American Journal of Botany 98(4): 638–653. 2011.

A molecular phylogenetic approach to western North America endemic *Artemisia* and allies (Asteraceae): Untangling the sagebrushes[1]

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- *Premise of the study*: *Artemisia* subgenus *Tridentatae* plants characterize the North American Intermountain West. These are landscape-dominant constituents of important ecological communities and habitats for endemic wildlife. Together with allied species and genera (*Picrothamnus* and *Sphaeromeria*), they make up an intricate series of taxa whose limits are uncertain, likely the result of reticulate evolution. The objectives of this study were to resolve relations among *Tridentatae* species and their near relatives by delimiting the phylogenetic positions of subgenus *Tridentatae* species with particular reference to its New World geographic placement and to provide explanations for the relations of allied species and genera with the subgenus with an assessment of their current taxonomic placement.
- *Methods*: Bayesian inference and maximum parsimony analysis were based on 168 newly generated sequences (including the nuclear ITS and ETS and the plastid *trnS*^{UGA}-*trnfM*^{CAU} and *trnS*^{GCU}-*trnC*^{GCA}) and 338 previously published sequences (ITS and ETS). Genome size by flow cytometry of species from *Sphaeromeria* was also determined.
- *Key results*: The results support an expanded concept and reconfiguration of *Tridentatae* to accommodate additional endemic North American *Artemisia* species. The monotypic *Picrothamnus* and all *Sphaeromeria* species appear nested within subgenus *Tridentatae* clade.
- *Conclusions*: A redefinition of subgenus *Tridentatae* to include other western North American endemics is supported. We propose a new circumscription of the subgenus and divide it into three sections: *Tridentatae, Filifoliae*, and *Nebulosae*. The position of the circumboreal and other North American species suggests that subgenus *Artemisia* is the ancestral stock for the New World endemics, including those native to South America.

Key words: Compositae; genome size; hybridization; polyploidy; reticulate evolution; sagebrush; *Sphaeromeria*; *Tridentatae*.

The genus *Artemisia* L. is the largest of tribe *Anthemideae* Cass. (Asteraceae Martinov), comprising around 500 species (Vallès and McArthur, 2001; Vallès and Garnatje, 2005), many of them ecologically and economically relevant. Some of them are important medicinal plants such as *Artemisia annua* L., whose component artemisinin is successfully used against malaria (Van der Meersch, 2005); others are used as condiments, as tarragon (*A. dracunculus* L.) or to make alcoholic beverages such as absinth (*A. absinthium* L.). *Artemisia* species are widely distributed in temperate areas in the northern Hemisphere (Bremer, 1994) but very sparsely in the southern Hemisphere, with fewer than 10 species there. Four or five subgenera are generally accepted: *Artemisia*, *Absinthium* (Mill.) Less., *Dracunculus* Besser, *Seriphidium* (Besser) Poljakov, and *Tridentatae* (Rydberg) McArthur; some treatments combine subgenera *Artemisia* and *Absinthium* in a single subgenus, *Artemisia* (Shultz, 2009). The classic subgeneric delimitations have been subject to rearrangement in the light of recent molecular studies, which in some cases do not support the traditional classifications and portray some of the classical subgenera as polyphyletic or paraphyletic (Watson et al., 2002; Vallès et al., 2003; Sanz et al., 2008; Tkach et al., 2008). Additionally, small segregate or monotypic genera are placed within *Artemisia* in these molecular phylogenies, at odds with traditional taxonomy. Processes like hybridization, introgression, and polyploidization, common in these plants, also complicate interpretation of relations at the molecular level (Ward, 1953; Estes, 1969; McArthur et al., 1981, 1988; Winward and McArthur, 1995; McArthur et al., 1998a; McArthur and Sanderson, 1999).

[1] Manuscript received 29 September 2010; revision accepted 20 December 2010.

The authors thank Dr. O. Hidalgo (Ohio University), I. Sánchez-Jiménez and L. Barres (Institut Botànic de Barcelona), and D. Vitales (Universitat de Barcelona) for helpful comments and support in phylogenetic analyses. Dr. L. M. Shultz (Utah State University) is acknowledged for providing leaf material of several accessions as well as for careful revision of the manuscript. Dr. M. P. Simmons and an anonymous reviewer are also thanked for their comments, corrections, and suggestions that improved the overall quality of the manuscript. Professor W. Greuter (Botanical Garden and Botanical Museum, Berlin) is thanked for his advice on taxonomical questions, and C. L. Christie is acknowledged for the pictures of Fig. 1. This work was subsidized by the projects CGL2007-64839-C02-01/BOS and CGL2007-64839-C02-02/BOS of the Spanish government and project 2009SGR00439 "Grup de Recerca Consolidat" from the Generalitat de Catalunya. S.G. was granted by the CSIC with a JAE-DOC contract and a short stay in the Shrub Sciences Laboratory (USDA) in Utah.

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doi:10.3732/ajb.1000386

The main speciation and diversification center of the genus *Artemisia* is Central Asia, and according to Tkach et al. (2008), 17 to 22 migrations may have occurred from Asia into North America and two to four from North America to Asia. The most representative group of North American endemic *Artemisia* is subgenus *Tridentatae*. The *Tridentatae* and allies characterize the landscape of western North America (sagebrush steppe) and are the most common shrubs in the western United States. They consist of 10 to 13 species (depending on the authority), all of them perennial, woody, and xerophytic, though as a group they occur over a broad habitat range because of their extraordinary variety of ecological specializations (West, 1983; Shultz, 2009). Sagebrushes form an intricate ecosystem crucial for the maintenance of its specific wildlife, including some endemic species such as the endangered sage grouse (genus *Centrocercus Swainson*) in which, under their cover, females incubate their eggs. The average life span of *Artemisia tridentata* Nutt., the most abundant *Tridentatae* species, ranges from 50 to 100 yr (Winward, 1970), but some individuals live more than 200 yr (Ferguson, 1964; McArthur and Stevens, 2004). The Intermountain West sagebrush steppe dates back to 12 Ma (Davis and Ellis, 2010). In fact, North America, particularly the Intermountain West, can be considered an evolutionary hotspot for the genus, given the richness and success of these species there.

On the basis of a synapomorphy (homogamous flower heads) together with its large size (ca. 130 species) and their endemicity to the Old World, the Asian species belonging to the subgenus *Seriphidium* have been suggested as probable ancestors of the *Tridentatae*, with migration to North America across Beringia (Beetle, 1960; Ling, 1991, 1995a, b). In counterpoint, McArthur and Plummer (1978) and McArthur et al. (1981), while agreeing with this migration route, proposed that the herbaceous members of the subgenus *Artemisia* could have been differentiated in North America during the Pleistocene in response to climatic changes, giving origin to the species of the subgenus *Tridentatae* and other endemics. Jeffrey (1995) and Shultz (2009) supported the origin of the *Tridentatae* from subgenus *Artemisia* species on a phytochemical basis.

