Phylogenetic relationships of *Artemisia* subg. *Dracunculus* (Asteraceae) based on ribosomal and chloroplast DNA sequences

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Abstract Molecular-phylogenetic analyses of a large sampling of *Artemisia* subg. *Dracunculus* were carried out under Bayesian and maximum parsimony criteria to circumscribe the subgenus and explore the relationships between the species in the core of the subgenus. The *Dracunculus* complex was resolved into two main clades: one core clade including most of the Eurasian representatives plus a few closely related North American endemic species, and a second, small clade including *A. salsoloides* and *A. tanaitica*. Segregation of the latter two species is proposed in order to keep the subgenus monophyletic. Within the core clade, the existence of different lineages such as the *A. dracunculus* or *A. campestris* complexes, as well as the relationships of *Mausolea*, *Neopallasia* and *Turaniphytum*, are scrutinized. Discordances between the molecular data and previous morphological taxonomic treatments are noted. The occurrence of polyploidy in the subgenus is explored using previously published and newly generated genome size data.

Keywords Bayesian inference; Compositae; ETS; genome size; hybridization; ITS; phylogenetic reconstruction; *trnS-trnC*; *trnS-trnfM*

Supplementary Material Figures S1 and S2 and Appendices 1 and 2 are available in the free Electronic Supplement to the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

■ INTRODUCTION

Several studies have been conducted on phylogenetic relationships within the genus *Artemisia* L. (Kornkven & al., 1998; Torrell & al., 1999; Watson & al., 2002; D'Andrea & al., 2003; Vallès & al., 2003; Sanz & al., 2008; Tkach & al., 2008a, b). These studies have highlighted the infrageneric complexity of the genus as well as some incoherence between traditional classification and molecular affinities. From Tournefort's early classification (1700) until the present day, many taxonomic rearrangements have been proposed (Vallès & McArthur, 2001; Vallès & Garnatje, 2005). *Artemisia* subg. *Dracunculus* (Besser) Rydb. was alternatively included in the genus *Artemisia* (e.g., Linnaeus, 1735) or described as an independent genus, *Oligosporus* (Cassini, 1817). The latter author segregated a group of taxa to include *Artemisia* species with capitula structured in functionally separate sexes, with outer female florets, and central functionally male florets with abortive ovaries. However, Besser (1829, 1832, 1834, 1835), Candolle (1837) and Rydberg (1916) again proposed its reinclusion in *Artemisia* as a separate section or subgenus. Currently, the subgenus *Dracunculus* includes about sixty to eighty taxa depending on the authors (Poljakov, 1961; Ling & al., 2006; Shultz, 2006), mainly distributed across the Northern Hemisphere growing in the arid and semi-arid regions of Europe, Asia, and North America. Despite the general agreement about the taxonomic

circumscription of subg. *Dracunculus*, consensus is still lacking about the infrageneric relationships between the species (Poljakov, 1961; Darijma, 1989; Ling, 1992; Ling & al., 2006). Furthermore, *Mausolea* Poljakov, *Neopallasia* Poljakov, and *Turaniphytum* Poljakov, previously considered congeneric with *Artemisia* (as, respectively, *A. eriocarpa* Bunge, *A. pectinata* Pall. and *A. eranthema* Bunge), are closely related to the subg. *Dracunculus* (Vallès & al., 2003; Sanz & al., 2008). The lack of a solid phylogenetic framework for the subgenus, however, does not allow an in-depth study of their particular position.

DNA sequence data have been utilized widely in plants to elucidate species relationships. The value of the plastid *trnS-trnC* and *trnS-trnfM* and nuclear ITS and ETS regions has been proved in several groups of Asteraceae (McKenzie & al., 2006; Fehrer & al., 2007; Mort & al., 2008; Sanz & al., 2008 and references therein). Previous studies regarding the systematics of *Artemisia* showed the monophyly of the main Eurasian group (Sanz & al., 2008; Tkach & al., 2008a) and the misplacement of the North American representatives (*A. filifolia* Torr., *A. pedatifida* Nutt., *A. porteri* Cronquist and *Picrothamnus desertorum* Nutt. [= *A. spinescens* D.C. Eaton]) (Sanz & al., 2008), previously included in subg. *Dracunculus* sensu Shultz (2006). More recent taxonomical (Shultz, 2009) and phylogenetic (Garcia & al., 2011) revisions of the New World endemics have referred these former *Dracunculus* species to the subg. *Tridentatae* (Rydb.) McArthur.

The integration of data resulting from phylogenetic studies with those of other disciplines, such as genome size variation, may contribute to a better understanding and interpretation of the evolutionary trajectories within narrow plant groups (Ohri, 1998). In fact, genome size varies about 2400-fold across flowering plants, and has applications in many fields (e.g., Bennett & Leitch, 2005 and references therein). This parameter is directly influenced by polyploidy, which is a very common process in plants (e.g., Soltis & Soltis, 2000; Wendel, 2000; Cui & al., 2006; Chen, 2007; Soltis & al., 2010). As it is common in the genus *Artemisia*, subg. *Dracunculus* presents a high rate of polyploidy. Reported ploidy levels range from diploid to decaploid (Kawatani & Ohno, 1964; Rousi, 1969; Torrell & al., 2001; Pellicer & al., 2007b). The base chromosome number is $x = 9$ except for the annual *A. scoparia* Waldst. & Kitam., a species with the dysploid basic chromosome number $x = 8$ (Vallès & al., 2001 and references therein). Genome size studies devoted to specific complexes of *Artemisia* (Garcia & al., 2006, 2008; Pellicer & al., 2007a), as well as those with a more general viewpoint (Torrell & al., 2001; Garcia & al., 2004; Pellicer & al., 2010), have demonstrated an increased rate of proportional DNA loss correlated to polyploidy in the genus.

The main objectives of the present study are to establish a phylogenetic framework of the subgenus *Dracunculus* in order to (1) determine the taxonomic circumscription of the subgenus, (2) explore the interspecific relationships within the subgenus, as well as with related genera of Artemisiinae, and, (3) analyze the evolutionary implications of genome size and polyploidy using new and previously published data.

■ MATERIALS AND METHODS

Taxon sampling. — DNA sequences from 63 species of *Artemisia* belonging to subg. *Dracunculus* and three related genera of Artemisiinae (*Mausolea*, *Neopallasia*, *Turaniphytum*) were newly generated (56 ITS and 3′-ETS, 61 5′-ETS, *trnS-trnC* and *trnS-trnfM* sequences). Sampling of the subgenus and closely related genera was completed with 11 previously published ITS and 3′-ETS sequences (Torrell & al., 1999; Sanz & al., 2008; Tkach & al., 2008a). Additionally, in order to build a phylogenetic framework in which to circumscribe subg. *Dracunculus* within the genus and the subtribe, ITS and ETS sequences of 48 formerly studied species (Torrell & al., 1999; Vallès & al., 2003; Sanz & al., 2008; Tkach & al., 2008a; Pellicer & al., 2010) were used. Appendix 1 lists the species, population localities, herbarium vouchers and GenBank accession numbers for the newly studied species, as well as the GenBank accessions and references for the species for which data was previously published. Appendix 2 lists the population provenance and herbarium vouchers for the species used in flow cytometry analyses.

New plant material was obtained from field expeditions, from culture of achenes in the greenhouses at the Faculty of Pharmacy, University of Barcelona, and the Botanical Institute of Barcelona, and also from voucher specimens from the herbaria B, E, LE and W.

