flux la quantitat de DNA en 14 poblacions pertanyents a vuit tàxons (set espècies, una amb dues varietats) del gènere *Tripleurospermum* (*Asteraceae*, *Matricariinae*). Els valors 2C es troben en un rang entre 4,87 i 9,22 pg, i la quantitat de DNA per genoma haploide (1Cx), entre 1,99 i 2,75 pg. S'han trobat diferències estadísticament significatives segons el nivell de ploïdia, el cicle vital o factors ambientals com l'altitud. També s'ha vist que la mida del genoma es correlaciona positivament amb la longitud total del cariotip. La presència de rizoma es relaciona amb un contingut més elevat de DNA. No obstant, el petit nombre de dades analitzades no permet d'arribar a conclusions definitives sobre la variació en la quantitat de DNA en aquestes espècies, tot i que sí que significa una primera aproximació al coneixement del grup des d'aquest punt de vista, sobre el qual actualment estem aprofundint.

Genome size variation in some representatives of the genus *Tripleurospermum*

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Abstract

Genome size has been estimated by flow cytometry in 14 populations belonging to eight taxa (seven species, one of them with two varieties) of the genus *Tripleurospermum*. 2C nuclear DNA amounts range from 4.87 to 9.22 pg, and nuclear DNA amounts per basic chromosome set from 1.99 to 2.75 pg. Statistically significant differences depending on ploidy level, life cycle or environmental factors such as altitude have been found. Also, genome size is positively correlated with total karyotype length. The presence of rhizome is related to nuclear DNA content in these species.

Additional key words: *Asteraceae*, C-value, DNA amount, flow cytometry, *Matricariinae*, nuclear DNA content.

Introduction

The C-value of an organism, *i.e*. the amount of DNA in the unreplicated nuclear genome (Swift 1950), which is considered constant within a species, influences various cellular parameters, such as cell and nuclear volume and chromosome size, and developmental parameters like minimum generation time or duration of meiosis, among others (Price *et al.* 1981a,b, Bennett 1987). Many other important relationships have also been detected, *e.g*. with reproductive biology, ecology and plant distribution (Bennett 1998). Because of the number of biological correlations, C-value data can be considered a good predictor of phenotypic traits at multiple levels (Sparrow and Micksche 1961, Underbrink and Pond 1976). Thus, taxonomy, genome evolution, ecology, genomics, plant breeding, cell and molecular biology, conservation, physiology and development can all be better understood when C-value analysis is considered.

Genome size has been shown to vary over 1000-fold in angiosperms, ranging from *ca.* 0.10 pg in *Aesculus hippocastanum* L. to 127.4 pg in *Fritillaria assyriaca* Baker (Bennett and Smith 1976, 1991). Nonetheless, the true range of genome size variation is still unknown, and although knowledge of C-values is constantly increasing, the available data only represent approximately 1.5 % of the global angiosperm flora. Since 1976, a research group has been collecting any C-value estimate made in this period, and has assembled a database that encompasses all known information on plant C-values; it is available through an internet database (www.rbgkew.or.uk/cval/homepage.html, Bennett and Leitch 2003).

Tripleurospermum Sch. Bip. (*Asteraceae, Anthemideae, Matricariinae*) is a small genus of 38 species, and comprises plants often included in the genus *Matricaria* L. (Applequist 2002), there being some disagreements over the limits of the two genera (Bremer and Humphries 1993): some authors do not separate

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Tripleurospermum from *Matricaria* although the former has one adaxial and two lateral seed ribs and the latter four or five adaxial seed ribs (Bremer 1994). It belongs to subtribe *Matricariinae* which is the biggest in the *Anthemideae* in terms of the number of genera. It is mainly distributed in Europe (South East) and in temperate Asia (South West), with a few species also in North America and North Africa, and one widespread species (*Tripleurospermum perforatum*, often considered a synonym of *T. inodorum*, Applequist 2002). Some *Tripleurospermum* are used as ornamentals, others for medical purposes, like *T. maritimum*, which is claimed to repel fleas, beetles and other insects (Herrera 1995) or *T. decipiens*, in which saponines with a possible pharmacological application have been found (Mojab *et al.* 2003), whilst still others are invasive weeds (*T. inodorum / T. perforatum*, Buckley *et al.* 2001).

The principal aims of the study are: to increase the knowledge on C-values of this genus, to test the possibility of the existence of any relationship between genome size data and cytological, morphological or environmental factors and to analyze the scope of intraspecific and interspecific genome size variation within the genus, if any.

Materials and methods

Plants: The studied material includes eight taxa (seven species, one of them with two varieties) of the genus *Tripleurospermum* (Table 1). Vouchers for most materials are deposited in the Huseyin Inceer herbarium (Trabzon, Turkey). The chosen populations represent distinct life forms, ploidy levels and chromosome numbers. The two populations of *Tripleurospermum maritimum* were obtained from botanical gardens through Index Seminum, with known original location, whereas all other taxa were collected directly from natural populations. Seeds of *Petunia hybrida* Vilm. cv. PxPc6, used as internal standard for flow cytometric measurements, were obtained from the Institut des Sciences du Végétal (CNRS, Gif-sur-Yvette, France).

Karyology: Ripe achenes were germinated on wet filter paper in Petri dishes left in the dark at room temperature. Root tip meristems were pretreated with 0.05 % aqueous colchicine at room temperature for 2 h 15 min (diploid taxa) or 2 h 45 min (polyploid taxa), fixed in absolute ethanol and glacial acetic acid (3:1) for 2 - 4 h at room temperature and stored in the fixative at 4 ºC. Root tips were hydrolysed in 1 M HCl for 5 min at 60 ºC, stained in 1 % aqueous aceto-orcein for 2 - 12 h at room temperature, and squashed and mounted in a drop of 45 % acetic acid-glycerol (9:1). The preparations were observed with an optical microscope at a magnification of 1000×. The best metaphase plates were photographed. The counts and the calculation of total karyotype length were carried out using around five plates per population.

Flow cytometric measurements: DNA 2C-values of the tested species were estimated using flow cytometry. *Petunia hybrida* Vilm. cv. PxPc6 (2C=2.85 pg, Marie and Brown 1993) was used as an internal standard. Young, healthy leaf tissue from the target species and calibration standard (both cultivated in pots) were placed together in a plastic Petri dish and chopped in Galbraith's isolation buffer (Galbraith *et al.* 1983) with a razor blade. The amount of target species leaf (about 25 mm^2) was approximately twice that of the internal standard. The suspension of nuclei in the isolation buffer was filtered through a nylon mesh with a pore size of 30 µm, supplemented with 100 µg cm^{-3} ribonuclease A (RNase A, *Boehringer*, Meylan, France) and stained for 20 min with propidium iodide (*Sigma-Aldrich*, Alcobendas, Madrid, 60 µg cm^{-3}), the chosen fluorochrome (Johnston *et al.* 1999); tubes were kept on ice during staining and then left at room temperature until the measurement. For each population, five individuals were analyzed; two samples of each individual were extracted and measured independently. Measurements were made at the 'Serveis Cientificotècnics generals' of the Universitat de Barcelona using an *Epics XL* flow cytometer (*Coulter Corporation*, Hialeah, USA). The instrument was set up with the standard configuration: excitation of the sample was performed using a standard 488-nm air-cooled argon-ion laser at 15 mW power. Forward scatter (FSC), side scatter (SSC), and red (620 nm) fluorescence for propidium iodide were acquired. Optical alignment was based on optimized signal from 10-nm fluorescent beads (*Immunocheck*, *Epics Division*, *Coulter Corporation*). Time was used as a control for the stability of the instrument. Red fluorescence was projected onto a 1 024 monoparametric histogram. Gating single cells by their area versus peak fluorescence signal excluded aggregates. Acquisition was automatically stopped at 8 000 nuclei. The total nuclear DNA content was calculated by multiplying the known DNA content in *Petunia* by the quotient between the 2C peak positions of *Tripleurospermum* and the internal standard in the histogram of fluorescence intensities for the 10 runs, based on the assumption that there is a linear correlation between the fluorescence signals from stained nuclei of the unknown specimen and the known internal standard and the DNA amount. Mean values and standard deviations were calculated based on the results for the five individuals.

Statistics: Statistical analyses (analysis of variance and Pearson's product moment correlation) were carried out to evaluate the relationships between the studied variables

(DNA content and DNA per basic chromosome set *vs*. altitude and life cycle, among others). All the analyses were performed with the *Statgraphics Plus 5.0* program (*Statistical Graphics Corp*., Rockville, Maryland). In addition to the data obtained in the present study, those from the C-value database (www.rbgkew.org.uk/cval/homepage.html, Bennett and Leitch 2003) for the annual diploids *Matricaria chamomilla* (2n=18, 2C = 7.75 pg, Nagl and Ehrendorfer 1974), *M. discoidea* (2n=18, 2C = 4.90 pg, Nagl and Ehrendorfer 1974) and *M. matricarioides* (2n=18, 2C = 4.65 pg, Bennett 1972) were also used for the statistical analyses of the present work, bearing in mind the close affinity between the two genera, and the fact that many *Tripleurospermum* species had been previously classified as *Matricaria* members and *vice versa* (this closeness can be deduced from the complex synonymy of the species presented in Table 1).

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

Taxa	Origin of materials	Herbarium voucher
T. callosum (Boiss. & Heldr.) E. Hossain (*Chamaemelum callosum* Boiss. & Heldr.)	Turkey, Gumushane, Tekke koyu. 1100 m.	Hb. Inceer 120
T. callosum (Boiss. & Heldr.) E. Hossain (*Chamaemelum callosum* Boiss. & Heldr.)	Turkey, Rize, Ikizdere, Anzer koyu. 2200 m.	Hb. Inceer 136
T. callosum (Boiss. & Heldr.) E. Hossain (*Chamaemelum callosum* Boiss. & Heldr.)	Turkey, Bayburt, Kop Dagi. 2300 m.	Hb. Inceer 69
T. elongatum (Fischer & C. Meyer ex DC.) Bornm. (*Matricaria elongata* (Fischer & C. Meyer ex D.C.) Hand.-Mazz.)	Turkey, Gumushane, Torul. 1300 m.	Hb. Inceer 144
T. maritimum (L.) K. Koch (*Matricaria maritima* L.)	Iceland, Akureyri. Sandy banks near sea level.	Index Seminum Akureyri
T. maritimum (L.) K. Koch (*Matricaria maritima* L.)	Germany, Weimar, Hermstedt. 150 m.	Index Seminum Jena
T. melanolepis (Boiss.) Rech. f.	Turkey, Gumushane, Kose Dagi, 1700 m.	Hb. Inceer 113
T. oreades (Boiss.) Rech. f. var. *oreades* (*Matricaria oreades* Boiss.)	Turkey, Gumushane, Kose Dagi. 1800 m.	Hb. Inceer 106
T. oreades (Boiss.) Rech. f. var. *oreades* (*Matricaria oreades* Boiss.)	Turkey, Rize, Cat Koyu. 1150 m.	Hb. Inceer 109
T. oreades (Boiss.) Rech. f. var. *tchihatchewii* E. Hossain (*Chamaemelum tchihatchewii* Boiss.)	Turkey, Rize, Ikizdere, Sivrikaya, Koyu. 1750 m.	Hb. Inceer 104
T. repens (Freyn & Sint.) Bornm.	Turkey, Rize, Ikizdere, Cimil-Baskoy 1900 m.	Hb. Inceer 132
T. repens (Freyn & Sint.) Bornm.	Turkey, Rize, Ikizdere, Between Cimil and Baskoy. 1800 m.	Hb. Inceer 133
T. sevanense (Manden.) Pobed. (*Matricaria sevanensis* (Manden.) Rauschert, *Chamaemelum sevanense* Manden.)	Turkey, Gumushane, Kose Dagi. 1800 m.	Hb. Inceer 121
T. sevanense (Manden.) Pobed. (*Matricaria sevanensis* (Manden.) Rauschert, *Chamaemelum sevanense* Manden.)	Turkey, Gumushane, Kose Dagi. 1600 m.	Hb. Inceer 105a

Results and discussion

According to the existing data in the C-value database, this is the first study of seven of the eight taxa analysed. Previously, Nagl and Ehrendorfer (1974) used the Feulgen method (microdensitometry after Feulgen staining) to estimate the nuclear DNA amount of a diploid *T. maritimum* (2C = 5.50 pg). Indeed, this is the first flow cytometric investigation for DNA content assessment on the subtribe *Matricariinae* (Table 2).

Relationship with karyological characters: A statistically significant difference has been found between 2C values and ploidy level (mean 2C of diploids = 5.08 pg; mean 2C of tetraploids = 8.52 pg), as might be expected in a narrow group of species. This finding is quite clear and widespread. Fridlender *et al.* (2002) also detected a statistically significant difference between genome size and ploidy level in species from *Colchicum*. Similar relationships have been found in many other genera (*Achillea*, Dąbrowska 1992, *Artemisia*, Torrell and Vallès 2001, Garcia *et al.* 2004). In fact, genome size measurements are currently used as a reliable and fast method to establish ploidy level in groups of species in which the nuclear DNA amount of at least one diploid species is known (Vilhar *et al.* 2002). Moreover, data on

nuclear DNA amount in *Tripleurospermum* also show a positive correlation with karyotype length (r = 0.75, $P < 0.01$), as reported in *Echinops* by Garnatje *et al.* (2004).

We have found that genome size per basic chromosome set decreases with polyploidy: there is a significant difference ($P < 0.0005$) between diploids and tetraploids. For these two ploidy levels, diploids always present a higher nuclear DNA amount per basic chromosome set (mean genome sizes per basic chromosome set: 2.54 pg in diploids and 2.13 pg in tetraploids). A loss of nuclear DNA amount in the process of polyploidization is suggested to explain this observation (Leitch and Bennett 2004). Sharma and Sen (2002) considered that with polyploidization, chromosomes tend to diminish their size slightly, each one equally, and hypothesized that this 'strengthening' mechanism was a 'defence strategy' against the increased possibility of mutations with polyploidy (an increase in nuclear DNA amount increases the probability of mutations). Nuclear DNA loss per basic chromosome set in polyploids has been frequently reported, so genome downsizing following polyploid formation has been considered a widespread biological phenomenon (Leitch and Bennett 2004 and references therein). Some molecular processes for this genome downsizing have been suggested, such as the homoeologous pairing, which can lead to chromosome restructuring and deletions, and hence to a loss of DNA as a consequence of the breakdown in the postreplicative mismatch repair system (Comai 2000) or the selective gene loss (Ku *et al.* 2000, Simillion *et al.* 2002), among others. However, many of the underlying molecular mechanisms responsible for this phenomenon are still unknown.

Table 2. Nuclear DNA content and other karyological characters of the populations studied. Life cicle: A - annual, B - biennial; P - perennial; RP - rhizomatous perennial. 2C - nuclear DNA content (means ± SD of 10 samples). 1 pg = 978 Mbp (Doležel *et al.* 2003). TKL - total karyotype length. 2n - somatic chromosome number. 2C/p.l. - DNA per basic chromosome set (quotient between 2C nuclear DNA content and ploidy level). *Inceer and Beyazoglu (2005); **Nagl and Ehrendorfer (1974); *** Bennett (1972).

Taxa	Life cycle	2C [pg]	2C [Mbp]	TKL [μm]	2n	Ploidy level	2C/p.l.
T. callosum (Rize)	P	8.17±0.08	7 990.26	32.09±1.52	36	4x	2.04
T. callosum (Bayburt)	P	7.98±0.02	7 804.44	31.43±0.43*	36*	4x*	1.99
T. callosum (Gumushane)	P	8.18±0.10	8 000.04	32.09±1.33	36	4x	2.05
T. elongatum	B/P	4.87±0.14	4 765.86	14.00±0.54*	18*	2x*	2.44
T. maritimum (Germany)	A/B	5.50±0.05	5 379.00	-	18	2x	2.75
T. maritimum (Iceland)	A/B	9.22±0.18	9 017.16	-	36	4x	2.31
T. melanolepis	RP	4.88±0.04	4 772.64	13.15±0.75*	18*	2x*	2.44
T. oreades var. *oreades* (Gumushane)	RP	8.76±0.22	8 567.28	29.35±1.02	36	4x	2.19
T. oreades var. *oreades* (Rize)	RP	9.05±0.23	8 850.90	28.78±1.48	36	4x	2.26
T. oreades var. *tchihatchewii*	RP	8.91±0.57	8 713.98	24.78±0.64*	36*	4x*	2.23
T. repens (Rize, 1800 m)	P	8.54±0.44	8 352.12	39.30±1.23*	36*	4x*	2.14
T. repens (Rize, 1900 m)	P	8.32±0.18	8 136.96	40.50±1.52	36	4x	2.08
T. sevanense (Gumushane 1600 m)	P	8.26±0.30	8 078.28	26.15±0.75	36	4x	2.07
T. sevanense (Gumushane 1800 m)	P	8.34±0.21	8 156.52	25.12±1.13	36	4x	2.09
*Matricaria chamomilla***	A	7.75	7 579.50	-	18	2x	3.87
*Matricaria discoidea***	A	4.90	4 795.20	-	18	2x	2.45
*Matricaria matricarioides****	A	4.65	4 547.70	-	18	2x	2.32

Systematic implications: interspecific and intra-specific variability: The paucity of available data on C-value in subtribe *Matricariinae* makes it difficult to discuss about interspecific variation in these plants. Nevertheless, we have noted that the genus *Tripleurospermum* is quite homogeneous in terms of genome size: the ratio between maximum and minimum nuclear DNA amount and nuclear DNA amount per basic chromosome set is quite low in this group. This fact, and the absence of relevant morphological or ecological differences between the analysed plants, could also suggest homogeneity in the *Tripleurospermum* species. A high value of this ratio has been reported in other *Anthemideae* groups, such as in the genus *Artemisia* (particularly in subgenus *Artemisia*, Garcia *et al.* 2004).

Because C-value is considered constant within a species, the existence of variation in nuclear DNA amount under the specific level is controversial. Whilst some authors uphold the idea that the amount of nuclear genome can vary as a response to environmental changes (the genome plasticity or flexibility theory - Durrant and Jones 1971, Joarder *et al.* 1975, Ohri 1998), others

attribute this supposed variability to mistakes or methodological problems (Greilhuber 1997, 1998). Nonetheless, it is obvious that a certain degree of genuine intraspecific variation is always possible (Greilhuber 1998, Schmuths *et al.* 2004). Within some of the taxa of *Tripleurospermum* used in this study, different populations of the same species have been analyzed (Table 3). All but one of these species show a low percentage of intraspecific variation. Moreover, they belong to the same restricted geographical area (Turkey). Between the two varieties of *T. oreades* there also exists a low percentage of intraspecific variation, although a little bit higher than for the remaining species. In this taxon, we found a higher variation in nuclear DNA content between two populations of one variety than between both varieties (Table 3). Although the two populations analysed of *T. maritimum* belong to different ploidy levels (2x and 4x), and although the nuclear DNA loss per chromosome set in polyploids must be taken into account, the 19.56 % of intraspecific variation, calculated with the nuclear DNA per basic chromosome set, is still remarkable. The geographical distance between both populations and the remarkably different latitude of them, one coming from Germany (51 º) and the other from Iceland (65 º), could contribute to explaining the variability of the C-value in this species, as other authors have stated (Grime and Mowforth 1982, Ohri 1998).

Table 3. Nuclear DNA amount (2C) and intraspecific variability (calculated as a percentage of the quotient between the highest and the lowest nuclear DNA amount) of some populations of *Tripleurospermum.* [1]Nuclear DNA amount per basic chromosome set. [2]Percentage of the quotient between the highest and the lowest nuclear DNA amount per basic chromosome set.

Taxa	2C [pg]	Intraspecific variability [%]
T. callosum	7.98 (Bayburt) 8.17 (Rize) 8.18 (Gumushane)	2.51
T. maritimum	5.50 / 2.75[1] (Germany) 9.22 / 2.30[1] (Iceland)	19.56[2]
T. oreades var. *oreades*	8.76 (Gumushane) 9.05 (Rize)	3.31
T. oreades var. *tchihatchewii*	8.91	
T. repens	8.32 (Rize, 1900 m) 8.54 (Rize, 1800 m)	2.64
T. sevanense	8.26 (Gumushane, 1600 m) 8.34 (Gumushane, 1800 m)	0.97

Life cycle and environmental factors: Many studies have indicated a relationship between life cycle and nuclear DNA amount. It is believed that, in the framework of a genus, an increase in nuclear DNA amount implies a longer cell cycle. According to this, annual plants should have less nuclear DNA amount than perennials (Nagl and Ehrendorfer 1974, Rees and Narayan 1981, Bennett and Leitch 2003). In the present study, the *ANOVA* shows that annual and biennial taxa present a significantly higher genome size ($P < 0.05$) than perennials. It must be said, however, that most perennials of the subtribe *Matricariinae* are also tetraploid, while the majority of annuals and biennials are diploid. Thus, the increase in nuclear DNA amount of these perennial species comes from their tetraploid character rather than from their life cycle. In fact, the majority of polyploids are perennial plants (Jackson 1976). Another explanation could be that annual character is secondary and relatively recent in this group, so that the surplus DNA has not yet been eliminated from the genome. In summary, although many authors have found that perennials have significantly higher nuclear DNA amounts than annuals (Garnatje *et al.* 2004 and references therein), others have reported the opposite relationship (Martel *et al.* 1997, Jakob *et al.* 2004) or even the absence of a relationship between life cycle and genome size (Grime and Mowforth 1982, Garcia *et al.* 2004). The numerous exceptions clearly suggest that the correlation between these two parameters is not as clear as initially thought.

Nuclear DNA per basic chromosome set and altitude are negatively correlated ($r = -0.67$, $P < 0.01$) in the genus *Tripleurospermum*: there is a decrease in genome size with the altitude. This is in agreement with the negative correlation found between these two parameters in wild populations of *Arachis duranensis* (Temsch and Greilhuber 2001). On the other hand, these data do not support the observations of Bennett (1976) and Rayburn and Auger (1990), who suggested that increased nuclear DNA amount was an adaptation to altitude. An increase in nuclear DNA amount with increasing altitude was also detected in many different genera (Laurie and Bennett 1985, Godelle *et al.* 1993, Cerbah *et al.* 1999). Suda *et al.* (2003) suggested that the nuclear DNA amount of endemics to Tenerife was negatively correlated with altitude in genera distributed over a large altitudinal range, and genera with a limited range in altitude showed a positive correlation between DNA content and altitude. Again, the link between altitude and genome size is uncertain, and clearly varies diversely in different plant groups; a mechanism to explain this phenomenon is still lacking.

The diploid and tetraploid populations of *T. maritimum* show the highest nuclear DNA amounts within the diploid and tetraploid *Tripleurospermum* studied here. This species occupies sandy places near the sea, very dry areas which exhibit elevated salinity, implying relatively adverse conditions for the growth of vegetation. Various studies have shown that plants inhabiting arid or extreme environments (such as deserts and highly nitrogenated soils) tend to have an increased nuclear DNA amount in comparison with their relatives

living under more favourable environmental conditions (Garcia *et al.* 2004 and references therein). Consequently, it is conceivable that an adaptation to these extreme habitats increases genome size in *Tripleurospermum*. Nevertheless, more data will need to be collected before such a relationship can be clearly established.

Within the Turkish tetraploid species of *Tripleurospermum* studied, the rhizomatous ones were found to have a significantly higher nuclear DNA amount (P = 0.0005) than those without rhizome (mean 2C of rhizomatous ones = 8.90 pg; mean 2C of non rhizomatous ones = 8.26 pg). Rhizomes give plants the ability to colonize habitats, and it is plausible that this adaptive advantage could be related to the higher nuclear DNA amount in these species, as stated previously in relation to salinity. Possibly as a result of the presence of rhizomes, these plants show a lower incidence of sexual reproduction. Consequently, chromosomes and nuclear DNA amount are less important, and variations, such as mutations, deletions or an increase in genome size, can be easily tolerated.

Concluding remarks: This study represents the first relatively extensive survey of nuclear DNA amount in the genus *Tripleurospermum*, accounting for 20 % of the taxa. Although it does not permit absolute and definitive conclusions, it contributes as a first step towards a genome size analysis of this group of plants. A good phylogenetic context within which to analyse these data would be useful in order to study the systematics of the genus, and would help in achieving a better understanding of the results. Thus, further research in this area will be necessary.

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3. Recomptes nous o rars en tàxons de la subtribu *Artemisiinae* (*Anthemideae*, *Asteraceae*) de Mongòlia.

Sònia GARCIA, Teresa GARNATJE, Shagdar DARIIMAA, Shagdar TSOOJ i Joan VALLÈS. Botanical Journal of the Linnean Society 150: 203-210 (2006).

Aquest estudi comprèn 25 recomptes cromosòmics de 18 espècies de la subtribu *Artemisiinae* (*Anthemideae*, *Asteraceae*) de poblacions provinents de Mongòlia. La majoria (15 espècies) s'inclouen dins d'*Artemisia*, el gènere més gran de la subtribu, mentre que les altres són de dos gèneres que hi estan estretament relacionats: *Ajania* (dues espècies) i *Neopallasia* (una espècie). Onze recomptes són nous, tres no coincideixen amb dades prèvies i la resta confirmen informacions ja existents. La majoria d'espècies tenen x=9 com a nombre cromosòmic de base, tot i que també hi ha alguns tàxons amb x=8. Els nivells de ploïdia oscil·len entre el diploide i l'hexaploide. S'ha detectat la presència de cromosomes B en una espècie, *Ajania fruticulosa*.

Botanical Journal of the Linnean Society, 2006, **150**, 203–210. With 19 figures

New or rarely reported chromosome numbers in taxa of subtribe Artemisiinae (Anthemideae, Asteraceae) from Mongolia

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This study encompasses 25 chromosome counts of 18 species in the subtribe Artemisiinae (tribe Anthemideae) of the family Asteraceae, from Mongolia. Most (15 species) belong to *Artemisia*, the largest genus of the subtribe, whereas the others come from two genera very closely related to it: *Ajania* (two species) and *Neopallasia* (one species). Eleven counts are new reports, three are not consistent with previous reports and the remainder confirm scanty earlier information. The majority of the species have $x = 9$ as their basic chromosome number, but there are some taxa with $x = 8$. Ploidy levels range from $2x$ to $6x$. The presence of B-chromosomes was detected in *Ajania fruticulosa*. *Botanical Journal of the Linnean Society*, 2006, **150**, 203–210.

ADDITIONAL KEYWORDS: *Ajania* – *Artemisia* – B-chromosomes – cytotaxonomy – Compositae – dysploidy – karyology – *Neopallasia* – polyploidy.

INTRODUCTION

The Artemisiinae, the largest subtribe in the Anthemideae (Asteraceae), comprise a worldwide group of genera, which include many useful species. Many karyological surveys have been made on them (Watanabe, 2002, and references therein). The present paper focuses on the genus *Artemisia*, one of the largest in the family Asteraceae (comprising *c*. 500 taxa) and, to a lesser extent, two small genera, *Ajania* and *Neopallasia*, that are so phylogenetically close to *Artemisia* that some species have been classified as members of *Artemisia* (see the synonymy of some of the taxa studied given below). Since the earliest chromosome studies of the genus *Artemisia* in the first half of the 20th century (Weinedel-Liebau, 1928) many others have followed, enhancing the available cytogenetic and karyological data, not only on this genus, but also on its allies (Eherendorfer, 1964; Kawatani & Ohno, 1964; Korobkov, 1972; McArthur, Pope & Freeman, 1981; Ouyahya & Viano, 1981, 1988; Vallès & Siljak-Yakovlev, 1997; Vallès *et al*., 2005, and references therein). However, chromosomal data are still scarce or non-existent for numerous Artemisiinae taxa. Additionally, chromosome reports on Mongolian populations (some of them endemic) of these taxa are also limited, and because this region is regarded as one of the most outstanding speciation and diversification points of the subtribe, the value of these data is high. This paper sets out to produce more. Many of the counts reported here are new; others confirm unique or very scarce reports and only three differ from those cited previously. Some evolutionary and systematic traits of these genera are also discussed in the light of these new cytogenetic data.

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MATERIAL AND METHODS

Root-tip meristems were obtained from wild-collected achenes by germinating them on wet filter paper in Petri dishes at room temperature. They were pretreated with 0.05% aqueous colchicine at room temperature for 2.5 h. The material was fixed in absolute ethanol and glacial acetic acid (3 : 1) for 2–4 h at room temperature and then stored in the fixative at 4 °C. Samples were hydrolysed in 1 N HCl for 2 min at 60 °C, stained in 1% aqueous aceto-orcein for 2–12 h at room temperature and squashed and mounted in a drop of 45% acetic acid/glycerol (9 : 1). The best metaphase plates were photographed on a Zeiss Axioplan microscope with an AxioCam MRc 5 digital camera and acquired with AxioVision AC v. 4.2 software (Carl Zeiss Vision, GmbH). Vouchers of the species studied are deposited in the herbarium of the Centre de Documentació de Biodiversitat Vegetal de la Universitat de Barcelona (BCN).

To assess the existence of published chromosome counts in the species studied we used the most common indexes of plant chromosome numbers (cited in Vallès, Torrell & Garcia-Jacas, 2001a), as well as the chromosome number databases Index to Plant Chromosome Numbers (Missouri Botanical Garden, http://mobot.mobot.org/W3T/Search/ipcn.html) and Index to Chromosome Numbers in the Asteraceae (Watanabe, 2002, http://www-asteraceae.cla.kobe-u.ac.jp/index.html; Watanabe, 2004).

RESULTS AND DISCUSSION

We present the data and comments on the different taxa grouped by genera in alphabetical order. In *Artemisia* we have considered the main sections (treated by several authors as subgenera) into which the genus is divided. The localities are given with the indication of Mongolian administrative divisions, aimag (province, written aimak in Russian language works) and sum (village, written somon in Russian language works).

GENUS *AJANIA* POLJAKOV

Ajania achilleoides (Turcz.) Poljakov [*Artemisia achilleoides* Turcz., *Chrysanthemum achilleoides* (Turcz.) Hand.-Mazz., *Hippolytia achilleoides* (Turcz.) Poljakov]

Mongolia, Dund (Central) Gobi aimag: Erdene-Dalai sum, 16 km north-east of the sum, dry steppe hills, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 5.ix.2004 (BCN 23793). $2n = 18$ (Fig. 1).

Mongolia, Dund (Central) Gobi aimag: Erdene-Dalai sum, 46 km north-east of the sum, dry steppe hills, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 5.ix.2004 (BCN 23794). $2n = 18$.

According to our data this is the first count on this species, endemic to Mongolia and China, confirmed in two very close populations. It is a diploid based on $x = 9$. This count confirms the basic number in the genus *Ajania*. The diploid level was also reported in *Ajania fastigiata* ($2n = 18$, Maltzeva, 1969). Other works on the relatively large genus *Ajania* (about 40 species) have dealt with non-Central Asian species (Sokolovskaya, 1966; Probatova & Sokolovskaya, 1990; Kondo *et al*., 1992; Abd El-Twab, Kondo & Hong, 1999) and reported different ploidy levels up to decaploid.

Ajania fruticulosa (Ledeb.) Poljakov [*Tanacetum fruticulosum* Ledeb. *Chrysanthemum fruticulosum* (Ledeb.) B. Fedtsch.]

Mongolia, Umnu (South) Gobi aimag: Mandal Oboo sum, 20 km south-east of the sum, desert steppe with *Anabasis*, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 4.ix.2004 (BCN 23795). $2n = 36+0–2–4B$ (Fig. 2).

This is the second report on chromosome number for this Central Asian species, and the first in a Mongolian population. It confirms the first, which was obtained from a Chinese population by Kondo *et al*. (1998). In our case, the presence of 2–4 B-chromosomes was detected in some cells (see chromosomes marked with arrows in Fig. 2). The presence of B-chromosomes in the subtribe is not unusual (Vallès & Garnatje, 2005). Tetraploidy is common in the genus, as many other counts of 36 chromosomes have been reported (e.g. in *A. przewalskii*, Kondo *et al*., 1992 and A. *latifolia*, Kondo *et al*., 1998).