Pursh (1814) described the first species now included in the *Tridentatae* (= sagebrush), *Artemisia cana* Pursh (Pursh, 1814; Torrey and Gray, 1843; Rydberg, 1916). Subsequently, sagebrush classification over and under the subgeneric level has been difficult and subject to periodic rearrangement (Table 1). Initially, *Tridentatae* was placed in subgenus *Seriphidium* (Rydberg, 1916). McArthur raised the *Tridentatae* to subgeneric status (McArthur et al., 1981) and posited that the similarity with *Seriphidium* was a result of convergent evolution. The treatment of *Seriphidium* and *Tridentatae* as two independent clades draws support from several phylogenetic surveys of the genus (Watson et al., 2002; Vallès et al., 2003; Sanz et al., 2008; Tkach et al., 2008). *Seriphidium* has occasionally been segregated as an independent genus (Bremer and Humphries, 1993; Ling, 1991, 1995a, b), including *Tridentatae* species, and more recently in taxonomic and molecular phylogenetic treatments of the subtribe Artemisiinae (Watson et al., 2002; Ling et al., 2006). However, the separation of *Seriphidium* from the genus *Artemisia* is not supported by cpDNA restriction sites or internal transcribed spacer (ITS) and external transcribed spacer (ETS) sequence phylogenies (Kornkven et al., 1998, 1999; Torrell et al., 1999; Sanz et al., 2008), nor on a morphological basis. Also, the *Tridentatae* occasionally have been treated at the generic level (Weber, 1984; and L. M. Shultz, Utah State University, Logan, Utah, USA, personal communication), but as for *Seriphidium*, the generic rank is considered unsuitable (Oberprieler et al., 2007; Funk et al., 2009).

The interspecific relations and boundaries of the *Tridentatae* and allies are complex and unresolved. It sometimes has been considered as a monophyletic group (Kornkven et al., 1998, 1999; Vallès et al., 2003), but the possibility of independent introductions of *Artemisia* from the Old World to the New World complicates New World *Artemisia* evolutionary history (Tkach et al., 2008). Two *Tridentatae* lineages have been proposed on the basis of some morphological and ecological characteristics (Ward, 1953; Beetle, 1960; Shultz, 1983; see Table 1), but the available molecular data do not support recognition of these lineages (Kornkven et al., 1998, 1999; Watson et al., 2002; Riggins, 2008). Moreover, several species have been included and subsequently excluded in numerous 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). Placement of species like *Artemisia bigelovii* A. Gray, *A. californica* Less., *A. filifolia* Torr., *A. palmeri* A. Gray, *A. pygmaea* A. Gray, and *A. rigida* (Nutt.) A. Gray is particularly problematic. Shultz (2009), in her recent monograph of the *Tridentatae*, advocates an extended concept of the subgenus, recognizing two sections: *Tridentatae* L. M. Shultz and *Nebulosae* L. M. Shultz, the latter created to accommodate some other North American endemic *Artemisia* on the basis of molecular studies (Watson et al., 2002). Molecular cytogenetics and genome size data (Garcia et al., 2007, 2008, 2009) also have shed light in particular cases and support a more restrictive concept of the *Tridentatae*, the "*Tridentatae* core" or *true sagebrushes*, which may be partly equivalent to section *Tridentatae* sensu Shultz (2009). Moreover, two North American endemic genera, the monotypic *Picrothamnus* Nutt. and *Sphaeromeria* Nutt. (nine species, with eight of them being worthy of rare and endangered species classification; Holmgren et al., 1976), also have appeared embedded in an endemic North American *Artemisia* clade in some recent studies (Watson et al., 2002; Vallès et al., 2003; Riggins, 2008; Sanz et al., 2008). Apart from many similar morphological and ecological features, these species share the presence of interxylary cork, typical of *Tridentatae* species (Holmgren et al., 1976).

Given the complexity that most researchers have faced when working in this group, our attempt to resolve its phylogenetic relations includes four molecular data sets. As nuclear gene regions, the rDNA ITS and ETS were chosen for study and analysis. The ITS region had been tested previously in the *Tridentatae* (Kornkven et al., 1998; Garcia et al., 2008), but we included this region again in this study, as all subspecific entities of the *Tridentatae*, most of the remaining North American endemic *Artemisia*, and two closely related genera (*Picrothamnus* and *Sphaeromeria*) are included for the first time in a unique data set. The virtues of this region (biparentally inherited, high rates of base substitution, ease of amplification with universal primers) have led to extensive ITS use by molecular systematists (Baldwin et al., 1995), though either complete or incomplete concerted evolution and lineage sorting, among other things, can be a source of problems in phylogenetic reconstructions (Mayol and Rosselló, 2001; Álvarez and Wendel, 2003; Nieto Feliner and Rosselló, 2007). Because for some recently evolved angiosperm lineages the ITS is not sufficiently informative as a result of insufficient sequence variation, we included data from a neighboring rDNA region, the ETS. It is generally longer than ITS, easily amplifiable with universal primers, and has proved

TABLE 1. Comparison of different hypotheses of interspecific relations within *Tridentatae*.

	Rydberg (1916)	Hall and Clements (1923)	Ward (1953)	Beetle (1960)	Shultz (1983)	Kornkven et al. (1999)	Shultz (2006a)	Shultz (2009)
Subgenus	**Subgenus *Seriphidium***	**-Section *Seriphidium***	**Subgenus *Tridentatae***	**Subgenus *Tridentatae***	**Subgenus *Tridentatae***	**Subgenus *Seriphidium***	**Subgenus *Tridentatae***	**Subgenus *Tridentatae***
-Section or lineage	**-Section *Pygmaeae***	*A. cana*	***-A. tridentata* lineage**[a]	***-A. tridentata* lineage**	***-A. tridentata* lineage**	**-Section *Tridentatae***		**-Section *Tridentatae***
	A. pygmaea	*A. palmeri*	*A. arbuscula*	*A. bigelovii*	*A. nova*	*A. arbuscula*	*A. arbuscula*	*A. arbuscula*
	-Section *Rigidae*	*A. pygmaea*	*A. arbuscula* subsp.	*A. longiloba*	*A. tridentata*	*A. bigelovii*[c]	*A. bigelovii*	*A. bigelovii*
	A. rigida	*A. rigida*	*longiloba*	*A. nova*	***-A. cana* lineage**	*A. cana*	*A. cana*	*A. cana*
	-Section *Tridentatae*	*A. tridentata*	*A. arbuscula* subsp.	*A. pygmaea*	*A. cana*	*A. longiloba*	*A. nova*	*A. nova*
	A. angusta	- subsp. *arbuscula*	*nova*	*A. tridentata*	*A. tripartita*	*A. nova*	*A. pygmaea*	*A. pygmaea*
	A. arbuscula	- subsp. *bolanderi*	*A. tridentata*	***-A. cana* lineage**		*A. pygmaea*	*A. rigida*	*A. rigida*
	A. bolanderi	- subsp. *nova*	***-A. cana* lineage**[b]	*A. cana*		*A. rigida*	*A. rothrockii*	*A. rothrockii*
	A. cana	- subsp. *parishii*	*A. cana*	*A. rigida*		*A. rothrockii*	*A. spiciformis*	*A. spiciformis*
	A. nova	- subsp. *rothrockii*	*A. tripartita*	*A. tripartita*		*A. tridentata*	*A. tridentata*	*A. tridentata*
	A. parishii	- subsp. *trifida*				*A. tripartita*	*A. tripartita*	*A. tripartita*
	A. rothrockii	- subsp. *typica*						**-Section *Nebulosae***
	A. spiciformis							*A. californica*
	A. tridentata							*A. filifolia*
	A. tripartita							*A. nesiotica*
	A. vaseyana							
Questionable placement	*A. palmeri*		*A. palmeri*		*A. pygmaea*	*A. californica*		*Sphaeromeria*
			A. pygmaea		*A. rigida*	*A. filifolia*[c]		
Hybrid origin			*A. rothrockii*	*A. arbuscula*	*A. arbuscula*			*A. arbuscula* subsp.
				A. rothrockii	*A. rothrockii*			*longicaulis*
								A. argilosa
								A. tridentata subsp.
								xericensis
Excluded taxa	*A. bigelovii*		*A. bigelovii*	*A. palmeri*	*A. bigelovii*	*A. palmeri*	*Sphaeromeria*	*A. papposa*
					A. palmeri			*A. pedatifida*
								A. porteri

[a] Seldom do root sprouts after fire; mostly tridentate leaves and xerophytic.