DNA extraction, amplification and sequencing. — Total genomic DNA was extracted from silica-dried leaves, herbarium material or fresh leaves using the CTAB method (Doyle & Doyle, 1987) as modified by Soltis & al. (1991) and Cullings (1992). The Nucleospin plant extraction kit (Macherey-Nagel, Pennsylvania, U.S.A) was used for those cases of poor-quality material. PCR was carried out in either GRI Labcare, MJ research PTC100 and G-STORM GS1 research thermal cyclers in 25 μl volume. ITS region (including 5.8S gene) was amplified with ITS1 as forward primer and ITS4 as the reverse one (White & al., 1990). The PCR amplification conditions used were 94°C, 2 min; 30× (94°C, 1 min; 55°C, 30 s; 72°C, 3 min); 72°C, 15 min, and storage at 4°C. The ETS region was amplified using ETS1f and 18SETS as forward and reverse primers respectively (Baldwin & Markos, 1998). The PCR profile used for amplification was 95°C, 5 min; 30× (94°C, 45 s; 50°C, 45 s; 72°C, 40 s); 72°C, 7 min, and 4°C for storage. Both forward and reverse primers were used for sequencing. For some taxa, internal primers AST1f and AST1R (Markos & Baldwin, 2001) were used for amplification (same PCR conditions) of shorter fragments. For phylogenetic analysis, only both 5′ and 3′ ends were used. The region $trnS^{UGA}$-$trnfM^{CAU}$ was amplified using $trnS^{UGA}$ and $trnfM^{CAU}$ primers (Shaw & al., 2005) with the following PCR conditions; 30× (94°C, 30 s; 62°C, 1 min 30 s; 72°C, 2 min); 72°C, 5 min, and storage at 10°C. The primers $trnS^{GCU}$ as forward and $trnC^{GCA}$ for reverse (Kim & al., 2005) were used for amplification of the $trnS^{GCU}$-$trnC^{GCA}$ region following the same PCR procedure as $trnS^{UGA}$-$trnfM^{CAU}$.

PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, California, U.S.A.) or with DNA Clean & Concentrator-5 D4003 (Zymo Research, Orange, California, U.S.A.). Direct sequencing of the amplified fragments was performed using Big Dye Terminator Cycle Sequencing v.3.1 (PE Biosystems, Foster City, California, U.S.A.) using the primers ITS4, 18SETS, ETS1f, $trnS^{UGA}$, $trnfM^{CAU}$ and $trnC^{GCA}$. Nucleotide sequencing was carried out at the Serveis Cientificotècnics (Universitat de Barcelona) using a ABI PRISM 3700 DNA Analyzer (PE Biosystems).

Sequence assembly, alignment and phylogenetic analyses. — Nucleotide sequences were edited using Chromas v.1.56 (Technelysium Pty, Tewantin, Australia) and subsequently assembled and edited using BioEdit v.7.0.9 (Hall, 1999). Alignments were made separately for each region with ClustalW (Thompson & al., 1997) using the default settings implemented in BioEdit, and gaps were manually adjusted to improve the alignments. A total of seven DNA matrices were prepared and analysed, including and excluding polyploid species, arranged in the following sets: (1) ITS1, ITS2 and the 3′ end of the ETS region to (a) circumscribe subg. *Dracunculus* within *Artemisia*, and (b) to confirm the inclusion of the segregated genera *Mausolea*, *Neopallasia* and *Turaniphytum* within the subgenus; (2) ITS (ITS1-5.8S-ITS2), both 5′ and 3′ ETS ends, $trnS^{UGA}$-$trnfM^{CAU}$ and $trnS^{GCU}$-$trnC^{GCA}$ to study in depth the phylogenetic relationships within the subgenus *Dracunculus*.

Bayesian inference (BI). — Analyses were carried out with MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003). The most appropriate nucleotide substitution models for the different

approaches (Tables 1 & 2) were chosen with MrModeltest v.2.3 (Nylander, 2004). For the circumscription of the subgenus *Dracunculus* (set 1) two runs of four Markov chains each were conducted simultaneously for 6×10^6 generations, and these were sampled every 100 generations. Data from the first 6000 generations were discarded as the "burn-in" period, after confirming that likelihood values were stabilized prior to the 6000th generation. In the second aproach (set 2), we used the same procedure but analyses were run for 3×10^6 generations (separated datasets), and 6×10^6 generations (combined dataset, ITS+ETS+*trnS-trnC*+*trnS-trnfM*), discarding the first 3000 and 6000 generations respectively. The 50% majority-rule consensus trees and posterior probabilities (PP) of nodes were calculated from the pooled samples.

Parsimony analysis. — Parsimony analyses (MP) using PAUP* v.4.0b10 (Swofford, 2003) were carried out after excluding non informative characters. Heuristic searches involved 100 random sequence addition replicates holding one tree at each step during the stepwise addition, and tree bisection reconnection (TBR) branch swapping with character states unordered and unweighted. Branch support of the tree nodes was evaluated by running either 100 replicates of heuristic search with simple taxon addition and TBR branch swapping (small datasets), or by faststep bootstrap implemented in PAUP* using 1000 replicates, 10 random sequence additions and no branch swapping. This second approach is an alternative to large dataset, and it provides similar estimates, although less, to those performed with branch swapping (Mort & al., 2000).

Table 1. Numerical results from the parsimony analysis and models selected for Bayesian inference of the ITS, ETS and the combined analyses from set 1.

Dataset		ITS	ETS	ITS+ETS
Number of taxa		115	115	115
Total characters		489	372	861
Number of informative characters		114	93	207
Tree length (number of steps)		378	224	619
Range of divergence: ingroup-outgroup (%)		3.00–12.80	1.89–14.15	2.97–13.33
Range of divergence: ingroup (%)		0–10.22	0–11.35	0–10.06
Consistency index (CI)[a]		0.451	0.533	0.410
Retention index (RI)[a]		0.858	0.925	0.884
Homoplasy index (HI)[a]		0.648	0.466	0.589
Rescaled consistency index (RC)[a]		0.386	0.493	0.362
Nucleotide substitution model	(AIC)	GTR+I+G	GTR+I+G	GTR+I+G
	(hLRT)	GTR+G	GTR+I+G	GTR+I+G

[a] Uninformative characters were excluded from the analyses.

Table 2. Numerical results from the parsimony analysis and models selected for Bayesian inference of the ITS, ETS, *trnS-trnC*, *trnS-trnfM* and the combined analyses from set 2.

Dataset		ITS	ETS	*trnS-trnC*	*trnS-trnfM*	ITS+ETS+*trnS-trnC*+*trnS-trnfM*
Number of taxa		56	56	56	56	56
Total characters		641	719	818	1120	3298
Number of informative characters		39	90	16	14	159
Tree length (number of steps)		82	149	23	22	307
Range of divergence: ingroup-outgroup (%)		3.00–7.00	0.60–7.20	0.60–2.5	0.70–5.70	0.90–5.55
Range of divergence: ingroup (%)		0–6.90	0–6.72	0–3.71	0–5.51	0–5.45
Consistency index (CI)[a]		0.632	0.718	0.739	0.636	0.589
Retention index (RI)[a]		0.901	0.936	0.850	0.913	0.879
Homoplasy index (HI)[a]		0.367	0.281	0.260	0.363	0.456
Rescaled consistency index (RC)[a]		0.569	0.672	0.6283	0.581	0.518
Nucleotide substitution model	(AIC)	SYM+G	HKI+I+G	GTR+I+G	F81+I+G	GTR+I+G
	(hLRT)	SYM+G	HKI+I+G	F81+I+G	F81+I+G	GTR+I+G

[a] Uninformative characters were excluded from the analyses.