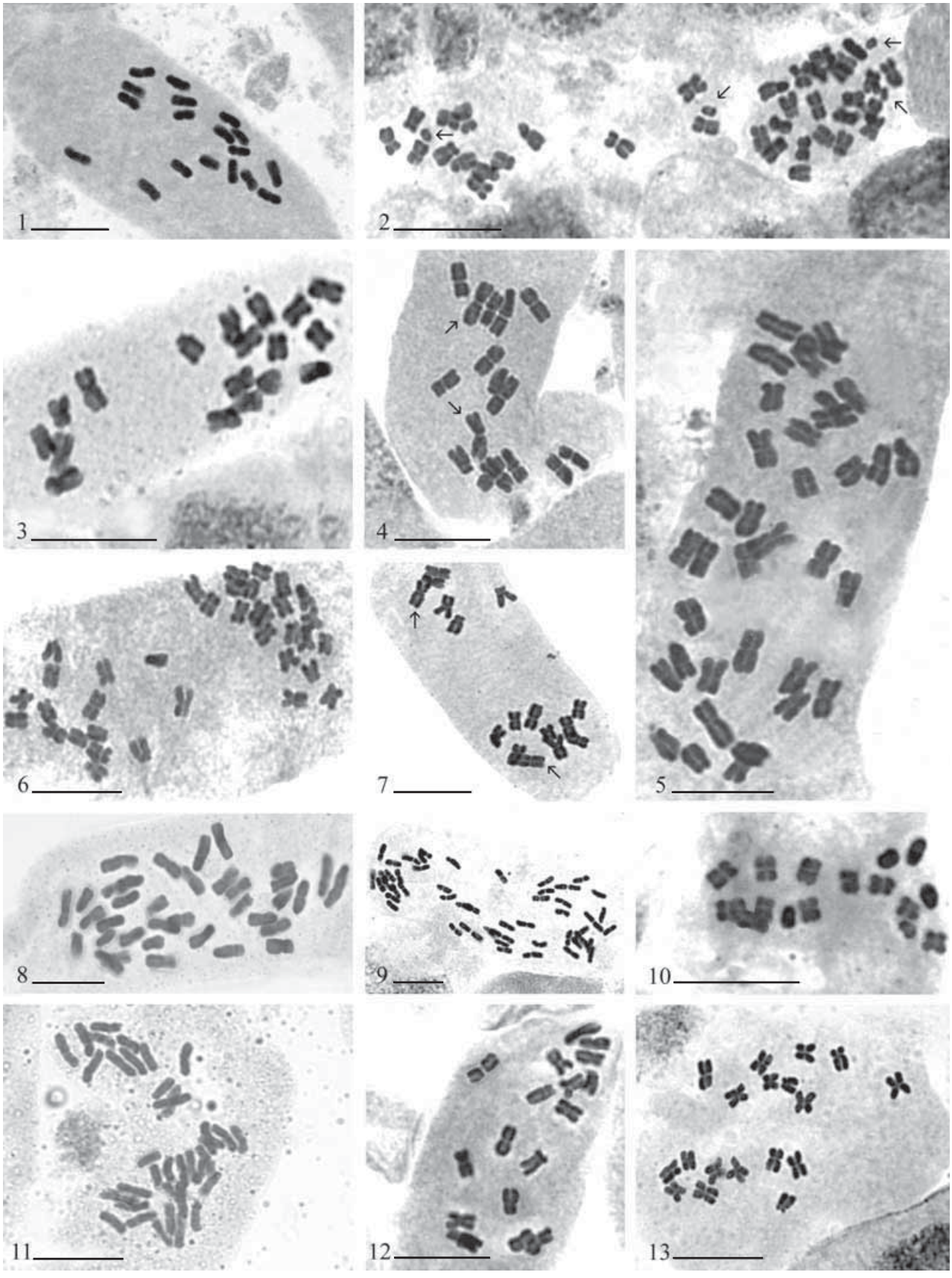
GENUS *ARTEMISIA* L.

SECTION *ARTEMISIA*

Artemisia adamsii Besser

Mongolia, Tuv (Central) aimag: Bayan Undjuul sum, 20 km north of the sum, dry steppe, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 5.ix.2004 (BCN 23815). $2n = 18$ (Fig. 3).

Figures 1–13. Somatic metaphases. Scale bars = 10 μm. Fig. 1. *Ajania achilleoides* ($2n = 18$). Fig. 2. *Ajania fruticulosa* ($2n = 36 + 4B$); arrows indicate the B-chromosomes. Fig. 3. *Artemisia adamsii* ($2n = 18$). Fig. 4. *A. leucophylla* ($2n = 16$); arrows indicate the long metacentric chromosome pair. Fig. 5. *A. medioxima* ($2n = 36$). Fig. 6. *A. messerschmidtiana* ($2n = 36$). Fig. 7. *A. obscura* ($2n = 16$); arrows indicate the long metacentric chromosome pair. Fig. 8. *A. transbaicalensis* ($2n = 36$). Fig. 9. *A. umbrosa* ($2n = 54$). Fig. 10. *A. anethoides* ($2n = 16$). Fig. 11. *A. depauperata* ($2n = 36$). Fig. 12. *A. macrocephala* ($2n = 18$). Fig. 13. *A. dolosa* ($2n = 18$).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This count confirms the only previous one, from a population collected in north-east China by Wang, Guan & Zhang (1999), on plant material from Mongolia. It represents a diploid level based on $x = 9$, the most common basic chromosome number in the genus (Vallès *et al*., 2005, and references therein).

Artemisia leucophylla (Turcz. ex Besser) C. B. Clarke (*Artemisia vulgaris* L. var. *leucophylla* Turcz. ex Besser)
Mongolia, Arkhangai aimag: Taryat sum, Khorgo-Terkh National Park, near lake Terkhen Sagan nur, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 27.viii.2004 (BCN 23796). $2n = 16$ (Fig. 4).

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 23797). $2n = 16$.

In this case, our counts, assessed in two well-separated populations, do not agree with the only earlier one ($2n = 18$), obtained from a Russian population (Krogulevich, 1978) from Eastern Sayana (Siberia). Many species of the genus, especially those belonging to sections *Artemisia* and *Absinthium* (Torrell *et al*., 1999), display the basic chromosome number $x = 8$, but less commonly than the dominant $x = 9$ (Torrell *et al*., 2001). Evidence is given in several studies (Vallès & Siljak-Yakovlev, 1997; Torrell *et al*., 2001) that $x = 8$ arises via a descending dysploidy process from $x = 9$, as a result of a chromosomal fusion, demonstrable by the existence of a long metacentric chromosome pair in $x = 8$ taxa such as *A. splendens* (Torrell *et al*., 2001). This long metacentric chromosome pair with some centromeric fragility, also confirmed in other species with the same phenomenon (Torrell *et al*., 2001; M. Torrell & J. Vallès, unpubl. data), could explain the former count of $2n = 18$ in this species. *Artemisia leucophylla* is related closely to *A. vulgaris* (up to the point to which it has been classified by some workers as a variety of this species, see the synonymy), *A. mongolica* and *A. obscura* (see below for this species), three taxa also with $2n = 16$ (Watanabe, 2002, and references therein).

Artemisia medioxima 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). $2n = 36$ (Fig. 5).

This is the first count carried out in this Mongolian and Russian endemic species. It is a tetraploid based on $x = 9$, the most common basic chromosome number in *Artemisia*, confirming the relevance of polyploidy in the genus (Vallès & Garnatje, 2005; Vallès *et al*., 2005, and references therein).

Artemisia messerschmidtiana Besser
Mongolia, Tuv (Central) aimag: Bayan Undjuul sum, Ikh Khairkhan mountains, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 5.ix.2004 (BCN 23798). $2n = 36$ (Fig. 6).

According to our literature research, this is the first report of the chromosome number in this species, which is endemic to Siberia, Mongolia and the north of China.

Artemisia obscura Pamp.
Mongolia, Umnu (South) Gobi aimag: Bulgan sum, E Gurvan Saikhan mountains, canyon, *Sh. Dariimaa, Sh. Tsooj, J. Vallès & E. Yatamsuren*, 2.ix.2004 (BCN 23799). $2n = 16$ (Fig. 7).

This is the first count reported for this species, with a distribution restricted to Mongolia and China, and together with *Artemisia leucophylla* and many other members of the *A. vulgaris* group (Vallès & Garnatje, 2005, and references therein) it confirms the existence of the basic chromosome number $x = 8$ in this complex of the genus (discussed for *Artemisia leucophylla*, see above).

Artemisia transbaicalensis Leonova
Mongolia, Uvur-Khangai aimag: Kharkhorin sum, 2 km west of the sum, margin of *Betula* and *Larix* forest, 1900 m, steppe, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 26.viii.2004 (BCN 23800). $2n = 36$ (Fig. 8).

Again, this is the first report on the chromosome number of this species, which grows in the Baikal Lake basin (Siberia and Mongolia) and whose basic chromosome number ($x = 9$) and ploidy level are widespread in the genus.

Artemisia umbrosa Turcz. ex DC.
Mongolia, Selenge aimag: Shaamar sum, Tujiin Nars, 5 km east of the sum, *Sh. Dariimaa, Sh. Tsooj, J. Vallès & E. Yatamsuren*, 9.ix.2004 (BCN 23789). $2n = 54$ (Fig. 9).

According to our literature search, this record is the second count for this species, and does not agree with the first by Hoshi *et al*. (2003), who reported $2n = 50$ from Russian material. The existence of this hexaploid cytoype is evidence of a high polyploidization activity as an evolutionary mechanism of the genus in this area. Similarly to *A. obscura* and *A. leucophylla*, the species belongs to the *A. vulgaris* group, but in this case the basic chromosome number is $x = 9$, the original in the genus (Vallès & Garnatje, 2005).

SECTION *ABSINTHIUM* DC.

Artemisia anethoides Matff.
Mongolia, Selenge aimag: Shaamar sum, 3 km west of the sum, Buureg Tolgoi hills, near river Orkhon,

700 m, *Sh. Dariimaa, Sh. Tsooj, J. Vallès & E. Yatamsuren*, 7.ix.2004 (BCN 23790). $2n = 16$ (Fig. 10).

To our knowledge, this is the second count on this species, an endemic of China and Mongolia, and agrees with the previous report, of plants from north-east China by Wang *et al*. (1998). It confirms the existence of the basic chromosome number $x = 8$ in section *Absinthium*.

Artemisia macrocephala Jacquem. ex Besser
Mongolia, Arkhangai aimag: Tsenkher sum, near river Urd-tamir, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 26.viii.2004 (BCN 23801). $2n = 18$ (Fig. 12).

Mongolia, Uvur Khangai aimag: Arvaykheer city, ruderal in streets, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 30.viii.2004 (BCN 23802). $2n = 18$.

Mongolia, Dund (Central) Gobi aimag: Erdene-Dalai sum, 6 km north-east of the sum, steppe, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 4.ix.2004 (BCN 23803). $2n = 18$.

Verified in three well-separated populations, the present one confirms two previous counts, one from a Siberian population (Republic of Tuva, Russia, Krogulevich & Rostovtseva, 1984) and the other from Tadzhikistan (Astanova, 1989). These are the first counts in Mongolian populations of this species, which has a large distribution throughout Central and Eastern Asia.

SECTION *DRACUNCULUS* BESSER

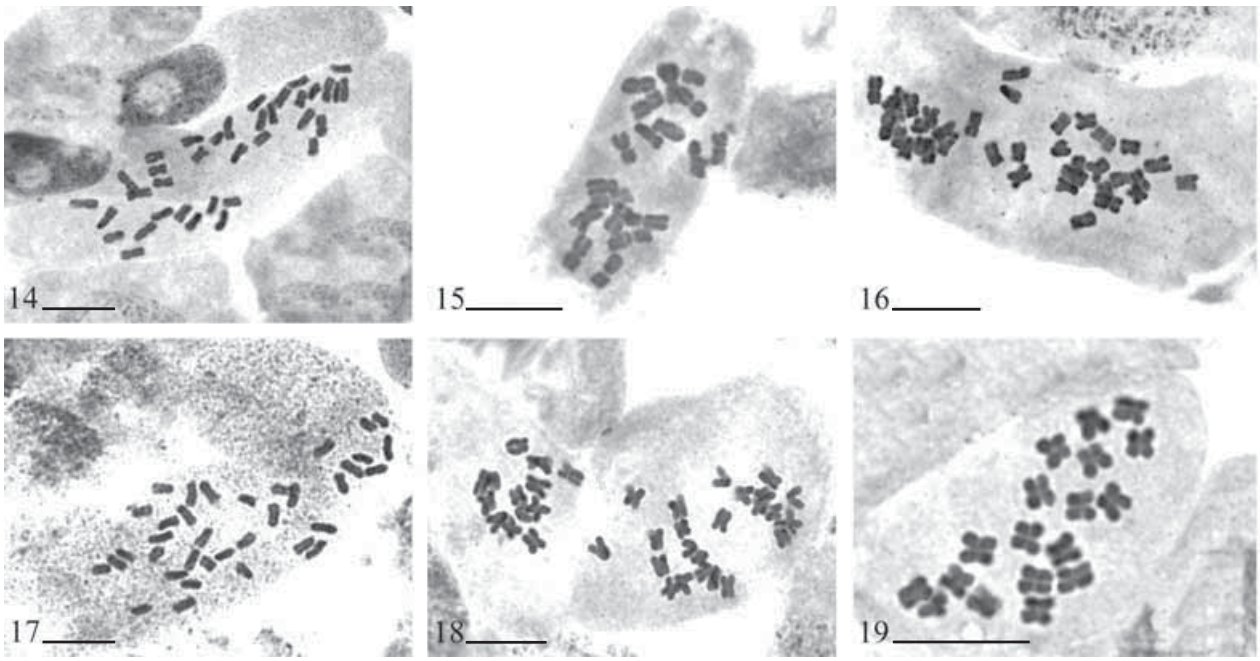
Artemisia depauperata Krasch.
Mongolia, Arkhangai aimag: Tsetserleg city, Sagaan-Davaa pass, 2200 m, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 26.viii.2004 (BCN 23804). $2n = 36$ (Fig. 11).

Our report agrees with the only previous one and confirms the tetraploid cytotype of this species, reported by Krogulevich (1978) from a Russian population (Eastern Sayana, Siberia). It is the first count in a Mongolian population of the species, which is endemic to Mongolia and Russia.

Artemisia dolosa Krasch.
Mongolia, Uvur Khangai aimag: Khujirt sum, 20 km south of the sum, meadow steppe, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 30.viii.2004 (BCN 23805). $2n = 18$ (Fig. 13).

Mongolia, Tuv (Central) aimag: Mungunmort sum, 20 km W of the sum, W slope of Mungun mountain, *Sh. Dariimaa, Sh. Tsooj, J. Vallès & E. Yatamsuren*, 7.ix.2004 (BCN 23791). $2n = 36$ (Fig. 14).

These are the first two counts reported in this species, endemic to Mongolia and the Altai mountains of Russia, one population being diploid and the other tetraploid. Both the basic chromosome number $x = 9$ and the two ploidy levels are common in the genus. The existence of diploid and tetraploid cytotypes has been confirmed separately by flow cytometry (S. Garcia



Figures 14–19. Somatic metaphases. Scale bars = 10 μm. Fig. 14. *Artemisia dolosa* ($2n = 36$). Fig. 15. *A. giraldii* ($2n = 18$). Fig. 16. *A. klementzae* ($2n = 36$). Fig. 17. *A. monostachya* ($2n = 36$). Fig. 18. *A. xanthochroa* ($2n = 36$). Fig. 19. *Neopallasia pectinata* ($2n = 18$).

et al., unpubl. data). The occurrence of a high degree of diversification (with a relevant role played by polyploidy) in the studied area is also supported by this finding.

Artemisia giraldii Pamp.
Mongolia, Bulgan aimag: Sansar sum, north-east slope of Khugunkhaan mountain, steppe near *Betula* and *Pinus* forest, 2000 m, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 25.viii.2004 (BCN 23806). $2n = 18$ (Fig. 15).

According to our data, this is the first report of the chromosome number in this species, endemic in China and Mongolia. This is a taxon very closely related to *A. dracunculus* L., which presents a polyploid series based on $x = 9$, starting at the diploid level as in *A. giraldii*, but reaching the decaploid level (Vallès *et al*., 2001a, and references therein).

Artemisia klementzae Krasch.
Mongolia, Bulgan aimag: Sansar sum, Khugunkhaan mountain, sandy steppe, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 25.viii.2004 (BCN 23807). $2n = 36$ (Fig. 16).

Again, we present a new chromosome count in a Mongolian endemic species.

Artemisia monostachya Bge. ex Maxim. [*A. pubescens* Ledeb. var. *monostachya* (Bge. ex Maxim.) Y.R. Ling]
Mongolia, Arkhangai aimag: Ikh Tamir sum, 30 km north-west of the sum, Khoer Davaa pass, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 26.viii.2004 (BCN 23808). $2n = 36$ (Fig. 17).

Mongolia, Arkhangai aimag: Taryat sum, Khorgo-Terkh National Park, rocky mountain slope near lake Terkhen Sagan nur, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 27.viii.2004 (BCN 23809). $2n = 36$.

To our knowledge, the chromosome number of this taxon has not been reported before. This first count is confirmed in two close populations of this species, endemic to Russia, China and Mongolia.

Artemisia xanthochroa Krasch.
Mongolia, Uvur-Khangai aimag: Tugrug sum, 40 km south of the sum, desert steppe with *Caragana*, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 31.viii.2004 (BCN 23810). $2n = 36$ (Fig. 18).

This is the first chromosome count for this species, endemic to China and Mongolia.

GENUS *NEOPALLASIA* POLJAKOV

Neopallasia pectinata (Pall.) Poljakov (*Artemisia pectinata* Pall.)
Mongolia, Umnu (South) Gobi aimag: Bulgan sum, 10 km south of the sum, *Sh. Dariimaa, D. Samjid, Sh. Tsooj & J. Vallès*, 26.viii.2004 (BCN 23811). $2n = 18$ (Fig. 19).

Mongolia: Ulaanbaatar, ruderal in the city, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 7.ix.2004 (BCN 23812). $2n = 18$.

Confirmed in two separate Mongolian populations, our record validates one of the previous counts on this Central Asian species, reported under its original name of *Artemisia pectinata* (Suzuka, 1952; Kawatani & Ohno, 1964; Qiao, Yan & Zhang, 1990). A tetraploid population of this taxon has also been reported (Vallès *et al*., 2005) from Chinese material.

CONCLUDING REMARKS

The results obtained prove again the existence of two basic chromosome numbers in *Artemisia* (Vallès & Garnatje, 2005). All the studied taxa but three have $x = 9$, the most common basic chromosome number in the genus, subtribe, tribe and family (Solbrig, 1977; Schweizer & Ehrendorfer, 1983; Oliva & Vallès, 1994; Vallès & Siljak-Yakovlev, 1997). Of all the taxa considered, three present $x = 8$ as their basic chromosome number, evidence for descending dysploidy resulting from chromosome fusion that occurs in the genus (Vallès & Siljak-Yakovlev, 1997; Torrell *et al*., 2001; Vallès & Garnatje, 2005). This has also been reported in many other genera of the Asteraceae (Fernández Casas & Susanna, 1986; Garcia-Jacas, Susanna & Ilarslan, 1996; Siljak-Yakovlev, 1996; Vallès & Siljak-Yakovlev, 1997; Torrell *et al*., 2001; Vallès *et al*., 2001a, b), and is considered to be an important evolutionary mechanism. Another remarkable and well-known evolutionary mechanism in plants is polyploidy (Bretagnolle *et al*., 1998; Soltis & Soltis, 1999; Soltis *et al*., 2004), and the material studied in this paper is a good example of its relevance in the Artemisiinae. In our sample, ten of the 19 taxa analysed are polyploid, nine being tetraploid and one hexaploid. In the genus *Artemisia*, many of the species colonizing extremely arid habitats are polyploid, supporting the hypothesis that there is a connection between ecological tolerance and polyploidy in many plant groups (Otto & Whitton, 2000). Additionally, many authors have proposed that polyploids have radiated and expanded more than diploids and our data seem to confirm this, because the species in this study were chosen randomly and more than half of them are polyploid. The present data also agree with the premise that polyploids usually cover larger territories than related diploids (Ehrendorfer, 1980), although there are many exceptions to this rule (Bretagnolle *et al*., 1998).

Finally, the abundance of polyploids and the existence of species with the two basic chromosome numbers found in the genus *Artemisia*, together with the large number of species from this genus and the subtribe which inhabit the Mongolian area, confirm that Mongolia represents a speciation and diversification

centre, both for the genus and for the subtribe Artemisiinae as a whole.

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4. Variació de la mida del genoma en el complex d'*Artemisia arborescens* (*Asteraceae*, *Anthemideae*) i les seves cultivars.

Sònia GARCIA, Teresa GARNATJE, John D. TWIBELL i Joan VALLÈS.
Genome 49: 244-256 (2006).

Diferents poblacions mediterrànies silvestres *d'Artemisia arborescens*, de diverses localitats que representen la seva distribució geogràfica, així com algunes de les seves cultivars més ben conegudes i alguns espècimens cultivats com a ornamentals en jardins, carrers, carreteres o vivers han estat analitzats per a estimar-ne la seva quantitat de DNA. Altres espècies estretament relacionades amb aquesta i endèmiques de la Macaronèsia, *Artemisia argentea*, *A. canariensis* i *A. gorgonum*, també han estat analitzades i s'ha relacionat la seva quantitat de DNA amb la biogeografia d'aquest grup. A més, també es van mesurar cinc poblacions d'*Artemisia absinthium*, una altra espècie propera, per a establir comparacions. Les mesures, adquirides per citometria de flux, oscil·len entre els 8,29 i els 11,61 pg (valors 2C). S'han detectat diferències estadísticament significatives en quantitats de DNA depenent de factors com la insularitat o la domesticació. No obstant, la variabilitat intraespecífica per a la majoria d'aquestes espècies és baixa. Aquest estudi aborda també el possible origen híbrid i possibles identificacions incorrectes d'algunes suposades cultivars d'*A. arborescens*.

Genome size variation in the *Artemisia arborescens* complex (Asteraceae, Anthemideae) and its cultivars

Sònia Garcia, Teresa Garnatje, John D. Twibell, and Joan Vallès

Abstract: Different wild Mediterranean populations of *Artemisia arborescens* from diverse locations representing its geographical distribution, as well as some of its well-known cultivars and some specimens cultivated as ornamentals in gardens, streets, roads and nurseries, were analysed for genome size. Other closely related species endemic to Macaronesia, *Artemisia canariensis*, *Artemisia argentea*, and *Artemisia gorgonum*, were also analysed, and their nuclear DNA amount has been related to the biogeography of this group of species. Additionally, 5 populations of the closely related *Artemisia absinthium* were analysed to establish comparisons. Measurements acquired by flow cytometry ranged from 8.29 to 11.61 pg for 2C values. Statistically significant differences of 2C nuclear DNA amounts with respect to factors such as insularity or domestication have been detected. However, quite a low intraspecific genome size variation has been found in these species. Furthermore, the study also addressed the possible hybrid origins and possible misidentifications of some of the supposed cultivars of *A. arborescens*.

Key words: Artemisia arborescens, *Artemisia absinthium*, *Artemisia argentea*, *Artemisia canariensis*, *Artemisia gorgonum*, C value, Compositae, cultivar, domestication, flow cytometry, genome size, hybridization, interspecific variation, intraspecific variation, speciation.

Résumé : Plusieurs populations sauvages d'*Artemisia arborescens* provenant de différentes localités représentant toute sa distribution géographique, ainsi que quelques-uns de ses cultivars les plus connus et quelques spécimens cultivés en tant que plantes ornementales dans des jardins, rues, routes et pépinières, ont été analysés pour estimer la taille de leurs génomes. La quantité d'ADN nucléaire a été également mesurée chez d'autres espèces fortement rattachées à celle-ci et endémiques de la région Macaronésienne, *Artemisia canariensis*, *Artemisia argentea* et *Artemisia gorgonum*, et les résultats ont été mis en rapport avec la biogéographie de ce groupe d'espèces. De plus, cinq populations d'une autre espèce proche de ce complexe, *Artemisia absinthium*, ont été aussi analysées afin d'établir des comparaisons. Les mesures, obtenues par la méthode de cytométrie en flux, vont de 8,29 à 11,61 pg pour les valeurs 2C. Des différences statistiquement significatives de la quantité d'ADN nucléaire ont été détectées, en rapport avec des facteurs tels que l'insularité ou la domestication. Cependant, nous n'avons décelé qu'une assez faible variation intraspécifique de la taille du génome chez ces espèces. L'hypothèse d'une origine hybride et la mauvaise identificaction de quelques cultivars d'*A. arborescens* sont aussi discutées.

Mots clés : Artemisia arborescens, *Artemisia absinthium*, *Artemisia argentea*, *Artemisia canariensis*, *Artemisia gorgonum*, Compositae, cultivar, cytométrie en flux, domestication, hybridation, spéciation, taille du génome, valeur C, variation interspécifique, variation intraspécifique.

Introduction

The fast-growing shrub *Artemisia arborescens* L., commonly named tree wormwood, silver wormwood, or shrubby wormwood, is a morphologically variable species, tolerant of a wide range of climates and soil conditions (it grows spontaneously or naturalized near human residences), which colonizes a wide geographical range, inhabiting the whole Mediterranean region, across the coastal mainlands and islands. Typically, it is a xerophytic plant of rocks, cliffs, and

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pastures near the sea and forms an upright mound of fine silvery-grey leaves with a mild camphor fragrance. According to popular folklore, the plant was spread by Moors and Knights Templar during the times leading up to and including the Crusades and it is frequently found around old fortified sites (Twibell 1992). Medicinal properties, such as antibacterial, anti-inflammatory, antihistamine, anticatarrhal, choleretic, and mucolytic are attributed to its essential oils, which are extracted and commercialized (Sheppard-Hanger 1995). The medicinal and ornamental uses confer a certain economic interest to this species. It is phylogenetically close to *Artemisia absinthium* L. (both species belonging to the same subgenus of *Artemisia*, *Absinthium*) with which it shares morphological affinity and ecology, although important chemical differences have been detected between them. Additionally, several cultivars of *A. arborescens*, such as 'Brass Band', 'Faith Raven', 'Huntington', 'Little Mice', 'Porquerolles', and 'Powis Castle', are grown in gardens and are highly prized owing to their ever-silver filigree leaves, hardiness, and ease of reproduction by cuttings. There is a certain amount of confusion within these cultivars (Twibell 1992, 1994), some of them being considered as simple varieties or forms of *A. arborescens*, while others are thought to be hybrids between *A. arborescens* and *A. absinthium* (Twibell 1992). Others are in fact misidentifications of cultivars from other *Artemisia* species that tend to be perpetuated within the nursery trade.

There are some species closely related to *A. arborescens* inhabiting Macaronesia, namely *Artemisia argentea* L'Hér., endemic to Madeira (and found on all islands in the archipelago: Madeira, Porto Santo, and Desertas); *Artemisia canariensis* Less. (syn. *Artemisia thuscula* Cav.), endemic to the Canary Islands (where it is found on every island except Lanzarote and Fuerteventura); and *Artemisia gorgonum* Webb, endemic to the Cape Verde archipelago and detected on the islands of São Antão, Santiago, and Fogo (Hansen and Sunding 1993). These taxa are considered the vicariant species of *A. arborescens* in Macaronesia, given that *A. arborescens* itself is not present on these islands and that places it usually colonizes are occupied by the above local endemics. The Azores form an exception, however, as no *A. arborescens* or similar species has been found there (Hansen and Sunding 1993). *Artemisia arborescens* grows on the North African coasts and it is likely that the presence of these similar taxa in the archipelagos (where they would have differentiated from their ancestor) is due to colonization events from the mainland, as has been described in other genera of Macaronesia (e.g., *Cheirolophus*, Garnatje 1995).

Data on genome size can help to clarify many of these aspects. The nuclear DNA content, or C value, is considered constant within a species: the "C" in C value stands for "constancy" (Swift 1950). Countless studies have been carried out that reveal the existence of a relationship between the nuclear DNA content of a particular species and its ecology, distribution, life cycle, biomass production, resistance, habit, and several other features (Bennett 1998; Bennett and Leitch 2005). Among these investigations, many workers have focused on re-examining this supposed species-specific constancy, some reporting a considerable intraspecific genome size variation (Rayburn et al. 1985; Thibault 1998) and others supporting intraspecific C-value stability (Bennett et al. 2000; Auckland et al. 2001). Up to now, the extent of intraspecific genome size variation has been hotly debated (Greilhuber 2005) and some authors attribute such variation only to methodological errors or taxa misidentification (Greilhuber 1998; Ohri 1998). However, factors such as changes in repetitive DNA and retrotransposon activity (Bennetzen and Kellogg 1997); chromosomal phenomena such as duplications, aneuploidy, and the presence of B chromosomes (Poggio et al. 1998); or even the existence of dimorphic sex chromosomes (Costich et al. 1991) can be a source of variation within a species, among other possibilities. It is believed that changes in genome size within a species can be a true indicator of ongoing processes of speciation or genetic divergence (Price 1976; Murray 2005), although it is also true that speciation can take place without any change in amounts of nuclear DNA (Bennett and Leitch 2005). Consequently, intraspecific C-value variation is considered in certain cases to be taxonomically significant, because variation in nuclear DNA amounts can be indicative of reproductive isolation and morphological diversification (Bennett and Leitch 2005). The availability of rapid techniques like flow cytometry has allowed us to study a considerable set of populations, geographically distant to the taxa mentioned above (and embracing almost the whole area of distribution of a single species, *A. arborescens*). The possibility that nuclear DNA content differs among the wild and cultivated populations and cultivars of this species and the similar Macaronesian taxa is tested in this study, given the above mentioned features of this core of species. Moreover, we have tried to solve the previously mentioned confusion within the cultivars of *A. arborescens* from the analysis of genome size data.