[b] Root sprouts and layers after fire; leaves entire or deeply divided and mesophytic.

[c] These species were excluded from section *Tridentatae* in Kornkven et al. (1998).

useful at low taxonomic levels (Linder et al., 2000), including in recent studies in *Artemisia* (Sanz et al., 2008; Tkach et al., 2008; Pellicer et al., 2010a), though, as with the ITS region, it is not free from problems related to concerted evolution.

Since a 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. On the basis of the pioneering work of Shaw et al. (2005), we selected *trnS*^{UGA}-*trnfM*^{CAU} and *trnS*^{GCU}-*trnC*^{GCA}, the latter exclusive to the Asteraceae because of two inversions in the LSC region of the chloroplast DNA (Kim et al., 2005). These two fragments occur between the regions considered to provide the greatest number of potentially informative characters across all phylogenetic lineages (Shaw et al., 2005).

In summary, the current study attempts to resolve phylogenetic relations for these taxa, with these specific goals: (1) to assess the circumscription, boundaries, and internal relations of members of subgenus *Tridentatae* in the genus *Artemisia* and to hypothesize their likely ancestral stock; (2) to explain the relation with genera *Sphaeromeria* and *Picrothamnus* and to assess their generic independence and evaluate their present taxonomy; and (3) to identify the (subgeneric) placement of the other North American endemic *Artemisia* and of some other taxa of likely hybrid origin. Finally, the paper aims to contribute and analyze genome size data for the first time for genus *Sphaeromeria*, complementing the representation of the North American endemic Artemisiinae (Garcia et al., 2008).

MATERIALS AND METHODS

Taxon sampling and data sets used*—First data set (sagebrushes)*—Plant material for 42 populations was obtained (Table 2), including all subgenus *Tridentatae* species, subspecies (22 taxa), and some taxa of likely hybrid origin (*A. argilosa* Beetle, *A. arbuscula* Nutt. subsp. *longicaulis* Winward et McArthur, and *A. tridentata* Nutt. subsp. *xericensis* Winward ex R. Rosentreter et R. G. Kelsey following Shultz [2009]), as well as many North American endemic *Artemisia* that had been considered related to or included in the *Tridentatae* in previous research efforts (seven taxa), eight *Sphaeromeria* (unfortunately, we were not able to extract DNA from *Sphaeromeria martirensis* [Wiggins] A. H. Holmgren, L. M. Shultz et Lowrey, which would have completed the representation of the genus), and one population of *Picrothamnus desertorum* Nutt. Because previous phylogenetic approaches to the genus *Artemisia* (Torrell et al., 1999; Vallès et al., 2003; Sanz et al., 2008; Tkach et al., 2008) showed unclear sister-group relations for the *Tridentatae*, we followed the criteria of adding complementary species representing each subgenus of *Artemisia* and not being endemic to North America (four taxa). Phylogenetic analyses have been performed with and without subspecies/hybrids, but since clades did not change significantly, we kept results of the complete set of taxa. All nuclear and chloroplast DNA sequences (168) were newly generated. Table 2 shows the provenance of all the species investigated and the GenBank accession numbers for DNA sequences. All cited taxa were sampled for DNA sequencing, and, in addition, the eight *Sphaeromeria* species also were sampled for nuclear DNA content assessment.

Second data set (global)—To establish the placement of subgenus *Tridentatae* within the genus *Artemisia* and determine the more closely related groups, our first data set (excluding subspecies and putative hybrid taxa) was supplemented with ITS and ETS sequences of the *Artemisia* and allies, available at GenBank (110 species, 220 sequences). Sequences belong to previous molecular systematic approaches to *Artemisia* (Torrell et al., 1999; Vallès et al., 2003; Sanz et al., 2008; Tkach et al., 2008; Pellicer et al., 2010b), and GenBank accession numbers can be consulted in Appendix S1 (see Supplemental Data with the online version of this article).

Molecular techniques*—DNA extraction, amplification, and sequencing*—We extracted total genomic DNA using either the CTAB method of Doyle and Doyle (1987) as modified by Soltis et al. (1991) or the Nucleospin Plant (Macherey-Nagel, GmbH et 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, CSIC; Facultat de Farmàcia, Universitat de Barcelona), or herbarium material (see Table 2). Polymerase chain reaction (PCR) was performed by using either GRI Labcare (Essex, UK) or MJ Research Inc. (Watertown, Massachusetts, USA) thermal cyclers in a 25-μL volume. Subsequently, PCR products were purified with either the QIAquick PCR purification kit (Qiagen, Valencia, California, USA) or the DNA Clean and Concentrator-5 D4003 (Zymo Research, Orange, California, USA). Direct sequencing of the amplified DNA segment was performed with the Big Dye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, California, USA). Nucleotide sequencing was carried out at the Serveis Cientificotècnics (Universitat de Barcelona) on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City, California, USA).

ITS region—Double-stranded DNA of the ITS region (including ITS1, 5.8S gene, and ITS2) 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 94°C 2 min; 30 × (94°C 1 min 30 s; 55°C 2 min; 72°C, 3 min); 72°C 15 min. Because the mean length of this region is relatively short (653 bp), only the ITS4 primer was used in sequencing in most cases, though both forward primers were used at times when necessary.

ETS region—Double-stranded DNA of the 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 95°C, 5 min; 30 × (94°C, 45 s; 50°C, 45 s; 72°C, 40 s); 72°C, 7 min. Because of the mean length of this region (1624 bp), both ETS1f and 18SETS were used as sequencing primers, and also the internal primers AST1F and AST1R (Markos and Baldwin, 2001) were used occasionally.

trnS^{UGA}-*trnfM*^{CAU}—This region was amplified with *trnS*^{UGA} (forward) and *trnfM*^{CAU} (reverse) primers (Demesure et al., 1995). The amplification parameters were 80°C, 5 min; 30 × (94°C, 30 s; 62°C 1 min 30 s; 72°C 2 min) 72°C, 5 min. *trnS*^{UGA} was used as the sequencing primer, but occasionally *trnfM*^{CAU} was also needed. The mean length of this region was 1077 bp.

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 the primer *trnC*^{GCA}R, though *trnS*^{UGA} was occasionally needed. The mean length of this region was 841 bp.

DNA cloning—Although for most taxa, direct sequencing performed well, yielding clean and unambiguous sequences, in a very few cases (ETS of *Artemisia deserti* Krasch. and ETS and ITS of *Sphaeromeria capitata* Nutt.), DNA sequences were difficult or impossible to read. In these cases, we conducted cloning of PCR products with the TOPO TA Cloning kit from Invitrogen (Carlsbad, California, USA), following the manufacturer's instructions. There were no significant changes between clones in both cases (clades posterior probability [PP] = 0.98–1.0, BS = 96–100% in preliminary analyses), so only one clone per species was selected for the final analyses.

Flow cytometry measurements—For genome size estimation, flow cytometry was used. *Petunia hybrida* Vilm. 'PxPc6' (2C = 2.85 pg, Marie and Brown, 1993) was used as the internal standard. Fresh leaf tissue of the standard and the target species was chopped together in 600 μL of Galbraith's isolation buffer (Galbraith et al., 1983) supplemented with 100 μg/mL ribonuclease A (RNase A, Boehringer, Meylan, France), stained with 36 μL of 1 mg/ml propidium iodide (Sigma-Aldrich Química, Alcobendas, Spain) to a final concentration of 60 μg/mL, and kept on ice for 20 min. We sampled five individuals for each population, and two replicates of each individual were independently processed. The flow cytometer used was an Epics XL (Coulter Corporation, Hialeah, Florida, USA) at the Serveis Cientificotècnics (Universitat de Barcelona). More details about the method we followed are described in Garcia et al. (2008).