Genome size assessments. — Leaf tissue of five individuals for the 31 populations (22 species) studied was chopped in 600 µl of LB01 isolation buffer (Doležel & al., 1989) with a razor blade, together with the chosen internal standard, and supplemented with 100 µg/ml ribonuclease A (RNase A, Boehringer, Meylan, France). For each individual, two independent samples were extracted to be processed under the cytometric assessment. Samples were subsequently stained with 36 µl of propidium iodide (1 mg/ml) to a final concentration of 60 µg/ml (Sigma-Aldrich Química, Madrid, Spain), kept on ice for 20 min and measured in an Epics XL flow cytometer (Coulter Corporation). *Petunia hybrida* Vilm. "PxPc6" (2C = 2.85 pg) and *Pisum sativum* "Express long" (2C = 8.37 pg), from the Institut des Sciences du Végétal (CNRS) at Gif-sur-Yvette (France), were used as internal standards. Measurements were made at the "Serveis Cientificotècnics" of the Universitat de Barcelona. To ascertain that the instrument showed a linear response across the range of genome sizes studied, we performed several assays which included both internal standards and one of the populations with the highest genome size at the same time. The difference between the obtained results with respect to each standard was negligible (less than 2% of deviation) hence we can certify the linearity of the flow cytometer in this interval and the convenience of the use of the chosen internal standards.

■ **RESULTS**

Bayesian analyses : Circumscription of subg. *Dracunculus*. — Phylograms displaying the boundaries of subg. *Dracunculus* within *Artemisia* are presented in Fig. 1 (including both diploid and polyploid species) and in Fig. S1A (including only diploid species). Tree topologies among datasets have been revealed consistent. As a result, the major clades recognized in both analyses are nearly identical, with similar node PP and BS values.

Bayesian analyses: Phylogenetic relationships within subg. *Dracunculus* s.str. — A second BI approach has been performed to study in greater depth the relationships of the representatives nested in the main clade defining subg. *Dracunculus* (Fig. 1, clade A; PP = 1.00, BS = 91%), which is basically composed of Eurasian representatives (Fig. 2). As previously done, a second phylogenetic analysis has been carried out as well excluding the polyploid species of the group (Fig. S1B). The fact that we have decided to remove the polyploid species and those whose ploidy level have ever been reported has resulted in a widely reduced sampling. Although the main clades 1, 2 and 3 are yet consistent (Fig. 2; Fig. S1B), subclade 1d is not resolved in the diploid approach and representatives previously included in subclade 1b have not been sampled in this diploid analysis.

In both approaches when the Akaike Information Criterion (AIC) and hierarchical Likelihood Ratio Test (hLRT) criteria presented different models that best fitted to our datasets (see Tables 1 & 2), independent analyses were performed for each model. After checking that no inconsistencies existed between

the resulting trees, we have only presented the trees extracted from the AIC model, as this has proved to be more advantageous than hLRT model selection (Posada & Buckley, 2004).

Parsimony analyses. — Tables 1 and 2 summarize the numerical data related to the tree in Fig. 1 (ITS, ETS and combined), and also in Fig. 2 (both independent and combined analyses) which include the dataset characteristics, i.e., number of accessions, range of sequence divergence, tree lengths and data on character fitness (consistency, retention, homoplasy and rescaled consistency indexes) of the most-parsimonious trees (MPT).

Congruence between trees and conflictive species (Figs. 1, 2 & S2). — As the BI turned out to be of better resolution than parsimony, and tree topologies showed no incongruence between the significantly supported branches, only the Bayesian trees of the combined datasets have been presented (but including both node PP [BI] and BS [MP] values). The partition homogeneity test carried out prior to regions combination indicated that certain degree of incongruence between nuclear and plastid regions existed ($P < 0.05$). Even so, the topologies of the trees obtained from independent and partially combined matrices were evaluated for congruence prior to combining all the datasets, showing no general conflicts between significantly supported clades of the chloroplast and nuclear regions, and some of the discordances observed where, however, not supported, probably due to the low phylogenetic signal of the plastid regions. Even though, discordant positions among trees were detected in *A. crithmifolia* L., *A. kuschakewiczii* Winkl., *A. nanschanica* Krasch., *A. pamirica* C. Winkl. and *A. sosnovskyi* Krasch. (Fig. S2), and therefore were excluded from the combined analysis (subg. *Dracunculus* s.str.; Fig. 2; Fig. S1B).

Genome size in subg. *Dracunculus*. — Table 3 gives the 2C- and 1Cx-values, and ploidy levels for the populations studied. For calculations, previously published data on genome size in the subgenus have been also used (Torrell & Vallès, 2001; Garcia & al., 2004, 2008; Pellicer & al., 2007a, 2010). The 2C DNA contents (restricted to clade A, Fig. 1) vary about 7.1-fold, from *A. capillaris* Thunb. (mean 2C = 3.35 pg; $2n = 2x = 18$) to *A. dracunculus* L. (mean 2C = 23.90 pg; $2n = 10x = 90$; Torrell & Vallès, 2001; Pellicer & al., 2007a). Monoploid genome size varies 1.9-fold, from $1Cx = 1.67$ pg in *A. capillaris* to $1Cx = 3.27$ pg in *A. arenaria* DC. (Pellicer & al., 2010). *Artemisia salsoloides* Willd., which is phylogenetically isolated from the remaining subg. *Dracunculus* species (Fig. 1, clade E), also has a significant bigger genome size ($1Cx = 5.70$ pg).
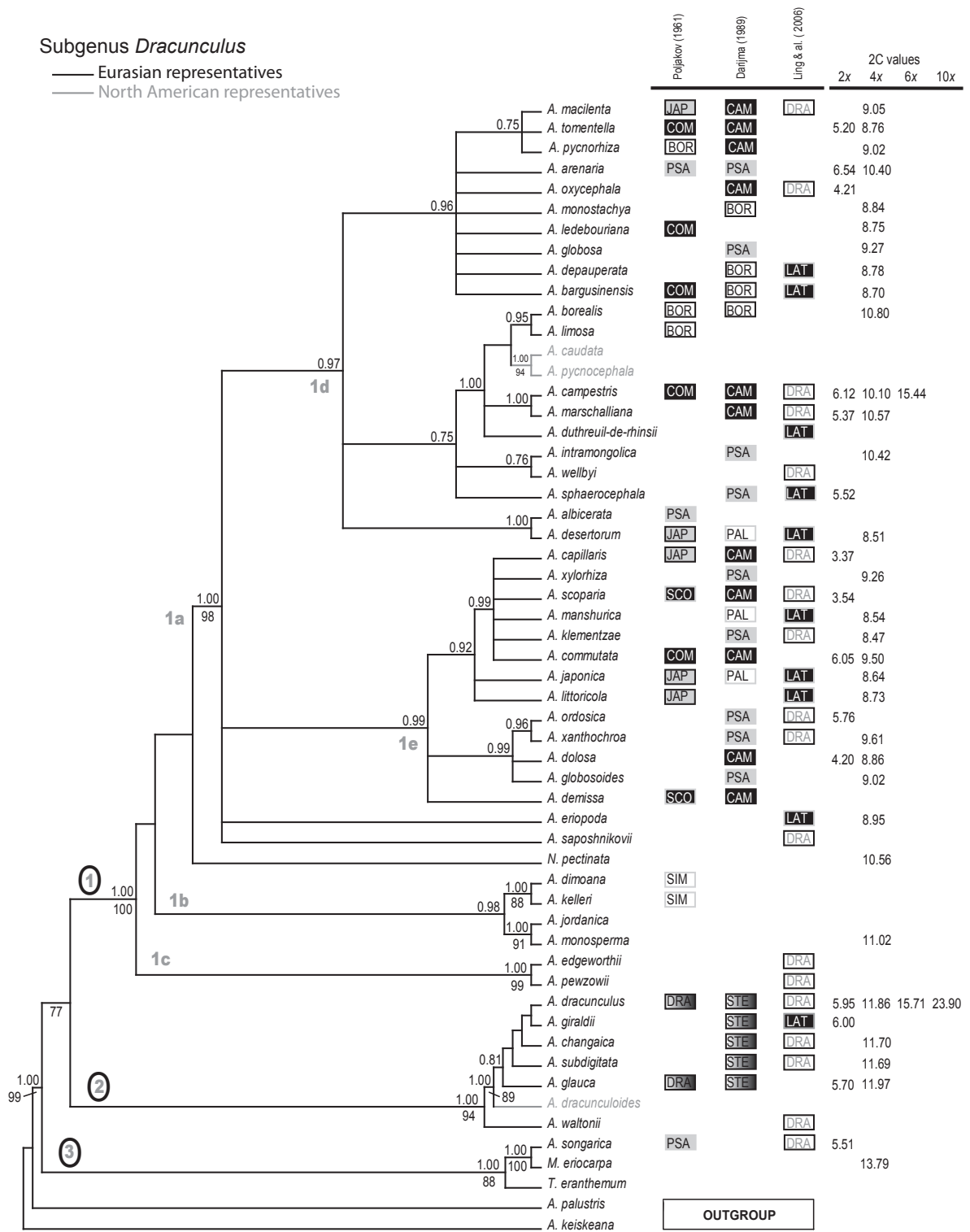
■ **DISCUSSION**