Materials and methods

Plant material

Young fresh leaf tissue was extracted from the studied plants for sample preparation. Some of the seeds of the analysed *A. arborescens* were sampled from Mediterranean populations (including both island and continental representatives), the majority of which were collected from natural sites and one that was obtained via *Index Seminum*. They were then germinated in pots and cultivated under stable conditions in a greenhouse. Other samples were obtained from adult specimens of *A. arborescens*, some of them also collected from wild populations, some found cultivated in gardens or acquired via plant nurseries or from the National Council for the Conservation of Plants and Gardens (NCCPG) *Artemisia* collection. Leaf tissue from the other species, *A. canariensis*, *A. argentea*, *A. gorgonum*, and *A. vallesiaca* All., as well as from the cultivars, was also obtained from specimens in botanical and nursery gardens or from the NCCPG *Artemisia* collection. Table 1 shows the populations studied, with an indication of their origin and herbarium voucher information. The NCCPG *Artemisia* collection holds numerous plants from this genus of known wild or domesticated provenance, which are grown as living specimens. The collection endeavours to maintain the continuity of living specimens and their genetic variability by vegeta-

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

Taxa	Locality, collector(s), and date	Herbarium voucher[a]
A. arborescens (populations cultivated in gardens, streets, roads or nurseries)	France, Paris. Purchased in a market. J. Vallès, II-2005.	BCN 28633
	France, Catalonia, El Portús. Cultivated as ornamental in a garden. J. Vallès, 28-III-2005.	BCN 28638
	Morocco, Rif. Cultivated in a private garden. O. Hidalgo & A. Romo, 20-VI-2005.	BCN 28628
	Spain, Catalonia, Barcelona, Montjuïc. Historical Botanic Garden. M. Veny, III-2005.	BCN 28637
	Spain, Catalonia, Barcelona, Montjuïc. Cultivated as ornamental in the nursery "Tres Pins". T. Garnatje, III-2005.	BCN 28636
	Spain, Catalonia, La Garrotxa, Besalú. Planted in a roundabout of the road from Girona to Besalú, near the "Pont Vell". J. Vallès, 26-VI-2005.	BCN 28627
	Spain, Madrid. Botanic Garden. J. Vallès, 16-V-2005.	BCN 28641
	Spain, València. Purchased in the nursery "Pro Agri". J. Vallès, 1-VI-2005	BCN 28630
A. arborescens (wild populations)	Algeria. National Park of Gouraya, Bejaia. J. Vallès, 11-X-2004.	BCN 28635
	Algeria. Alger, Botanic Garden Essais Hamma. From a wild locality of Algeria. J. Vallès, 21-IX-2004.	BCN 28642
	France, Corsica, Bonifaziu. Over the walls of the ancient Citadelle. M. Bosch & M. R. Orellana, 9-V-2005.	BCN 28640
	France, Porquerolles. Maritime sands. From the *Index Seminum* of the Museum of Natural History of Paris, III-2004.	BCN 28643
	Greece, Corfu, Armenodes. NCCPG *Artemisia*. L. Chilton 1995.	NCCPG 1995/76
	Greece, Crete. Kalyves beach. GR-128. T. Garnatje & J. Luque, 15-VIII-2002.	BCN 28644
	Greece, Rhodes. Mount Filerimos. NCCPG *Artemisia*. B. Tickner, IV-1993.	NCCPG 1994/68
	Italy, Sardinia, Alguero. Wayout urban nucleous by Fertilia road margins, T. Garnatje & J. Vallès, 7-XII-1999.	BCN 25512
	Italy, Sicily, Sciacca. Gole della Tardara, river Carboi. V. Ilardi, G. Domina & C. Blanché. 10-V-2005.	BCN 28645
	Spain, Alacant, La Encina. Near "La Casa de los Corrales". T. Garnatje & R. Vilatersana, 23-V-2005.	BCN 28646
	Spain, Balearic Islands, Formentera. T. Garnatje & R. Vilatersana, 18-IV-04.	BCN 28647
	Spain, Balearic Islands, Formentera. T. Garnatje & R. Vilatersana, 18-IV-04.	BCN 28648
	Spain, Balearic Islands, Mallorca. S'Alqueria Blanca, "Santuari de la Consolació", enclosure margins, at 200 m. J. Vicens. 24-VI-1997.	BCN 22321
	Spain, Balearic Islands, Menorca, Maó. Cliffs near the harbour, 2 km from Es Castell. A. Gómez, M.A. Ribera, J.A. Seoane & J. Vallès 19-I-1997.	BCN 22115
	Spain, Menorca. Cliffs at Binibeca. NCCPG *Artemisia*. J. Twibell, VIII-1995.	NCCPG 1995/74
	Spain, Catalonia, Tarragona. Road from Roquetes to Alfara de Carles (TV3422), km 8 (near the canal), enclosure of a house. T. Garnatje & R. Vilatersana, 26-V-2005.	BCN 28631
	Turkey, Hatay, Samandag, Çevlik, Kral Mezarlarý Mevkii. Rocky cliffs, 10 m above sea level. Fadime Gumusboga, 7-VI-2005.	BCN 28629
Cultivars of *A. arborescens*		
'Brass Band'	England, United Kingdom, NCCPG *Artemisia*. Geoff Hamilton, 1989.	NCCPG 1989/01
'Faith Raven' type 1	England, United Kingdom, NCCPG *Artemisia*. Faith Raven, 1990.	NCCPG 1990/03
'Faith Raven' type 1	England, United Kingdom, NCCPG *Artemisia*. Ex John or Faith Raven (1969). Via J. Goulsbra, 1993.	NCCPG 1993/04
'Faith Raven' type 2	England, United Kingdom, NCCPG *Artemisia*. Ex John or Faith Raven (circa 1970). Via Dr. Jamison, XII-1993.	NCCPG 1993/05
'Huntington'	England, United Kingdom, from Heronswood Nursery Seattle USA. Via N Pope (Hadspen House Nursery, UK) and R. Mort, 1999.	NCCPG 1999/01
'Little Mice'	England, United Kingdom, NCCPG *Artemisia* 2005/29 from Chilton Quality Plants nursery. J. Twibell, 2005.	NCCPG 2005/29
'Little Mice'	France, Theix. Purchased in the nursery "Le Clos d'Armoise". S. Garcia, V-2005.	BCN 28649

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

Taxa	Locality, collector(s), and date	Herbarium voucher[a]
'Porquerolles'	France. Conservatoire Botanique de Porquerolles, near Hyères, form of unknown origin selected from trial stock of *A. arborescens* variants. NCCPG *Artemisia*. J. Simmons (Royal Botanical Gardens, Kew), 28-X-1986.	NCCPG 1990/05
'Powis Castle'	England, United Kingdom. Blooms of Bressingham. NCCPG *Artemisia* J. Twibell, 1988.	NCCPG 1988/08
'Powis Castle'	Spain, Catalonia, Barcelona, Sant Andreu de Llavaneres. Purchased in the nursery "Sala-Graupera", S. Garcia & J. Vallès, III-2005.	BCN 28639
Other related species and cultivars		
A. absinthium	Armenia. NCCPG *Artemisia*. J. Vallès, 1997.	NCCPG 1997/54
A. absinthium	Iran. Seed from Teheran Botanic Garden. J. Twibell, 1994.	NCCPG 1994/54
A. absinthium	Spain, Andalusia. Sierra Nevada. Near Alburgue and northern road barrier Picos de Valetta. NCCPG *Artemisia*. J. Twibell, XI-2000.	NCCPG 2000/84
A. absinthium	Spain, Catalonia, Girona, Maçanet de Cabrenys. J. Vallès, IV-2005.	BCN 28632
A. absinthium	France, Mèze. Purchased in the nursery "Pépinière Filippi". S. Garcia, VI-2005.	BCN 28650
A. argentea	Portugal, Madeira. Wasteland next to an old wall between Madaleno do Mar and Ponta do Sol. NCCPG *Artemisia*. Bernard Tickner, 1994.	NCCPG 1994/90
A. canariensis	Spain, Catalonia, Barcelona, Montjuïc. Botanic Garden of Barcelona. S. Garcia, M. Veny & T. Garnatje, III-2005.	BCN 28634
A. gorgonum	Portugal. Cape Verde Isles, São Antão, upper part of Ribeira da Torre 1400 m above sea level (seed via Bonn Botanic Garden, NCCPG *Artemisia*). W. Lubin, 13-IX-1994.	NCCPG 1995/09
A. vallesiaca	England. NCCPG *Artemisia*. J. Twibell, 27-VI-2005.	NCCPG 1988/30

[a]Vouchers deposited in the herbarium of the Centre de Documentació de Biodiversitat Vegetal de la Universitat de Barcelona (BCN) and in the NCCPG *Artemisia* Collection, Elsworth.

tive propagation. Collection plants are available for research purposes or for possible future re-introduction.

Seeds of *Pisum sativum* 'Express Long', used as internal standard for flow cytometry measurements, were obtained from the Institut des Sciences du Végétal (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France).

DNA content assessment

DNA 2C values of the tested species were estimated using flow cytometry. *Pisum sativum* 'Express Long' (2C = 8.37 pg, Marie and Brown 1993) was used as an internal standard. Young healthy leaf tissues from the species to be studied and calibration standard were placed together in a plastic Petri dish and chopped in Galbraith's isolation buffer (Galbraith et al. 1983) with a razor blade. The amount of target species leaf (about 25 mm^2) was approximately twice that of the internal standard. The suspension of nuclei in the isolation buffer 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 Química, Alcobendas, Madrid, Spain), the chosen fluorochrome, supplemented with 100 µg/mL ribonuclease A (Boehringer, Meylan, France); tubes were kept on ice during staining and then left at room temperature until the measurement. For each population, 5 individuals were analyzed; 2 samples of each individual were extracted and measured independently. Measurements were made at the Serveis Cientificotècnics Generals de la Universitat de Barcelona using an Epics XL flow cytometer (Coulter Corporation, Hialeah, Fla.). The instrument was set up with the standard configuration: excitation of the sample was done using a standard 488 nm air-cooled argon-ion laser at 15 mW power. Forward scatter (FSC), side scatter (SSC), and red (620 nm) fluorescence for propidium iodide were acquired. Optical alignment was based on optimized signal from 10 nm fluorescent beads (Immunocheck, Epics Division, Coulter Corporation, Hialeah, Fla). Time was used as a control of the stability of the instrument. Red fluorescence was projected on a 1024 monoparametrical histogram. Gating single cells by their area versus peak fluorescence signal excluded aggregates. Acquisition was automatically stopped at 8000 nuclei. The total nuclear DNA content was calculated by multiplying the known DNA content in *Pisum* by the quotient between the 2C peak positions of the target species and the chosen internal standard in the histogram of fluorescence intensities for the 10 runs, based on the assumption that there is a linear correlation between the fluorescence signals from stained nuclei of the unknown specimen and the known internal standard and the DNA amount. Mean values and standard deviations were calculated based on the results for the 5 individuals.

Statistics

Statistical analyses were carried out to evaluate the relationships between the studied variables. All analyses were performed with the program Statgraphics Plus 5.0 (Statistical Graphics Corp., Rockville, Md.).

Results and discussion

The results for each population are presented in Table 2. The analyses were of good quality with a mean HPCV (half peak coefficient of variation) of 2.72%. This is the third study on *Artemisia* genome size conducted by our research

Table 2. 2C Nuclear DNA contents of the populations studied.

Taxa	2C±s.d. (pg)[a]	C value (Mbp)[b]
Populations of *Artemisia arborescens*		
Algeria– Gouraya	11.46±0.09	5603.94
Algeria–Essais Hamma	11.32±0.08	5535.48
France–Corsica	11.37±0.12	5559.93
France–Paris	10.97±0.15	5364.33
France–Porquerolles	11.17±0.13	5462.13
France– El Portús	10.37±0.17	5070.93
Greece–Corfu	10.67±0.08	5217.63
Greece–Crete	11.43±0.11	5589.27
Greece–Rhodes	11.22±0.11	5486.58
Italy–Sardinia	11.30±0.19	5525.7
Italy–Sicily	11.41±0.07	5579.48
Morocco–Rif	11.07±0.07	5413.23
Spain–Alacant–La Encina	11.22±0.17	5486.58
Spain–Balearic Islands–Formentera (1)	11.20±0.05	5476.8
Spain–Balearic Islands–Formentera (2)	11.28±0.15	5515.92
Spain–Balearic Islands–Mallorca	11.61±0.15	5677.29
Spain–Balearic Islands–Menorca–Binibeca	11.15±0.06	5452.35
Spain–Balearic Islands–Menorca–Maó	11.61±0.23	5677.29
Spain–Catalonia–Barcelona–Nursery "Tres Pins"	10.74±0.24	5251.86
Spain–Catalonia–Barcelona–Historical Botanic Garden	10.85±0.15	5305.65
Spain–Botanic Garden of Madrid	10.80±0.13	5281.2
Spain–Catalonia–Besalú	10.35±0.05	5061.15
Spain–Catalonia–Roquetes	11.23±0.10	5491.47
Spain–València– ursery "Pro Agri"	11.15±0.23	5452.35
Turkey–Samandag	11.18±0.11	5467.02
Cultivars		
'Brass Band'	10.14±0.20	4958.46
'Faith Raven' type 1 (from J. Goulsbra, UK)	10.14±0.20	4958.46
'Faith Raven' type 1 (from J. Twibell, UK)	10.19±0.07	4982.91
'Faith Raven' type 2 (UK)	11.11±0.10	5427.9
'Huntington'	9.86±0.07	4821.54
'Little Mice' (UK)	9.73±0.05	—
'Little Mice' (France–Theix)	9.74±0.20	—
'Porquerolles'	11.11±0.14	5432.79
'Powis Castle' (UK)	10.29±0.04	5031.81
'Powis Castle' (Spain–Catalonia–Barcelona)	10.36±0.10	5066.04
Other related species		
A. absinthium (Spain–Catalonia)	8.94±0.04	4371.66
A. absinthium (Armenia)	8.39±0.40	4102.71
A. absinthium (Iran–Teheran)	8.29±0.10	4053.81
A. absinthium (Spain–Sierra Nevada)	8.79±0.13	4298.31
A. absinthium (France–Mèze)	8.79±0.28	4298.31
A. argentea	10.30±0.05	5036.7
A. canariensis	10.63±0.17	5198.07
A. gorgonum	10.24±0.17	5007.36
A. vallesiaca	9.81±0.13	2398.55

[a]2C nuclear DNA content (mean value ± standard deviation of 10 samples).
[b]1 Mbp = 1.0224×10^{-3} pg (Doležel et al. 2003).

team (Torrell and Vallès 2001; Garcia et al. 2004), but the first focused on intraspecific variation and plant domestication within a single species. Previously, other works on genome size reported C values for 7 *Artemisia* species (Nagl and Ehrendorfer 1974; Geber and Hasibeder 1980; Greilhuber 1988; Bennett and Smith 1991). According to the categories of genome size (Soltis et al. 2003), the species reported in this study should be considered intermediate (< 3.5 to 14.0 pg).

In the present work, 2C nuclear DNA content was determined for the first time for *A. arborescens*. Flow cytometry was used to analyse 35 populations of this species, which represent most of its distribution in the whole Mediterranean

basin, as well as some cultivated forms and cultivars (17 wild populations, 8 cultivated in gardens or roads, and 10 populations of 6 cultivars of *A. arborescens*). The highest 2C values are those of the population from Mallorca and a population from Menorca (11.61 pg); the lowest value is the one corresponding to 'Little Mice' (9.73 pg). Additionally, values obtained for *A. absinthium*, *A. argentea*, and *A. canariensis* are consistent with those from previous studies, namely 8.52 pg in Torrell and Vallès (2001) and 9.06 pg in Garcia et al. (2004) for *A. absinthium* (conversely, Nagl and Ehrendorfer (1974) gave a lower estimate for this species, 7.30 pg), 10.25 pg for *A. argentea* (Greilhuber 1988), and 10.52 pg for *A. thuscula* (syn. *A. canariensis*) in Torrell and Vallès (2001). In accordance with the data from the plant DNA C-value database (Bennett and Leitch 2004), these are also first estimates of 2C value for the Cape Verde endemism *A. gorgonum*, and for *A. vallesiaca*, which has been analysed to compare with 'Little Mice' (this point will be discussed later). All species studied share the same chromosome number, $2n = 18$, according to Kawatani and Ohno (1964), Borgen (1975), and Torrell et al. (1999, 2001), with the exception of *A. vallesiaca*, with $2n = 36$ (Kawatani and Ohno 1964).

The rank of variation within *A. arborescens* is 7.8% within the cultivated populations, 14% within the studied cultivars, and up to 8.8% within the wild populations. This percentage of intraspecific genome size variation within *A. arborescens* is not especially high and, strictly speaking, the variation detected for this species should be referred to that found for the wild populations. For the populations of *A. absinthium* analysed, the intraspecific variation is even lower (6%); although they come from very different and distant geographical locations, it must be outlined that only 5 populations have been studied (because the study was not focused on this species).

Some of the specimens analysed came directly from natural populations, although most of them were grown in a greenhouse under controlled conditions. The fact that not all of the plants were grown under identical conditions could have caused errors owing to the differential presence of cytosolic compounds. However, given that all specimens occupy fairly similar environments in the Mediterranean region, the moderately low degree of intraspecific variation found (which does not reach 10%, excluding the cultivars), and the absence in *Artemisia* of some of the compounds that are known to have caused biases in the genome size assessments (such as chlorogenic acid or caffeine, Noirot et al. 2000, 2003), we believe that the effect of interference with DNA staining of cytosolic compounds, if present, is not quite meaningful in this particular case.

Compared with other studies, similar variation in genome size within a single species has been detected. For instance, there was a 1.1-fold difference between accessions of *Arabidopsis thaliana* in a study performed by Schmuts et al. (2004). In that work, significant differences ($p < 0.05$) were found between all measurements of the 5 largest diploids and the 3 accessions with the smallest genome size. Also, in *Silene latifolia*, divergences between male and female individuals from the same population and between geographically separated populations have been reported (Meagher and Costich 1994 1996; Meagher et al. 2005). On the other hand, many studies show a lower percentage of intraspecific variation. In a study on nuclear DNA amounts of different and geographically isolated populations of *Sesleria albicans*, even though only 1.6% variation had been detected, the authors found it to be statistically significant (Lysák et al. 2000). In *Hordeum spontaneum*, where genome size for populations representing wide ecological and geographical differences were measured, statistically significant variation up to 5% was found (Turpeinen et al. 1999). This is also the case in *Armeria maritima*, in which genome size variation of 7% was related to geographic origin (Vekemans et al. 1996).

Although many of the examples of intraspecific C-value variation have, lately, shown to be artefacts of the measurement methods (Greilhuber 2005; Murray 2005) many reports continue to be published that document genuine intraspecific C-value variation where the appropriate controls and standards have been used (Bennett and Thomas 1991; Reeves et al. 1998; Hall et al. 2000; Moscone et al. 2003). Since it is known that the estimated 25 000 to 50 000 genes encoded in a eukaryotic genome make up only approximately 0.12 pg of DNA (Narayan 1998), that the variation probably takes place in the non-coding component of the genome, which is mainly formed by repetitive DNA (Barakat et al. 1997; Flavell et al. 1997), and that several molecular mechanisms are known that could be responsible for a decrease or an increase in genome size, such as the presence of B chromosomes or transposable elements (more than 60% of some plant genomes are comprised of transposable elements and mostly their defunct remnants (Bennetzen and Kellogg 1997)), it is possible that the C value of a species, although fairly constant, allows a certain reasonable degree of variation, and thus may not be strictly constant. All things considered, given that measurements were always made with the same internal standard and with the same flow cytometer for each taxon, we believe that the differences detected in this study reflect authentic intraspecific variation.

Effect of domestication, hybrid origin, or both

As has been stated before, we have studied 17 wild populations, 8 populations corresponding to specimens of *A. arborescens* found cultivated in gardens, roads or nurseries and 10 corresponding to 6 different cultivars of this species. Among the cultivars of *A. arborescens* there are certain points of confusion:

'Powis Castle' — Although many nurseries sell this as a form of *A. arborescens*, some others suggest that this is a hybrid between *A. arborescens* and *A. absinthium* (this will be detailed further on).

'Brass Band' — This plant is believed to be identical to 'Powis Castle' (Twibell 1992).

'Faith Raven' — There are 2 different plants in circulation under this epithet, namely type 1, which is also thought to be the same as 'Powis Castle', and type 2, which is closer to typical *A. arborescens* (Twibell 1994).

'Porquerolles' — A compact form of unknown origin selected from a trial of *A. arborescens* variants at the Porquerolles Botanic Garden near Marseille (France).

'Huntington' — Another form of intermediate character (or possible hybrid) somewhere between *A. absinthium*

and *A. arborescens*. This plant supposedly originated from the Huntington Botanic Garden (San Marino, Calif.), but is not officially recognised there.

'Little Mice' — Known as "the little brother" of *A. arborescens* in the nursery trade and sold as a cultivar of this species; however, morphological evidence supports that 'Little Mice' is closer to species from the *Artemisia* subgenus *Seriphidium*, particularly to *A. vallesiaca*, than to those from subgenus *Absinthium*, to which *A. arborescens* belongs.

To reveal the existence of intraspecific genome-size variation according to the degree of domestication between the different populations of *A. arborescens*, an ANOVA was performed that resulted in statistically significant differences ($P = 0.0000$). The populations studied were classified in 3 categories: wild, cultivated, and cultivars (Fig. 1). The wild populations had significantly larger genome sizes than the cultivated populations (approximately 5%), and were also larger than the known cultivars (nearly 3%) such as 'Powis Castle', 'Faith Raven', 'Porquerolles', and 'Huntington' according to the means comparison test (based on Tukey's honestly significant difference procedure, HSD). Another ANOVA was performed excluding the most doubtful cultivars of *A. arborescens*, namely 'Little Mice' and 'Huntington' (Fig. 2), and statistically significant differences were found between the 3 groups ($P = 0.0000$), although means for the cultivars (10.48) and cultivated (10.78) were not significantly different in the means comparison test. Finally, an additional ANOVA was done considering 'Porquerolles' and 'Faith Raven' type 2 as cultivated, given their higher morphological affinity to *A. arborescens* than to the other cultivars; again, statistically significant differences ($P = 0.0000$) between the groups were detected, with all of the means significantly different from each other in the means comparison test (Fig. 3).

Given these results, it is conceivable that the process of domestication in *A. arborescens* has lead to a progressive diminution of its genome. In fact, some of the cultivars of this species show a clearly different aspect from wild *A. arborescens*, with a smaller size or a more compact and silvery-grey foliage and a slightly different odour. The essence of these plants has been analysed by Twibell (1992), who found divergences in their vapour profiles. The decrease in genome size of the cultivars with respect to the original species has also been detected in other plants, particularly in crops. Nagato et al. (1981) and Yamamoto and Nagato (1984) reported that in Asian rice and soybean, genome size of cultivars was smaller than that of their wild progenitors.

On the other hand, a likely hybrid origin of some of the cultivars could explain these differences in genome size, especially those between the wild populations and the cultivars. According to Thomas (1982), the variety 'Powis Castle' is reputed to be a hybrid of *A. absinthium* and *A. arborescens*, but contrary to popular opinion, it did not originate in the National Trust garden at Powis Castle (Wales, UK). The plant was actually taken as a cutting from a plant in a garden (open under the National Gardens Scheme) by Jimmy Hancock (circa 1969–1971), who later (in 1972) became the Head Gardener at Powis Castle (Hancock 1991, private communication). The source of the original plant remains unclear. As Twibell (1992) explains, one possible

Fig. 1. Box-and-whisker plot of the statistical analysis (ANOVA) of 2C nuclear DNA content (pg) vs. domestication. Statistically significant differences ($P = 0.0000$) between cultivars and cultivated and wild *Artemisia arborescens*. The data are divided into 4 equal areas of frequency (quartiles). A box encloses the middle 50 percent, where the median is drawn as a vertical line inside the box. The + symbol shows the place of the mean value of the sample. Horizontal lines (whiskers) extend from each end of the box. The left (or lower) whisker is drawn from the lower quartile to the smallest point within 1.5 interquartile ranges from the lower quartile. The other whisker is drawn from the upper quartile. The external points are those exceeding more than 1.5 interquartile ranges, and they are represented with the symbol ■.

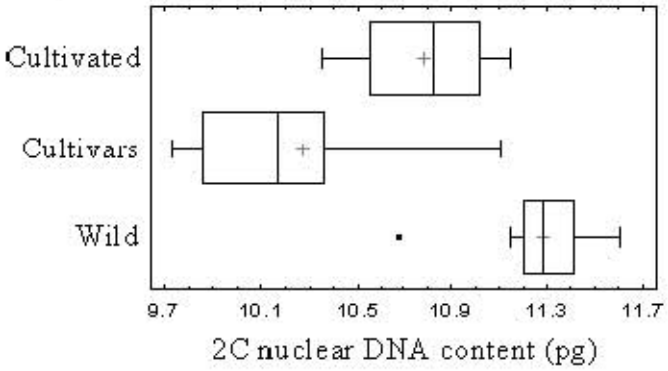
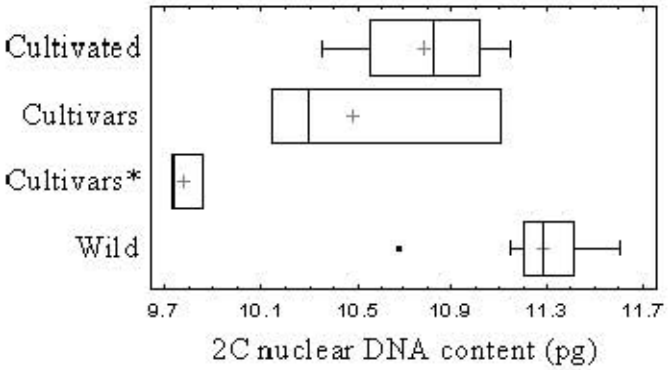
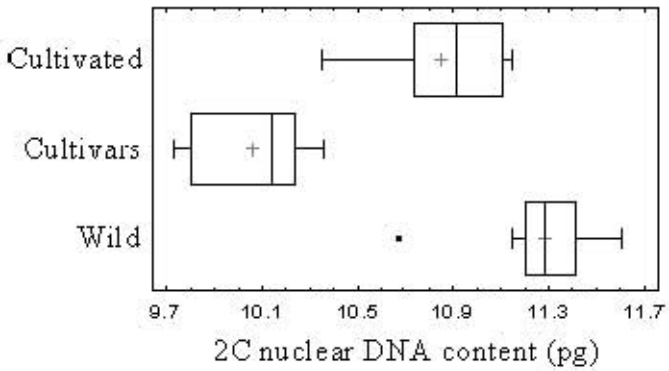


Fig. 2. Box-and-whisker plot of the statistical analysis (ANOVA) of 2C nuclear DNA content (pg) vs. domestication. Statistically significant differences ($P = 0.0000$) between cultivars, doubtful cultivars (denoted with an asterisk, including 'Little Mice' and 'Huntington'), and cultivated and wild *Artemisia arborescens*. Boxes were plotted as described in the caption for Fig. 1.



basis for the hybrid origin theory of this and other cultivars might derive from their behaviour if severely cut back during the growing season, conditions in which the plants produce simpler greener leaves to maximize photosynthesis. These leaves are fairly similar to those of *A. absinthium*, and the characteristic silver filigree leaves of *A. arborescens* develop subsequently. Additionally, most of these varieties are essentially non-flowering forms (Twibell 1992), another sign of their possible hybrid origin. In this sense, if we calculate the mean value of genome size data (2C values) for wild *A. absinthium* (8.60) and wild *A. arborescens* (11.28), and also the mean between these figures — which would correspond to, more or less, the expected value for their hybrid — the resulting number (9.94) is close to the nuclear DNA

Fig. 3. Box-and-whisker plot of the statistical analysis (ANOVA) of 2C nuclear DNA content (pg) vs. domestication. Statistically significant differences ($P = 0.0000$) between cultivars and cultivated (including 'Porquerolles' and 'Faith Raven' type 2) and wild *Artemisia arborescens*. Boxes were plotted as described in the caption for Fig. 1.

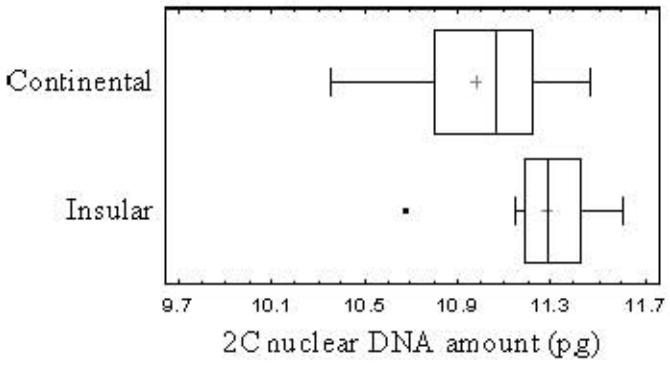


amount shown by some of the cultivars. On the one hand, 'Powis Castle', 'Faith Raven' type 1, and 'Brass Band' show very similar genome sizes (mean 2C nuclear DNA amount = 10.22), a fact that would support them being the same cultivar but with different names; on the other hand, 'Huntington' has a slightly smaller genome size, only 9.86 pg. The hypothesis of the hybrid origin of some of these cultivars is supported by these findings, the former ones being closer to *A. arborescens* and the latter closer to *A. absinthium* on the basis of their genome sizes. 'Little Mice' has not been included here for the reasons mentioned earlier; however, its genome size (2C = 9.73 pg) is closer to that of *A. vallesiaca* (2C = 9.81 pg) than to those of the wild or cultivated *A. arborescens*. This, the fact that no hybrid origin is suspected for 'Little Mice', and also the very close morphological affinity with *A. vallesiaca*, lead us to the conclusion that this is not, indeed, a true cultivar of the tree wormwood.

Influence of insularity

Many of the wild populations of *A. arborescens* analyzed come from Mediterranean islands, and others are of continental origin. It is thought that insular selection pressures can propitiate smaller genome sizes; in this sense, Suda et al. (2003) postulated in a recent work that selection pressures acting on Macaronesian archipelagos favoured small 2C values. The same findings have been reported in the insular representatives of the genus *Cheirolophus* (T. Garnatje, S. Garcia, and M.Á. Canela, unpublished). To test this hypothesis, an ANOVA was performed between the insular and continental *A. arborescens* (considering both wild and cultivated, see Fig. 4). Insular species were found to have significantly higher genome sizes (2C values) than continental species ($P = 0.0182$). However, the fact that all of the cultivated species are continental could have biased the analysis, this difference being a consequence of the domestication process rather than a genuine difference between island and continental populations. Therefore, the ANOVA was carried out considering only the wild populations, and the difference was no longer significant ($P = 0.9799$). In other words, island and continental populations are quite equal in terms of genome size, reinforcing the notion that genome size is fairly constant within a species.

Fig. 4. Box-and-whisker plot of the statistical analysis (ANOVA) of 2C nuclear DNA content (pg) vs. insularity. Statistically significant differences ($P = 0.0182$) between continental and insular populations of *Artemisia arborescens*. Boxes were plotted as described in the caption for Fig. 1.



Evidence of speciation

Comparing the mean genome size of the wild populations of *A. arborescens* with those of the species occupying the same ecogeographical placement in some of the islands of Macaronesia (i.e., *A. argentea* in Madeira, *A. canariensis* in the Canary Islands, and *A. gorgonum* in Cape Verde), it is noticeable that the latter have considerably less nuclear DNA than the former. As has been previously stated, it is quite possible that these Macaronesian species are the vicariants of *A. arborescens*, as all of them have high morphological affinity and the same chromosome number and also all occupy the same ecological niche that *A. arborescens* would fill if present in Macaronesia. In other words, these Macaronesian taxa could have undergone a process of speciation, which probably has been reflected in a decrease in genome size owing to stronger selective constraints in the Atlantic islands compared with the Mediterranean islands, where the studied populations of *A. arborescens* grow. As previously stated, insular selection pressures in Macaronesia have favoured smaller genome sizes. Because none of Macaronesian islands were part of a continent, the native plants probably reached the island by long-distance dispersal. Given that *A. arborescens* is also present on the North African coast (3 of the populations studied come from North Africa: 2 from Algeria and 1 from Morocco), it is likely that seeds from these continental populations propagated to Macaronesian archipelagos, and subsequently differentiated into separated species, as an adaptive response to each of the various islands' environments (this mechanism has also been described for other Macaronesian taxa, Garnatje 1995). Actually, it has been shown that evolutionary phenomena of speciation and adaptive radiation occur faster in insular ecosystems, particularly in oceanic islands, than on continents (García-Talavera 1999). On the other hand, the fact that none of these taxa are present on Lanzarote or Fuerteventura, which are the closest islands to the North African coast, is probably due to the high volcanic activity on these islands in the past (which could have caused massive species extinction) and their particularly arid climatic conditions.

Speciation may occur without any detectable change in 2C values (Bennett and Leitch 2005), but there are available examples of considerable intraspecific genome-size variation that, together with clear morphological and ecological differences, can lead to species split (the case of *Lachnagrostis littoralis*, Murray 2005). Amidst the known mechanisms that can lead to a genome size decrease there are the processes of unequal intrastrand homologous recombination, the illegitimate recombination or the loss of DNA during the repair of double-stranded breaks. The available studies suggest that deletion mechanisms may play a more prominent role in genome size evolution than previously thought (Bennett and Leitch 2005).

Concluding remarks

Although moderately low, genuine intraspecific variation has been found within the different populations of *A. arborescens* and *A. absinthium* analysed. It has been clearly shown that genome size varies greatly across plant species and that both increases and decreases can be related to evolutionary events (Bennett and Leitch 2005; Cullis 2005). Although evolution can point to a gain in total nuclear DNA amount in a certain group of taxa, in some others the tendency is to reduce it; consequently, it is extremely difficult, if not impossible, to establish a general pattern of change in genome size. As Bennett and Leitch (2005) stated, variation in DNA amount between species begins with changes within species. This implicitly recognizes that C values are fairly constant, but allows a certain degree of intraspecific variation. If we accept the existence of a certain degree of intraspecific variation, and also accept that genome-size diversification is an important process during speciation in plants (Greilhuber 1998; Soltis et al. 2003), we must wonder what percentage of variation is acceptable within a single species. There is a similar dilemma in the issue of establishing a sufficient amount of genetic differentiation that can be associated with speciation, because there are studies showing that extensive amounts of genetic differentiation are related with speciation, although not all the variability observed can be directly related to the speciation process (Hancock 2003). Ultimately, what is questioned here is the concept of a species. Additional comparative studies on genome size variation within a species in relation to morphological, environmental, or ecogeographical differences between its populations are needed to establish such a criterion.

Additionally, this study has also helped to clarify the confusion existing within the different cultivars of *A. arborescens* using data on nuclear DNA amounts. From our findings, we conclude that 'Powis Castle' and 'Huntington' originated from a hybrid between *A. arborescens* and *A. absinthium*, that 'Brass Band' and 'Faith Raven' type 1 are the same as 'Powis Castle', and that 'Porquerolles' and 'Faith Raven' type 2 are simply cultivated forms of *A. arborescens*. Finally, 'Little Mice' is not a cultivar of *A. arborescens* at all; from its morphological appearance and genome size, it is closer to species from the subgenus *Seriphidium*.

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5. Colocalització extensiva del DNA ribosòmic (18S-5,8S-26S i 5S) en les artemísies endèmiques d'Amèrica del Nord (subgènere *Tridentatae*, *Artemisia*, *Asteraceae*) revelada per FISH.

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Plant Systematics and Evolution, en premsa.

S'han dut a terme bandatges amb cromomicina A_3 i assaigs d'hibridació *in situ* fluorescent en sis espècies d'*Artemisia*, amb especial èmfasi en el subgènere *Tridentatae*. S'han calculat les dades morfomètriques dels cariotip i s'han construït idiogrames amb les posicions de les regions riques GC i els *loci* del DNA ribosomal 18S-5.8S.26S i 5S. Tots aquests *loci* són colocalitzats i, fins allà on sabem, aquesta és la primera vegada dins de la família de les *Asteraceae* i en les angiospermes en general que es detecta una colocalització dels dos tipus de DNA ribosòmic en tots els *loci* marcats. A més, s'ha avaluat l'activitat de les regions organitzadores nucleolars (NORs) mitjançant la tinció amb nitrat de plata. El subgènere *Tridentatae* es pot considerar, des d'aquest punt de vista, un grup citogenèticament homogeni, la qual cosa suggereix que l'evolució d'aquestes espècies no ha estat acompanyada de grans reorganitzacions cariotípiques. No obstant això, algunes espècies que són taxonòmicament conflictives també mostren diferències substancials respecte del patró general. Finalment, es detecta pèrdua en el nombre de *loci* de DNA ribosòmic en un tàxon tetraploide respecte als diploides estudiats. Aquestes dades ajuden a comprendre millor les relacions interespecífiques entre els tàxons estudiats, així com les relacions sistemàtiques i evolutives en el subgènere *Tridentatae* en conjunt.