Data analyses*—Construction and editing of sequence matrices*—DNA sequences were edited with Chromas Lite 2.01 (Technelysium PTy, Tewantin, Queensland, Australia) and subsequently assembled with Bioedit Sequence Alignment Editor 7.0.9.0 (Hall, 1999), aligned with ClustalW Multiple Alignment v. 1.4 (Thompson et al., 1994), and corrected manually. The DNA data sets generated included the following: (1) the first data set (the sagebrushes)

Table 2. Origin of specimens, herbarium vouchers, and GenBank accession numbers for ITS, ETS, *trnS*^{GCU}-*trnC*^{GCA}, and *trnS*^{UGA}-*trnfM*^{CAU} newly generated sequences.

Taxon	Collection data and herbarium voucher	ITS	ETS	*trnS-trnC*	*trnS-trnfM*
Artemisia absinthium L.	Setcases, Catalonia, Spain (BCN 12313)	HQ019033	HQ018991	HQ019075	HQ019117
Artemisia arbuscula Nutt. subsp. *arbuscula*	Corn Creek Canyon, Millard Co., Utah, USA (SSLP-EDM 2877)	HQ019034	HQ018992	HQ019076	HQ019118
Artemisia arbuscula Nutt. subsp. *longicaulis* Winward et McArthur	Toulon, Pershing Co., Nevada, USA (SSLP-EDM 2860)	HQ019035	HQ018993	HQ019077	HQ019119
Artemisia arbuscula Nutt. subsp. *thermopola* Beetle	Yellowstone, National Park, Teton Co., Wyoming, USA (SSLP-EDM 3032)	HQ019036	HQ018994	HQ019078	HQ019120
Artemisia argilosa Beetle	Coalmont, Jackson Co., Colorado, USA (SSLP-EDM 3034)	HQ019037	HQ018995	HQ019079	HQ019121
Artemisia bigelovii A. Gray	Emery Co., Utah, USA (SSLP-EDM 2869)	HQ019038	HQ018996	HQ019080	HQ019122
Artemisia californica Less.	Santa Clarita, Los Angeles Co., California, USA (SSLP-EDM 3039)	HQ019039	HQ018997	HQ019081	HQ019123
Artemisia cana Pursh subsp. *bolanderi* (A. Gray) G. H. Ward	Bridgeport, Mono Co., California, USA (SSLP-EDM 3047)	HQ019040	HQ018998	HQ019082	HQ019124
Artemisia cana Pursh subsp. *cana*	Sheridan, Sheridan Co., Wyoming, USA (SSLP-EDM 2128)	HQ019041	HQ018999	HQ019083	HQ019125
Artemisia cana Pursh subsp. *viscidula* (Osterh.) Beetle	Warner Pass, Lake Co., Oregon, USA (SSLP-EDM 2436)	HQ019042	HQ019000	HQ019084	HQ019126
Artemisia deserti Krasch.	Semnan, Iran (BCN 13322)	HQ019043	HQ019001	HQ019085	HQ019127
Artemisia dracunculus L.	Kharkhorin, Khengai Aimag, Mongolia (BCN 17750)	HQ019044	HQ019002	HQ019086	HQ019128
Artemisia filifolia Torr.	Kanab, Kane Co., Utah, USA (BCN 13332)	HQ019045	HQ019003	HQ019087	HQ019129
Artemisia frigida Willd.	Kyzyl, Tüva, Russia (BCN 16421)	HQ019046	HQ019004	HQ019088	HQ019130
Artemisia longiloba (Osterh.) Beetle	Corral Creek, Grand Co., Colorado, USA (Linda Sanders 3)	HQ019047	HQ019005	HQ019089	HQ019131
Artemisia ludoviciana Nutt. subsp. *ludoviciana*	Zion National Park, Washington Co., Utah, USA (BCN 13955)	HQ019048	HQ019006	HQ019090	HQ019132
Artemisia nesiotica P. H. Raven	San Clemente Island, Los Angeles Co., California, USA (SSLP-EDM 3090)	HQ019049	HQ019007	HQ019091	HQ019133
Artemisia nova A. Nelson	Tunnel Spring, Desert Experimental Range, Millard Co., Utah, USA (SSLP-EDM 2876)	HQ019050	HQ019008	HQ019092	HQ019134
Artemisia nova A. Nelson subsp. *duchesnicola* Welsh et Goodrich	Tridell Road, Uintah Co., Utah, USA (SSLP-EDM 3029)	HQ019051	HQ019009	HQ019093	HQ019135
Artemisia palmeri A. Gray	Los Peñasquitos Canyon Preserve, San Diego, San Diego Co., California, USA (SSLP-EDM 3044)	HQ019052	HQ019010	HQ019094	HQ019136
Artemisia pedatifida Nutt.	North of Point Rocks, Sweetwater Co., Wyoming, USA (SSLP-EDM 1138)	HQ019053	HQ019011	HQ019095	HQ019137
Artemisia porteri Cronquist	Fremont Co., Wyoming, USA (SSLP-EDM 3094)	HQ019054	HQ019012	HQ019096	HQ019138
Artemisia pygmaea A. Gray	Juab Co., Utah, USA (BCN 14116)	HQ019055	HQ019013	HQ019097	HQ019139
Artemisia rigida (Nutt.) A. Gray	Malheur Reservoir, Malheur Co., Oregon, USA (SSLP-EDM 2859)	HQ019056	HQ019014	HQ019098	HQ019140
Artemisia rothrockii A. Gray	Reed Flats, White Mountains, Inyo Co., California, USA (L. M. Shultz 19803)	HQ019057	HQ019015	HQ019099	HQ019141
Artemisia tridentata Nutt. subsp. *parishii* (A. Gray) Hall et Clements	West of Rosamond, Kern Co., California, USA (SSLP-EDM 3037)	HQ019058	HQ019016	HQ019100	HQ019142
Artemisia tridentata Nutt. subsp. *spiciformis* (Osterh.) Kartesz et Gandhi	Ford Ridge, Bristle Cone Scout Camp, Carbon Co., Utah, USA (SSLP-EDM 2839)	HQ019059	HQ019017	HQ019101	HQ019143
Artemisia tridentata Nutt. subsp. *tridentata*	Salt Creek Canyon, Juab Co., Utah, USA (SSLP-EDM 2871)	HQ019060	HQ019018	HQ019102	HQ019144
Artemisia tridentata Nutt. subsp. *vaseyana* (Rydb.) Beetle	Salt Creek Canyon, Juab. Co., Utah, USA (SSLP-EDM 2872)	HQ019061	HQ019019	HQ019103	HQ019145
Artemisia tridentata Nutt. subsp. *wyomingensis* Beetle et A. L. Young	Gordon Creek, Carbon Co., Utah, USA (SSLP-EDM 2886)	HQ019062	HQ019020	HQ019104	HQ019146
Artemisia tridentata Nutt. subsp. *xericensis* Winward ex R. Rosentreter et R. G. Kelsey	Mann Creek Reservoir, Washington Co., Idaho, USA (SSLP-EDM 2858)	HQ019063	HQ019021	HQ019105	HQ019147
Artemisia tripartita Rydb. subsp. *rupicola* Beetle	Pole Mountain, Albany Co., Wyoming, USA (SSLP-EDM 3033)	HQ019064	HQ019022	HQ019106	HQ019148
Artemisia tripartita Rydb. subsp. *tripartita*	Dubois Sheep Station, Clark Co., Idaho, USA (SSLP-EDM 2845)	HQ019065	HQ019023	HQ019107	HQ019149
Picrothamnus desertorum Nutt.	Winton Road, Sweetwater Co., Wyoming, USA (SSLP-EDM 2403)	HQ019066	HQ019024	HQ019108	HQ019150
Sphaeromeria argentea Nutt.	Point of Rocks, Sweetwater Co., Wyoming, USA (BCN 74770)	HQ019067	HQ019025	HQ019109	HQ019151
Sphaeromeria cana (D. C. Eaton) A. Heller	Elery Lake, Mono Co., California, USA (BCN 74775)	HQ019068	HQ019026	HQ019110	HQ019152

TABLE 2. Continued.