Systematic circumscription of subg. *Dracunculus* within *Artemisia* (Figs. 1 & S1A). — The analysis of the combined nuclear DNA matrices, including both diploid and polyploid species (Fig. 1) and the additional one restricted to known diploids (Fig. S1A), resulted in major compatible topologies. Thus, both phylogenetic approaches presented a supported backbone structure with correspondence between the major clades of
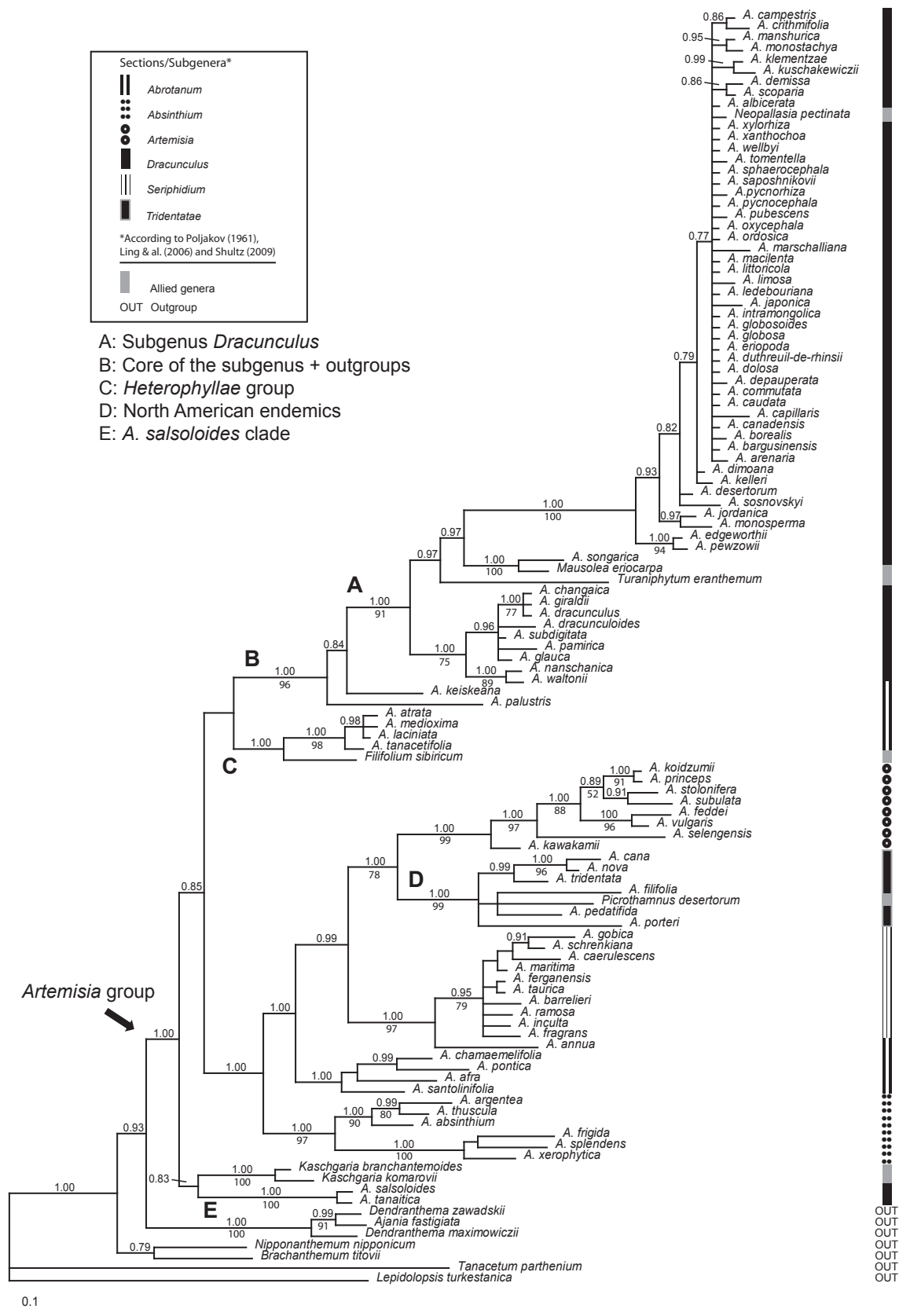
Fig. 1. 50% majority-rule consensus tree from Bayesian inference of the combined ITS+3′ETS data set. Posterior probability values (PP) are indicated above branches and parsimony bootstrap values (BS) are indicated below branches (PP and BS values below 0.75 and 75% respectively are not depicted). A traditional classification under sectional/subgeneric criteria of *Artemisia* is also mapped in the tree.

Fig. 2. *Artemisia* subg. *Dracunculus* core (see Fig. 1, clade B) majority-rule consensus tree (50%) based on Bayesian inference of the combined nuclear (ITS, ETS) and plastid (*trnS*[UGA]-*trnfM*[CAU] and *trnS*[GCU]-*trnC*[GCA]) regions. Coded indels have been added to the analysis. Posterior probability values (PP) are indicated above branches and parsimony bootstrap values (BS) are indicated below branches (PP and BS values below 0.75 and 75% respectively are not depicted). Clade 1, Core of the subg. *Dracunculus*; clade 2: *Artemisia dracunculus* complex; clade 3, related genera.