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

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

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

Introduction

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

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

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

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

Materials and methods

Plant material. Ripe achenes from adult plants were collected from wild populations of each taxon. Root tip meristems from seedlings were obtained by germinating them on wet filter paper in Petri dishes in the dark at room temperature. An indication of the provenance of the species studied is shown in Table 1.

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

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

The chromosome preparations for fluorochrome banding, silver staining and *in situ* hybridization were done using the air-drying technique of Geber and Schweizer (1987), with some modifications: root tips were washed with agitation in citrate buffer (0.01 M citric acid – sodium citrate, pH = 4.6) for 15 minutes, excised, and incubated in an enzyme solution [4% cellulase Onozuka R10 (Yakult Honsha), 1% pectolyase Y23 (Sigma) and 4% hemicellulase (Sigma)] at 37°C for 20 to 25 minutes, depending on the species and meristematic thickness. The lysate of 8–10 root-tips was centrifuged twice in 100 µl buffer and once in

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

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

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

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

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

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

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

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

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

Results

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

Table 2. Karyological data

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

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

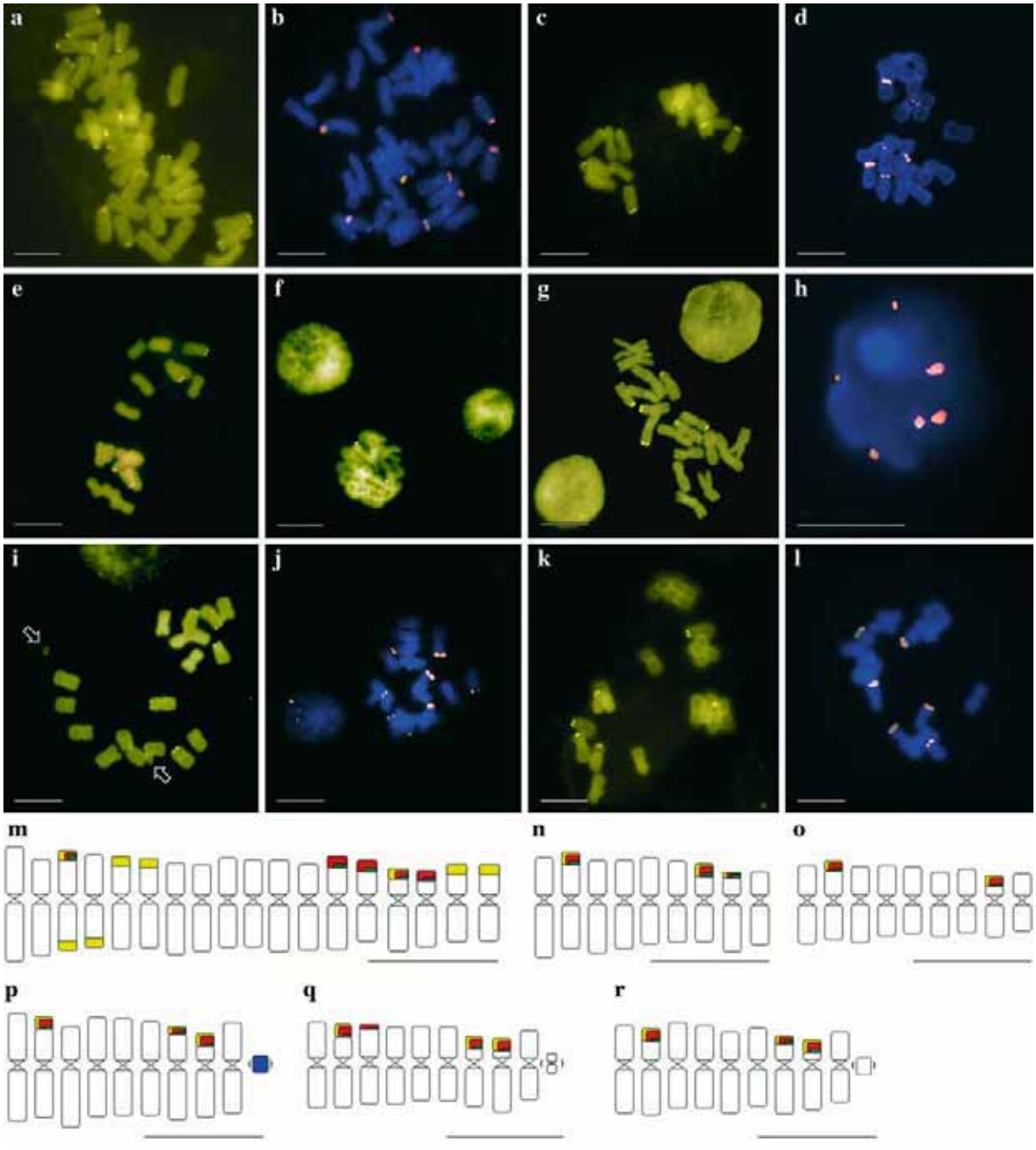

Fig. 1. Fluorochrome banding with chromomycin (**a**, **c**, **e**, **f**, **g**, **i**, **k**), fluorescent *in situ* hybridization (**b**, **d**, **h**, **j**, **l**) and haploid idiograms (**m-r**) of the different taxa studied. Scale bars = 10 μm for photographs and idiograms. (a, b, m) *A. argillosa*.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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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6. Implicacions evolutives i ecològiques de la mida del genoma en les artemísies (*sagebrushes*) endèmiques nord-americanes (*Artemisia*, *Asteraceae*) i espècies afins.

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Biological Journal of the Linnean Society, en premsa.

Mitjançant citometria de flux s'ha determinat la quantitat de DNA per a 51 poblacions de 19 espècies pertanyents al subgènere *Tridentatae*, altres artemísies endèmiques d'Amèrica del Nord i alguns tàxons híbrids, i s'han analitzat aquestes dades en un marc filogenètic. Hem obtingut resultats similars per a la majoria de *Tridentatae*, amb l'excepció de tres espècies taxonòmicament conflictives. Aquesta homogeneïtat en les dades de quantitat de DNA podria donar suport, juntament amb l'elevada afinitat morfològica, química i cariològica, a un procés de diversificació recent en aquest petit grup, on múltiples processos de reticulació haurien estat freqüents. Les *Tridentatae* i totes les altres artemísies endèmiques d'Amèrica del Nord mostren quantitats de DNA significativament més elevades en comparació amb els altres subgèneres. En base a aquestes observacions i ànalisis comparatives incloent dades ecològiques i morfològiques, es suggereix un canvi evolutiu en l'estratègia vital d'aquestes espècies, lligat a una expansió del genoma, en el qual hi estarien involucrats processos d'acumulació de DNA deixalla o egoista. Per contra, quantitats de DNA més baixes estarien relacionades amb un comportament invasor o de "mala herba" de determinades espècies d'*Artemisia*. A part, hem obtingut també dades en híbrids homoploides i poliploides, i, mentre que en el primer cas la quantitat de DNA és propera a la mitjana esperada de les espècies progenitores, en el segon és inferior a l'esperada, per efecte de la poliploïdia.

Evolutionary and ecological implications of genome size in the North American endemic sagebrushes and allies (*Artemisia,* Asteraceae)

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Abstract

Genome size of 51 populations of 19 species of the North American endemic sagebrushes (subgenus *Tridentatae*), related species and some hybrid taxa were assessed by flow cytometry, and analysed in a phylogenetic framework. Results were similar for most *Tridentatae* species, with the exception of three species taxonomically conflictive: *A. bigelovii*, *A. pygmaea* and *A. rigida*. Genome size homogeneity (together with the high morphological, chemical and karyological affinities as well as low DNA sequence divergence) could support a recent diversification process in this geographically restricted group, thought to be built upon a reticulate evolutionary framework. The *Tridentatae* and the other North American endemic *Artemisia* show a significantly higher genome size as compared with the other subgenera. Our comparative analyses including genome size results and different kinds of ecological and morphological traits suggest an evolutionary change in lifestyle strategy linked to genome expansion, in which junk or selfish DNA accumulation might be involved. Conversely, weed or invasive behaviour in *Artemisia* is coupled with lower genome sizes. Data for both homoploid

and polyploid hybrids were also assessed. Genome sizes are close to the expected mean of parental species for homoploid hybrids, but lower than expected in the allopolyploids, a phenomenon previously documented related with polyploidy.

Key words: C-value; Compositae; hybridization; polyploidy; r-K selection; reticulate evolution; selfish DNA; speciation; *Tridentatae*; weed.

Introduction

The sagebrushes (subgenus *Tridentatae*, *Artemisia, Asteraceae*) are probably the most common woody plants in terms of area occupied and number of individual plants in the western USA, profusely distributed from Canada to Mexico west of 100º W longitude (McArthur and Sanderson, 1999). They comprise about a dozen species (and 20 taxa all together including subspecific entities; Shultz, 2005) of landscape-dominant, xerophytic shrubs, endemic to North America. The base chromosome number is exclusively $x = 9$ (there are other *Artemisia* $x = 8$-based, although $x = 9$ is the most widespread in the genus), and ploidy levels range from $2x$ to $8x$ (but mostly $2x$ and $4x$; McArthur and Sanderson, 1999). Based on evidence from different sources, the North American endemic sagebrushes can be considered a monophyletic and fairly homogeneous group, but taxonomic questions still remain unresolved (Kornkven et al., 1998; Vallès et al., 2003; Garcia et al., unpublished).

The most abundant and widespread species of this subgenus is *A. tridentata* Nutt., including its five subspecies. The subgenus can be considered a large species complex (*sensu* Clausen, 1951) centered on *A. tridentata*, because hybridization between most taxa at all levels appears to be possible (McArthur et al., 1979; McArthur et al., 1988). Some other species are also ecologically important and landscape-dominant, i.e. *A. arbuscula* Nutt., *A. cana* Pursh, and *A. nova* A. Nelson. The remaining *Tridentatae* [*A. argillosa* Beetle, *A. bigelovii* A. Gray, *A. longiloba* (Osterh.) Beetle, *A. pygmaea* A. Gray, *A. rigida* A. Gray, *A. rothrockii* A. Gray, and *A. tripartita* Rydb. and *A. spiciformis* (Osterh.)] are more restricted in distribution. *Artemisia* species from other subgenera are also endemic to western North America [*A. californica* Lessing., *A. filifolia* Torrey, *A. ludoviciana* Nutt., *A. nesiotica* P. H. Raven, *A. palmeri* A. Gray, *A. papposa* S.F. Blake and Cronquist, *A. pedatifida* Nutt. and *A. porteri* Cronquist]; other species, also present in North

America, have a more holoartic distribution (*A. absinthium* L., *A. campestris* L., *A. frigida* Willd., and *A. vulgaris* L.).

Genome size study has application in many plant research fields, e. g. ecology, evolutionary biology, systematics, taxonomy, or biogeography (Bennett and Leitch, 2005a, 2005b, 2005c). The relationships between the nuclear DNA amount and cytological traits, reproductive biology, ecology, environmental features, distribution, biomass production and many other plant characteristics have been widely investigated and established in many plant groups. Additionally, the possibility of genome size variation, at specific or subspecific levels, has been studied and it is object of controversy (Greilhuber, 2005; Murray, 2005), and needs additional study and synthesis.

We undertook genome size analysis on the *Tridentatae* and allies to: a) exploit the nuclear DNA amount information for taxonomic purposes, i.e. to identify evolutionary relationships between these plants together with the available phylogenetic data existing for them; b) detect any relationship between the nuclear DNA amounts and morphological traits of these plants, their surrounding environmental features, their geographic distribution, and weed characteristics, among other features; c) study the scope of genome size variation at species/population level; d) observe genome size changes linked to hybridization processes; and d) increase general knowledge in *Artemisia* C-values, particularly to complete the survey of the genome size in the *Tridentatae* and in other North American endemics of this genus, as a complement to previous research (Torrell and Vallès, 2001; Garcia et al., 2004).

Materials and Methods

***Plant material*---** Table 1 lists the 51 populations studied, along with their site of origin and collection information. Twelve *Tridentatae* species, with 13 subspecific entities (which constitute a complete representation of the North American endemic sagebrushes), four populations of hybrids and eight closely related *Artemisia* species were included.

***Flow cytometry measurements*---** DNA 2C-values of the tested species were estimated using flow cytometry. *Pisum sativum* L. 'Express long', and *Petunia hybrida* Vilm. 'PxPc6' (2C=8.37 pg, 2.85 pg respectively, Marie and Brown, 1993) were used as internal standards to cover the range of 2C-values found (HPCV= 0.3% and 0.6%, respectively). To assure that the flow cytometer shows a linear respose across the range of genome sizes studied, linearity of the instrument was assessed with the two standards and the taxon of the highest ploidy level in a

single measurement, and the same values were obtained for this one (less than 2% of deviation between results) with respect to each standard. The method followed is described in Garcia et al. (2004).

DNA amplification and sequencing strategies--- With the purpose of analyzing genome size variation in a phylogenetic framework, a phylogenetic tree was generated, which included all the *Tridentatae*, the other North American endemic *Artemisia* of this study, and a representation of each *Artemisia* subgenus. The analysis was based in the sequences of the ITS1 and ITS2 regions (internal transcribed spacer) of the nuclear ribosomal DNA. Most sequences have been published previously (Kornkven et al., 1998; Vallès et al., 2003) and are available from Genbank; to complete the *Tridentatae* representation, however, sequences for 7 taxa were newly generated (see Table 1 for Genbank accession numbers). Double-stranded DNA ITS region was amplified with primers 1406f (Nickrent et al. 1994) and ITS4 (White et al. 1990). The profile used for amplification is the same as in Vallès et al. (2003). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, California, U.S.A.). ITS4 was used as sequencing primer, and direct sequencing of the amplified DNA segment was performed using the Big Dye Terminator Cycle sequencing v3.1 (PE Biosystems, Foster City, California, U.S.A.). Nucleotide sequencing was carried out at the Serveis Cientificotècnics at the Universitat de Barcelona, on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City, California, U.S.A.). DNA sequences were edited by Chromas 1.56 (Technelysium PTy, Tewantin, Queensland, Australia) and aligned visually. We were not able to amplify DNA of *A. frigida*, and this species is not present in the phylogenetic analysis. The sequence alignment matrix is available from the corresponding author.

Data analysis

Model selection and Bayesian inference analysis--- To determine models under the Akaike Information Criterion (AIC) (Posada and Buckley, 2004) the data set was analysed using MrModeltest 2.2 (Nylander, 2004). The model SYM+G+I best fitted our data, and was used to perform a Bayesian analysis with MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001). Four Markov chains were run simultaneously for 1,000,000 generations, and these were sampled every 100 generations. Data from the first 1,000 generations were discarded as the burn-in period, after confirming that likelihood values had stabilized prior to the 1000th generation. Posterior probabilities were estimated through the construction of a 50% majority rule consensus tree. The outgroup species, *Kaschgaria brachanthemoides* (Winkler) Polj. and *Nipponanthemum nipponicum* (Franchet ex Maxim.) Kitam., were chosen on the basis of previous work (Vallès et al., 2003; Sanz et al., submitted).

Statistical analyses--- The analyses of the differences between the mean DNA amount and all the other parameters were performed using both the phylogenetically based generalized least squares (PGLS) algorithm, as implemented in the PHYLOGR R package (R Project 2005), and ANOVA for comparative purposes. Genome size data from previous work was also used for these analyses (Table 2).

Results

Table 3 presents 2C DNA amounts estimated for the sampled taxa, together with other karyological data. The range of variation was 3.72-fold for 2C values and 1.65-fold in monoploid genome size. The analyses were of good quality (half peak coefficient of variation, HPCV= 1.96%). This is our fourth study on *Artemisia* nuclear DNA amounts by flow cytometry, but the first especially focused on subgenus *Tridentatae* and other North American endemic *Artemisia*. For two subspecies of *Artemisia arbuscula* (*longicaulis* and *thermopola* Beetle), *A. argillosa, A. cana* subsp. *bolanderi, A. californica, A. longiloba*, *A. ludoviciana*, *A. nova* subsp. *duchesnicola, A. nesiotica, A. papposa*, *A. pedatifida, A. rigida*, *A. spinescens,* three subspecies of *A. tridentata* (*parishii, wyomingensis* and *xericensis*) and two subspecies of *A. tripartita* (*tripartita* and *rupicola* Beetle) the present work is the first estimate of their nuclear DNA amounts. Although we previously estimated nuclear DNA amounts in the remaining *Tridentatae* taxa (see Torrell and Vallès, 2001 and Garcia et al., 2004 for details) we made a complete subgeneric analysis in this study in order to assess interspecific, intraspecific and interpopulation genome size differences. With the data presented here, genome sizes are known for all the species and subspecies of the subgenus *Tridentatae* as well as most North American *Artemisia* endemic species.

Discussion

Inter- and intraspecific genome size differences

Except for three taxa (*Artemisia bigelovii*, *A. rigida* and *A. pygmaea*, discussed later in more detail), similar nuclear DNA amounts have been obtained at the same ploidy level. Such homogeneity in genome size data might be a reflection of the limited genetic differences that characterize the group, which can also favour hybridization and backcrossing among taxa. Their fairly homogeneous karyotype morphology (McArthur et al., 1981; Garcia et al., in press) and the low levels of genetic divergence detected in different regions of nuclear and chloroplastic DNA found in the present study and in previous ones (Kornkven et al., 1998, 1999; Stanton et al., 2002) might also respond to the same phenomenon. Our current data and previously published reports (Ward, 1953; McArthur et al., 1981, 1988) suggest that reticulate evolution is an important factor in this group of plants.

Nevertheless, variation in DNA amount between species could begin with changes within species, as speciation itself might start with slight morphological or ecological changes. Low interspecific differences have been found in our taxa, together with low intraspecific ones (indeed, interpopulational) at the same ploidy level. In a general sense, the extent of intraspecific genome size variation is controversial; some authors attribute differences to methodological errors or taxa misidentification (Greilhuber, 1998; Ohri, 1998). However, factors like changes in repetitive DNA (Rabinowicz, 2000) or retrotransposon activity (Bennetzen et al., 2005) can be a source of true variation within a taxon. Doležel and Bartoš (2005) state that differences of 5% should be considered acceptable in some groups. Among the different populations of the same taxon which have been assessed in this study and in the previous ones (Torrell and Vallès, 2001; Garcia et al., 2004; Garcia et al., 2006), low percentages of difference have been detected for the majority of species (most ranging from 1 to 2%). Even in the case of a population of *A. tridentata* subsp. *parishii*, with clearly segregating flowering phenotypes, one upright and the other drooping (McArthur, 2005; collections 3037 and 3038, respectively, of Table 2), nuclear DNA amounts differences between phenotypes are negligible. Morphological differences, however, do not necessarily imply changes in the genome sizes even in closely related taxa. On the other hand, considerable differences were found in *A. pygmaea* (5.98%) and in *A. tridentata* subsp. *spiciformis* (10.02%) even though populations of these species show little morphological differences.

Can genome size discriminate the sagebrushes amidst Artemisia*?*

Although taxonomic limits of subgenus *Tridentatae* are fuzzy, the sagebrushes form a natural group of species based on habit, morphology, anatomy, chemistry and cytology (McArthur, 1979), clearly distinct from the other subgenera. Our genome size research reported herein supports the separation of the *Tridentatae* from the other subgenera. Statistical analyses show a significant difference between mean genome sizes of the *Tridentatae* with respect to that of the non-*Tridentatae Artemisia*. The *Tridentatae* genome size is larger than that of the other subgenera (*Tridentatae* mean 2C=8.98 pg, vs. non *Tridentatae* mean 2C=7.48 pg), as previously reported by Garcia et al. (2004) on a limited data set (1Cx mean values for each subgenera: *Dracunculus* 1Cx=2.67 pg, *Artemisia* 1Cx=3.05 pg, *Absinthium* 1Cx=3.56 pg, *Seriphidium* 1Cx=3.89 pg, *Tridentatae* 1Cx=4.08 pg). These results would support separation of

the *Tridentatae* from the subgenus *Seriphidium*, which included them in traditional taxonomic treatments (Rydberg, 1916; Ward, 1953; Ling, 1991). Differences in monoploid genome size between ploidy levels are also statistically significant (means: 4.22, 3.94, 3.80 and 3.29 for 2x, 4x, 6x and 8x respectively, p=0.058) as previous studies have reported for other taxa (Leitch and Bennett, 2004), hence a decreasing monoploid genome size is detected with increasing ploidy levels, confirming the general phenomenon of nuclear DNA loss during polyploidization.

Many authors support the hypothesis that ancestral angiosperm species possess low genome sizes (Leitch et al., 1998; Soltis et al., 2003), and others also suggest that evolutionary trend is toward increasing genome sizes (Bennetzen, 2002; Devos et al., 2002; Bennetzen, 2005). In fact, both increases and decreases have been found in the evolutionary history of different plant genera (Bennett and Leitch, 2005c). We believe that the larger genome size of the *Tridentatae* in respect to the other *Artemisia* subgenera is evidence of a derived phylogenetic position. The origin of the genus likely lies in Asia, rich in the number of *Artemisia* species and home to most of its *Anthemideae* relatives. Indeed, subgenus *Tridentatae* is thought to have evolved from the less phylogenetically advanced circumboreal subgenera *Artemisia* or *Dracunculus* (on the basis of distribution, flower morphology and secondary woodiness; McArthur, 1983), and *Tridentatae* ancestral stock most likely bridged the Bering Strait from Asia to North America (McArthur and Plummer, 1978; McArthur et al., 1981). Some species, such as *A. frigida* and *A. dracunculus* naturally occur in both areas. Their low genome sizes (5.25 pg and 5.94 pg respectively, Garcia et al., 2004; Pellicer et al., 2007) might also suggest a possible role of species like these or any ancestor as the ancestral stock for the *Tridentatae*. In this sense, the tree topology of Figure 1 would suggest that species from subgenus *Dracunculus* are the sister group of all other *Artemisia* (although with insufficient statistical support).

Genome size and colonizing ability

The ability of the *Tridentatae* to colonize extensive areas reflects competitive success, suggesting that the larger genomes characterising this subgenus has not been a constraint, there. Such a genome expansion without increasing ploidy level could be explained by activation of transposable elements (Kellogg and Bennetzen, 2004) or presence of B-chromosomes (these have been yet detected in the *Tridentatae*, McArthur et al., 1981; McArthur and Sanderson, 1999; Garcia et al., in press), although more studies are necessary to

definitively establish such a connection in these plants. However, polyploidy is one of the mechanisms that best explains genome size increase, and sagebrushes display abundant polyploidy, e.g. 2/3 of the populations of *A. nova* are tetraploid and 1/3 diploid, and the populations of *A. rothrockii* or *A. argillosa* are entirely polyploid (McArthur and Sanderson, 1999, Mahalovich and McArthur, 2004). At the other extreme of the genome size spectrum we could cite the case of many island colonizers species, where a significant reduction in genome size has been detected, presumably in response to insular selection pressures (Suda et al., 2003; Garcia et al., 2006; Garnatje et al., 2007). We hypothesize that the reduced competition pressure in *Tridentatae* habitats allows expansion of genome size (whether by transposable elements activation, increase in the frequency of supernumerary chromosomes, polyploidization or any other mechanisms), while in environments subject to competitive constraints, the pattern followed is the decrease in total nuclear DNA amount. Indeed, molecular mechanisms are known which can lead to genome size increase or decrease (Petrov et al., 2000; Bennetzen et al., 2005). This hypothesis fits well with renewed theories about selfish and junk DNA, which postulate that the C-value of a species is merely a by-product of the persistent accumulation of phenotypically neutral DNA (driven by genetic drift, by mutation pressure resulting in duplications of genomic segments, or by the maintenance of extinct genes whose function has been lost), that is excised only when it becomes too costly (Gregory and Hebert, 1999, and references therein). Recent studies, however, confer more importance to junk DNA, which should be regarded as a major player in many of the processes that shape the genome and control the activity of its genes (Biémont and Vieira, 2006).

Fitness, adaptatation and genome size

The r-K selection theory (MacArthur and Wilson, 1967) posits that evolutionary systems must choose whether they invest more resources in reproduction or development, a choice dependent on the selective environment. In a changing or disturbed context, selection for abundant offspring will prevail (r-selection), whereas selection for development is suitable in predictable conditions, with adequate supply of resources (K-selection). In the light of previous data and our present results, we suggest that the genus *Artemisia* in North America displays a continuum from one evolutionary strategy to the other, during its speciation and diversification processes, which is coupled with a considerable genome size increase (however,

some authors support that both strategies are exclusive; Flegr, 1997). In the proposed scenario, the *Tridentatae* (together with the other North American endemics) arose *in situ* in North America from an ancestor coming from subgenera *Artemisia* or *Dracunculus*, when alternating moist and dry climates during the Pleistocene provided opportunity to fill large new niches (McArthur, 1983). Given the abundance and present distribution of *Artemisia s. l.* in North America, they might have spread profusely at initial stages of the colonization. Most likely, species with more r-selection traits (profuse seed production, more herbaceous habit, shorter generation times, smaller sizes, etc.) in this genus might have pioneered this colonization. This role could have been played by species such as *A. dracunculus* or *A. frigida*, by other counterparts in their subgenera (*A. campestris* or *A. vulgaris*, for example), or by some ancestral taxa with similar features. These species show reduced genome sizes as compared with the *Tridentatae*, as well. These taxa are all present in Eurasia and also cover large areas of North America (this four species are listed as weeds or invasive plants in the USA; see below for further discussion about this topic). Hence, such species would be prime candidates for ancestral stock, and the *Tridentatae* could have arisen from subsequent evolutionary processes in any of those species, resulting in an optimal adaptation to their environment (together with a genome size increase), and thus acquiring traits that would class them as more K-strategists. Although an organism mainly adopts one strategy, the majority would fall between these ecological extremes, and can show features representatives of both ends of the r/K range.

To test the hypothesis that this change in lifestyle strategy is linked with a significant genome size increase, we collected environmental and morphological data on different characteristics of these species (environmental, morphological, etc.) that could have a bearing on r- or K-selection. These data have been extracted from the abundant literature existing for the *Tridentatae* and other *Artemisia* (McArthur et al., 1979; Cronquist, 1994; McArthur and Stevens, 2004; Shultz, 2006; Plants database of the United States Department of Agriculture, http://plants.usda.gov/, 12/2006). Some cautions/premises were established to develop these analyses: 1) only diploid taxa have been used to avoid biased results to monoploid genome downsizing in polyploids (except for *A. argillosa* and *A. rothrockii*, only known at tetraploid level); 2) when there are several subspecific entities for a species, only one has been chosen for consistency in analysis and to avoid uneven representation; 3) since taxonomic nomenclature of *Tridentatae* is often confusing, we have considered taxa at the species level any taxon which has been formally treated at this level at least once; 4) to prevent bias due to phylogenetic

relationships among taxa, the statistical analyses performed take their phylogeny into account (see Methods and Figure 1), although the results of the ordinary ANOVA test are also given for comparative purposes. The ecological and morphological traits evaluated are summarized in Table 4. A discussion of every trait for all these groups follows next (see Table 5 for statistical analyses and comparisons between groups):

Elevation range: This variable was included as it may reflect an ability to colonize different environments and habitats. Nor significant correlations neither meaningful differences between groups were observed. However, we note that *Artemisia frigida* exhibits an altitudinal gradient from 900 to 3,500 m, and presents one of the lowest in genome sizes of perennial *Artemisia* inhabiting North America. Its small genome size may well be involved in this broad adaptability. In contrast, *A. pygmaea*, with the largest genome size of the species studied, only inhabites a narrow elevation range.

Mean annual precipitation and drought tolerance: Differences between groups are nonsignificant in all cases. However, the largest genome sizes in the *Tridentatae* are found in the species group inhabiting areas with lowest mean annual precipitation, which also coincides with the highest genome size found in the most drought tolerant, confirming previous research (Garcia et al., 2004).

Mean plant height: In all groups, but particularly in the *Tridentatae*, lower statured species tend to show larger genome sizes. The differences are significant in the ordinary test and almost significant in the PLGS. The r/K theory asserts that K-strategists should be larger in size. These lower statured species, however, tend to show a woody habit (which implies more biomass, i. e. selection for development). This trait is particularly outstanding in *A. pygmaea*, the smallest of all sagebrushes, but with a dwarf shrub habit, and the largest genome size. Indeed, seeds and seedlings of pygmy sagebrush are the largest of the whole subgenus; this is probably another sign of selection for development. Additionally, researches have found that particularly in the *Tridentatae*, polyploids tend to show lower sizes than diploids of the same species (Barker and McKell, 1986; Sanderson et al., 1989; McArthur and Sanderson, 1999).

Seed production: The hypothesis states that plants with r-selection traits tend to produce more seeds than K-selectors. Hence, according to our prediction species with profuse seed set should show lower genome sizes than less seed producers. However, we were unable to find either statistical support or meaningful differences between genome sizes of the different groups which could support this premise. Nevertheless, one of the highest seed producers is

again the low-genome-sized *A. frigida*: each 2.5 cm length of inflorescence contains approximately 1,000 seeds (Harvey, 1981), with about 10 million cleaned seeds per kg (Plummer et al., 1968).

Fire ecology: Species which layer or stump sprout after fire show smaller genome sizes than those who are entirely killed by fire. The differences are not significant, but the trend is the consistent in the three groups. The ability to colonize disturbed environments linked to r-strategists could also be related to this lower genome size in these species.

Growth rate: The differences between slow and fast growing species are significant in both statistical analyses, and in all groups the slow growing ones have increased genome sizes. Indeed, smaller genomes are usually correlated with short life cycles (which imply fast growth). And usually, slow growth is linked with long-lived plants, a trait which better fits the K-strategy growth.

Vegetative reproduction: Species that can reproduce vegetatively show larger genome sizes than those that cannot, although there is no statistical significance in any test. Because meiosis can be disregarded, it does not exert any control on the genome size of the individual, it is possible that accumulation of selfish or junk DNA is more likely in species that present asexual reproduction than in those that do not.

Salinity tolerance: Differences are nonsignificant again in all groups. However, a particularly halofilous species, *A. filifolia*, which inhabits exclusively dunes or sandhills, presents one of the lowest genome sizes of the North American endemics. Most traits of this species (profuse seed set, quick growth and maturation, ability to resprout vigorously after fire, relatively tall stature but less woodiness than common sagebrushes) would class this *Artemisia* as an r-strategist. However, we cannot set a link between genome size and salinity tolerance from these data.

Distribution: In all groups studied, plants showing more extensive distribution have lower genome sizes. The differences are statistically significant when all species are included in the analysis. Species with wider distribution are usually r-strategists (hence, with lower genome sizes according to our hypothesis), whereas more restricted species tend to be K-strategists, with higher genome sizes. Again, the case of *A. pygmaea* (2C=11.19 pg, 2*n*=18), with a restricted, scattered distribution on the cold desert of the Great Basin and fringe areas and the highest genome size of all the sagebrushes, might represent a model of this hypothesis. *Artemisia frigida* (2C=5.25 pg, 2*n*=18) would be placed on the other end of the r/K gradient: this is probably the most widely distributed and abundant species of all the genus, whose range

extends from Mexico in the south, through most of the Western United States, Western Canada, and Alaska in the north, and into Siberia, Mongolia, and Kazakhstan (USDA, 1937; Harvey, 1981).

From all these data and statistical analyses it is clear that neither one group (the *Tridentatae*) nor the other (the remaining *Artemisia*) meet exactly all the conditions that would shape a r- or a K-strategist, although many species can be safely included in one or the other category (as previously noted, it is known that a given species will mainly adopt one strategy, even though traits of the other can be present). However, from the trend outlined from these relationships: it seems that fast-growing, less drought tolerant, bigger (but less woody), and more widely distributed species tend to show lower nuclear DNA amounts, and would be more easily classified as r-strategists, whereas slow-growing, more drought tolerant, smaller but more woody species, and more restricted in distribution tend to have higher DNA amounts (and would be *more* K-strategists). Apart from the species included in this study we note that the uncommon annual *Artemisia* (*A. annua*, *A. scoparia*, for instance) also fit better in the r-selection category, showing smaller genome sizes (see Torrell and Vallès, 2001 and Garcia et al., 2004), although exceptions can be found.

Weed behaviour and genome size

Studies have shown that weeds and invasive species (the model of r-strategists) tend to show lower genome sizes as compared with their counterparts of their genera (Bennett and Leitch, 2005a). In contrast, species appearing in the red list of endangered species (Vinogradov, 2004) mostly show high nuclear DNA amounts. From the U.S. Invasive Plants List (http://plants.usda.gov, 12/2006), eleven *Artemisia* species are cited (*A. absinthium*, *A. annua*, *A. biennis*, *A. campestris*, *A. cana*, *A. dracunculus*, *A. filifolia*, *A. frigida*, *A. ludoviciana*, *A. tridentata* and *A. vulgaris*). Except from *A. ludoviciana*, which is only known as a tetraploid, and *A. cana* subsp. *cana*, an octaploid (although other subspecies of *A. cana* are known at the diploid level, but the list does not mention which of these behaves as a weed) all the other never exceed 9.01 pg. This finding also supports that a high genome size might be a charge inhibitory of such a weediness (hence, r-strategist) behaviour.