Taxon	Collection data and herbarium voucher	ITS	ETS	*trnS-trnC*	*trnS-trnfM*
Sphaeromeria capitata Nutt.	Lookout Mountains, Moffat Co., Colorado, USA (BCN 74773)	HQ019069	HQ019027	HQ019111	HQ019153
Sphaeromeria compacta (H. M. Hall) A. H. Holmgren, L. M. Shultz et Lowrey	Spring Mountains, limestone rock crevices, Clark Co., Nevada, USA (BCN 74768)	HQ019070	HQ019028	HQ019112	HQ019154
Sphaeromeria diversifolia (D. C. Eaton) Rydb.	Santiaquin Canyon, Uinta National Forest, Utah Co., Utah, USA (BCN 74774)	HQ019071	HQ019029	HQ019113	HQ019155
Sphaeromeria potentilloides A. Heller	Near Reese River, Lander Co., Nevada, USA (BCN 74771)	HQ019072	HQ019030	HQ019114	HQ019156
Sphaeromeria ruthiae A. H. Holmgren, L. M. Shultz et Lowrey	Refrigerator Canyon, Zion National Park, Washington Co., Utah, USA (BCN 74769)	HQ019073	HQ019031	HQ019115	HQ019157
Sphaeromeria simplex A. Heller	Moss Agate, Carbon Co., Wyoming, USA (BCN 74772)	HQ019074	HQ019032	HQ019116	HQ019158

Note: BCN = Herbarium of the Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona; EDM = E. Durant McArthur collection numbers, vouchers in the SSLP (Shrub Sciences Laboratory Herbarium), and other particular collection accession numbers.

included the whole ITS (ITS1-5.8S-ITS2), the ETS (excepting some limited regions of ambiguous alignment in the middle of the region), and the complete chloroplast *trnS*^{UGA}-*trnfM*^{CAU} and *trnS*^{GCU}-*trnC*^{GCA} sequences; (2) the second data set (global) addressed to delimit *Tridentatae* and allies within the genus *Artemisia* as a whole included the ITS1, ITS2, and the 3′ side of ETS. Independent and combined nuclear and plastid matrices were generated and analyzed, though results were kept only for the combined ITS+ETS sets ("sagebrushes" and global) and *trnS*^{UGA}-*trnfM*^{CAU}+*trnS*^{GCU}-*trnC*^{GCA} ("sagebrushes" only). The sequence matrices are available in Appendices S2 to S7 (see Supplemental Data with the online version of this article).

Phylogenetic analyses—For the first data set (the sagebrushes), the complementary species added to delimit the subgenus were *A. absinthium*, *A. deserti*, *A. dracunculus*, and *A. frigida* Willd. (following the criteria of [1] choosing a representation of each subgenus of *Artemisia* and [2] not being endemic to North America). For the second data set (global), complementary species were those used as outgroup taxa following previous phylogenetic analyses of the group (Sanz et al., 2008, and Tkach et al., 2008): *Ajania fastigiata* (C. Winkler) Poljakov, *Brachanthemum titovii* Krasch., *Chrysanthemum maximowiczii* V. Komarov, *Ch. zawadskii* Herbich, *Elachanthemum intricatum* (Franch.) Y. Ling et Y. R. Ling, *Hippolytia megacephala* (Rupr.) Poljakov, *Lepidolopsis turkestanica* (Regel et Schmalh.) Poljakov, *Nipponanthemum nipponicum* (Franchet ex Maxim) Kitam, *Tanacetum parthenium* (L.) Sch. Bip., and *Turaniphytum eranthemum* (Bunge) Poljakov. To increase the information contributed by plastid data, gaps in chloroplast DNA (21) were coded in a binary matrix (0: absence / 1: presence), following the simple indel coding method (Simmons and Ochoterena, 2000) that was added to the combined *trnS-trnfM* and *trnS-trnC* data set. Nuclear gaps were not coded, as according to our observations and previous experience (Sanz et al., 2008; Pellicer et al., 2010b), these data did not provide significant information.

Model selection and Bayesian inference analysis—Evolutionary models were selected with MrModeltest 2.3 (Nylander, 2004) under the Akaike Information Criterion (AIC; Akaike, 1974) and the hierarchical Likelihood Ratio Tests (hLRT) (Posada and Buckley, 2004). The chosen models were subsequently used to perform Bayesian MCMC analyses (Yang and Rannala, 1997) with MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Four Markov chains were run simultaneously for 1 to 6 $\cdot 10^6$ generations (depending on the data set), and these were sampled every 100 generations. Data from the first 1000 to 6000 generations were discarded as the "burn-in" period, after confirming that likelihood values had stabilized before the 1000th to 6000th generation. Posterior probabilities (PP) were estimated through the construction of a 50% majority rule consensus tree. The output trees were edited with FigTree v. 1.2.2. (Edinburgh, UK) and Adobe Photoshop CS3 Extended v. 10.0.1 (Dublin, Ireland).

Parsimony analysis—These analyses involved heuristic searches conducted with PAUP* 4.0b10 (Swofford, 2003) under the maximum parsimony criterion. Uninformative characters were excluded from the analyses, and outgroups were defined. The standard parameters were as follows: MulTrees 100 random taxon additions, with Tree Bisection Reconnection (TBR) branch swapping, one tree held at each step and characters equally weighted. Posterior heuristic searches were developed with the constraint of saving no more than 1000 trees larger or equal to the tree lengths. To obtain estimates of support for branches of tree nodes, faststep bootstrap analyses (Felsenstein, 1985) as implemented in PAUP* were carried out with the use of 1000 replicates, 10 random sequence additions per replicate, and no branch swapping. This is an alternative for large data sets, providing similar estimates to those performed with branch swapping (Mort et al., 2000).

Split decomposition—Given that processes like hybridization, introgression, and polyploidy can be important factors in the evolution of species diversity (reticulate evolution), it is possible that a bifurcating tree may not explain the whole evolutionary history of a given group (Winkworth et al., 2005). Although mostly bifurcating trees are used here to explain and analyze relations among these species, we also have included a Neighbor-Net splits graph analysis (with uncorrected p-distances), as implemented in SplitsTree 4 (Huson and Bryant, 2006).

Statistical analyses—The nonparametric Mann-Whitney test was carried out to evaluate significance of genome size differences among groups, after testing for normality of the data set with negative result. Analyses were performed only on 1Cx values, corrected for the polyploids on the basis of results of Garcia et al. (2008), to avoid the influence of the genome downsizing effect that polyploids may suffer (Leitch and Bennett, 2004). In addition to data obtained in the current study, those from a previous paper on *Artemisia* genome size (Garcia et al., 2008) have been included in the analyses for comparative purposes. Statistical analyses were performed with STATA v.10 (StataCorp LP, College Station, Texas, USA).

RESULTS

Phylogenetic analyses—The resulting phylogenetic analyses produced the trees shown in Fig. 1 (ITS+ETS reconstruction of the reduced data set), Fig. 2 (ITS1+ITS2+3′ETS reconstruction of the global data set), and Fig. S8 (*trnS-trnC+trnS-trnfM* regions of the reduced data set; see Supplemental Data with the online version of this article). The splits graph analysis (corresponding to the same data set and regions of Fig. 1 but excluding subspecies and presumed hybrid taxa) is shown in Fig. 3.