Poljakov (1961); Series: DRA *Dracunculi*, SIM *Simplicifoliae*, PSA *Psammophilae*, JAP *Japonicae*, COM *Commutatae*, BOR *Boreales*, SCO *Scopariae*
Darijma (1989); Section: PAL *Paleodracunculus*, STE *Stenophyllae*, CAM *Campestres*, BOR *Boreales*, PSA *Psammophilae*
Ling & al. (2006); Section: DRA *Dracunculus*, LAT *Latilobus*

interest (clades A–E). These results confirm the highly supported monophyly of the main lineage of subg. *Dracunculus* (Fig. 1, clade A), but fail to resolve specific relationships within some groups, as evidenced by the presence of a large polytomy. The presence of polytomies in phylogenetic trees might be the result of either character discrepancies or short branch lengths. Their origin has been related to methodological artifacts and also to the existence of evolutionary histories which are more congruent with a multifurcating pattern of speciation (Whitfield & Lockhart, 2007; Calviño & al., 2008). Processes such as hybridization or genome introgression, not seldom linked to rapid diversification times, might be related to the lack of

resolution and therefore, to complicate diversification histories (e.g., *Armeria*: Fuertes Aguilar & Nieto Feliner, 2003; *Eryngium*: Calviño & al., 2008). It is difficult to discriminate if there have been simultaneous diversification episodes of multiple taxa in subg. *Dracunculus*, but the antecedents of a complicate evolutionary history reported in the genus (Garcia & al., 2011 and references therein) might be indicating that this is the case.

As stated in the results section, the group is nested in a very robust clade (Fig. 1; Fig. S1A; PP = 1.00; BS > 90%), and embeds the crown clade, including most of the Eurasian representatives (with the exclusion of *A. salsoloides* and *A. tanaitica* Klokov [clade E], later discussed) plus a few endemic North

Table 3. Nuclear DNA content and karyological information of the populations studied.

Taxa	Chromosome number	Ploidy level	2C (SD)[a]	2C (Mbp)[b]	1Cx (pg)[c]	HPCV[d] (%)	Standard
A. arenaria	36	4x	10.38 (0.04)	10,151.64	2.60	2.25	*Pisum*
A. borealis	36	4x	10.95 (0.26)	10,709.10	2.74	2.98	*Pisum*
A. borealis	36	4x	10.65 (0.15)	10,415.70	2.66	3.01	*Pisum*
A. campestris subsp. *variabilis*	54	6x	15.44 (0.11)	15,100.32	2.57	3.25	*Pisum*
A. capillaris	18	2x	3.25 (0.03)	3,178.50	1.63	1.75	*Pisum*
A. capillaris	18	2x	3.44 (0.04)	3,364.32	1.72	1.98	*Pisum*
A. crithmifolia	54	6x	14.79 (0.09)	14,464.62	2.47	3.98	*Pisum*
A. crithmifolia	54	6x	15.08 (0.15)	14,748.24	2.51	3.68	*Pisum*
A. crithmifolia	54	6x	15.04 (0.11)	14,709.12	2.51	3.95	*Pisum*
A. crithmifolia	54	6x	15.55 (0.13)	15,207.90	2.59	3.67	*Pisum*
A. crithmifolia	54	6x	15.04 (0.08)	14,709.12	2.51	3.59	*Pisum*
A. desertorum	36	4x	8.61 (0.12)	8,420.58	2.15	2.21	*Petunia*
A. dolosa	18	2x	4.20 (0.08)	4,107.60	2.10	1.36	*Petunia*
A. dolosa	36	4x	8.86 (0.23)	8,665.08	2.22	1.98	*Petunia*
A. dracunculus	36	4x	11.82 (0.10)	11,559.96	2.96	3.01	*Pisum*
A. dracunculus	36	4x	11.93 (0.11)	11,667.54	2.98	2.87	*Pisum*
A. eriopoda	36	4x	8.95 (0.12)	8,753.10	2.24	2.54	*Petunia*
A. glauca	18	2x	5.75 (0.04)	5,623.50	2.88	3.20	*Petunia*
A. intramongolica	36	4x	10.42 (0.09)	10,190.76	2.61	3.68	*Pisum*
A. japonica	36	4x	8.64 (0.13)	8,449.92	2.16	2.10	*Petunia*
A. klementzae	36	4x	8.47 (0.13)	8,283.66	2.12	3.56	*Petunia*
A. littoricola	36	4x	8.73 (0.19)	8,537.94	2.18	2.36	*Petunia*
A. manshurica	36	4x	8.54 (0.05)	8,352.12	2.14	3.65	*Petunia*
A. ordosica	18	2x	5.76 (0.11)	5,633.28	2.88	2.98	*Petunia*
A. pycnocephala	18	2x	6.22 (0.15)	6,083.16	3.11	3.64	*Pisum*
A. salsoloides	18	2x	11.40 (0.04)	11,149.20	5.70	2.74	*Pisum*
A. songarica	18	2x	5.51 (0.15)	5,388.78	2.76	2.96	*Petunia*
A. tomentella	18	2x	5.20 (0.08)	5,085.60	2.60	3.02	*Petunia*
A. tomentella	36	4x	8.76 (0.13)	8,567.28	2.19	3.25	*Petunia*
A. xanthochroa	36	4x	9.61 (0.08)	9,398.58	2.40	2.56	*Pisum*
A. xylorhiza	36	4x	9.26 (0.15)	9,056.28	2.32	2.99	*Pisum*

[a] Nuclear DNA content in pg [2C value (standard deviation)].
[b] Nuclear DNA content in Mbp; 1 pg = 978 Mbp (Doležel & al., 2003).
[c] Monoploid genome size.
[d] Half Peak Coefficient of Variation.

American representatives (*A. canadensis* Michx., *A. caudata* Michx., and *A. pycnocephala* DC.) that are closely related to their Old World congeners (Fig. 1, clade A).

Until recently, *A. filifolia*, *A pedatifida*, *A. porteri* and *P. desertorum* (formerly *A. spinescens*), which are included in the North American endemic lineage (Fig. 1, clade D), had been traditionally considered within subg. *Dracunculus* because they display the characteristic heterogamous capitula structure of the group, with outer florets female and the central ones hermaphrodite but functionally male (Shultz, 2006 and references therein). Recent taxonomical and molecular re-evaluations of the New World endemics (Shultz, 2009; Garcia & al., 2011), have concluded their inclusion within subg. *Tridentatae*. Therefore, (1) the synapomorphy of the presence of functionally male central florets should no longer be considered as a restricted trait to subg. *Dracunculus* and (2), the origin of these taxa is obviously different from the remaining *Dracunculus*, and likely mediated by subg. *Tridentatae* representatives. The close relationship of this group of species with the subg. *Tridentatae* has been suggested previously based on karyological, cytological and phytochemical similarities (Beetle, 1960; McArthur & Pope, 1979). In the case of *A. filifolia*, its phylogenetic position has been previously reported by Kornkven & al. (1998), from chloroplast restriction site data, and also Watson & al. (2002) and Sanz & al. (2008) from nuclear ribosomal sequences. All of these data indicate its close relationship with the *Tridentatae* group, despite the lack of morphological resemblances.

As mentioned previously, *A. salsoloides* and *A. tanaitica* are phylogenetically isolated from the subg. *Dracunculus* clade, being robustly grouped (clade E; PP = 1.00; BS = 100%) but with an undetermined position in both phylogenetic approaches (Figs. 1 & 2). The linkage of these two species supports Čerepanov (1995) who considered them synonyms. Leonova (1988) proposed the segregation of *A. salsoloides* into the new monotypic sect. *Salsoloides* Leonova (retained within subg. *Dracunculus*) based on the presence of star-like hairs in young stems, not found in any other group within the genus. Our results have suggested a possible sister relationship between the latter species and *Kaschgaria* Poljakov, but this cannot be confirmed due to weak node support (Fig. 1; Fig. S1A; PP < 0.85). Although there is no strong morphological evidence for such a relationship, the same singular hair structure found in *A. salsoloides* has been reported in *Kaschgaria* (Ling & al., 2006), and the genome size data seems to provide support for this relationship as well. While genome size of *A. salsoloides* (2C = 11.40 pg) could be considered an outlier within subg. *Dracunculus*, as clearly differs from the range of 2C values found in this group (2C = 3.25 to 6.54 pg; Pellicer & al., 2010), it falls closer to the previously reported genome size of *K. brachanthemoides* (C. Winkl.) Poljakov (2C = 14.09 pg; Garcia & al., 2004).