Hybrid formation

Owing to their widespread and sympatric or tightly parapatric distribution, to their wind pollination and to their genetic similarity, *Tridentatae* taxa tend to hybridize. The data set in this study includes genome size data for both polyploid and homoploid hybrids. Both diploid (homoploid) and a tetraploid offspring were produced from an experimental hybridization of diploid *A. tridentata* subsp. *tridentata x A. tridentata* subsp. *vaseyana* (McArthur et al., 1998; McArthur and Sanderson, 1999). A second experimental hybrid combination resulting from the crossing of an octoploid *A. cana* subsp. *cana* with a tetraploid *A. tridentata* subsp. *wyomingensi*s produced hexaploid hybrid progeny (McArthur et al., 1998 and McArthur and Sanderson, 1999). Nuclear DNA amounts of both sets of the hybrids are consistent with the expected means corresponding to their parents' genome sizes, although the tetraploid and hexaploid offsprings show a little less than that mean, most likely due to their polyploid nature. In both these polyploid hybrids, a similar genome size decrease is detected. This could also reflect rapid genome reorganization after hybridization (which is coupled with ribosomal DNA loss in some cases, Garcia et al., unpublished).

Taxa of questionable taxonomic position and genome size

Genome size variation at species level has been considered as a predictor of taxonomic heterogeneity and as an indicator of incipient speciation in process (Murray, 2005). Hence, a critical study of genome size can contribute to the clarification of taxonomic placement between closely related species. The monophyly of subgenus *Tridentatae* has been supported by several independent studies (Kornkven et al., 1998, Torrell et al., 1999; Vallès et al., 2003); however, *A. bigelovii*, *A. pygmaea*, and *A. rigida* classically included in this group have been subject of controversy, with countless studies proposing either their inclusion or exclusion.
If mean genome sizes of the traditional *Tridentatae* (sensu Shultz, 2006 -Flora of North America) are aligned from the lowest to the highest (at diploid level), *A. bigelovii* and *A. rigida* appear at the lowermost end (8.00 pg and 8.23 pg, respectively) and *A. pygmaea* at the uppermost (11.19 pg), while the remaining converge in the narrow range between 8.54 and 9.24 pg. This exercise may be revealing about the potential use of genome size in this field, but some other data about these three species makes us have bearing on they placement within the *Tridentatae*.

In the case of *A. bigelovii*, its floral morphology (the only *Tridentatae* with heterogamous capitula), molecular phylogenetic data (Kornkven et al., 1998), essential oil composition (Holbo and Monzingo, 1965; Geissman and Irwin, 1974) and our own results on molecular cytogenetics by FISH and molecular phylogenetics (Garcia et al., unpublished) do not support its inclusion in *Tridentatae*. *Artemisia bigelovii* has been considered to occupy an unclear position between the true sagebrushes (*Tridentatae*) and subgenus *Artemisia*, but generally treated as a *Tridentatae* on the basis of many characters such as wood anatomy, leaf form, karyotype morphology, RAPD genetic markers and cpDNA restriction site analyses (McArthur et al., 1981, 1998; Kornkven et al., 1999). We believe that these seemingly disparate results may have a basis in morphological convergence and a past chloroplast capture by hybridisation with core *Tridentatae*.

A second case is that of *A. pygmaea*. This is a dwarf, depressed shrub, with different leaf morphology and larger seeds as compared with the other *Tridentatae* (Cronquist, 1994; McArthur and Stevens, 2004). It is a relatively uncommon species and occurs on dry alkaline sites, probably due to the numerous morphological adaptations that incorporates for the extremely xeric sites which inhabits, where few other species occur (deserts of Nevada, Utah and Arizona). Based on these specialized features, Rydberg (1916) placed *A. pygmaea* in a separated section (sect. *Pygmaea* Rydb.) in subgenus *Seriphidium*. Overall karyotype morphology of *A. pygmaea* is shared with the traditional *Tridentatae*, although it has bigger chromosomes and the habitual presence of a B-chromosome intensely stained with DAPI, findings which could account for its higher genome size (Garcia et al., in press). Essential oil composition also supports exclusion from the core of the true sagebrushes (Holbo and Monzingo, 1965; Geissman and Irwin, 1974). Additionally, molecular biology studies have placed this species as sister to the other *Tridentatae* (Kornkven et al., 1998; Watson et al., 2002; Garcia et al., unpublished).

The third case is that of *A. rigida*. This species, particularly well adapted to restricted scabland habitats also displays specialized morphological and anatomical modifications to the conditions of aridity of Western North America (Hall and Clements, 1923; Shultz, 1983). Similar to pigmy sagebrush, *A. rigida* was also placed alone in another section within *Seriphidium*, sect. *Rigidae* Rydb. (1916). Holbo and Mozingo's (1965) chromatographic characterization point to its exclusion from the *Tridentata*e, as well as our findings with *in situ* hybridization, which detected two additional loci carrying ribosomal DNA (Garcia et al., in press) with respect to the other

Tridentatae analysed. Many studies have claimed for retention of this three species within the *Tridentatae* (Hall and Clements, 1923; Ward, 1953; Beetle, 1960; McArthur et al., 1981; Bremer and Humphries, 1993; Kornkven et al., 1998, 1999), but our present findings put again a question mark about their taxonomic placement; although they are not definitely excluded, the degree of relationship remains elusive (Figure 1).

Other North American endemic Artemisia

This study also reports genome size data for some other non-*Tridentatae*. These species, assigned to other *Artemisia* subgenera are also endemic to North America and share some morphological traits as well as overlapping distribution. These commonalities led us to consider these taxa for comparative purposes with the *Tridentatae*. These are (1) *Artemisia palmeri* is a large woody plant endemic to the coastal area near San Diego (California). It has been treated as a member of the the subgenus *Seriphidium* (Ward, 1953) and also considered in an independent genus, *Artemisiastrum* (Rydberg, 1916). However, it is disjunct from other *Seriphidium* and displays a growth, leaf form and floral characters reminiscent of its subgenus *Artemisia* congeners, especially of *A. ludoviciana* (Shultz, 1993; McArthur, 2005). Another species, (2) *A. californica* and (3) *A. nesiotica*, which is sometimes considered as a subspecies of the later belong to subgenus *Artemisia* although these species are woody unlike most members of subgenus *Artemisia*. (4) *Artemisia filifolia,* (5) *A. pedatifida* and (6) *A. spinescens* belong to subgenus *Dracunculus* (the latter has sometimes been considered a separate monotypic genus, *Picrothamnus desertorum*). *Artemisia californica* and *A. filifolia* grossly resemble one another in that they are of similar size with a willowly, filiform appearance and both share cpDNA segments with the *Tridentatae* (Kornkven et al., 1999; McArthur, 2005). *Artemisia filifolia* also has other affinities with the *Tridentatae* (karyotype morphology; McArthur and Pope, 1979; and similarities in secondary chemistry; Kelsey and Shafizadeh, 1979). Interestingly, each of these North American endemics show substantially increased genome sizes with respect to the mean of their subgenera at the same ploidy level (see Garcia et al., 2004). This would also support our hypothesis of genome size expansion linked to absence of competitive constraints and diversification when colonizing North America.

Conclusion

The higher genome size not only of the *Tridentatae* but also of the other North American *Artemisia* endemics, together with other shared traits, characterizes what we could call in a wide sense the "North American *Artemisia*" group, which is consistent with a recent molecular phylogeny of the whole genus (Sanz et al., submitted). Apart from the exceptions previously discussed, the core of North American sagebrushes forms a homogeneous group of species (also visible in their similar genome sizes) which are probably undergoing diversification and speciation processes. All this might point to a process of reticulate evolution, a hypothesis reinforced by the difficulty (and incongruences) of many authors to establish a clear phylogenetic framework for the *Tridentatae*. Finally, a change in lifestyle strategy linked to genome size gain in the North American *Artemisia* is suggested, on the basis of morphological, ecological traits, and geographical distribution. The developmental-reproduction trade-off (r-K selection) that these species might face *in the struggle for life* appears coupled with significant changes in nuclear DNA amounts, in which selfish and junk DNA might probably be involved. As Gregory and Hebert (1999) stated, it will be now critical to ascertain wheter these changes arose via the gradual accumulation or deletion of small segments of DNA or wheter a more punctuated pattern of change predominates.

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

Taxa	Origin of materials	Coll n.[1]
Subgenus *Tridentatae*		
A. arbuscula subsp. *arbuscula*	Corn Creek Canyon, Millard Co.Utah. 1,830 m.	2877
A. arbuscula subsp. *arbuscula*	South of Jordanelle Reservoir, Wasatch Co., Utah. 1,890 m.	3027
A. arbuscula subsp. *arbuscula*	Sage Junction, Lincoln Co., Wyoming. 1,930 m.	3028
A. arbuscula subsp. *longicaulis*	Toulon, Pershing Co. Nevada. 1,335 m.	2860
A. arbuscula subsp. *longicaulis*	Bruneau, Owyhee Co. Idaho. 1,012 m.	2855
A. arbuscula subsp. *thermopola*	East bank of Snake River, Yellowstone.	3032
A. argillosa	Coalmont, Jackson Co., Colorado. 2,497m.	3034
A. bigelovii	Emery Co. Utah. 1,801 m.	2869
A. bigelovii	15 km east of Fremont Junction. Emery Co. Utah. 1,777 m.	3050
A. bigelovii	Padre Canyon, Cocconino Co., Arizona. 1,799 m.	3051
A. cana subsp. *bolanderi*	17 km northwest of Bridgeport, Mono Co., California. 2,270 m.	3047
A. cana subsp. *cana*	Sheridan, Sheridan Co. Wyoming. 1,140 m.	2128
A. cana subsp. *viscidula*	Strawberry Valley, Wasatch Co. Utah. 2,374 m.	2844
A. cana subsp. *viscidula*	Soldier Summit, Wasatch. Co. Utah. 2,255 m.	2875
A. cana subsp. *viscidula*	Fossil Butte National Monument, Lincoln Co. Wyoming, 1,650 m.	2851
A. cana subsp. *cana* x	Pleasant Grove Plots, Uinta National Forest, Utah Co.,	2759
A. tridentata subsp.	Utah. 1,734 m.	2760
wyomingensis[2]		
A. longiloba	Evanston, Uinta Co., Wyoming. 2,067 m.	3025
A. nova	Tunnel Spring, Desert Experimental Range.	2876
A. nova	Millard Co. Utah. 2174 m. Pine Valley Pass, Millard Co.	2873
	Utah. 1820 m.	
A. nova	Birch Springs Road, Mount Borah, Custer Co., Idaho. 2,120 m.	3053
A. nova subsp. *duchesnicola*	Tridell Road, Uintah Co., Utah. 1,702 m.	3029
		3030
A. pygmaea	Yuba Dam Road, Juab Co. Utah. 1,535 m.	2870
A. pygmaea	San Rafael Swell, Emery Co., Utah. 2,195 m.	
A. rigida	Malheur Reservoir, Malheur Co. Oregon. 1,035 m.	2859
A. rothrockii	Reed Flats, White Mountains, Inyo Co., California. 3072 m	19803[3]
A. spiciformis	Ford Ridge, Bristle Cone Scout Camp, Carbon Co. Utah. 2,856 m.	2839
A. tridentata subsp. *parishii*[4]	West of Rosamond, Kern Co., California. 722 m.	3037/3038
A. tridentata subsp. *tridentata*	Salt Cave Hollow, Salt Creek Canyon, Juab Co. Utah. 1,870 m.	2871

A. tridentata subsp. *tridentata*	Beaver, Beaver Co. Utah. 1780 m.	s. n.
A. tridentata subsp. *vaseyana*	Salt Cave Hollow, Salt Creek Canyon, Juab Co., Utah. 1,878m.	2872
A. tridentata subsp. *vaseyana*	Hobble Creek Canyon, Utah Co. Utah. 1,555 m.	2874
A. tridentata subsp. *vaseyana*	Spring City, Sanpete Co. Utah. 1,950 m.	2879
A. tridentata subsp. *wyomingensis*	Gordon Creek, Carbon Co. Utah. 1,980 m.	2880
A. tridentata subsp. *xericensis*	Mann Creek Reservoir, Washington Co.,Idaho. 929 m.	2858
A. tripartita subsp. *rupicola*	Pole Mountain, Albany Co., Wyoming. 2,647 m.	3033
A. tripartita subsp. *tripartita*	Dubois Sheep Station, Clark Co., Idaho. 1,650 m.	2845
A. tripartita subsp. *tripartita*	Birch Springs Road, Mount Borah, Custer Co., Idaho. 2,191 m.	3054

Hybrids

A. cana subsp. *cana* x	Pleasant Grove Plots, Uinta National Forest, Utah Co., Utah. 1,734	2759
A. tridentata subsp.	m.	2760
wyomingensis[2]		
A. tridentata subsp. *tridentata* x	Orem, Utah Co., Utah. 1,474 m.	3049
A. tridentata subsp. *vaseyana*		
A. tridentata subsp. *tridentata* x	Shrub Sciences Laboratory. Provo, Utah. 1,374 m.	3048
A. tridentata subsp. *vaseyana*		

Other *Artemisia*

A. californica	Santa Clarita, Los Angeles Co., California. 487 m.	3039
A. californica	Los Peñasquitos Canyon Preserve, San Diego, San Diego Co., California. 70 m.	3043
A. nesiotica	San Clemente Island, Los Angeles Co., California.	3090
A. filifolia	Moccasin, Mohave Co. Arizona. 1,530 m.	2868
A. ludoviciana	Salt Cave Hollow Road, Uinta National Forest, Salt Creek Canyon, Juab Co., Utah. 2,084 m.	3087
A. palmeri	Los Peñasquitos Canyon Preserve, San Diego, San Diego Co., California. 70 m.	3044
A. papposa	Milepost 130, U. S. Highway 20, 16 km west of Hill City. Elmore Co.Idaho. 1,679 m.	3077
A. pedatifida	North of Point of Rocks, Sweetwater Co., Wyoming.	1138
A. spinescens	Winton Road, Sweetwater Co., Wyoming.	2403

[1] E. Durant McArthur collection numbers; vouchers are deposited in the herbarium of the Rocky Mountain Research Station, Provo, Utah (SSLP).

[2] Synthetic hybrids (see McArthur et al., 1998 and McArthur and Sanderson, 1999).

[3] Leila M. Shultz collection number.

[4] Separate floral morphologies (see McArthur, 2005).

TABLE 2. Previous genome size measurements of *Tridentatae*.

Taxa	2C (s.d.) (pg)[1]	2C (Mbp)[2]	2n[3]	P. L.	2C/P.I.[4]	Standard[5]
A. arbuscula subsp. *arbuscula**	9.22 (0.11)	9017.16	18	2	4.61	*Petunia*
*A. bigelovii***	15.49 (0.10)	15149.22	36	4	3.87	*Pisum*
A. cana subsp. *cana**	25.65 (0.61)	25085.70	72	8	2.57	*Pisum*
A. cana subsp. *viscidula***	8.54 (0.09)	8352.12	18	2	4.27	*Petunia*
*A. nova***	6.37 (0.14)	6229.86	18	2	3.19	*Petunia*
*A. pygmaea***	11.54 (0.18)	11286.12	18	2	5.77	*Petunia*
*A. spiciformis**	8.18 (0.30)	8000.04	18	2	4.09	*Pisum*
A. tridentata subsp. *tridentata***	8.17 (0.08)	7990.26	18	2	4.09	*Petunia*
A. tridentata subsp. *vaseyana***	8.66 (0.07)	8469.48	18	2	4.33	*Petunia*

Note: The data belong to previous studies in nuclear DNA amount in the genus *Artemisia*: * Torrell and Vallès, 2001; ** Garcia et al., 2004. Data of the *A. nova* population is not at all consistent with those of the five populations studied in the present paper, so that we believe that it could be the product of a misidentification. [1]2C nuclear DNA content (mean value and standard deviation of the samples). [2]1 pg = 978 Mbp (Doležel et al., 2003). [3]Somatic chromosome number. [4]Monoploid genome size (2C value divided by ploidy level, Greilhuber et al., 2005). All chromosome counts have been carried out in the populations studied in the present paper. [5]Internal standard used in each case (see text for details about *Pisum* and *Petunia*).

TABLE 3. Nuclear DNA content and other karyological characters of the populations studied.

Taxa	2C (s.d.) (pg)[1]	2C (Mbp)[2]	2n[3]	P. L.	2C/P.l.[4]	Standard[5]
Subgenus *Tridentatae*						
A. arbuscula subsp. *arbuscula* (2877)	9.21 (0.06)	9007.38	18	2	4.61	*Petunia*
A. arbuscula subsp. *arbuscula* (3027)	9.04 (0.13)	8841.12	18	2	4.52	*Petunia*
A. arbuscula subsp. *arbuscula* (3028)	15.55 (0.35)	15207.9	36	4	3.89	*Pisum*
A. arbuscula subsp. *longicaulis* (2855)*	22.85 (0.18)	22347.3	54	6	3.81	*Pisum*
A. arbuscula subsp. *longicaulis* (2860)*	23.10 (0.39)	22591.8	54	6	3.85	*Petunia***
A. arbuscula subsp. *thermopola* (3032)*	9.47 (0.13)	9261.66	18	2	4.73	*Pisum*
A. argillosa (3034)*	15.77 (0.65)	15423.06	36	4	3.94	*Petunia***
A. bigelovii (3051)	8.00 (0.10)	7824.00	18	2	4.00	*Petunia*
A. bigelovii (3050)	15.06 (0.13)	14728.68	36	4	3.76	*Pisum*
A. bigelovii (2869)	15.32 (0.09)	14982.96	36	4	3.83	*Pisum*
A. cana subsp. *bolanderi* (3047)*	9.01 (0.09)	8811.78	18	2	4.50	*Petunia*
A. cana subsp. *cana* (2128)	27.04 (0.42)	26445.12	72	8	3.38	*Pisum*
A. cana subsp. *viscidula* (2844)	8.73 (0.24)	8537.94	18	2	4.37	*Petunia*
A. cana subsp. *viscidula* (2851)	8.51 (0.13)	8322.78	18	2	4.26	*Petunia*
A. cana subsp. *viscidula* (2875)	8.58 (0.19)	8391.24	18	2	4.29	*Petunia*
A. longiloba (3025)*	16.62 (0.45)	16254.36	36	4	4.15	*Pisum*
A. nova (3053)	9.09 (0.06)	8890.02	18	2	4.51	*Petunia*
A. nova (2873)	17.25 (0.15)	16870.5	36	4	4.31	*Pisum*
A. nova (2876)	17.10 (0.11)	16723.8	36	4	4.28	*Pisum*
A. nova subsp. *duchesnicola* (3029)*	22.90 (0.39)	22396.2	54	6	3.82	*Pisum*
A. nova subsp. *duchesnicola* (3030)*	22.43 (0.24)	21936.54	54	6	3.74	*Pisum*
A. pygmaea (2836)	10.89 (0.24)	10650.42	18	2	5.45	*Petunia*
A. pygmaea (2870)	11.14 (0.19)	10894.92	18	2	5.57	*Petunia*
A. rigida (2859)*	8.23 (0.13)	8048.94	18	2	4.12	*Petunia*
A. rothrockii (19803)*	16.41 (0.25)	16048.98	36	4	4.10	*Pisum*

A. spiciformis (2839)	9.00 (0.19)	8802	18	2	4.50	*Petunia*
A. tridentata subsp. *parishii* (3037)*	16.61 (0.27)	16244.58	36	4	4.15	*Pisum*
A. tridentata subsp. *parishii* (3038)*	16.32 (0.17)	15960.96	36	4	4.08	*Pisum*
A. tridentata subsp. *tridentata* (1996)	8.42 (0.27)	8234.76	18	2	4.21	*Petunia*
A. tridentata subsp. *tridentata* (2871)	8.24 (0.25)	8058.72	18	2	4.12	*Petunia*
A. tridentata subsp. *vaseyana* (2879)	15.12 (0.37)	14787.36	36	4	3.78	*Petunia***
A. tridentata subsp. *vaseyana* (2872)	8.89 (0.20)	8694.42	18	2	4.45	*Petunia*
A. tridentata subsp. *vaseyana* (2874)	8.85 (0.22)	8655.3	18	2	4.43	*Petunia*
A. tridentata subsp. *wyomingensis* (2880)*	15.07 (0.19)	14738.46	36	4	3.77	*Petunia*
A. tridentata subsp. *xericensis* (2858)*	16.24 (0.13)	15882.72	36	4	4.06	*Pisum*
A. triparitita subsp. *rupicola* (3033)*	8.68 (0.19)	8489.04	18	2	4.34	*Petunia*
A. tripartita subsp. *tripartita* (3054)	8.85 (0.08)	8655.30	18	2	4.42	*Petunia*
A. tripartita subsp. *tripartita* (2845)*	15.32 (0.18)	14982.96	36	4	3.83	*Petunia***
Hybrids						
A. cana subsp. *cana x A. tridentata* subsp. *wyomingensis* (2759)	19.15 (0.68)	18728.70	54	6	3.19	*Pisum*
A. cana subsp. *cana x A. tridentata* subsp. *wyomingensis* (2760)	18.72 (0.35)	18308.16	54	6	3.12	*Pisum*
A. tridentata subsp. *tridentata x A. tridentata* subsp. *vaseyana* (3048)	15.71 (0.14)	15364.38	36	4	3.93	*Pisum*
A. tridentata subsp. *tridentata x A. tridentata* subsp. *vaseyana* (3049)	8.52 (0.25)	8332.56	18	2	4.26	*Petunia*
Other *Artemisia*						
A. californica (3039)*	8.38 (0.22)	8195.64	18	2	4.19	*Petunia*
A. californica (3043)*	8.57 (0.12)	8381.46	18	2	4.28	*Petunia*
A. filifolia (2868)	7.26 (0.06)	7100.28	18	2	3.63	*Petunia*
A. nesiotica (3090)	8.38 (0.15)	8195.64	18	2	4.19	*Petunia*
A. ludoviciana (3087)*	13.82 (0.17)	13515.95	36	4	3.45	*Pisum*

A. palmeri (3044)*	7.14 (0.07)	6982.92	18	2	3.57	*Pisum*
A. papposa (3077)*	8.44 (0.17)	8254.32	18	2	4.22	*Petunia*
A. pedatifida (1138)*+	8.86 (0.09)	8665.08	18	2	4.43	*Petunia*
A. spinescens (2403) *+	7.58 (0.20)	7413.24	18	2	3.79	*Petunia*

Note: (*) Taxa for which genome size has been estimated for the first time; (**) It was not possible to use a internal standard closer to the value of these populations; however, the linearity of the flow cytometer has been assessed and guarantees a fluctuation threshold lower than 2% in this range of data (see Methods); (+) Only two individuals have been measured [1]2C nuclear DNA content (mean value and standard deviation of the samples). [2]1 pg = 978 Mbp (Doležel et al., 2003). [3]Somatic chromosome number. [4]Monoploid genome size (2C value divided by ploidy level, Greilhuber et al., 2005). All chromosome counts have been carried out in the populations studied in the present paper. [5]Internal standard used in each case (see text for details about *Pisum* and *Petunia*).

TABLE 4. Environmental, ecological and morphological characteristics, together with the mean values of genome size data and ploidy levels of the taxa included in the analyses.

Taxa	Group[1]	2C[2]	P. L.[3]	Dist.[4]	E. R.[5]	M. A. P.[6]	D. T.[7]	P. H.[8]	S. P.[9]	F. E.[9]	G. R.[10]	V. R.[11]	S. T.[12]
A. arbuscula subsp. *arbuscula*	AT	9.24	2	3	3	3	1	1	1	Y	S	N	1
A. bigelovii	AT	8.00	2	2	2	2	3	2	3	N	S	N	2
A. cana subsp. *viscidula*	AT	8.61	2	3	3	3	1	3	3	Y	F	Y	1
A. nova	AT	9.09	2	3	3	1	3	2	3	N	S	N	2
A. pygmaea	AT	11.19	2	2	1	1	3	1	2	N	S	N	2
A. rigida	AT	8.23	2	2	2	2	3	1	1	Y	S	N	2
A. spiciformis	AT	8.59	2	3	3	3	1	3	3	Y	F	Y	1
A. tridentata subsp. *vaseyana*	AT	8.8	2	3	3	1	2	3	3	N	S	N	1
A. tripartita subsp. *tripartita*	AT	8.85	2	3	2	3	2	3	3	Y	S	Y	2
A. longiloba	AT	9.21	2	2	1	2	3	1	2	-	S	Y	3
A. argillosa	AT	15.77	4	2	2	3	2	2	3	-	-	Y	3
A. rothrockii	AT	16.41	4	1	1	2	3	2	1	N	S	Y	2
A. californica	ANT	8.48	2	2	2	2	2	3	3	N	S	Y	1
A. filifolia	ANT	7.2	2	3	-	3	2	3	3	Y	F	Y	3
A. palmeri	ANT	7.14	2	1	-	2	2	3	3	Y	F	N	1

A. papposa	ANT	8.44	2	1	-	2	2	1	2	N	S	N	2
A. spinescens	ANT	7.58	2	3	3	1	3	1	3	-	S	Y	3
A. pedatifida	ANT	8.86	2	2	2	1	3	1	-	-	S	N	3
A. nesiotica	ANT	8.38	2	1	1	1	2	2	3	N	S	Y	1
A. ludoviciana	ANT	13.82	4	3	3	3	2	3	3	Y	F	Y	2
*A. vulgaris**	ANT	6.08	2	3	1	2	2	3	2	N	F	Y	2
*A. fragrans**	ANT	5.35	2	-	1	1	3	2	2	-	-	N	3
*A. herba-alba**	ANT	6.57	2	-	1	1	3	2	2	-	-	N	3
*A. arborescens**	ANT	11.61	2	-	1	2	2	3	3	-	-	Y	2
*A. absinthium**	ANT	8.64	2	3	1	3	2	2	3	Y	F	Y	2
*A. campestris**	ANT	5.87	2	3	3	1	3	2	2	Y	F	N	2
*A. chamaemelifolia**	ANT	6.04	2	-	1	3	1	2	2	-	-	Y	1
*A. dracunculus**	ANT	5.94	2	3	1	2	2	2	2	-	-	Y	2
*N. nipponicum**	O	11.87	2	1	-	-	-	2	2	-	-	-	-
*K. brachanthemoides**	O	14.09	2	1	1	1	2	1	1	-	-	-	2

* Peviously published genome size data for these species in Torrell and Valles (2001) and in Garcia et al. (2004).

[1]Group AT= *Tridentatae*; ANT= Non Tridentatae *Artemisia*; O= Outgroup (other subgenera).

[2]Mean 2C values of the populations measured in the present work and in the previously published.

[3]Ploidy level.

[4]Distribution. Values from 1= restricted (1-5 states in the USA); 2= medium (>5-10 states); 3= wide(>10 states).

[5]Elevational range. Values from 1= narrow (<1500 m); 2=medium (2000-2500 m); 3= wide (>2500 m).

[6]Mean annual precipitation. Values from 1=low (< 300 mm); 2= medium (300 – 400 mm); 3= high (500-1000 mm).

[7]Drought tolerance. Values from 1= bad drought tolerant to 3 = better drought tolerant.

[8]Plant height. Values from 1= dwarf (< 0.5 m); 2= subshrubs (0.5-1.5 m); 3= shrubs (>1.5 m).

[9]Seed production. Values from 1= low (<2000 seeds/g); 2= medium (2000-5000 seeds/g); 3= high (>5000 seeds/g).

[10]Fire ecology. Y= layers/ stumps sprout after fire; N= killed by fired.

[11]Growth rate. F=fast; S= slow.

[12] Vegetative reproduction, where Y = sometimes reproduces vegetatively and N= never reproduces vegetatively

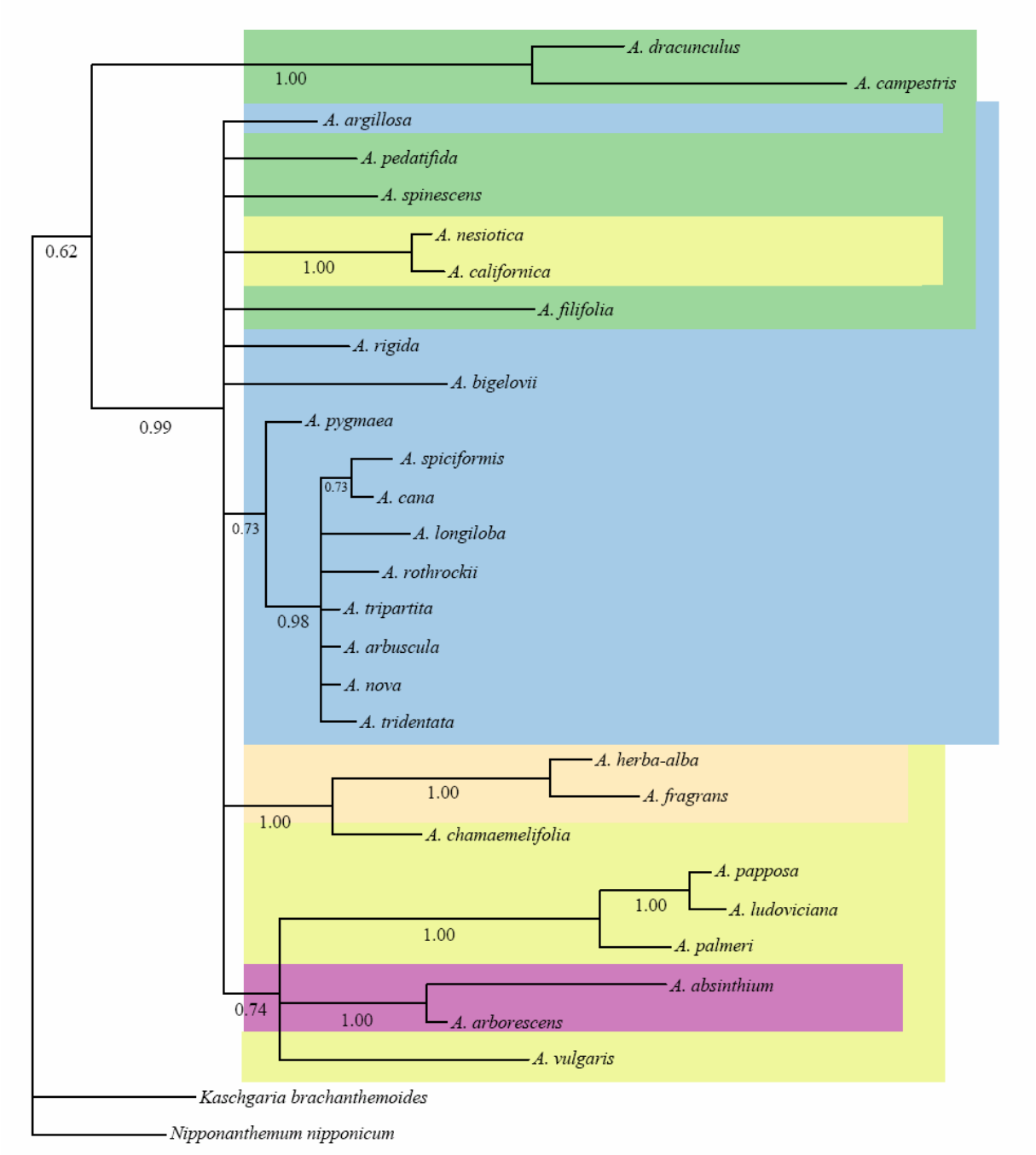
[13] Salinity tolerance. Values from 1= bad salinity tolerant to 3 = better salinity tolerant.

TABLE 5. Mean genome size and results of the comparisons, using t tests and phylogenetically based generalized least squares (PGLS) algorithm). Significances belong to the group AT+ANT.

	AT*	ANT*	AT+ANT	Ordinary test	PLS test
Group					
	8.98	7.48	8.08	p=0.014	p=0.092
Elevation Range					
1	10.2	7.33	7.90	Nonsignificant	Nonsignificant
2	8.36	8.67	8.49		
3	8.87	8.72	8.25		
Mean annual precipitation					
1	9.69	7.1	7.97	Nonsignificant	Nonsignificant
2	8.48	7.95	8.13		
3	8.82	7.29	8.17		
Drought tolerance					
1	8.81	6.04	8.12	Nonsignificant	Nonsignificant
2	8.82	7.99	8.14		
3	9.14	6.85	7.99		
Plant heigth					
1	9.47	8.29	8.96	P=0.038	p=0.087 (1-2)
2	8.55	6.68	7.09		
3	8.71	8.19	8.37		
Seed production					
1	8.73		8.73	Nonsignificant	Nonsignificant
2	10.2	6.33	6.93		
3	8.66	8.43	8.54		
Fire ecology					
Y	8.70	7.21	8.04	Nonsignificant	Nonsignificant
N	9.27	7.85	8.56		
Growth rate					
F	8.60	6.99	7.45	p=0.009	p=0.054

S	9.08	8.35	8.80		
Vegetative growth					
Y	8.81	7.77	8.09	Nonsignificant	Nonsignificant
N	9.09	7.04	8.06		
Salinity tolerance					
1	8.81	7.51	8.16	Nonsignificant	Nonsignificant
2	9.07	7.76	8.36		
3	9.21	7.11	7.46		
Distribution					
1	9.71	8.57	9.02	Nonsignificant	p=0.055 (1-2)
2	8.79	7.58	8.62		p=0.011 (1-3)
3	6.75		6.75		

FIGURE 1. Phylogram from Bayesian phylogenetic of ITS1 and ITS2 sequence data for 28 *Artemisia* and two outgroup species. Bayesian clade-credibility values (posterior probability; >0.5) below branches (green=subgenus *Dracunculus*; blue=subgenus *Tridentatae*; Yellow=subgenus *Artemisia*; pink=subgenus *Seriphidium*; violet=subgenus *Absinthium*).