Separated and combined data set analyses—We tested congruence among nuclear and chloroplast data sets with the incongruence length difference test (ILD) (Farris et al., 1994), implemented in PAUP* as the partition homogeneity test (Swofford, 2003). The test did not find significant incongruence between the chloroplast regions ($P = 0.23$) but did report significant incongruence between the nuclear ones ($P < 0.01$) in all data sets. However, as none of the apparent topological conflict was between well-supported clades in the different analyses performed, nuclear regions were nevertheless combined (see trees in Figs. 1 and 2) after analyzing the nuclear regions separately, as in previous studies (Acevedo-Rosas et al., 2004; Hoggard et al., 2004; Li et al., 2007; Hidalgo et al., 2008; Englund et al., 2009). Although these nuclear regions form part of the same transcriptional unit, ETS apparently evolves faster than ITS (Baldwin and Markos, 1998; Bena et al., 1998), which could explain some of the incongruence, considering that the ILD test does not distinguish whether incongruence comes from different phylogenetic histories or from different rates of evolution (Li et al., 2007). Differential unequal concerted evolution

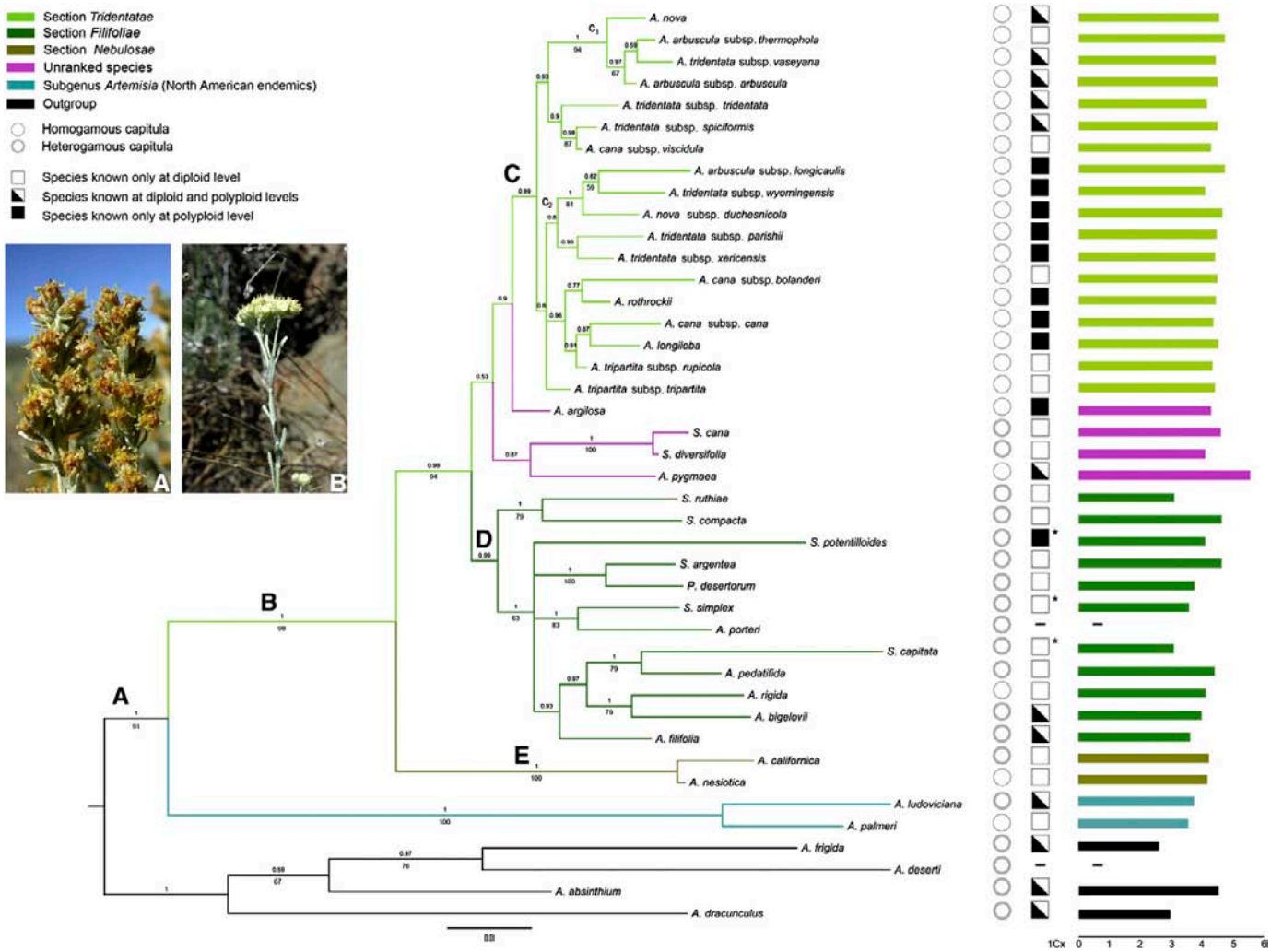

Fig. 1. Phylogenetic reconstruction (midpoint phylogram) obtained through combined analysis of ITS and ETS sequence data for 42 taxa (reduced set, "sagebrushes"). Majority rule consensus tree (50%) based on Bayesian MCMC inference with Bayesian clade-credibility values (posterior probability >0.5) above branches and parsimony bootstrap percentages (>50%) below branches. Flower heads composition and ploidy levels known for each species are depicted and monoploid genome sizes (1Cx) represented as bar graphs: note homogeneous genome size data for species from section *Tridentatae* and heterogeneous values for section *Filifoliae*. Asterisk (*) indicates ploidy levels inferred from known DNA amounts. Clades discussed in the text are indicated by letters. Scale bar indicates number of substitutions per site. Different inflorescence types are shown in the photographs: (A) Racemiform synflorescence of *Artemisia tridentata* subsp. *vaseyana*; (B) corymbiform synflorescence of *Sphaeromeria cana.* Both specimens are from White Mountains, Inyo Co., California (2003 Christopher L. Christie; photos reproduced with permission).