A more in-depth revision of these species will be needed to confirm their phylogenetic placement. Until then, we propose (1) to exclude *A. salsoloides* from subg. *Dracunculus* and (2) to reconsider the generic status of Poljakov's *Kaschgaria* to accommodate their species within *Artemisia*, thus keeping the genus monophyletic (Fig. 1).

Phylogenetic relationships of subg. *Dracunculus* with the sister group (Figs. 1 & 2). — Previous studies placed *Filifolium sibiricum* (L.) Kitam. as sister taxon of subg. *Dracunculus* (Sanz & al., 2008). However, an enlarged sampling of the genus carried out in the present phylogeny has revealed a strong relationship of this species with representatives of the *Heterophyllae* group (clade C, sect. *Abrotanum* Besser, subg. *Artemisia*; *A. atrata* Lam., *A. laciniata* Willd. *A. medioxima* Krasch. ex Poljakov and *A. tanacetifolia* L.) sensu Darijma (1989), but without statistical support to confirm their sister position to the subgenus (Fig. 1, PP < 0.75; Fig. S1A, PP = 0.90). Although the analysis of ITS and ETS placed both *A. keiskeana* Miq. and *A. palustris* L. at sister position of subg. *Dracunculus* (Fig. 1, clade B; PP = 1.00, BS = 96%), the lack of resolution within the clade makes it complicate to discriminate between them. Even though, the presence of these two species close to subg. *Dracunculus* might indicate that subgenus was differentiated from an *Artemisia*-like ancestor, as has been hypothesized in other lineages (McArthur & Plummer, 1978).

Besides, the phytochemistry of the genus *Artemisia* can shed light on the knowledge of these species and their relationships. Greger (1988) pointed out that the distribution of secondary metabolites (e.g., polyacetylenes or coumarin sesquiterpene ethers) can provide valuable criteria when studying the systematics of *Artemisia*. This author found correlations regarding the occurrence of dehidrofalcarinone derivatives and other aromatic acetylenes in subg. *Dracunculus* and the *Heterophyllae* group (subg. *Artemisia*), and also pointed out the dominance of this dehydrofalcarinone pathway in *A. keiskeana*. Likewise, Belenovskaja (1996) indicated a close relationship between *A. palustris* and the *Heterophyllae* group on the basis of their characteristic phenolic composition. Keeping in mind the segregation of *A. salsoloides* and *A. tanaitica*, and the lack of support for the relationships between the subg. *Dracunculus* clades and the *Heterophyllae* plus *A. palustris* and *A. keiskeana*, Greger's findings provide further evidence to better understand the basal phylogenetic relationships of the subgenus with the latter ones.

Taxonomic conflicts within the core of the subgenus (Figs. 2, S1B, clade 1, Fig. S2). — As stated before, the nucleus of subg. *Dracunculus* appears embedded into a large and quite homogeneous lineage (sequence divergence <7.2%; see Table 2) which includes representatives mainly from Eurasia, as well as few from North America (Fig. 2). This group, strongly supported (PP = 1.00, BS > 95%), is basically split in three main subclades (1–3), which are not always in agreement with the classical taxonomic treatments and with biogeographic and morphological features (Poljakov, 1961; Darijma, 1989; Ling & al., 2006). Likewise, the lack of a strong complete infrageneric treatment of the subgenus and the heterogenic criteria found between the already published (see Fig. 2), makes difficult to establish comparisons between our results and the taxonomic aspects, but also provides a source of points that need to be discussed:

• *Subclade 1a*. – This large lineage is basically split in two minor clades (Fig. 2, clades 1d & 1e) with the exception of *A. eriopoda* Bunge, widely distributed across Central and

East Asia, and *A. saposhnikovii* Krasch. ex Poljakov, endemic to Kyrgyzstan and South West Xinjiang (People's Republic of China). The latter species was related by Poljakov (1961) to *A. albicerata* Krasch., *A. arenaria* and *A. songarica* Schrenk., among others, to perform the ser. *Psammophilae* Poljakov, but no molecular evidences for such relationship between them have been found. Although the phylogenetic position of many representatives embraced within these two clades shows no correspondence between molecular and morphological data, some clusters highlight diverse interesting relationships. The case of the *A. campestris* L. group is a good example. It is composed, apart from this species, basically of *A. borealis* Pall., *A. limosa* Koidz., *A. caudata*, *A. pycnocephala*, *A. marschalliana* Spreng. and *A. duthreuil-de-rhinsii* Krasch. (Fig. 2, PP = 1.00). The last species is newly included within the complex in this study, whereas the remaining had previously been studied at a morphological level in different floras (Poljakov, 1961; Shultz, 2006). *Artemisia caudata* and *A. pycnocephala* are the unique North American endemics included in the present work whose phylogenetic position correlates with the main circumscription of the subgenus. This fact can be easily understood because of the narrow relationship of these species with the worldwide distributed *A. campestris*. In fact, these taxa have been considered as subspecies (i.e., *A. campestris* L. subsp. *pycnocephala* (Less.) H.M. Hall & Clem. and *A. campestris* subsp. *caudata* (Michx.) H.M. Hall & Clem.). Similar explanations can be found for *A. borealis* and *A. marschalliana*, both formerly labelled as *A. campestris* subspecies, which appear related to the East Asian *A. limosa*, endemic to Sakhalin island (Poljakov, 1961), and to *A. campestris* respectively. Moreover, other related taxa appear segregated into separate clades (Fig. 2, clades 1d, 1e) such as *A. macilenta* (Maxim.) Krasch., *A. pycnorhiza* Ledeb., *A. oxycephala* Kitam., *A. commutata* Besser or *A. dolosa* Krasch., among others. If we look at the diploids tree (Fig. S1B), although we have to take into account that sample size has been reduced (clade d cannot be longer considered), the species included within clade e partially agree with sect. *Campestres* sensu Darijma (1989).

Some of the species clusters seem to fit with their geographic distribution, i.e., *A. ordosica* Krasch., *A. xanthochroa* Krasch., *A. dolosa* and *A. globosoides* Ling & Y.R. Ling, which are embedded into a well-supported clade (Fig. 2, PP = 0.99) that involves basically Mongolian endemics with the exception of *A. ordosica*, that also occurs in China (but restricted to Inner Mongolia and neighboring provinces). The relationship between the annual/biennial species *A. scoparia* and *A. demissa* Krasch. is not clear. While the nuclear markers (Fig. 1) embrace these to species, but without statistical support (PP = 0.86, BS = 70%), in agreement with Poljakov's (1961) inclusion under the ser. *Scopariae* Krasch, chloroplast data (Fig. S2B) place *A. scoparia* embedded into a clade with *A. capillaris* among others. Thus, our combined phylogenetic data do not confirm Poljakov's relationship (Fig. 2), but cluster *A. scoparia* and *A. capillaris* in the same lineage, which is complemented by their rather similar genome sizes, these being the smallest amounts reported for the subgenus (*A. capillaris* 2C = 3.35 pg; *A. scoparia* 2C = 3.54 pg).

As previously stated, the inclusion of species into unresolved polytomies might indicate either episodes of hybridization and/or reflect the low signal of the DNA regions used. Further in-depth studies with complementary DNA regions will be helpful to unravel and discriminate between the evolutionary forces leading to speciation in the genus.