7. Canvis en el DNA ribosòmic durant la formació de poliploides i híbrids en les artemísies endèmiques d'Amèrica del Nord (subgènere *Tridentatae*, *Artemisia*, *Asteraceae*).

Sònia GARCIA, Teresa GARNATJE, Jaume PELLICER, Joan VALLÈS, i Sonja SILJAK-YAKOVLEV.

El subgènere *Tridentatae* (*Artemisia*, *Asteraceae*), també conegut com el grup d'artemísies endèmiques d'Amèrica del Nord (anomenades *sagebrushes*) pot ser considerat un complex poliploide. L'autopoliploïdia i l'al·lopoliploïdia, així com diferents tipus d'hibridació i introgressió són factors ben documentats involucrats en l'evolució d'aquestes espècies. Hem utilitzat la hibridació *in situ* fluorescent i el bandeig amb fluorocroms per a detectar i analitzar els canvis en el DNA ribosòmic i heterocromàtic lligats als processos de poliploïdia en aquest grup. En alguns poliploides s'observa la pèrdua de *loci* de DNA ribosòmic, mentre que d'altres mostren el que caldria esperar respecte als seus diploides. Els patrons de bandatge (de DNA ric en G-C i A-T) també són diferents depenent del parell diploide-poliploide estudiat. Malgrat que la hibridació *in situ* genòmica (GISH) no aconsegueix distingir entre els genomes parentals d'un suposat al·lopoliploide, hi ha diferències en el marcatge de certs *loci* de DNA ribosòmic que suggereixen la dominància d'un dels progenitors sobre l'altre, en congruència amb les dades morfològiques i amb algunes de seqüències de DNA. A més, el valor sistemàtic de la hibridació *in situ* fluorescent i el bandatge de fluorocroms són utilitzats per a elucidar la posició sistemàtica d'*Artemisia bigelovi*i, una espècie clàssicament considerada com a membre del grup *Tridentatae* però taxonòmicament conflictiva, i en la qual es detecta un patró diferencial de marcatge respecte a la resta del subgènere. Finalment, la presència de la seqüència telomèrica de tipus *Arabidopsis* és detectada per primer cop en el gènere.

Ribosomal DNA changes during polyploid and hybrid formation in the North American endemic sagebrushes (*Tridentatae*, *Artemisia*).

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Abstract

Subgenus *Tridentatae* (*Artemisia*, Asteraceae), also known as the North American endemic sagebrushes, can be considered a polyploid complex. Autopolyploidy and allopolyploidy, as well as different kinds of hybridization and introgression processes are well documented factors involved in the evolution of these species. Fluorescent *in situ* hybridization and fluorochrome banding have been used to detect and analyze ribosomal and heterochromatic DNA changes linked to the process of polyploidization in this group; additionally, we also made use of FISH for detecting the presence of the *Arabidopsis*-type telomere repeat for the first time in the genus. Loss of rDNA loci was observed in some polyploids, whereas others showed the double expected from their diploid relatives, hence different models in polyploid evolution are detected for these species. Banding patterns (of GC- and AT- rich DNA) are also different depending on the diploid-polyploid species pair studied. Although GISH failed to distinguish between the parental genomes of a putative allopolyploid, differential labelling of paternal probes seems to indicate one progenitor's dominance over the other at some rDNA loci, consistently with plant morphology and some chloroplast DNA sequence data. Additionally, the taxonomic value of FISH and fluorochrome

banding is exploited for elucidating the position of *Artemisia bigelovii*, a species classically considered as a *Tridentatae* member but with a questionable systematic position, and a differential pattern with respect to the rest of the group is found.

Key words: *Arabidopsis*-type telomere, allopolyploidy, autopolyploidy, Compositae, FISH, fluorochrome banding, fluorescent *in situ* hybridization, fluorocrome banding, genome organization.

Introduction

The subgenus *Tridentatae* of genus *Artemisia* (Asteraceae) makes up a natural grouping of species called sagebrushes, which are endemic to western North America. Sagebrushes are about a dozen of landscape-dominant species, probably the most common woody plants in terms of area occupied and number of individual plants in the western region of the United States (McArthur and Sanderson, 1999). The *Tridentatae* have been the subject of countless research works from many points of view due to their ecological and economical interest, and classical cytogenetic studies are particularly abundant (see a review in McArthur and Sanderson, 1999). Additionally, reports on banding and fluorescent *in situ* hybridization of the 5S and 18S-5.8S-26S rDNA have been performed (Torrell et al., 2003; Garcia et al., in press), which have established a characteristic signal pattern of these taxa, as compared with the other subgenera.

Polyploidy in the sagebrushes

Interspecific hybridization and polyploidy are widespread phenomenon in flowering plants, considered some of the key elements involved in plant diversification (Levin, 1983; Stebbins, 1950). As many as 70% of plants may have experienced one or more genome duplication events in their evolutionary past (Masterson, 1994), and even *Arabidopsis thaliana*, one of the lowest-genome-sized plants, is thought to be a paleopolyploid (Vision et al., 2000; Bowers et al., 2003). The prevalence and success of polyploidy implies that it may confer some evolutionary advantage. This is usually attributed to the genetic variability of

polyploids, as they can harbour greater genetic diversity than their diploid relatives, especially in the case of allopolyploids (Doyle et al., 1999). Nevertheless, the mechanisms by which polyploidy contributes to this variation are not yet well understood, partly because a number of genetic and epigenetic phenomena are involved (Soltis et al., 2004, and references therein).

Particularly in the *Tridentatae* both processes are believed to have facilitated their speciation and diversification (McArthur et al., 1981; McArthur and Sanderson, 1999). From the results of many of previous studies it became evident that *Tridentatae* species exhibit abundant polyploidy, a feature that is also widespread in *Artemisia* as a whole (Ehrendorfer, 1964; Estes, 1969; Stahevitch and Wotjas, 1988; Garcia et al., 2006; Pellicer et al., 2007). All the most abundant species (*A. arbuscula*, *A. cana*, *A. nova* and *A. tridentata*) and others less common or less widely distributed (*A. bigelovii*, *A. longiloba*, *A. rigida*, *A. rothrockii*, and *A. tripartita*) include diploid and polyploid populations, and it seems that the *Tridentatae* exhibit broad polyploidy and hybridization phenomena which are also evident at ecotonal interfaces and within populations (McArthur and Sanderson, 1999). Hence, given their extensive and overlapping areas of distribution in many cases, it could be stated that *Artemisia* subgenus *Tridentatae* fits well in the definition of a polyploid complex, in which interrelated and interbreeding plants with different ploidy levels can allow genetic exchanges between species, creating a complex network of interrelated taxa (Babcok and Stebbins, 1938).

Fluorescent *in situ* hybridization (FISH) of rDNA loci and fluorochrome banding of heterochromatic DNA constitute reliable landmarks for chromosome identification (Castilho and Heslop-Harrison, 1995), and can contribute elucidating genomic rearrangements in polyploids (Heslop-Harrison, 1991, 2000). As the number of rDNA chromosomal loci mostly vary between related taxa, this can also help revealing interspecific relationships (Maluszynska and Heslop-Harrison, 1991; Cerbah et al., 1998). Indeed, the use of FISH and fluorochrome banding has yet contributed interesting data to the study of evolutionary relationships in the genus *Artemisia* (Vallès and Siljak-Yakovlev, 1997; Torrell et al., 2001, 2003; Garcia et al., in press) and close genera. Moreover, many other chromosomal regions of repetitive DNA are also susceptible to be revealed by FISH, such as telomeres, transposable elements, or intersimple sequence repeats (ISSR) among others, which can provide more characteristics for the description of the karyotypes. The telomeres are repeated sequences

highly conserved which stabilise and protect chromosome ends, and most plant groups posses the *Arabidopsis*-type telomere $(TTTAGGG)^n$, although this sequence is not ubiquitous to all flowering plants (see Fajkus et al., 2005, for a review on this topic), and to our knowledge, its presence has never been assessed in *Artemisia*, although it has in a related and close genus, *Chrysanthemum* (Abd-el-Twab and Kondo, 2006).

Genomic *in situ* hybridization (GISH), is a useful technique that can shed light on the origin of hybrid taxa by the use of fluorescently labelled DNA probes of the putative progenitors to paint metaphase plates of their presumed hybrid progeny (Parokonny and Kenton, 1995). Bennett (1995) described the GISH technique as a "dream come true", and the ability to "paint" genomes with different colours has enhanced understanding of cellular organization and evolutionary pathways in recent years (Borgen et al., 2003); the application of GISH for such evolutionary studies is illustrated in several works on species from the genus *Nicotiana* (Lim et al., 2000, 2005, and references therein).

Hence, the application of techniques like these is essential in any cytogenetical approach to solve questions related to chromosomal evolution. With all these ideas in mind, we conducted fluorochrome banding and *in situ* hybridization assays with the objectives of: a) determining the number and distribution of GC-rich bands and rDNA loci (5S and 18S-5.8S-26S genes) in diploid and polyploid representatives of subgenus *Tridentatae*; b) detecting in *Artemisia* the presence of the most usual plant telomeric repeat $(TTTAGGG)_n$ and of the intersimple sequence repeat (ISSR) $(GT_{12})^n$ isolated from representatives of a close genus (*Chrysanthemum*, Yang et al., 2006), in order to provide more elements for the description of their karyotypes; c) describing changes in number and signal strength of ribosomal DNA loci or any chromosomal rearrangement related with ploidy level increase; d) establishing, with the help of GISH, the hybrid origin of the presumed allopolyploid *A. arbuscula* ssp. *longicaulis*, whose putative parents are *A. arbuscula* ssp. *arbuscula* and *A. tridentata* ssp. *wyomingensis* and observing possible genomic changes related with allopolyploidy in this taxon, e) linking observed changes in genome size in increasing ploidy levels (Garcia et al., submitted) with the signal pattern differences of these species.

Material and methods

***Plant material*---** Ripe achenes from adult plants were collected from wild populations of each taxon. Root tip meristems from seedlings were obtained by germinating them on wet filter paper in Petri dishes in the dark at room temperature. An indication of the provenance of the species studied is shown in Table 1.

***Chromosome preparations*---** for FISH assays, root tips were pretreated either with 0.05% aqueous colchicine or with a saturated aqueous solution of Gammexane® (hexachlorocyclohexane; Sigma Aldrich) at room temperature, for 2 hours 30 minutes to 4 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.

Most chromosome preparations for fluorochrome banding and *in situ* hybridization were done using the air-drying technique of Geber and Schweizer (1987), with some modifications: root tips were washed with agitation in citrate buffer (0.01 M citric acid – sodium citrate, pH = 4.6) for 15 minutes, excised, and incubated in an enzyme solution [4% cellulase Onozuka R10 (Yakult Honsha), 1% pectolyase Y23 (Sigma) and 4% hemicellulase (Sigma)] at 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 4000 rpm for 5 minutes for each centrifugation, and removing the supernatant each time. The final pellet was resuspended in 50 µl of fixative, about 10 µl were dropped onto a clean slide, and air-dried. Alternatively, some chromosome squashes were prepared following the enzymatic softening of material, as described in Leitch et al. (2001).

***Fluorochrome banding*---** in order to reveal GC-rich DNA bands, chromomycin A_3 was used, following the protocol described in Vallès and Siljak-Yakovlev (1997).

***Fluorescent* in situ *hybridization*---** DNA hybridization was carried out according to the protocol described in 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). Plant telomeric repeat probe $(TTTAGGG)^n$ was also labelled with digoxigenin-11-dUTP and the intersimple sequence repeat (ISSR) $(GT_{12})^n$ with biotin; in these cases the protocol was carried out as described in Leitch et al. (1994), with modifications as described in Lim et al. (1998). The preparations were counterstained with Vectashield (Vector Laboratories), a mounting medium containing DAPI (4'-6 diamidino-2 phenylindole).

***Genomic* in situ *hybridization*---** The hybridization mixture contained 8 µg ml^{-1} digoxigenin-labeled *A. tridentata* ssp. *wyomingensis* DNA and 8 µg ml^{-1} biotinlabelled *A. arbuscula* ssp. *arbuscula* DNA (probes obtained by nick translation following the instructions of the manufacturer). After overnight hybridization at 37ºC, slides were washed in 20% (v/v) formamide in 0.1XSSC at 42•C at an estimated hybridization stringency of 80–85%. Sites of probe hybridization were detected by using 20 µg ml^{-1} fluorescein-conjugated anti-digoxigenin IgG (Roche Biochemicals) and 5 µg ml^{-1} Cy3-conjugated avidin (Amersham Biosciences). Chromosomes were counterstained with 2 µg ml^{-1} DAPI in 4XSSC, mounted in Vectashield.

The best plates were photographed with a digital camera (AxioCam MRc5 Zeiss) coupled on a Zeiss Axioplan microscope and images were analyzed with Axio Vision Ac software version 4.2. FISH preparations

were observed with an epifluorescent Zeiss Axiophot microscope with different combinations of Zeiss excitation and emission filter sets (01, 07 and 15). Hybridization signals were analysed and photographed using the highly sensitive CCD camera (Princeton Instruments), and an image analyser software (Metavue, version 4.6, Molecular Devices Corporation). Alternatively, for telomere and ISSR FISH and for GISH assays, plates were examined using a Leica DMRA2 epifluorescence microscope (Wetzlar, Germany) and photographed with an Orca ER camera (Hamamatsu Photonics, Welwyn Garden City, UK). Images were processed for colour balance, contrast and brightness uniformity with Adobe Photoshop (version 7.0.1). Graphics of the haploid idiograms were performed with PowerPoint (Microsoft Office XP Professional v. SP3) based in the results from Garcia et al. (in press).

Results

Chromosome complements are $2n=2x=18$ for diploids, $2n=4x=36$ for tetraploids and $2n=6x=54$ for the only hexaploid studied (Table 2 summarizes results obtained for both $2x$ and $4x$ populations). In every taxon analyzed, FISH signals belonging to 5S and 18S-5.8S-26S rDNA genes appear always colocalized, located at the distal ends of the short arms of either metacentric or submetacentric chromosomes (Figure 1). Chromomycin (GC-rich DNA) stains always the same loci that rDNA, although in some cases, particularly in the polyploids, additional chromomycin rich bands are present. Diploid *A. bigelovii* has two loci (four sites) of both rDNA located at the distal ends of the short arms of one metacentric and one submetacentric chromosome pair. The remaining diploids show different number of marked loci: *A. tridentata* ssp. *tridentata* shows metaphase plates either with five or six sites, and both *A. nova* and *A. tripartita* ssp. *tripartita* show always six sites (three loci). Particularly in the first and the second some loci are larger than the other, which allows distinguishing homologues at metaphase. In regard to tetraploids, *A. bigelovii* shows four rDNA loci, two weakly stained and the other two more strongly; *A. tridentata* ssp. *tridentata* presents 18 bands stained with chromomycin (one chromosome pair marked in both ends) of which 11 sites also present rDNA FISH signals. The tetraploid *A. nova* presents 16 GC-rich bands, of which one chromosome pair is also stained at both ends, and 12 of these sites also show both rDNA. The tetraploid *A. tripartita* ssp. *tripartita* follows a different pattern, with 12 GC-rich bands, 6-8 of which are also colocalized with both rDNAs. The hexaploid *A. nova* ssp. *duchesnicola* present 8 rDNA loci and DAPI used as a counterstaining in hybridization

experiments revealed 30 bands in this taxon, but as these bands were not visible in all the species they have not been considered for systematic purposes.

To assess the existence of *Arabidopsis*-type telomeres in *Artemisia*, we conducted an *in situ* hybridization assay in the tetraploid *A. nova*, where the plant-type telomere probe efficiently hybridized to the ends of chromosomes, although the signal strength was quite faint in most cases (Figure 2, B). Additionally, with the purpose to characterize a novel tandem repeat for *Artemisia*, an attempt was done in order to isolate the intersimple sequence repeat (ISSR) $(GT_{12})^n$ from a representative of the family, *Chrysanthemum indicum*, obtained from Yang et al. (2006). This species is quite close to our genus, as it belongs to the same tribe (Anthemideae) and even to the same subtribe (Artemisiinae) (*sensu* Bremer and Humphries, 1993); however, we were unsuccessful and this ISSR appears dispersed throughout the whole chromosome complement of *A. nova* (results not shown).

GISH is used to recognize the genomic origin of the chromosomes in allopolyploids (Heslop-Harrison, 1992; Gill and Friebe, 1998). We conducted a GISH assay in a taxon of putative hybrid allopolyploid origin, *A. arbuscula* ssp. *longicaulis*, however, this assay has also been unsuccessful, as it is unable to distinguish each parental genome in the metaphase plates of the hybrid. Nevertheless, ribosomal DNA signals from *A. arbuscula* ssp. *arbuscula*, appear stained stronger than those from the other parent in the merged image (Figure 2, C to F).

Discussion

This study confirms what previous findings on *Artemisia* (Torrell et al., 2003; Garcia et al., in press) cytogenetics had pointed, namely, that repeated units of the two different rDNA multicopy families analyzed appear always overlapped, colocalized with GC-rich regions, and always located at telomeric position. This unusual organization of ribosomal DNA (for a review on this subject see Garcia et al., in press), confirmed by PCR assays and more recently by Southern blot hybridization (A. Kovarik, pers. comm.) is not only exclusive of *Artemisia* (also reported in Abd El-Twab and Kondo, 2006), but seems to be common in the branch holding the tribes Anthemideae and Gnaphalieae (*sensu* Bremer and Humphries, 1993; Kubitzki, 2007), and ongoing studies will determine the precise distribution of rDNA units,

the possible existence of different gene families and the extent of this trait in the Asteraceae (Garcia et al., in preparation).

Signal pattern and systematic position: the case of A. bigelovii

The signal pattern of *A. bigelovii* (two rDNA loci in the diploid and four in the tetraploid) differs from the structure of the *Tridentatae* karyotype, which typically shows six rDNA sites at diploid level (Table 2). This finding contributes additional evidence to the possible misplacement of this species within the true sagebrushes, as floral morphology, essential oil composition, and molecular phylogenetics (Holbo and Monzingo, 1965; Geissman and Irwin, 1974; Kornkven et al., 1998; Garcia et al., unpublished) also suggest. Actually, *A. rigida*, another species classically included in the *Tridentatae* but with problematic taxonomic placement from many points of view, also revealed a differential signal pattern with respect to the typical of the sagebrushes, which argued for exclusion of this group (Garcia et al., in press). *Artemisia bigelovii*, although generally treated as a *Tridentatae* on the basis of other characters (wood anatomy, leaf form, karyotype morphology, RAPD genetic markers and cpDNA restriction site analyses; McArthur et al., 1981, 1998; Kornkven et al., 1999), it has also been considered to occupy an unclear position between the true sagebrushes and subgenus *Artemisia*, particularly because floral morphology, previously mentioned, is remarkably different from the *Tridentatae*, their flower heads being heterogamous instead of homogamous. Indeed, FISH on *A. annua*, from subgenus *Artemisia*, revealed the same signal distribution (Torrell et al., 2003). Additionally, *Artemisia filifolia*, a species classically considered a member from subgenus *Dracunculus* but closely related with the sagebrushes, also shows the same two loci (Garcia et al., in press) and a molecular phylogeny based on chloroplast DNA restriction site variation had placed both species together in the same clade (Kornkven et al., 1999). Molecular phylogenetic studies based on different nuclear and chloroplastic DNA regions may shed light on the relationships between these species (Garcia et al., unpublished).

Change of rDNA loci sites and polyploidization

The other diploid taxa of the study, *A. nova*, *A. tridentata* and *A. tripartita* present three rDNA loci, as usual in the sagebrushes *sensu stricto* (Garcia et al., in press). This confirms the existence of a typical banding and FISH pattern, for which, together with their increased genome and chromosome size (Torrell et al., 2003; Garcia et al., unpublished), the *Tridentatae* could be cytogenetically recognized and differentiated from the remaining *Artemisia* subgenera. In *A. tridentata*, however, cells with either five or six sites have been found (Figure 1, E, only shows the cytotype with 5 sites); although uncommon, a gene loss process could account for a missing site in this species. Alternatively, instead of occasional deletion of one locus, it could be that this site was very small and hardly visible due to size differences in rDNA loci (probably as a consequence of outbreeding, hence higher heterozigosity). If this was the case, however, we would see more pronounced differences in rDNA size in the other homologous loci, but conversely, homologous loci can be paired up and no size differences are apparent, so we believe that deletion of one locus might have taken place (K. Lim, pers. comm.). With respect to this, studies have shown that polymorphism in the number and chromosomal distribution of 5S and 25S rDNA can occur both among different subspecies and varieties of a given species, and within a population (Hasterok et al., 2006).
As one of the main goals of the present paper was to characterize and discuss rDNA change linked to polyploidy (whether in number, whether in signal strength), the corresponding tetraploids of these species, and a hexaploid taxon of *A. nova* (*A. nova* subsp. *duchesnicola*) have also been analysed. In all cases, genome size loss is detected with ploidy increase (Table 2), confirming the general trend towards genome downsizing with increasing ploidy level (Leitch and Bennett, 2004), already reported in *Artemisia* (Garcia et al., 2004; Pellicer et al., 2007) and particularly in the *Tridentatae* (Garcia et al., unpublished). However, in some other *Asteraceae* genera, genome sizes of polyploid taxa present additivity with respect to diploid genome sizes (Price and Bachmann, 1975; Marchi et al., 1983; Cerbah et al., 1999).

In some cases the decrease of DNA amount is higher than in others, a fact that could be related with the age of the polyploid, although rapid genomic rearrangements in few generations involving a considerable loss of genome size have also been found (reviewed in Levy and Feldman, 2002; Leitch and Bennet, 2004). With regards to the number of rDNA loci of the polyploids with respect to the diploids, two tendencies seem present in the studied

species. On the one hand, the number of rDNA sites of the tetraploids is twice the number found in diploids in the cases of *A. bigelovii* and *A. nova*. An additive pattern of both parental rDNA loci had been previously reported for the allopolyploid *Nicotiana rustica* with gene conversion to the parental rDNA type (Matyasek et al., 2003; Kovarik et al., 2004). In *Tragopogon*, another Asteraceae genus, the number and distribution of the rDNA loci in some of the polyploids studied were also additive of those observed in the diploids (Pires et al., 2004). Nevertheless, some weaker signals are detected in both polyploid taxa, hence loss of gene copies might be taking place at these loci. Additionally, translocations have occasionally been detected in *A. nova* (Figure 1, D –chromomycin stained, arrow).

On the other hand, there is a decrease in the expected number or rDNA sites during polyploid formation: the uncommon case of *A. tridentata* ssp. *tridentata*, where only eleven sites are present instead of the twelve expected, might be a consequence of hybridization between the diploid cytotype carrying 5 sites and the habitual cytotype with 6 sites (also present in another subspecies, *A. tridentata* ssp. *spiciformis*; see Torrell et al., 2003). Conversely, *A. tripartita* shows a different behaviour, as cells with either six or eight rDNA loci are found in the polyploid taxon where twelve were also expected; hence a dramatic decrease in rDNA amount per haploid chromosome set has taken place in this polyploid. This could be related with the higher percentage of genome size loss for this diploid-polyploid pair (see Table 2), although this data have to be taken with caution, as genome size for the tetraploid corresponds to a different population (population 2845 of *A. tripartita* ssp. *tripartita* was initially described as diploid, and genome size estimates available for this one correspond to the diploid cytotype; Garcia et al., unpublished). Finally, the single hexaploid studied, *A. nova* ssp. *duchesnicola*, shows 16 rDNA sites, instead of the 18 expected from the diploid *A. nova*. rDNA loci loss after polyploidization has also been reported in other Asteraceae genera such as *Xeranthemum* (Garnatje et al., 2004), and in other plant groups, i.e. *Sanguisorba* (Mishima et al., 2002) and *Zingeria* (Kotseruba et al., 2003), among others. rDNA loci loss in polyploids is probably a widespread phenomenon which might well be involved in genome size loss in polyploids with respect to the monoploid chromosome complement of diploids, and in the diploidization of the new polyploid cytotypes.

The case of *A. tripartita*, where both the diploid and tetraploid cytotypes belong to the same population has yet been detected in *Tridentatae* populations, and it is thought to

be quite common (McArthur et al., 1981) among the sagebrushes. Indeed, previous work had suggested the autopolyploid nature of many of the *Tridentatae* (McArthur et al., 1981), although the wide occurrence of hybridization processes including allopolyploidy is another major force in the evolution of this group. It is suspected that there is a relative frequency of unreduced gametes formed in such populations, one of the most common mechanisms for the production of polyploids (Lewis, 1980). Actually, the possibility of *in situ de novo* production of 4*x* plants in 2*x* populations has been verified in *A. tridentata* ssp. *vaseyana*. In some populations of this species the recent origin of the polyploid cytotype has been supported by RAPD (2*x* and 4*x* individuals had the same RAPD profile, apart from being morphologically and chemically identical; McArthur et al., 1998). We have no such available data for both *A. tripartita* of this study, but as this population was originally described as diploid, we might suggest the recent origin of the tetraploid cytotype, as well. Was this the case, however, rapid genomic rearrangements, probably resulting in genome imbalance following hybridization could have contributed to such loci loss, as suggested by different authors in other plant genera (Hasterok et al., 2006). For comparative purposes, it will be interesting to dispose on data of a recognized and long-term tetraploid *A. tripartita* population.

GC-rich, AT-rich DNA and polyploidy

Chromomycin positive bands, those which reveal GC-rich DNA, are always colocalized with rDNA in diploids, and in the same number of those. The interspersion of 18S rDNA genes in chromomycin positive regions corresponds to nuclear organizing regions (NORs), which are codified by 18S-5.8S-26S rDNA, are linked to GC-rich DNA (Siljak-Yakovlev et al., 2002), although not all GC-rich DNA bands include NORs. In the tetraploids, however, different patterns in its distribution seem to occur. The case of *A. bigelovii* and *A. tripartita*, with double number of GC-rich DNA bands with respect to the diploids, contrasts with both *A. nova* and *A. tridentata* tetraploids, showing 16 and 18 bands, respectively, and a special chromosome pair with GC-rich DNA bands in both ends. A similar case was previously described for *A. argillosa*, another *Tridentatae* tetraploid showing 16 chromomycin positive bands and also two chromosomes marked in both ends (Garcia et al., in press). In this previous paper, this high number of GC-rich DNA was considered

unexpectedly high as compared with previous findings not only in the *Artemisia* but also in the Asteraceae. Moreover, a high number of AT-rich DNA was also detected for *A. nova* ssp. *duchesnicola* (around 30), similarly to that found in *A. argillosa* (26 bands); and always at distal position (for both types of heterochromatin). Unfortunately we do not have information about the number of AT-rich DNA bands in the other tetraploids of this paper, as no DAPI positive bands were visible. However, our present results add evidence to the previous, and it seems that an expansion of both kinds of repetitive DNA might be linked with polyploid formation in these species, but its biological significance is still to uncover, although differentiation leading to diploidization of their karyotypes (to prevent from hybridization, i. e., enhancing reproductive isolation), or a protective function of chromosome ends were previously suggested as possible causes (Garcia et al., in press).

GISH of the putative allopolyploid A. arbuscula *ssp.* longicaulis*: possible gene conversion?*

Hybridization and introgression have been important in the evolutionary history of *Tridentatae* taxa (Ward, 1953; Beetle, 1960; McArthur et al., 1981, 1988), and many taxa are thought to have originated as hybrids, such as *A. arbuscula* ssp. *longicaulis*, *A. argillosa*, *A. spiciformis*, *A. tridentata* ssp. *xericensis* and *A. tridentata* ssp. *wyomingensis*, among others. We investigated in this study the hypothesis of hybrid origin for the first of these taxa, the putative allohexaploid *A. arbuscula* ssp. *longicaulis* (2*n*=54). Well documented ecological, morphological, chemical and cytological evidence points that diploid *A. arbuscula* ssp. *arbuscula* (2*n*=18) is one putative parent and tetraploid *A. tridentata* ssp. *wyomingensis* (2*n*=36) is the other, and general morphology and ecology suggest that it is best treated as a subspecies of *A. arbuscula* (see figure in Winward and McArthur, 1995). Additionally, both putative parental species share distribution areas. Genome size data are also consistent with the possibility of hybrid origin of this taxon and subsequent genome downsizing (*A. arbuscula* ssp. *arbuscula*, mean 2C=9.16 pg, *A. tridentata* ssp. *wyomingensis*, mean 2C=15.07 pg, *A. arbuscula* ssp. *longicaulis*, mean 2C=22.97 pg, expected mean= 24.23 pg; from Garcia et al., unpublished). However, no discrimination of the parental origin of the chromosomes could be detected by GISH. Few studies have succeeded in distinguishing between genomes of congeneric Asteraceae species (reviewed in Borgen et al., 2003). The recent

divergence of these taxa and the lack of enough genomic differentiation between them may have prevented GISH to work properly.

Nevertheless, this assay might have been useful in elucidating gene conversion, which can have influenced the structure of rDNA loci in this taxon. In metaphase plate of *A. arbuscula* ssp. *longicaulis* (Fig. 2, C to F) it seems that the probe corresponding to *A. arbuscula* ssp. *arbuscula* genomic DNA (red, Figure 1, D) paints rDNA loci stronger than the probe corresponding to the other parent (green, Figure 1, E). In the composite images this difference is also apparent, and particularly two decondensed rDNA loci are clearly *more* red than green (Figure 1, F, arrows). Therefore, it is possible that *A. tridentata* ssp. *wyomingensis* rDNA is being converted by that of *A. arbuscula* ssp. *arbuscula*, and the fact that those decondensed rDNA loci are preferentially stained in red suggests that also the most active rDNAs come from the last one. However, low levels of nucleotide sequence divergence are found between these three taxa in rDNA regions, although some shared polymorphic sites and a 15 bp indel in the *trn*S-*trn*C region of the chloroplastic DNA by both the hybrid and *A. arbuscula* ssp. *arbuscula* would suggest that the last one might be the maternal genome donor (recent studies, however, have documented a surprising amount of variation in the inheritance patterns of chloroplast DNA; see Hansen et al., 2007). The nucleocytoplasmatic interaction hypothesis states that the paternal genome of newly formed allopolyploids is most likely to reorganize to accommodate an alien cytoplasmatic background (Gill, 1991), which would be consistent with our suggestions, although other studies show that the maternal genome donor undergoes the greatest change (Volkov et al., 1999; Matyasek et al., 2003; Lim et al., 2000). Anyway, the possibility of a different model might not be underestimated, as this hybrid could have a multiple allopolyploid origin and a different behaviour could be observed in another population. In this sense, multiple origins of *Tragopogon* allopolyploids have occurred since the introduction of the diploid species into America (Soltis and Soltis, 2000).

Detection of the plant-telomere repeat

As far as we are aware, there is no available data regarding characteristics of telomeres in *Artemisia*, although Abd-el-Twab and Kondo (2006) detected telomere repeats in one species close to our genus, *Chrysanthemum zawadskii*. As in that case, hybridization of the *Arabidopsis*-type telomere probe took place exclusively at chromosome ends, although

telomere repeats may be present in a lower copy number than in *Chrysanthemum*, as the faint signal strength detected seems to suggest (Figure 1, B). The absence of such sequences at centro- or pericentromeric positions also indicates that chromosome fissions or fussions might not have been involved in the karyotype evolution of these species. Previous karyological studies in this group do not suggest any of these rearrangements in the typical *Tridentatae* karyotype (although translocations are occasionally observed, see above); however, this goes beyond the goal of the present study, which was mainly detecting the presence of the typical plant telomere. Such studies in other subgenera of *Artemisia* would be desirable, particularly in those that have *x*=8 as basic chromosome number (*Absinthium*, *Artemisia*, and *Dracunculus*), which is thought to come from a descending disploidy process involving chromosome fusion (Vallès and Siljak-Yakovlev, 1997).