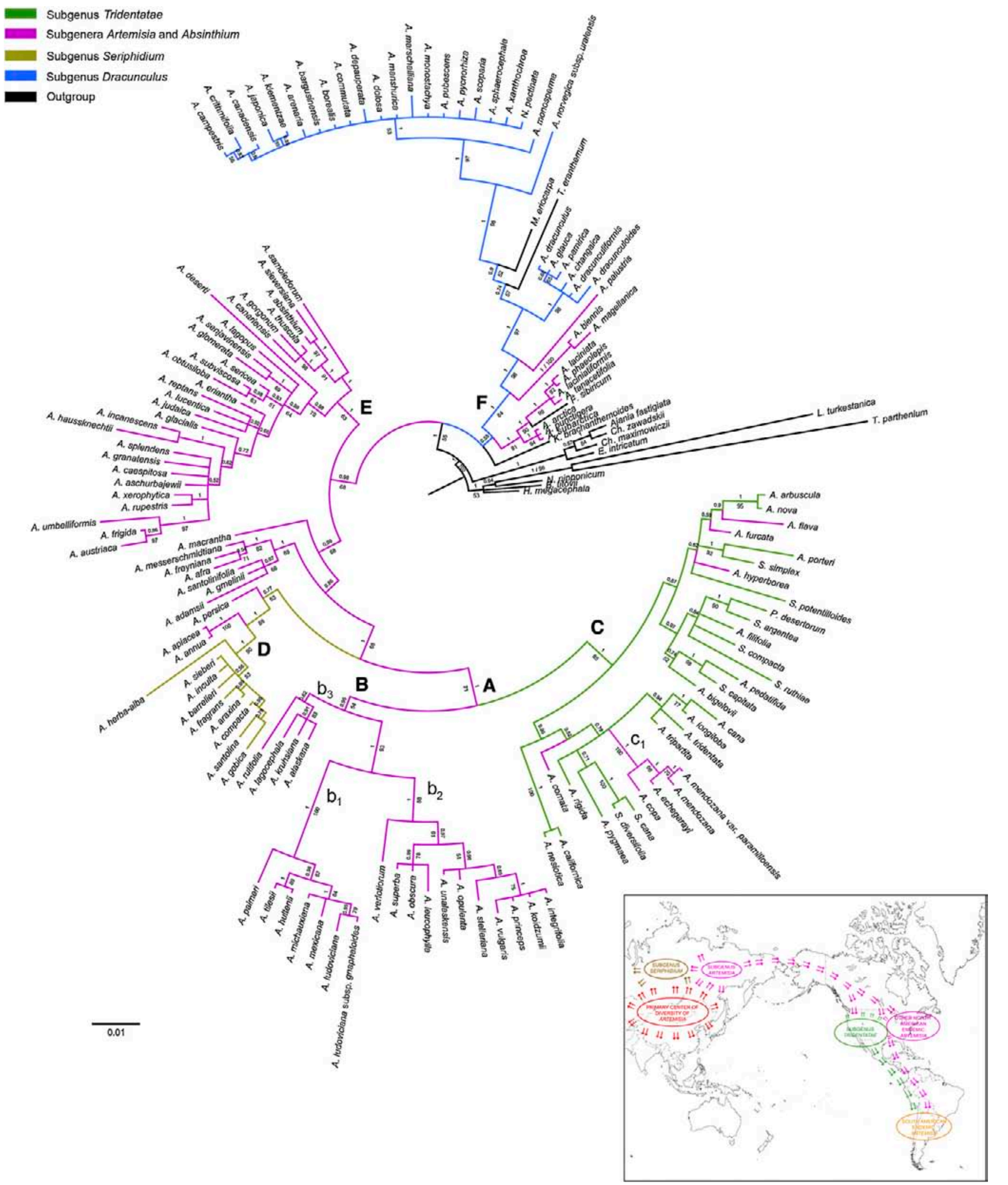

Fig. 2. Phylogenetic reconstruction (midpoint phylogram) obtained through combined analysis of ITS1, ITS2, and 3′ ETS sequence data for 152 taxa (global set, genus *Artemisia*). Majority rule consensus tree (50%) based on Bayesian inference with Bayesian clade-credibility values (posterior probability >0.5) above branches and parsimony bootstrap percentages (>50%) below branches. Clades discussed in the text are indicated by letters. Bar indicates number of substitutions per site. The map shows our hypothesis on the colonization of genus *Artemisia* through the Bering Strait to North America and its subsequent diversification in the New World.

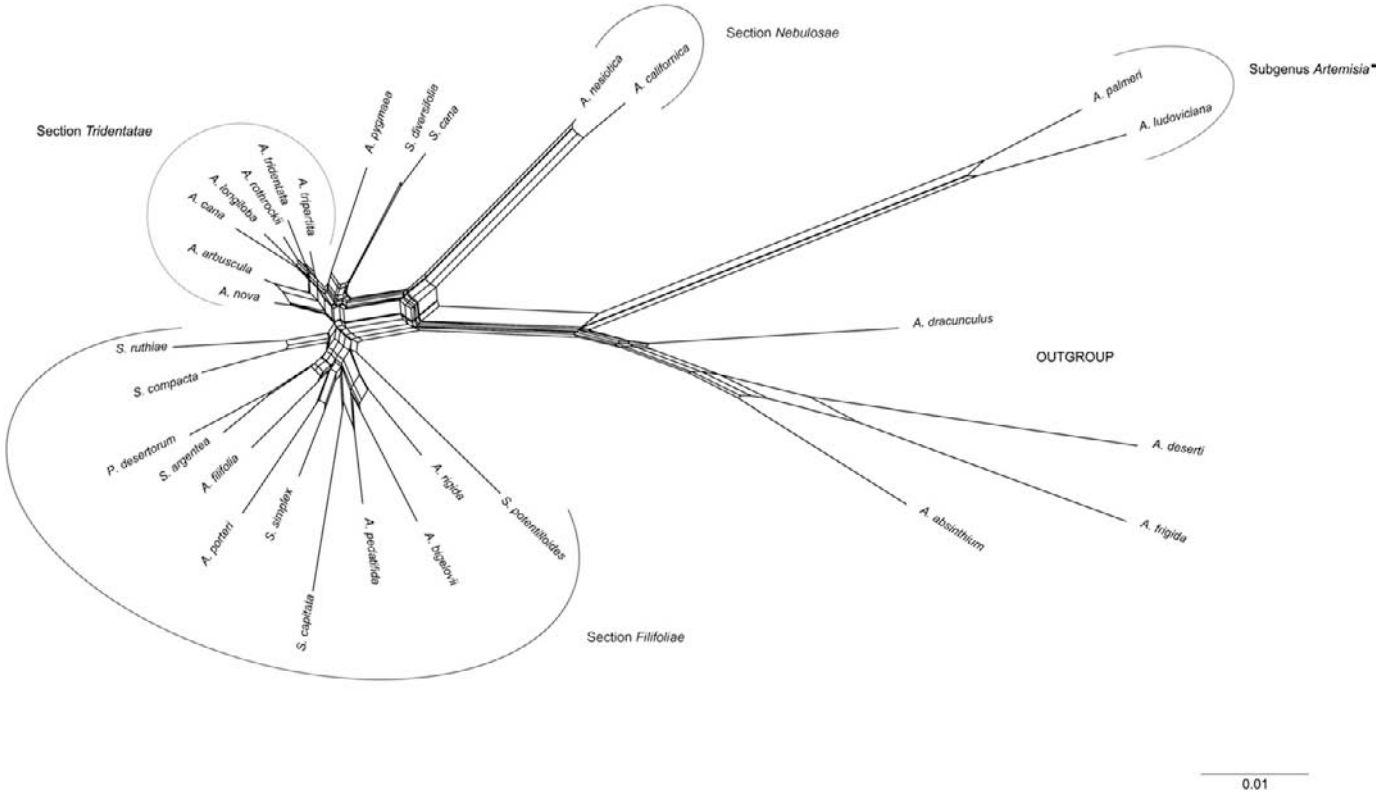

Fig. 3. Neighbor-net based on ITS+ETS sequences from the reduced set (subspecific and putative hybrid entities excluded) with uncorrected p-distances. Scale bar indicates number of expected changes. Asterisk (*) indicates North American endemic.

or recombination could be factors explaining differences in evolutionary rates of these regions as well. Moreover, the ILD test is also prone to inaccurate estimations of incongruence if the data sets are very different in size (Dowton and Austin, 2002; Li et al., 2007), as in the case of our study (653 vs. 1624 bp in the ITS+ETS reduced data set). Additionally, both of the combined ITS+ETS phylogenetic trees had better resolution than when the sequences were analyzed separately, an approach that has also been used to increase phylogenetic resolution in other plant groups (Whitten et al., 2000; Hall et al., 2002; Hoggard et al., 2004). The chloroplast data set (both regions, gaps coded) was not combined with the nuclear one because of some conflicts and also because it slightly lowered the phylogenetic resolution of the ITS+ETS data set. Nevertheless, the chloroplast combined sequence tree is shown (Appendix S8, see Supplemental Data with the online version of this article), as it supports monophyly of the subgenus *Tridentatae* and illustrates some interesting associations.