• *Subclade 1b.* – This well-supported lineage (Fig. 2, PP = 0.98) is split into two subclades which embed *A. dimoana* M. Pop. and *A. kelleri* Krasch. on one side (PP = 1.00, BS = 88%), and *A. monosperma* Delile and *A. jordanica* Danin (PP = 1.00, BS = 91%) on the other side, being this clade only supported by nuclear data since the signal of the plastid regions does not resolve such relationship (Fig. 2SB). They all show a preference to inhabit sandy desert areas of Central Asia (*A. dimoana*, *A. kelleri*) and Southwest Asia (*A. jordanica*, *A. monosperma*). This confirms Poljakov's (1961) suggestions in his revision of *Artemisia* for the *Flora of the USSR*, placing together *A. dimoana* and *A. kelleri* into ser. *Simplicifoliae* Krasch. because of the presence of simple (partly lobed) leaves. *Artemisia monosperma* and *A. jordanica* share many morphological characters, and present a high degree of resemblance (Danin, 1999). Notwithstanding, while the first one is distributed from Egypt to Israel reaching Lebanon, the second one is present in South Jordan, Saudi Arabia and the Southwest of Iraq, without overlapped territories, and being the unique representatives of the subgenus in the zone. Previous works regarding *Artemisia* systematics and cytogenetics (Torrell & al., 1999 for phylogeny; Torrell & al., 2001 for cytogenetics) pointed out a narrow relationship between *A. monosperma* and the *A. campestris* complex also based on their morphological and ecological traits. Our findings, based on a more representative sample of the subgenus, clearly disagree with these suggestions, having seen its phylogenetic position.

• *Subclade 1c.* – The two annual-biennial species *Artemisia edgeworthii* Balakr. and *Artemisia pewzowii* C. Winkl. perform a very robust clade (Fig. 2, clade 1c; PP = 1.00, BS = 99%). Although *A. pewzowii* has a more reduced distribution than *A. edgeworthii*, both species overlap some of their Chinese territories (e.g., provinces of Qinghai, Xinjiang and Xizang). There are no significant morphological traits supporting this clustering; indeed pollen data reflect the presence of different subtypes (*Anomalae* for *A. edgeworthii*, *Sacrorum* for *A. pewzowii*; sensu Jiang & al., 2005).

The *Artemisia dracunculus* complex (Fig. 2, clade 2). — This complex is composed of about 10 species that are characterized by the presence of simple, linear to linear-lanceolate, leaves. This morphological trait marks the main difference between these species and the rest of the subgenus, which present basically pinnatisect leaves (Poljakov, 1961), with scarce exceptions such as *A. jordanica* (Danin, 1999). Polyploidy in this complex is of great prevalence, as in general for the subgenus, with series of $2n = 18, 36, 54, 72, 90$ chromosomes in species such as *A. dracunculus* (Kreitschitz & Vallès, 2003 and references therein). The complex includes the worldwide distributed *A. dracunculus* and other closely related taxa such as *A. giraldii* Pamp., *A. changaica* Krasch., *A. subdigitata* Mattf., *A. glauca* Pall., *A. pamirica* (see Fig. S2) and *A. dracunculoides* Pursh.

They are mainly endemic to Central Asia (with the exception of the North American endemic *A. dracunculoides*), but with more discrete distributions than the type species *A. dracunculus*.

The group has revealed itself strongly monophyletic (Fig. 2, clade 2; PP = 1.00, BS = 94%). Our results have also provided additional information as the inclusion of *A. waltonii* J.R. Drump ex Pamp. and *A. nanschanica* Krasch. (see Fig. S2) within this complex had never been reported to date. Notwithstanding, the phylogenetic placement of *A. nanschanica* and *A. pamirica* were incongruent between nuclear and chloroplastic trees (Fig. S2), so we decided to exclude both species from the combined tree.

The specific relationships between these taxa are not deeply resolved in the combined tree (Fig. 2), only pointing to the sister position of *A. waltonii*. From the combined ITS+ETS tree (Figs. 1 & S2A) a close relationship between *A. dracunculus*, *A. changaica* and *A. giraldii* can be deduced, but the lack of signal in the chloroplastic tree (Fig. S2B), which only succeeds to solve the main clade of the complex, does not allow us to make strong conclusions about phylogenetic relationships in the complex.

Disagreement about the taxonomic consideration of some of these plants can also be found in the literature. In the revisions of Poljakov (1961) and Darijma (1989), these taxa were considered at species level, while Ling & al. (2006) relegated the taxonomic rank of some of them to varieties of *A. dracunculus* on the basis of their close morphological similarity. Furthermore, Ling (1987) proposed the consideration of *A. subdigitata* as a variety of *A. dubia* Wall. ex Besser, traditionally included by Besser (1832) within sect. *Abrotanum* s.l. (incl. sect. *Artemisia*). He reviewed voucher specimens and concluded that the former could be considered as a variety of *A. dubia*, as both presented the capitula structure typical of subg. *Dracunculus*. The position in our tree of *A. subdigitata* within the *A. dracunculus* complex is more likely congruent with their morphological traits, rather than with those of *A. dubia*, which we sequenced from herbarium of the Natural History Museum Vienna (W), and resulted phylogenetically close to the *A. vulgaris* complex (data not shown).

***Mausolea*, *Neopallasia* and *Turaniphytum*: A close relationship with *Artemisia*. —** The close phylogenetic relationship existing between the genus *Artemisia* and several other Artemisiinae genera is well known, some of them previously nested in the genus justified under molecular (Vallès & al., 2003; Oberprieler & al., 2007; Sanz & al., 2008), or geographical criteria (Kadereit & Jeffreys, 2007). Vallès & al. (2003) and Sanz & al. (2008) pointed out the inclusion of *Mausolea* and *Turaniphytum* in subg. *Dracunculus*, but were unable to study in greater depth the relationships of these genera within the group. Our results confirm this fact. *Mausolea* and *Turaniphytum* are completely merged within *Artemisia* subg. *Dracunculus* (Figs. 1 & 2). This inclusion is supported by the fact that those genera present heterogamous capitula (with functionally male central florets), characteristic in the subgenus. Not only the combined analysis (Fig. 2), but also the nuclear and plastid independent Bayesian analyses (Fig. S2) revealed a strong relationship between *A. songarica*, *M. eriocarpa* and

T. eranthemum Bunge (Poljakov) (Fig. 2, clade 3; PP = 1.00, BS = 88%). From the morphological standpoint, we are not able to state which are the main traits defining this lineage, should it exist, but the strong congruence found between both ribosomal and chloroplast data does make us look for other explanations for such a linkage. Those three species inhabit desert and semidesert areas of Western Asia (Afghanistan, Iran, Kazakhstan) reaching Central Asia in the cases of *A. songarica* and *Mausolea*. The new relationship found between the latter two species might be better understood on the basis of a geographical effect, because of the coincidental distribution areas.

Neopallasia is another annual endemic genus from Central Asia composed of three species which was segregated from *Artemisia* by Poljakov (1955) despite the fact that its floral characters (heterogamous capitula with functionally male central florets) recommend its re-inclusion within subg. *Dracunculus* (Sanz & al., 2008). Our results also place the only species studied, *N. pectinata* (Pall.) Poljakov, clearly embedded into a well-supported (PP = 1.00, BS = 100%) and large clade containing most representatives of the subgenus (Fig. 2, clade 1). Despite our large sampling, the phylogenetic position of this species and the relationship with the remaining species of *Dracunculus* still lacks complete resolution. This fact might be influenced mainly by the poor phylogenetic signal of the chloroplast data (Fig. S2B), which places the species at undetermined position.