Conclusions

The present study confirms a typical *Tridentatae* signal pattern at the diploid level, with three rDNA loci located at telomeric positions of metacentric and submetacentric chromosomes and that *Artemisia* presents the *Arabidopsis*-type telomere repeat. At the tetraploid level, congruent similarities with previous research have been reported (Garcia et al., in press), and processes involving locus gain and loss of GC-rich (and possibly AT-rich) DNA are found to be linked to polyploid formation. Given the differential signal pattern of *A. bigelovii* with respect to the other *Tridentatae*, additional support is provided to the hypothesis of an improper placement of this species in the core of the *Tridentatae*, as previous research claimed. GISH failed to ascertain the hybrid origin of the putative allohexaploid of this study. However, it would be interesting to carry out more detailed studies on these species involving other techniques such as Southern blot hybridization, which would help revealing the hidden processes linked to polyploidy which may involve homogeneization, gene conversion or epigenetic silencing, among others, and this study contributes a first step to this purpose.

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FIGURE 1. Fluorochrome banding with chromomycin, fluorescent *in situ* hybridization genomic *in situ* hybridization and a diagrammatic representation of the chromosomes carrying rDNA loci of the different taxa studied (the chromosome marked with asterisk does not represent a chromosome pair, but a single one). (A, B) *A. bigelovii*; (C, D) *A. nova* (arrows in the chromomycin plates indicate a chromosomal translocation); (E, F) *A. tridentata* ssp. *tridentata*; (G, H) *A. tripartita* ssp. *tripartita*.

Scale bars = 10 µm.

Chromomycin 18S-5.8S-26S rDNA loci 5S rDNA loci

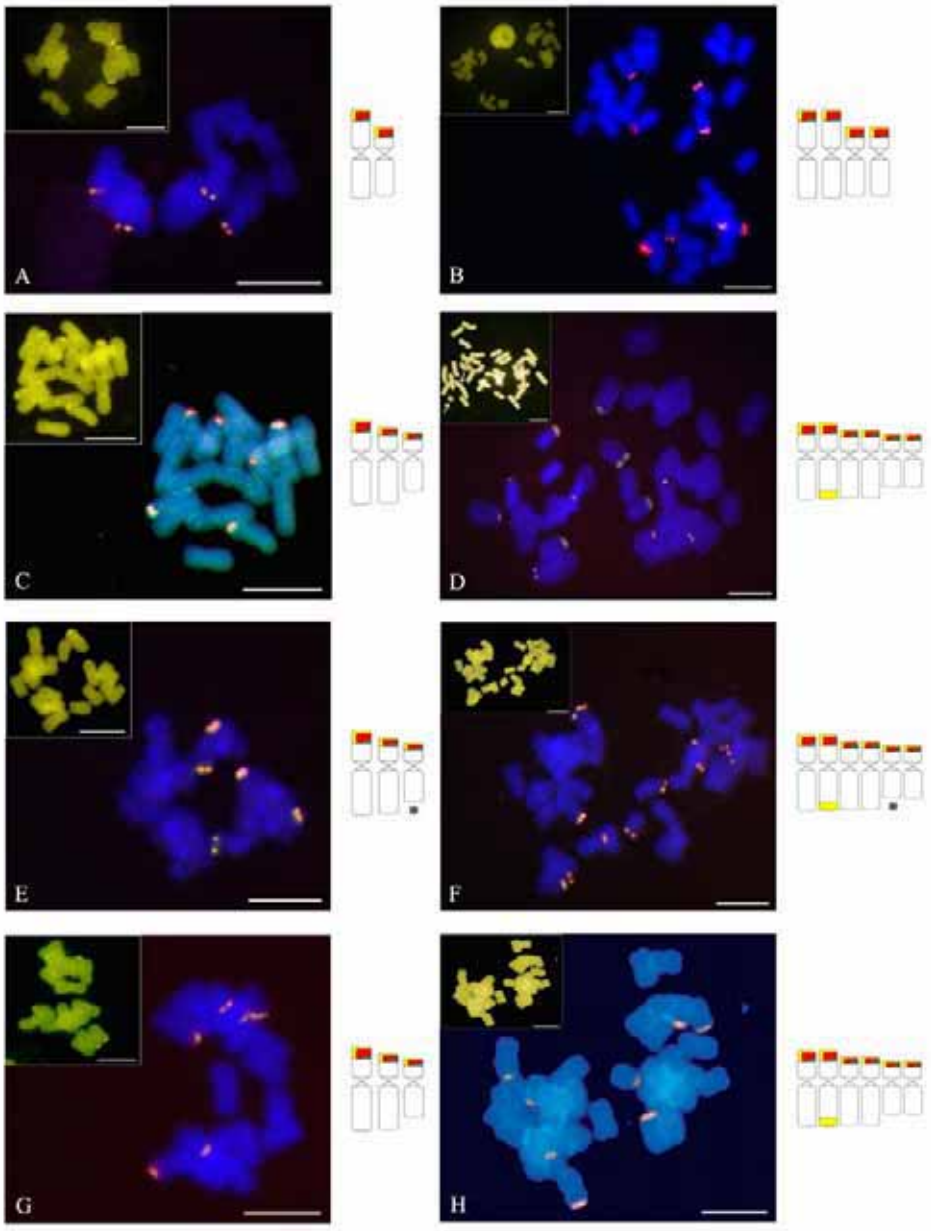

FIGURE 2. (A) Fluorescent *in situ* hybridization of *A. nova* ssp. *duchesnicola* and a diagrammatic representation of the chromosomes carrying rDNA loci; (B) *Arabidopsis*-type telomere probe hybridized in a metaphase plate of *A. nova* (4x); genomic *in situ* hybridization in a metaphase plate of *A. arbuscula* ssp. *longicaulis*, (C) DAPI staining, (D, E) labelling of putative parternal genomes, *A. arbuscula* ssp. *arbuscula* (red) and *A. tridentata* ssp. *wyomingensis* (green), (F) composed images; arrows indicate decondensed rDNA chromosome ends.
Scale bars = 10 µm.

Chromomycin 18S-5.8S-26S rDNA loci 5S rDNA loci

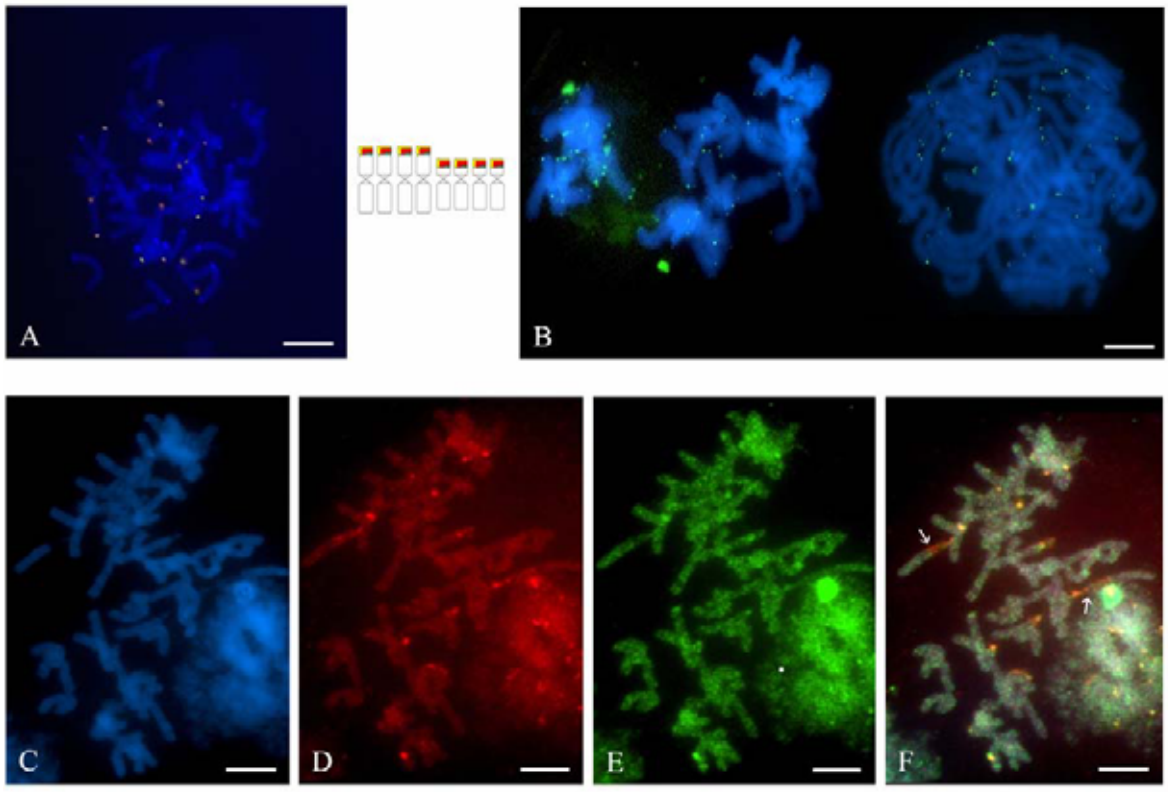

TABLE 1. Provenance of the populations of *Artemisia* studied. The superscript indicates: [1] E. D. McArthur collection numbers; vouchers are deposited in the herbarium of the Rocky Mountain Research Station, Provo, USA (SSLP). (*) These populations were originally described as diploid, but spontaneous tetraploid cytotypes have also been found.

Taxa	Origin of materials	Ploidy level	Coll n.[1]
A. arbuscula subsp. *longicaulis* Winward & McArthur	Bruneau, Owyhee Co. Idaho. 1012 m.	6*x*	2855
A. bigelovii Gray	Emery Co. Utah. 1801 m.	2*x*	2869
A. bigelovii Gray	15 km east of Fremont Junction. Emery Co. Utah. 1777 m.	4*x*	3050
A. nova A. Nels.	Birch Springs Road, Mount Borah, Custer Co., Idaho. 2120 m.	2*x*	3053
A. nova A. Nels.	Tunnel Spring, Desert Experimental Range.	4*x*	2876
A. nova var. *duchesnicola* Welsh & Goodrich	Tridell Road, Uintah Co., Utah. 1702 m.	6*x*	3029
A. tridentata Nutt.	Salt Cave Hollow, Salt Creek Canyon, Juab Co. Utah. 1870 m.	2*x*	2871
A. tridentata Nutt.	Juab Co. tri.	4*x*	U74
A. tripartita Rydb.	Dubois Sheep Station, Clark Co. Idaho. 1650 m.	2*x* and 4*x**	2845

TABLE 2. Chromomycin positive bands, rDNA loci and genome size of the diploid-tetraploid pairs of species studied. Data on genome sizes corresponds to the mean values of the populations assessed up to now (Torrell and Vallès, 2001; Garcia et al., 2004; Garcia et al., unpublished). (*) Between parenthesis, the percentage of diminution of monoploid genome size (1Cx) of tetraploids with respect to diploids.

	2*x*			**4*x***		
	CMA	**FISH**	**2C**	**CMA**	**FISH**	**2C***
A. bigelovii	4	4	8.00	8	8 (4 weak)	15.32 (4.25%)
A. nova	6	6	9.09	16	12 (6 weak)	17.10 (5.9%)
A. tridentata	5/6	5/6	8.24	18	11	15.87 (3.7%)
A. tripartita	6	6	8.85	12	6-8	15.32 (13.44%)

8. Estudis de sistemàtica molecular en el subgènere *Tridentatae* (*Artemisia*, *Asteraceae*) i espècies afins: discordança entre les filogènies nuclears i cloroplàstiques.

Sònia GARCIA, Jaume PELLICER, Joan VALLÈS i Teresa GARNATJE.

La taxonomia i la sistemàtica del subgènere *Tridentatae* no gaudeixen d'un consens clar, possiblement a causa d'una manca de caràcters morfològics per a distingir les seves espècies inequívocament, amb múltiples confusions en el nombre i la circumscripció dels seus membres. El punt de partida d'aquest treball fou analitzar dues regions nuclears (ITS i ETS) i dues de cloroplàstiques (*trnS-trnfM* i *trnC-trnS*) amb la idea d'obtenir una filogènia molecular en la qual es pogués analitzar-ne la història evolutiva. El treball inclou, a més d'una mostra completa de totes les espècies i subespècies del subgènere, una representació de la majoria d'artemísies d'altres subgèneres endèmiques també d'Amèrica del Nord i de dos gèneres estretament relacionats amb aquest, *Sphaeromeria* i *Picrothamnus*. Els arbres filogenètics generats a partir de l'anàlisi de l'ITS i l'ETS, per una banda i d'ambdues regions cloroplàstiques per l'altra mostren filogènies moleculars incongruents la qual cosa suggereix una història complexa d'evolució reticulada en el subgènere. Possiblement fenòmens d'hibridació, poliploïdia i introgressió, tan freqüents en aquest grup, hi estiguin relacionats. No obstant, i malgrat també la poca resolució, àrees de congruència entre els arbres donen suport a clares conclusions taxonòmiques respecte d'espècies històricament problemàtiques. A més, el gènere *Sphaeromeria* és parafilètic, i conjuntament amb el monotípic *Picrothamnus*, apareix immers dins del gènere *Artemisia* i estretament relacionat amb les seves espècies americanes. La sistemàtica molecular d'aquest grup exemplifica els problemes que poden aparèixer en intentar abordar la reconstrucció filogenètica de tàxons tan estretament relacionats i d'evolució relativament recent. D'acord amb els resultats obtinguts, és possible que no s'arribi a desenvolupar un marc filogenètic robust perquè la història evolutiva probablement contingui un excés de reticulació i les relacions entre espècies, més que ajustar-se a una topologia d'arbre, s'entenguin millor com a part d'una xarxa.

Molecular systematics of subgenus *Tridentatae* (*Artemisia*, Asteraceae) and allies: discordance between nuclear and chloroplast phylogenies

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Abstract

A molecular phylogeny based on two nuclear (ITS and ETS) regions has been performed for all members of *Artemisia* subgenus *Tridentatae*, as well as all the other North American endemic *Artemisia*, and also including the monotypic genus *Picrothamnus* and a representation of the genus *Sphaeromeria*, both closely related to *Artemisia*. Additionally, two chloroplastic (*trn*S^{UGA}-*trn*fM^{CAU} and *trn*S^{GCU}-*trn*C^{GCA}) DNA regions were sequenced with the purpose of improving the resolution and statistical supports of the phylogenetic inference. However, the phylogeny obtained with these two regions was incongruent with that produced by nuclear ribosomal DNA. On the basis of the ITS-ETS combined molecular phylogeny some relationships among *Tridentatae* and other *Artemisia* taxa appear to be clearer. The resulting phylogeny advocates for the monophyly of the *Tridentatae* with the exclusion of various species whose position has been historically questioned; hence, this paper claims for a more restrictive concept of this subgenus. From these data, there is no evidence of the existence of different lineages within the *Tridentatae*, despite notable morphological differences. Some North American endemic taxa classically embedded in the subgenus *Artemisia* appear also closely related between them, forming a well supported clade in the ITS phylogeny and separated from the *Tridentatae*; however, subgeneric boundaries with other subgenera of *Artemisia* are less well defined for the North American endemic taxa. Genus *Sphaeromeria* appears nested within the *Artemisia* clade and does not constitute a monophyletic association, with some of its representatives closely related

with the monotypic *Picrothamnus*, also embedded in the *Artemisia* clade. Processes involving reticulate evolution may contribute to difficult the interpretation of relationships among these species and genera.

Key words: Compositae, chloroplastic DNA, diversification, evolution, external transcribed spacer (ETS), incongruence, internal transcribed spacer (ITS), molecular phylogeny, reticulation, sagebrushes, speciation, *trnS*^{GCU}*-trnC*^{GCA}, *trnS*^{UGA}*-trnfM*^{CAU}.

Introduction

The genus *Artemisia* L. is the largest of the tribe Anthemideae (Asteraceae), comprising around 500 species (Vallès and McArthur, 2001; Vallès and Garnatje, 2005), many of them ecologically and economically relevant. It is widely distributed along the Northern hemisphere, with five subgenera currently accepted by most authors: *Artemisia*, *Absinthium*, *Dracunculus*, *Seriphidium* and *Tridentatae*, although subgeneric classification is still subject of rearrangements in the light of recent molecular studies (Watson et al., 2002; Vallès et al., 2003; Sanz et al., submitted). The exclusive North American *Artemisia* are of particular interest in the evolutionary history of the genus, in which the most representative group is its endemic subgenus *Tridentatae*, also known as sagebrushes. These species characterize the landscape of Western North America, and are probably the most common shrub plants in the whole United States. Sagebrushes consist in about a dozen species (depending on the authors), all of them perennial, woody and xerophytic, although they can colonize a broad ecological range, from dry valley floors to mesic mountain tops with higher precipitations (West, 1983).

Systematic and taxonomic problems of the Tridentatae

Since early works of Pursh, who described the first *Tridentatae* species, *A. cana* Pursh (Torrey and Gray, 1843; Rydberg, 1916), sagebrushes classification over and under subgeneric level has been classically difficult. Subgeneric placement is controversial, because traditionally, section *Tridentatae* has been placed in subgenus *Seriphidium*, based on floral morphology (Rydberg, 1916), although we follow McArthur and Plummer (1978) and McArthur et al. (1981) who raised the *Tridentatae* to subgeneric status, and explained the similarity between both groups as a result of convergent evolution, which

is consistent with their appearance as two independent clades in a first phylogenetic survey of the genus (Vallès et al., 2003). Indeed, *Seriphidium* itself has sometimes been segregated as a genus (Bremer and Humphries, 1993; Ling, 1991, 1995), although its separation from *Artemisia* is not supported by neither cpDNA restriction site nor ITS sequence phylogenies (Kornkven et al., 1998, 1999; Torrell et al., 1999).

The interspecific relationships and boundaries of the sagebrushes are unclear, although it is generally considered as a monophyletic group (Kornkven et al., 1998, 1999). Indeed, two lineages had been proposed within the subgenus (Ward, 1953; Beetle, 1960; Shultz, 1983; see Table 2), but the available molecular and morphological data do not support their recognition. Moreover, several species have been included and subsequently excluded in countless studies based on different approaches such as morphological, cytogenetical, and chemical (Rydberg, 1916; Hall and Clements, 1923; Ward, 1953; Beetle, 1960; Holbo and Mozingo, 1965; Kornkven et al., 1998, 1999). *Artemisia bigelovii*, *A. californica*, *A. filifolia, A. palmeri*, *A. pygmaea*, and *A. rigida* are particularly renowned from this point of view. *In situ* hybridization assays and genome size data (Garcia et al., in press; Garcia et al., unpublished) may shed light in some particular cases. Additionally, two North American endemic subgenera closely related with *Artemisia*, *Picrothamnus* and *Sphaeromeria*, have also appeared embedded in the North American endemic *Artemisia* clade in recent works (Watson et al., 2002; Vallès et al., 2003; Sanz et al., submitted). Unfortunately, and despite continuous research efforts, we have not yet an adequate phylogenetic framework in which to analyze relationships among these species and relatives.

Molecular approach

Given the above-cited problems that most researchers have faced when working in this group, our attempt to resolve its phylogenetic relationships includes a combined set of molecular data, as well as considering recent works including genome size and *in situ* hybridization data (Garcia et al., in press; Garcia et al., unpublished).

As nuclear gene regions, the rDNA internal and external transcribed spacers (ITS and ETS) have been our choice. The ITS region had been previously tested in the *Tridentatae* (Kornkven et al., 1998), although it yielded no high resolution of interspecific relationships within the group; however, we decided to try this region again as all subspecific entities of the *Tridentatae*, most of the remaining North American endemic

Artemisia and two closely related genera (*Picrothamnus* and *Sphaeromeria*) were included for the first time in a single molecular phylogenetic analysis. The virtues of this region (biparentally inherited, high rates of base substitution, ease of amplification with universal primers) have made the ITS extensively used by molecular systematists (Baldwin et al., 1995), although either complete or incomplete concerted evolution can be a source of problems in phylogenetic reconstructions (Mayol and Rosselló, 2001; Álvarez and Wendel, 2003; Nieto Feliner and Rosselló, in press). However, for some recently evolved angiosperm lineages, the ITS is not enough informative due to insufficient sequence variation (Francisco-Ortega et al., 1997; Susanna et al., 1999). Hence we decided to include data from another rDNA region, the ETS, which represents a good candidate for augmenting or replacing the ITS (Baldwin and Markos, 1998; Bena et al., 1998). It is generally longer than ITS, easily amplifiable with universal primers and has been proved useful at low taxonomic levels (Linder et al., 2000), with recent examples in the family Asteraceae (Hidalgo et al., 2006; Garnatje et al., 2007), although, as the ITS region, it is not free from problems related with concerted evolution.

Since the combination of different genomes is considered one of the best tools for phylogenetic reconstructions (Qiu et al., 1999), we decided to add chloroplast sequence data to our molecular study. Even though many noncoding chloroplastic regions are known, the majority of phylogenetic investigations based in cpDNA have basically used *trn*L-*trn*F and *trn*K/*mat*K regions, although in comparative studies their phylogenetic utility at low taxonomic levels is often limited with respect to other regions (Sang et al., 1997; Small et al., 1998). Previous work with *trn*L-*trn*F in subgenus *Tridentatae* (Stanton et al., 2002) and in subtribe *Artemisiinae* also proved uninformative (Vallès et al., 2003). Based on the pioneering work of Shaw et al. (2005), *trn*S^{UGA}-*trn*fM^{CAU} and *trn*S^{GCU}-*trn*C^{GCA} were selected. Although three genes are embedded in this region, *trn*S^{UGA}-*trn*fM^{CAU} has recently shown high variability in some plant groups (McKenzie et al., 2006; Mort et al., 2007), but its use is restricted to date. The second is exclusive of the Asteraceae, and has never been used with phylogenetic purposes up to now. The Asteraceae present two DNA inversions in the LSC region of the chloroplastic DNA, originated simultaneously during its early evolution (Kim et al., 2005). The region *trn*S^{GCU}-*trn*C^{GCA} corresponds to part of the regions *rpo*B-*trn*C^{GCA} and *trn*S^{GCU}-*trn*G^{UUC}-*trn*G^{UUC} in subfamily Barnadesioideae (Asteraceae) and in the rest of angiosperms. Indeed, this two last regions, together with *trn*S^{UGA}-*trn*fM^{CAU}, are three of the five regions providing the

greatest number of potentially informative characters across all phylogenetic lineages (Shaw et al., 2005).

On the basis of this knowledge, we thought that both *trn*S^{UGA}-*trnf*M^{CAU} and *trn*S^{GCU}-*trn*C^{GCA} could be a good choice. We also tried to use for the phylogenetic reconstruction a single copy nuclear gene, the chloroplast-expressed glutamine synthetase (ncpGS), as different studies stated its potential application for resolving relationships among closely related species, particularly at lower taxonomic levels (Emshwiller and Doyle, 1999). However, a preliminar approach did not yield enough variation to infer phylogenetic relationships (data not shown).

In summary, the present study attempts to resolve phylogenetical relationships for these taxa, with the specific goals of: a) examining the monophyly and circumscription of subgenus *Tridentatae*; b) studying the link with the close genera *Sphaeromeria* and *Picrothamnus* with respect to the genus *Artemisia*; c) resolving interspecific relationships; d) identifying the subgeneric placement of the other North American endemic *Artemisia*; e) examining congruence between nuclear and chloroplastic phylogenies, in order to assess possible interspecific gene flow.

Material and Methods

***Plant material*---** Plant material for 42 populations was obtained, including at least a representation of every species and subspecies of Artemisia subgenus Tridentatae as well as all the remaining North American endemic Artemisia, five Spaheromeria species and one population of the monotypic genus Picrothamnus. Additional representatives of each subgenera which were not endemic from North America were chosen as outgroups. Table 1 shows the provenance of all the species investigated.

***Molecular techniques*---** Total genomic DNA was extracted using either the CTAB method of Doyle and Doyle (1987) as modified by Soltis et al. (1991), or the Nucleospin Plant (Macherey-Nagel, GmbH & Co., Düren, Germany), depending on the quality of the vegetal material, either from silica gel dried leaves collected in the field, fresh leaves of plants cultivated in greenhouses (Institut Botànic de Barcelona; Facultat de Farmàcia, Universitat de Barcelona) or herbarium material (see Table 1). PCR was performed using either GRI Labcare or MJ research thermal cyclers in 25 µl volume. A key for the PCR parameters is as follows: initial denaturing step (temperature, time); number of repetitions of the amplification cycle [#X (denaturing temperature, time; primer annealing temperature, time; chain extension temperature, time)]; final extension step (temperature, time). All reactions ended with a final 10ºC hold step. Subsequently, PCR products were purified with either the QIAquick PCR purification kit (Qiagen, Valencia, California, U.S.A.) or the DNA Clean & Concentrator-5 D4003 (Zymo Research, Orange, U.S.A.). Direct sequencing of the amplified DNA segment was performed using the Big Dye Terminator Cycle sequencing v3.1 (PE Biosystems, Foster City, California, U.S.A.). Nucleotide sequencing was carried out at the Serveis

Cientificotècnics (Universitat de Barcelona) on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City, California, U.S.A.). DNA sequences were edited by Chromas 1.56 (Technelysium PTy, Tewantin, Queensland, Australia) and aligned visually. This matrix is available fom the corresponding author. The deposite of sequences at GenBank is pending.

ITS region: Double-stranded DNA of ITS region (including ITS1, ITS2 and 5.8S gene) was amplified by PCR with either 1406F (Nickrent et al., 1994) or ITS1 (White et al., 1990) as forward primers, and ITS4 (White et al., 1990) as the reverse primer. The PCR profile used for amplification was: 94ºC, 2 min; 30X (94ºC, 1 min 30 s; 55ºC, 2 min; 72ºC, 3 min); 72ºC, 15 min. Because the average length of this region is relatively short (around 800 bp), only the ITS4 primer was used in sequencing in most cases, although both forward primers have also been used some times when necessary.

ETS region: Double-stranded DNA ETS region was amplified with the ETS1f as forward and the 18SETS as reverse primers (Baldwin and Markos, 1998), and occasionally, also with the 18S2L as reverse primer (Linder et al., 2000). The PCR profile used for amplification was: 95ºC, 5 min; 30X (94ºC, 45 s; 50ºC, 45 s; 72ºC, 40 s); 72ºC, 7 min. Due to the length of this region (around 1700 bp) both strands had to be sequenced, mostly using the ETS1f and 18SETS as sequencing primers, although AST1R and AST1F were used some times. However, only the most conserved regions at the 3' and 5' ends were utilized for the phylogenetic reconstruction.

trnS^{UGA}*-trnfM*^{CAU}: This region was amplified with *trnS*^{UGA} (forward) and *trnf*M^{CAU} (reverse) primers (Demesure et al., 1995). The amplification parameters were 80ºC, 5 min; 30X(94ºC, 30 s; 62ºC, 1min 30 s; 72ºC, 2 min) 72ºC, 5 min. The trnS^{UGA} was used as sequencing primer, but occasionally the trnfM^{CAU} was also needed.

trnS^{GCU}*-trnC*^{GCA}: The primers *trnS*^{GCU} (Shaw et al., 2005), as forward, and *trnC*^{GCA}R (modified by Shaw et al., 2005, from Ohsako and Ohnishi, 2000), as reverse, were used to amplify this region. The PCR parameters were the same as for *trnS*^{UGA}*-trnfM*^{CAU}. This fragment was sequenced with *trnS*^{UGA} primer, although the *trnC*^{GCA}R was occasionally needed.

Data analysis--- The outgroup species were *A. absinthium*, *A. deserti*, *A. dracunculus* and *A. frigida* (following the criteria of choosing a representation of each subgenera of *Artemisia* and not being endemic to North America).

Parsimony analysis: These analyses involved heuristic searches conducted with PAUP 4.0b4a (Swofford, 1999) with Tree Bisection Reconnection (TBR) branch swapping and character states specified as unordered and unweighted. All most parsimonious trees were saved. To locate other potential islands of most parsimonious trees (Maddison, 1991), we performed 100 replications with random taxon addition, also with TBR branch swapping. Bootstrap analysis (BS) (Felsenstein, 1985) was carried out to obtain an estimate of support of the branches. Bootstrap analysis was performed using 100 replicates of heuristic search. Calculations were performed with PAUP.

Model selection and Bayesian inference analysis: To determine models under the Akaike Information Criterion (AIC) and hLRT (Posada and Buckley, 2004), data set was analysed using MrModeltest 2.2 (Nylander, 2004), subsequently used to perform a Bayesian analysis with MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001). Four Markov chains were run simultaneously for 1,000,000 generations, and these were sampled every 100 generations. Data from the first 1000 generations were discarded as the burn-in period,

after confirming that likelihood values had stabilized prior to the 1,000th generation. Posterior probabilities were estimated through the construction of a 50% majority rule consensus.

Results and Discussion

Combined data set analysis and incongruences among gene trees

Table 3 summarizes the characteristics of the data sets studied. Phylogenies produced with ITS and ETS regions (both in separate and combined analyses, Fig. 1) agreed to some extent with the results obtained in previous work (morphological, chemical, phylogenetic and cytogenetic studies; Kornkven et al., 1998, 199; Watson et al., 2002; Vallès et al., 2003) much better than with chloroplastic DNA phylogeny; both phylogenies had several points of incongruence, so we did not combine the data from chloroplastic DNA regions (Fig. 2). The controversial results could be due to past events of chloroplast capture, previously suggested as a cause of incongruence (Kornkven et al., 1999) or past hybridization events. Other causes such as taxon density, sampling error, convergence and heterogeneity of evolutionary rates can potentially produce erroneous phylogenetic reconstructions leading to incongruence between phylogenetic trees based on different genomes (Soltis and Kuzoff, 1995).

By and large, results are consistent with previous work on the whole genus, which characterized a North American endemic group including species from subgenus *Tridentatae* and the other genera *Picrothamnus* and *Sphaeromeria* (Watson et al., 2002; Vallès et al., 2003; Sanz et al., submitted), this time with a complete representation of all the *Tridentatae*, most of the other North American endemic *Artemisia* and a larger set of *Sphaeromeria* representatives.

The group of true sagebrushes and their hybrids

The clade embracing the *Tridentatae s. s.* is supported in the combined ITS+ETS analysis (Fig. 1) with a value of 98% of PP; we believe this is a natural group, as all the species within this clade are recognized *Tridentatae* members whose placement within this subgenus has never been put into question. So, according to these results, the monophyly of subgenus *Tridentatae* is supported, with the exclusion of *A. bigelovii*, *A. rigida* and *A. pygmaea* (*sensu* Shultz, 2006), three species classically included within the true sagebrushes. ITS and ETS inference propose two potential sister groups to the

subgenus *Tridentatae s. s.*: *A. argillosa*, and a clade whose composition is difficult to explain on morphological basis, including *A. californica*, *A. nesiotica*, *A. pygmaea* and two members of genus *Sphaeromeria*, *S. cana* and *S. diversifolia*; however, although this is a highly supported clade, with 97% PP in Bayesian inference, maximum parsimony analysis of the ITS region (Fig. 3) shows that *A. californica* and *A. nesiotica* are separated from the remaining species of this group (88% BS, not shown), and appear in a more basal position within the North American endemic *Artemisia*.

The close relationship between *A. argillosa* and the *Tridentatae* s. s. suggested in our phylogenetic inference is also supported by the fact that this taxa presents the morphological characteristics defining this subgenus (shrubs, with leaves in lateral fascicles, discoid, homogamous heads with hermafrodite flowers and glabrous receptacle). The specific status of this taxon is not yet currently accepted, and it appears as a synonym of *A. cana* subsp. *viscidula* in the Flora of North America (Shultz, 2006). However, *A. argillosa* is never closely related to any other species in the distinct phylogenies produced, which would reject previous hypotheses about its hybrid origin between *A. cana* subsp. *viscidula* and *A. longiloba* (Beetle, 1959). Additionally, the groups more closely related to *A. argillosa* are well supported and segregate from this species. Together with previous findings (Garcia et al., in press), these results also claim for the uniquity of this taxon and indeed to its best consideration at the specific status.

However, ETS and ITS combined phylogeny does not fully resolve interspecific relationships within this group, with subspecific entities of the same taxon (*A. arbuscula*, *A. cana*, *A. tridentata*) spread within well supported clades. Previous work (Ward, 1953; Beetle, 1960; Shultz, 1983) had suggested the existence of different lineages within the sagebrushes (Table 2), mostly based on leaf morphology, habitat preference and ability to layer and root-sprout after fire. Later work based on molecular phylogenetic analyses (Kornkven et al., 1998, 1999), however, failed to discriminate any of these groupings, as does ours. Actually, many subspecies in the *Tridentatae* are suggested to be hybrid combinations between two taxa (Beetle, 1960; Winward and McArthur, 1995), which may explain the odd groupings within this clade *prima facie*. Nevertheless, there is a well differentiated clade (95% PP) and some supported assemblages. On the one hand, the association between *A. arbuscula* subsp. *arbuscula* and *A. arbuscula* subsp. *thermopola* (99% PP) is not unexpected, being subspecific entities of the same taxon (general appearance and flowering period shared by both subspecies), and could be related with the presumed hybrid origin of *A. arbuscula* subsp. *thermopola* (*A. arbuscula* subsp.

arbuscula x A. tripartita subsp. *tripartita*; Beetle, 1960); in this case, the comparison of chloroplastic DNA sequences of the three taxa does not reject this hypothesis (although the possibility of other progenitor species is not dismissed), as 75% of polymorphic sites are shared with *A. arbuscula* subsp. *arbuscula*, and 25% with *A. tripartita* subsp. *tripartita*.