Genome size evolution and polyploidy in subg. *Dracunculus*. — The distribution of genome size data for tetraploid representatives seems to be consistent with some of the different lineages (Fig. 2). Thus, while the phylogenetically closely related clades 1d and 1e account for quite similar mean 2C values (clade 1d, 2C = 9.42 ± 0.82 pg; clade 1e, 2C = 8.95 ± 0.41), clade 2, which is more distant and embeds the *A. dracunculus* complex, is also segregated on the basis of its mean 2C value (2C = 11.80 ± 0.13 pg). This latter lineage is quite homogeneous in terms of 2C values, as well as from the karyological and palynological viewpoints, and that prevents us from finding differences between the species other than the morphological ones, and makes a conclusion about the appropriate taxonomic rank more difficult. It is also interesting to point that some of the species included in clade 1d, *A. campestris* and relatives, account for larger 2C values than the remaining tetraploid representatives (Fig. 2), although the lack of a larger sample does not permit to make statistical tests.

Recent research addressed to study the genome size changes related to polyploidization events in *Artemisia* (Pellicer & al., 2010), has revealed an increased ratio of DNA elimination along ascending ploidy levels as a mechanism of response, possibly, to the control of genome obesity after polyploidization events (Bennetzen & al., 2005). These results can be observed in the large polyploid species *A. dracunculus* (Fig. 3). While the genome size data observed at the lowest polyploid level (4*x*) and expected are similar, in ascending ploidy levels, differences become larger, demonstrating an increase in the portion of nuclear DNA which is being removed during polyploidization. Similar changes take place in *A. campestris*, the differences being more patent since the first polyploid levels (Fig. 3). Slight

changes at low polyploid levels (4*x*) have also been detected (e.g., *A. dolosa* or *A. glauca*; Fig. 3). A heterogenic behavior towards DNA gain or genome downsizing in polyploids of the same genus has been reported in literature (Leitch & al., 2008), as the result of different individual evolutionary histories. In the case of *Artemisia*, contrary to other plant groups (Leitch & Bennett, 2004; Leitch & al., 2008), conclusive hypotheses about the erosive effects of time after polyploidization cannot be stated because of the unknown origin of these polyploidy species.

Taxonomic implications: First steps to return some allied genera within *Artemisia*. — Based on the results obtained in the present work, and taking into account our previous work on Artemiisinae (Vallès & al., 2003; Sanz & al., 2008), the classification as segregate genera of *Mausolea* and *Filifolium*, and possibly also of *Turaniphytum* and *Neopallasia* (both containing three species), should be reconsidered. Both *Mausolea* and *Filifolium* species are clearly embedded in *Artemisia* in the present phylogeny, confirming the previous findings (Vallès & al., 2003; Sanz & al., 2008). Thus, they should be recognized as *Artemisia* species. For *Mausolea*, the solution is to return to the original name, *A. eriocarpa* Bunge. *Filifolium sibiricum* was originally described as *Tanacetum sibiricum* L. (non Falk = *T. vulgare* L.) and afterwards combined into *Artemisia*, as *A. sibirica* (L.) Maxim., a status which is consistent with the present molecular findings. Although the lack of material of some of the species of *Neopallasia* and *Turaniphytum* does not allow us to make fully conclusive decisions, we have presented new molecular insights indicating the treatment of *N. pectinata* and *T. eranthemum* as species of *Artemisia*, i.e., *A. pectinata* and *A. eranthema*. Indeed, the type of *Turaniphytum* is nowadays

considered as a species of *Artemisia* (*A. kopetdaghense* (Poljakov) Y.R. Ling) and the remaining one (*T. condringtonii* (Rech. f.) Podlech) was originally described as *A. condringtonii* Rech. f. Similar taxonomic rearrangements concern *Neopallasia*: while *N. tibetica* Y.R. Ling was newly described in this genus, *N. yunnanensis* (Pamp.) Y.R. Ling was originally described as *A. pectinata* Pall. var. *yunnanensis* Pamp. The present molecular data clearly support their inclusion in the genus *Artemisia*, implying reduction of these genera to synonymy under *Artemisia*.

■ CONCLUDING REMARKS

The monophyly of *Artemisia* subg. *Dracunculus* is confirmed in the phylogenetic reconstructions based on nuclear and chloroplastic DNA regions, but excluding *A. salsoloides* and *A. tanaitica* and including the presently considered related genera *Mausolea*, *Neopallasia* and *Turaniphytum*. *Artemisia keiskeana* and *A. palustris* are closely related to the subgenus, both being strong candidates to constitute the sister group of subg. *Dracunculus*, with a close phytochemical relationship with the subgenus. Three independent lineages can be distinguished within the subgenus for the first time in the present work. Data from genome size in polyploid species seem to be congruent with these major groups, and indicate, as it is common in the genus, a reduction of monoploid genome sizes that increase in higher ploidy levels. Furthermore, the inclusion of some related genera into *Artemisia* leads us to propose taxonomic reorganizations for these taxa, many of them previously labeled under this genus.
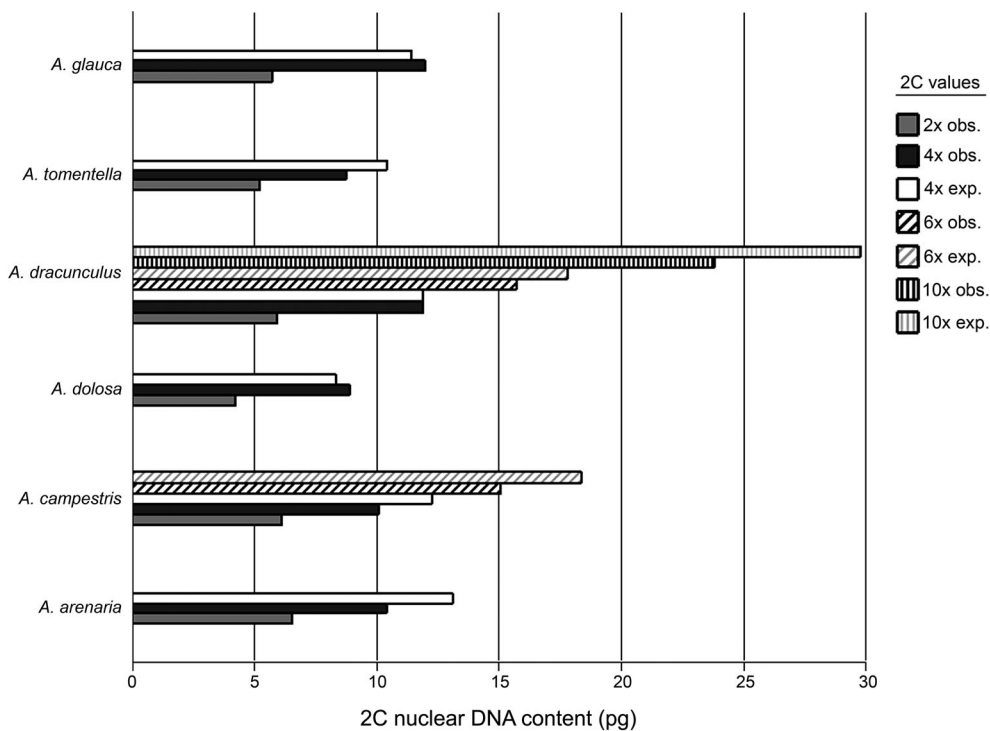
Fig. 3. Evolution of nuclear DNA content between ploidy levels in some species of *Artemisia*.

■ ACKNOWLEDGMENTS

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