On the other hand, *A. nova* and *A. tridentata* subsp. *vaseyana* appear also closely related (98% PP): both species share distribution area, and are widely distributed; moreover, the first has sometimes been described as a subspecies of *A. tridentata*, and also as a subspecies of *A. arbuscula* which, likewise, has been described as subsp. of *A. tridentata*. The close relationship between these three entities, *A. arbuscula*, *A. nova* and *A. tridentata*, foreshadowed by all those nomenclatural reordenations, explains the high support of the clade that groups them (99% PP, 89% BS).

Additionally, *A. cana* subsp. *bolanderi* and *A. rothrockii* are grouped (99% PP) probably because they share geographic distribution and due to the likely hybrid origin of *A. rothrockii* from a cross between representative(s) of the *A. cana* "complex" with other(s) from the *A. tridentata* "complex" (Beetle, 1960). If this was the case, we think that *A. cana* subsp. *bolanderi* would be one putative parent and *A. tridentata* subsp. *xericensis* would be the other, as the ITS phylogeny groups *A. tridentata* subsp. *xericensis* with *A. rothrockii* (99% PP, Fig. 3). Chloroplast DNA sequence comparison does not provide evidence about the putative maternal genome donor, as *A. rothrockii* shares 50% of polymorphic sites with either presumed progenitor.

Another well supported clade is the one joining *A. arbuscula* subsp. *longicaulis* with *A. tridentata* subsp. *wyomingensis* and *A. nova* subsp. *duchesnicola* (Fig. 1, 98% PP). The relationship of the two first taxa could also be explained as a result of hybridization (*A. arbuscula* subsp. *longicaulis* = *A. arbuscula* subsp. *arbuscula* x *A. tridentata* subsp. *wyomingensis*; Winward and McArthur, 1995); the link with *A. nova* subsp. *duchesnicola* is not surprising; as previously commented, *A. nova* has been described as a subspecies of either *A. arbuscula* or *A. tridentata*.

Repeated episodes of hybridization, which seem to be apparent in this group, complicate the interpretation of phylogenetic relationships from sequence patterns (Suárez-Santiago et al., 2007). Additionally, if the hybrid hypotheses here presented were true, gene conversion of rDNA to one parent's type would be also probably taking place, as in most cases the presumed hybrid shows a close relationship with one of the putative progenitors whereas the other putative progenitor appears well segregated.

Placement of conflictive species

Although the monophyly of subgenus *Tridentatae* has also been supported by several independent studies (Kornkven et al., 1998; Torrell et al., 1999; Vallès et al., 2003), *A. bigelovii*, *A. pygmaea*, and *A. rigida* classically included in this group, have always been subject of controversy, with countless studies proposing either their inclusion or exclusion. Moreover, neither hybrids nor subspecific entities of these three taxa have ever been described (differently from the other *Tridentatae*). In the case of *A. bigelovii*, its floral morphology (the only *Tridentatae* with heterogamous capitula; Hall and Clements, 1923; Ward, 1953; Shultz, 1983; Ling, 1991, 1995), molecular phylogenetic data (Kornkven et al., 1998), essential oil composition (Holbo and Monzingo, 1965; Geissman and Irwin, 1974) and our own results on molecular cytogenetics using FISH (Garcia et al., unpublished) clearly differentiate it from the *Tridentatae* s. s. This species has been generally treated as a *Tridentatae* on the basis of many characters such as wood anatomy, leaf form, karyotype morphology, RAPD genetic markers and cpDNA restriction site analyses (McArthur et al., 1981, 1998a; Kornkven et al., 1999). However, our present results add evidence to the segregation of *A. bigelovii* from the true sagebrushes. The combined ITS and ETS molecular phylogeny places this species well separated from the core of the *Tridentatae*, in a particularly surprising and highly supported grouping (98% PP, Fig. 1) together with another conflictive sagebrush species, *A. rigida* (commented further on), other North American members of subgenus *Dracunculus*, the monotypic genus *Picrothamnus* (the species of which has a nomenclatural synonymy with *Artemisia spinescens*, considered a member of subgenus *Dracunculus*,) and two *Sphaeromeria* species. Excepting *A. rigida*, all members of this assemblage present heterogamous capitula (although disk flowers are perfect and fertile in *A. bigelovii* in contrast to the sterile, functionally male disk flowers of subgenus *Dracunculus*). Chloroplast DNA phylogeny does not clearly indicate any relationship, although *A. bigelovii* appears grouped with most *Tridentatae* species and one member of subgenus *Dracunculus*, *A. pedatifida*, but with no significant statistical support (PP 56%, Fig. 2).

Artemisia rigida is a species particularly well adapted to restricted habitats and displays specialized morphological and anatomical modifications to extreme conditions of aridity (Hall and Clements, 1923; Shultz, 1983). It was placed alone in another section within subgenus *Seriphidium*, sect. *Rigidae* Rydb. (Rydberg, 1916). Holbo and Mozingo's

(1965) chromatographic characterization point to its exclusion from the *Tridentatae*; as well as our findings with *in situ* hybridization, genome size studies (Garcia et al., in press) and the present results of this molecular phylogeny (ITS+ETS, Fig. 1), which place it well separated from the core of the sagebrushes and which also suggest a tight relationship with *A. bigelovii* (98% PP). Kornkven et al. (1999) stated that *A. rigida* may have diverged early in the evolution of the subgenus, with its placement as sister to the core *Tridentatae* species, in addition to its morphological specialization.

Artemisia pygmaea is a dwarf shrub with different leaf morphology and larger seeds as compared with the other *Tridentatae* (Cronquist, 1994; McArthur and Stevens, 2004). It is a relatively uncommon species, limited to calcareous soils in the desert areas of central and western Utah, eastern Nevada and northern Arizona (Ward, 1953). Based on the specialized features that requires its ecology, Rydberg (1916) also placed *A. pygmaea* in a separated section (sect. *Pygmaea* Rydb.) in subgenus *Seriphidium*. Essential oil composition (Holbo and Mozingo, 1965; Geissman and Irwin, 1974), some differences in karyotype morphology (Garcia et al., in press) and a significantly larger genome size (Garcia et al., unpublished), also differentiate it from the core of the true sagebrushes. Previous molecular biology studies have placed this species as sister to the other *Tridentatae* (Kornkven et al., 1998; Watson et al., 2002). The results produced by the present ITS+ETS phylogenetic analysis would also place *A. pygmaea* in a sister group of the true sagebrushes, in a clade (97% PP, Fig. 1) together with other North American endemic *Artemisia* and some *Sphaeromeria* species.

However, we cannot ignore the association suggested by the cpDNA phylogeny, where *A. rigida* and *A. pygmaea* are grouped together with some other *Tridentatae* species, in a supported clade (95% PP, Fig. 2); this is also consistent with previous findings of a molecular phylogeny based on chloroplast DNA restriction site variation (Kornkven et al., 1999), where *A. rigida* appeared within the *Tridentatae* clade, but in an isolated position, and *A. pygmaea* together with other *Tridentatae* members. Maybe an ancient hybrid origin of these two taxa in the early evolution of the subgenus is responsible for these apparent incongruences concerning these taxa between both phylogenies, although we are hesitant to suggest such a possibility before additional molecular evidence is provided to support this hypothesis.

Other North American endemics

Several North American endemic species of *Artemisia* are traditionally embedded in different subgenera on the basis of floral morphology and other morphological traits, but sometimes their placement has been discussed, and the boundaries between subgenera are not so clear within North American endemics as within other *Artemisia* from other regions. Particularly, the position of *A. palmeri* has been controversial: it is a herbaceous perennial endemic to the California coast which was placed into a segregate monotypic genus, *Artemisiastrum palmeri* Rydb. based on the presence of receptacular bracts (Rydberg, 1916), but the presence of homogamous flower heads lead various authors to subsume it in sect. *Tridentatae* (Hall and Clements, 1923; Ling, 1991, 1995). As previous work (Kornkven et al., 1999) our present findings place it well separated not only from the *Tridentatae* but also from many other *Artemisia* and the close genera *Sphaeromeria* and *Picrothamnus*. Indeed, the tree corresponding to the analysis of the ITS region alone (Fig. 3) displays a clear relationship with other members from subgenus *Artemisia*, particularly with those forming the *A. vulgaris* complex (100% PP); unfortunately, we were not able to obtain ETS sequences for *A. carruthii*, *A. papposa* and *A. vulgaris*, but the combined ITS+ETS phylogeny also shows a clear relationship with *A. ludoviciana* subsp. *ludoviciana*, another typical member of this group (100% PP, 100% BS). On the other hand, the relationship between *A. californica* and *A. nesiotica* is clear (the last one was first described as *Crossostephium insulare* Rydb. and further combined as *A. californica* Less. var. *insularis* (Rydb.) Munz.) and confirmed with a high statistical support (100% PP, 100% BS) according to our phylogeny. The connection of these two taxa with respect to their subgenus, however, is not that clear, as these two species appear segregated with respect of the remaining subgenus *Artemisia* members (commented in the previous paragraph). The chloroplast DNA molecular phylogenetic reconstruction (Fig. 2) also places *A. californica* and *A. nesiotica* well separated of the other subgenus *Artemisia* members (only *A. ludoviciana* and *A. palmeri* in this tree, as we could not obtain cpDNA sequences of *A. carruthii*, *A. papposa* and *A. vulgaris*). Similar results regarding this subgenus have been obtained in comprehensive studies of the whole genus, in which species traditionally assigned to the subgenus *Artemisia* appear well spread all over the phylogenetic tree (Torrell et al., 1999; Vallès et al., 2003); this is probably another sign of the artificial traditional classification prevailing in *Artemisia*. Moreover, the fact that both *A. californica* and *A. nesiotica*, fairly similar in overall appearance, show different floral

morphologies (*A. californica* capitula are heterogamous and *A. nesiotica*'s are homogamous) indicates that this character, classically used for distinguishing between subgenera would not have systematic value at this level, as it seems that homogamous flower heads have appeared several times during the evolution of *Artemisia*.

The position of *Artemisia filifolia* had also been questioned, some authors suggesting a close relationship with the subgenus *Tridentatae*: it has some cytological, chloroplast DNA and chemical similarities with the sagebrushes (Kelsey and Shafizadeh, 1979; McArthur and Pope, 1979; Kornkven et al., 1999), although it is different in floral characteristics, wood anatomy (absence of interxylary cork; Moss, 1940) and ITS data sequence (Kornkven et al., 1998). *Artemisia filifolia* is traditionally assigned to subgenus *Dracunculus* on the basis of floral morphology. The results produced by this molecular phylogeny place it well separate from the core of the *Tridentatae*, although the subgeneric placement of *Artemisia filifolia* cannot be entirely resolved from the present data.

Sphaeromeria *and* Picrothamnus*: are they disguised* Artemisia*?*

Sphaeromeria and *Picrothamnus* are the only genera with exclusive North American distribution within the *Artemisiinae* (apart from *Artemisiastrum*, which is not frequently considered as commented above; Bremer and Humphries, 1993), or according to the most recent classification of the Asteraceae, within the *Artemisia* group (Oberprieler et al., 2007). Previous phylogenetic approaches (Watson et al., 2002; Vallès et al., 2003; Sanz et al., submitted) did not fully resolve their actual placement, although all agreed indicating their close relationship with *Artemisia*. Our own results add evidence to the close link uniting these three genera. The present molecular phylogenetic analysis (ITS+ETS, Fig. 1) places both genus well embedded in highly supported clades shared with other *Artemisia* members. Moreover, *Sphaeromeria* does not form a monophyletic group, with the five taxa studied (the genus consists of nine species) grouped in segregated clades. Additionally, the monotypic genus *Picrothamnus* is also immersed in a clade (98% PP) uniting other *Artemisia* and *Sphaeromeria*; particularly, *Picrothamnus desertorum* joins *Sphaeromeria argentea* in a highly supported clade (100% PP, 100% BS). These two taxa share distribution area and part of the blooming period (Shultz, 2006) which could account for intergeneric hybridization for explaining such a close relationship. Finally, although chloroplast DNA phylogeny does not resolve any grouping

involving species of these genera, it also places all *Sphaeromeria* species and *Picrothamnus desertorum* within all the other North American endemic *Artemisia* (98% PP, Fig. 2).

Morphological appearance of *Sphaeromeria* is remarkably different from that of *Artemisia* species, indeed many of its species were previously classified as members of *Tanacetum* (see Shultz, 2006). However, previous work of Holmgren et al. (1976) best treated *Sphaeromeria* closer to *Artemisia* than to *Tanacetum* on the basis of morphological traits; consistent results with these observations where obtained with RAPD analysis (McArthur et al., 1998b). The presence of interxylary cork is a characteristic shared by these three genera (Moss, 1940; Wood, 1966; Holmgren et al., 1976). On the other hand, morphological similarities of *Picrothamnus desertorum* with *Artemisia* members are clear; it was first described by Nuttall (1841), but later considered as a species from the genus *Artemisia*, *A. spinescens* D. C. Eaton. Hall & Clements (1923) consider it "in all essentials an *Artemisia* of the section *Dracunculus*", because of their functionally male central florets with reduced ovaries and fused style-branches. The cobwebby-pilose corollas and the spiny habit would be autoapomorphies of this species (Bremer and Humphries, 1993). In the molecular phylogeny obtained, *P. desertorum* appears grouped with other *Sphaeromeria* and *Artemisia*, mainly from subgenus *Dracunculus*, and with heterogamous flower heads (with the exception of *A. rigida*).

These results, i. e., that the genus *Sphaeromeria* is clearly paraphyletic in its current circumscription, and that together with *Picrothamnus* they are well immersed (with high statistical support) in the *Artemisia* group, would advise for a redefinition of these genera, including their species within *Artemisia*. They might form taxonomical sub-entities within *Artemisia* whose position cannot be determined due to the lack of resolution of this phylogenetic reconstruction. A complete data set including all *Sphaeromeria* species, as well as molecular cytogenetic studies, to date not performed neither in *Sphaeromeria* nor in *Picrothamnus*, might help uncover relationships within this group in *Artemisia*.

Concluding remarks

The results provided by this phylogenetic approach to this group of plants point that considering the geographic origin is very important in the systematic assessment of closely related species, as many of the associations shown are probably more congruent

with the geographic origin than with their subgeneric or even generic placement (Fuertes-Aguilar et al., 1999). With regards to subgenus *Tridentatae*, we think that it should be reduced to a limited number of species (*A. arbuscula*, *A. cana*, *A. longiloba*, *A. nova*, *A. rothrockii*, *A. tridentata* and *A. tripartita*) and their hybrids or subspecific entities. It is a group of closely related plants which share distribution areas and form wide populations where gene flow is common. Because of absence of reproductive barriers, reticulate events involving different kinds of hybridization among all these taxa (allopolyploidy, homoploid hybrid formation, introgression) may be abundant, giving birth to multiple and recurrent combinations which contribute to obscure true relationships among taxa and enhance the well-deserved reputation of this group of being taxonomically difficult. A different approach, probably involving studies at the population level and network analyses, will be useful in elucidating interspecific relationships in this group. *Artemisia argillosa* is potentially a member of the *Tridentatae* s. s., however, more data are required to confirm this point.

According to the ITS and ETS phylogenetic reconstruction, other species such as *A. bigelovii*, *A. rigida* and *A. pygmaea* may be segregated from the *Tridentatae s. s.* Otherwise, many recognized *Artemisia* members from other subgenera (*Artemisia* and *Dracunculus*), *Picrothamnus* and *Sphaeromeria*, should fit in this definition. As previously stated, many other data from different sources support the exclusion of these three controversial species from the core of sagebrushes (Garcia et al., in press, and references therein), although chloroplast DNA results placing *A. pygmaea* and *A. rigida* together with other *Tridentatae* cannot be neglected, and could point again to the reticulate history of this group. Additionally, neither *A. californica*, *A. filifolia* nor *A. nesiotica* should be included in the *Tridentatae*, as a previous study suggested, which also redefined *Artemisia* subgenus *Tridentatae* as comprising North American shrubs with both homogamous and heterogamous capitula (Shultz, 2005). Our proposal, on the contrary, is a restrictive one, limiting the concept of the *Tridentatae s. s.* to a small, homogeneous and hybridizing group of eight closely related species, in which reticulate relationships between them (i. e. subspecific entities of the same species spread in different clades) might contribute to their evolution and current species richness and diversity.

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TABLE 1. Origin and herbarium vouchers (BCF=herbarium of the Faculty of Pharmacy, University of Barcelona; BCN=herbarium of the University of Barcelona, Centre de Documentació de Biodiversitat Vegetal; EDM=E. Durant McArthur collection numbers; and other particular collection accession numbers). GenBank accession numbers for ITS, ETS, *trn*S^{UGA}-*trn*fM^{CAU} and *trn*S^{GCU}-*trn*C^{GCA} pending.

Taxon	Collection data and herbarium voucher
Artemisia absinthium L.	Setcases, Catalonia, Spain. (BCF 43821)
Artemisia arbuscula Nutt. subsp. *arbuscula*	Corn Creek Canyon, Millard Co., Utah., USA. (EDM 2877)
Artemisia arbuscula Nutt. subsp. *longicaulis* Winward & McArthur	Toulon, Pershing Co., Nevada, USA. (EDM 2860)
Artemisia arbuscula Nutt. subsp. *thermopola* Beetle	Yellowstone, National Park, Teton Co., Wyoming, USA. (EDM 3032)
Artemisia argillosa Beetle	Coalmont, Jackson Co., Colorado, USA. (EDM 3034)
Artemisia bigelovii A. Gray	Emery Co., Utah, USA. (EDM 2869)
Artemisia californica Less.	Santa Clarita, Los Angeles Co., California, USA. (EDM 3039)
Artemisia cana Pursh subsp. *bolanderi* (A. Gray) G. H. Ward	Bridgeport, Mono Co., California, USA. (EDM 3047)
Artemisia cana Pursh subsp. *cana*	Sheridan, Sheridan Co., Wyoming, USA. (EDM 2128)
Artemisia cana Pursh subsp. *viscidula* (Osterh.) Beetle	Warner Pass, Lake Co., Oregon, USA. (EDM 2436)
Artemisia carruthii Wood ex Carruth	Clear Creek Canyon, Sevier Co, USA. (EDM 1254)
Artemisia deserti Krasch.	Semnan, Iran. (BCN 13322)
Artemisia dracunculus L.	Kharkhorin, Khengai Aimag, Mongolia. (BCN 17750)
Artemisia filifolia Torr.	Kanab, Kane Co., Utah, USA. (BCN 13332)
Artemisia frigida Willd.	Kyzyl, Tüva, Russia. (BCN 16421).
Artemisia herba-alba Asso	Aranjuez, Madrid, Spain. (BCF 40435).
Artemisia longiloba (Osterh.) Beetle	Corral Creek, Grand Co., Colorado, USA (Linda Sanders 3)
Artemisia ludoviciana Nutt. subsp. *ludoviciana*	Zion National Park, Washington Co., Utah, USA. (BCN 13955)
Artemisia nesiotica P. H. Raven	San Clemente Island, Los Angeles Co., California, USA. (EDM 3090)

Artemisia nova A. Nelson	Tunnel Spring, Desert Experimental Range, Millard Co., Utah, USA. (EDM 2876)
Artemisia nova A. Nelson subsp. *duchesnicola* Welsh & Goodrich	Tridell Road, Uintah Co., Utah, USA. (EDM 3029)
Artemisia palmeri A. Gray	Los Peñasquitos Canyon Preserve, San Diego, San Diego Co., California, USA. (EDM 3044)
Artemisia papposa Blake & Cronquist	Owyhee Co., Idaho, USA. (Ann de Bolt, 1840)
Artemisia pedatifida Nutt.	North of Point Rocks, Sweetwater Co., Wyoming, USA. (EDM 1138)
Artemisia porteri Cronquist	Fremont Co., Wyoming, USA. (EDM 3094)
Artemisia pygmaea A. Gray	Utah, Juab Co., USA. (BCN 14116).
Artemisia rigida (Nutt.) A. Gray	Malheur Reservoir, Malheur Co. Oregon, USA. (EDM 2859)
Artemisia rothrockii A. Gray	Reed Flats, White Mountains, Inyo Co., California, USA. (L. Shultz 19803)
Artemisia tridentata Nutt. subsp. *parishii* (A. Gray) Hall & Clements	West of Rosamond, Kern Co., California, USA. (EDM 3037)
Artemisia tridentata Nutt. subsp. *spiciformis* (Osterh.) Kartesz & Gandhi	Ford Ridge, Bristle Cone Scout Camp, Carbon Co., Utah, USA. (EDM 2839)
Artemisia tridentata Nutt. subsp. *tridentata*	Salt Creek Canyon, Juab Co., Utah, USA. (EDM 2871)
Artemisia tridentata Nutt. subsp. *vaseyana* (Rydb.) Beetle	Salt Creek Canyon, Juab. Co., Utah, USA. (EDM 2872)
Artemisia tridentata Nutt. subsp. *wyomingensis* Beetle & A. L. Young	Gordon Creek, Carbon Co., Utah, USA. (EDM 2886)
Artemisia tridentata Nutt. subsp. *xericensis* Winward ex R. Rosentreter & R. G. Kelsey	Mann Creek Reservoir, Washington Co., Idaho, USA. (EDM 2858)
Artemisia tripartita Rydb. subsp. *rupicola* Beetle	Pole Mountain, Albany Co., Wyoming, USA. (EDM 3033)
Artemisia tripartita Rydb. subsp. *tripartita*	Dubois Sheep Station, Clark Co., Idaho, USA. (EDM 2845)
Artemisia vulgaris L.	Vila Nova de Gaia, Portugal. (BCN 15273)
Picrothamnus desertorum Nutt.	Winton Road, Sweetwater Co., Wyoming, USA. (EDM 2403)
Sphaeromeria argentea Nutt.	South of Chimney Rock, Sweetwater Co., Wyoming, USA. (Goodrich and Atwood, 22533)
Sphaeromeria cana A. Heller	North or Current, Broom Canyon, Nye Co., Nevada, USA (Goodrich, Smith and Tuhy 20075)
Sphaeromeria diversifolia Rydb.	Santiaquin Canyon, Utah. USA. (BCF 49505)

Sphaeromeria potentilloides A. Heller	South of Hill City, Camas Co., Idaho, USA. (EDM 2425).
Sphaeromeria ruthiae A. H. Holmgren, L. M. Shultz & T. K. Lowrey	Refrigerator Canyon, Zion National Park, Washington Co., Utah, USA. (EDM 1775)

TABLE 2. Comparison of the three hypotheses of interspecific relationships within subgenus *Tridentatae*. Two lineages have been proposed (1) *A. tridentata* lineage and (2) *A. cana* lineage.

	Ward (1953)	**Beetle (1960)**	**Shultz (1983)**
A. tridentata lineage Seldom root sprouts after fire Mostly tridentate leaves Xerophytic	*A. tridentata* *A. arbuscula* *A. arbuscula* subsp. *nova* *A. arbuscula* subsp. *longiloba*	*A. tridentata* *A. longiloba* *A. nova* *A. bigelovii* *A. pygmaea*	*A. tridentata* *A. nova*
A. cana lineage Root sprouts and layers Leaves entire or deeply divided Mesophytic	*A. cana* *A. tripartita*	*A. cana* *A. tripartita* *A. rigida*	*A. cana* *A. tripartita*
Questionable placement	*A. pygmaea* *A. palmeri*		*A. pygmaea* *A. rigida*
Hybrid origin	*A. rothrockii*	*A. arbuscula* *A. rothrockii*	*A. arbuscula* *A. rothrockii*
Excluded taxa	*A. bigelovii*	*A. palmeri*	*A. bigelovii* *A. palmeri*

TABLE 3. Summary of sequence data from ITS, ETS and *trn*S^{GCU}-*trn*C^{GCA}and *trn*S^{UGA}-*trn*fM^{CAU}. Consistency and homoplasy indexes are calculated excluding uninformative characters.

Data set	**ITS**	**ETS**	**Combined nrDNA**	**Combined cpDNA**
Number of taxa	43	39	39	39
Total characters	651	1456	2056	1996
Informative characters	76	220	296	28
Consistency Index (CI)	0.5106	0.6074	0.5602	0.6905
Homoplasy Index (HI)	0.4864	0.3926	0.4398	0.3095
Retention Index (RI)	0.7079	0.7139	0.6678	0.8267
Rescaled Consistency Index (RC)	0.3615	0.4359	0.3741	0.5708
Model selected (ModelTest)	GTR+I+G	GTR+I+G	GTR+I+G	GTR+I

FIGURE 1. Phylogenetic reconstruction obtained through combined analysis of ITS and ETS sequence data for 39 species. Majority rule consensus tree (50%) based on Bayesian inference with Bayesian clade-credibility values (posterior probability; >50%) above branches and parsimony bootstrap percentages (>50%) below branches.

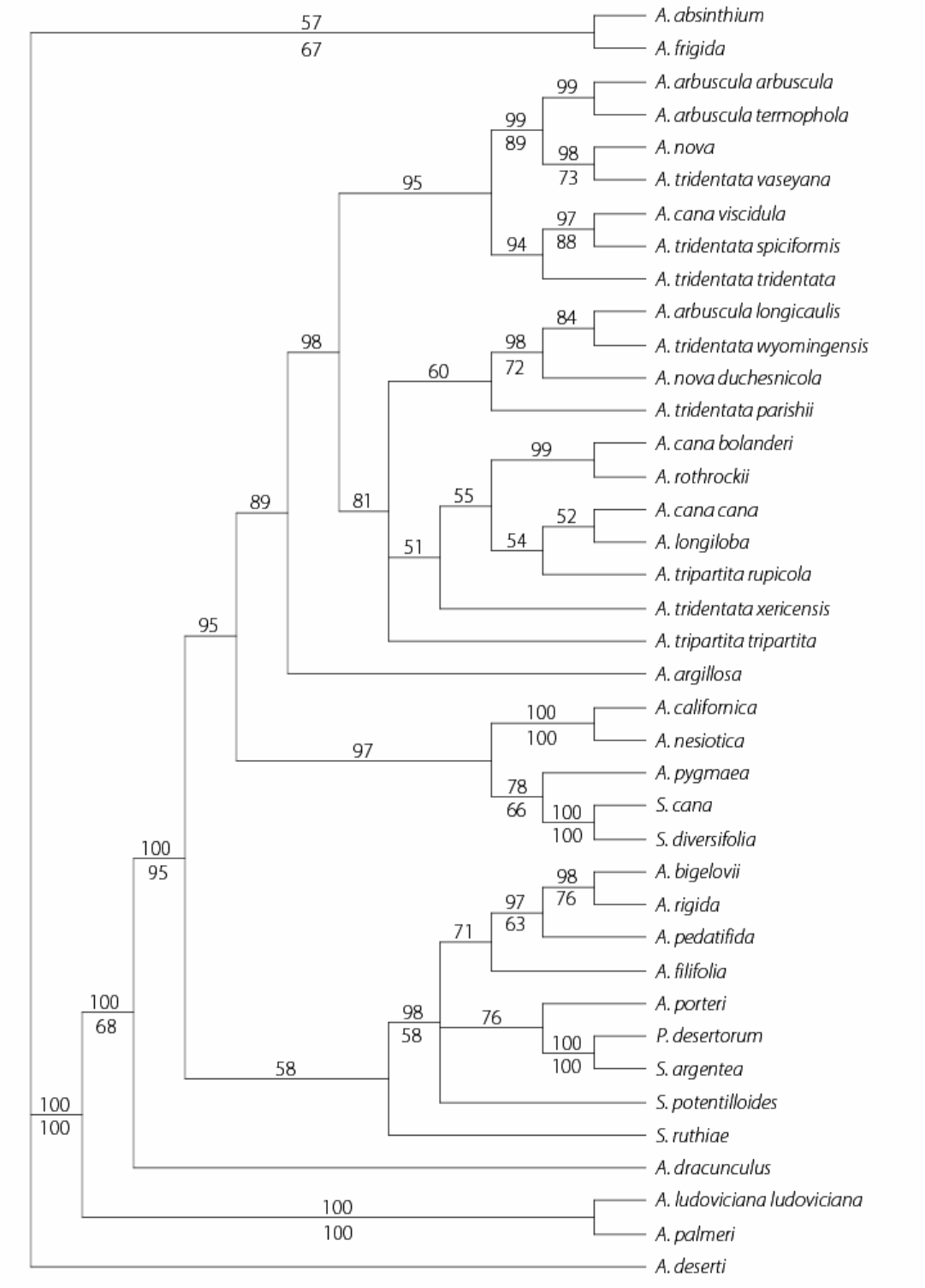

FIGURE 2. Phylogenetic reconstruction obtained through combined analysis of *trnS*^{GCU}-*trnC*^{GCA}and *trnS*^{UGA}-*trnf*M^{CAU}), for 39 species. Majority rule consensus tree (50%) based on Bayesian inference with Bayesian clade-credibility values (posterior probability; >50%) above branches.

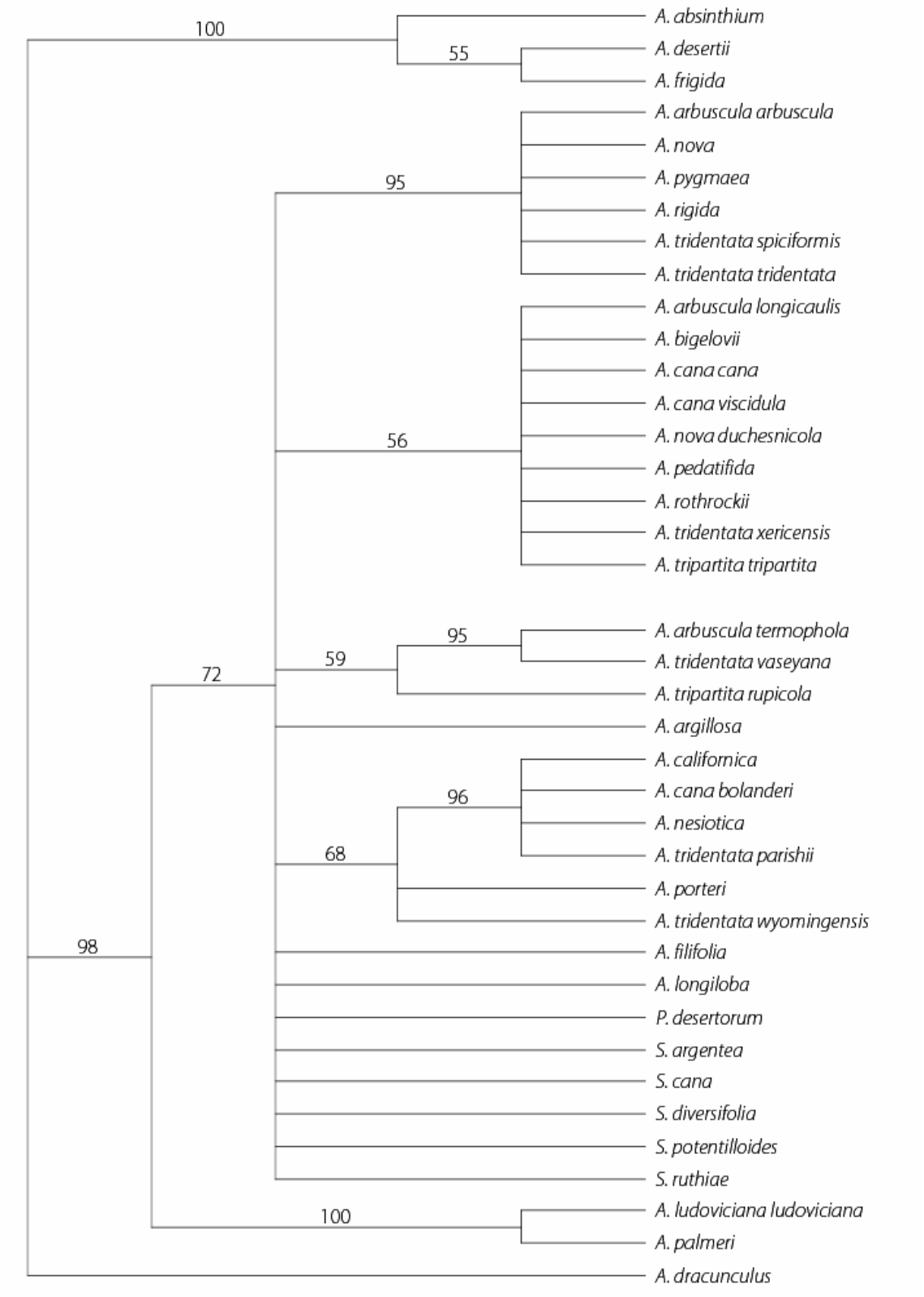

FIGURE 3. Phylogenetic reconstruction obtained through analysis of ITS sequence data for 43 species. Majority rule consensus tree (50%) based on Bayesian inference with Bayesian clade-credibility values (posterior probability; >50%) above branches.