Model selection and Bayesian analysis—Both AIC and hLRT criteria (implemented in MrModeltest 2.3; Nylander, 2004) agreed on the model GTR+I+G (General Time Reversible model, with gamma-distributed site-to-site variation and a proportion of invariable sites) for both nuclear data sets ("sagebrushes" and global). For the chloroplast data set, hLTR fit the model F81+I+G and AIC fit GTR+I+G. No inconsistencies were detected between the resulting trees. Therefore we show only the results obtained with the AIC model, as this approach presents several advantages over the hLRTs for model selection, including the simultaneous comparison of nested or nonnested models, the accounting for model selection uncertainty, and the ability to allow model-average inference (Posada and Buckley, 2004). Midpoint rooted phylograms are shown in Figs. 1, 2, and S8 (see Supplemental Data with the online version of this article), with PP values >0.5 above branches.

Parsimony analysis—Table 3 summarizes the data related to trees in Figs. 1, 2, and S2 (bootstrap support [BS] > 50% below branches; see Supplemental Data with the online version of this article), including the characteristics and nucleotide substitution models selected for each data set.

Genome size assessments—Newly generated genome size data are presented in Table 4. These are the first reports for *Sphaeromeria*. At the diploid level, values show a 1.5-fold variation. The lower value is for the diploid *Sphaeromeria ruthiae* A. H. Holmgren, L.M. Shultz et Lowrey (6.20 pg), and the upper value is for the tetraploid *S. potentilloides* A. Heller (15.12 pg). The measurements were of overall good quality, with a mean HPCV (half peak coefficient variation) of 0.71% for the studied species and 3.55% for the internal standard.

Monoploid genome size data have been used to discriminate between clades in the subgenus, as was done previously between subgenera (Appendix S9, see Supplemental Data with the online version of this article; data from Garcia et al., 2004, 2008) and to take advantage of the complete data for all the species in the present analysis. Comparisons have been done only between clades C and D (Fig. 1) because of the reduced sample size of clade E (2 species), which did not allow its inclusion in

TABLE 3. Summary of sequence data from ITS+ETS, and *trnS*^{GCU}-*trnC*^{GCA}+ *trnS*^{UGA}-*trnfM*^{CAU}. Ensemble consistency and homoplasy indexes are calculated excluding uninformative characters.

Data set	ITS+ETS (global)	ITS+ETS (reduced)	*trnS-C*+ *trnS-fM* (reduced)
Number of taxa	152	42	42
Total characters	862	2278	1918
Number of informative characters	260	319	27
Missing data codified as "N" (%)	1.55	1.03	0.89
Gaps codified as "-" (%)	5.99	27.03	6.74
Tree length (number of steps)	1451	839	138
Range of divergence: ingroup–outgroup (%)	2.17–13.35	29.49–51.11	0.55–1.03
Range of divergence: ingroup (%)	0–10.16	0.33–50.22	0–0.65
Ensemble consistency index (CI)	0.390	0.501	0.717
Ensemble retention index (RI)	0.793	0.599	0.512
Homoplasy index (HI)	0.610	0.499	0.283
Rescaled consistency index (RC)	0.309	0.300	0.368
Nucleotide substitution model AIC	GTR+I+G	GTR+I+G	GTR+I+G
hLTR	GTR+I+G	GTR+I+G	F81+I+G

statistical analyses. After testing for normality (with a negative result), we decided to use the nonparametric Mann-Whitney test, which gave a statistically significant difference ($U = 159.50$, $df = 1$, $P = 0.005$) between monoploid genome sizes of clades C and D (1C*x*, Fig. 1). Clade C (corresponding to section *Tridentatae*) has the largest mean genome size value: 1C*x* = 4.46 vs. 1C*x* = 3.92, respectively, for clades C and D.

DISCUSSION

Description and delimitation of subgenus* Tridentatae *and genome size differences between sections—By and large, results (Fig. 1) 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., 2008) in the same clade. However, because of incomplete sampling, these earlier authors were cautious about subsuming these in *Tridentatae*. On the basis of the phylogenetic relations presented in this study, we support a wider concept of subgenus *Tridentatae*, enlarging its traditional circumscription and building on the recent revision by Shultz (2009; see Table 1). In her monograph, Shultz (2009) recognized two sections: section *Tridentatae* (with homogamous capitula and including all the classically considered members of the subgenus, 10 species) and section *Nebulosae* (with heterogamous capitula, created to accommodate *A. californica* Less., *A. filifolia* Torr., and *A. nesiotica* P. H. Raven, species placed within the *Tridentatae* clade in independent molecular analyses).

Our nuclear (ITS+ETS) molecular phylogenetic reconstruction of *Tridentatae* and allies (Fig. 1) shows that all the North American endemics are grouped together (clade A, PP = 1.0, BS = 91%). The chloroplast tree (Fig. S2, see Supplemental Data with the online version of this article) also resolves the same clade (clade A) but with nonsignificant statistical support (PP = 0.79, BS = 50%). We define what we consider subgenus *Tridentatae* at clade B, both in the nuclear (PP = 1.0, BS = 98%) and in the chloroplast (PP = 0.99) reconstructions, therefore expanding subgenus *Tridentatae* to include some species previously treated in other subgenera. The sister group, highly supported in the nuclear phylogeny (PP = 1.0, BS = 100%) and in the chloroplast one as well (PP = 1.0, BS = 92%), is composed of two North American endemic species of subgenus *Artemisia*, *A. ludoviciana* Nutt. and *A. palmeri*, which were included in Watson et al.'s (2002) phylogenetic treatment as the "*A. vulgaris* group".

Sections and unranked species—Our description of subgenus *Tridentatae* is based on the nuclear reconstruction (Fig. 1), since the chloroplast phylogeny is basically unresolved, only well defining the limits of the subgenus. The first clade (C, PP = 0.99) is our section *Tridentatae*, e.g., the *Tridentatae* sensu stricto or *Tridentatae* core as recognized by Garcia (2007) and Garcia et al. (2007). Section *Tridentatae* is clearly a natural group, morphologically homogeneous and geographically defined; it is monophyletic, with all the species within this clade being typical *Tridentatae* members, all shrubby plants with homogamous capitula and whose placement has never been in question. This section is equivalent to section *Tridentatae* sensu Shultz (2009) with the exclusion of *A. bigelovii*, *A. pygmaea*, and *A. rigida*. These species, which have been classically included in the subgenus, have been the subject of taxonomic controversy, with many studies with different research emphases (morphological, ecological, cytogenetic) proposing either their inclusion or exclusion (discussed later). Section *Tridentatae* has also two particularities with respect to the rest of the tree: on the one hand, it is the group in which polyploidy is most frequent (note the squares in Fig. 1); on the other hand, this section presents shorter branches.

When Shultz (2009) erected the new section *Nebulosae* to accommodate three species that in previous molecular research had appeared closely related within subgenus *Tridentatae*, she chose the name both in reference to the still uncertain or "nebulous" boundaries of the proposed section and to allude to the range of those species forming a geographical cloud-like bordering of the Intermountain Region, the core distribution area for section *Tridentatae*. She also noted that species from *Sphaeromeria* and maybe other non-*Tridentatae Artemisia* might be part of the "nebulous complex" but did not propose a transfer until more species of these groups were studied with molecular data. The current study meets this requirement and expands knowledge not only for all but one *Sphaeromeria* species but also for the monotypic *Picrothamnus* and for other taxa of uncertain position. Following this reasoning, the next clade with significant support (clade D, PP = 0.99), a grouping of several North American endemic *Artemisia*, *Picrothamnus*, and *Sphaeromeria*, would correspond to an expansion of this "nebulous complex" concept. However, it would be taxonomically incorrect to use the name of section *Nebulosae* for clade D, since the type species selected by Shultz in describing this section is *A. californica*, which is not included in this clade but which forms a separate, well-supported grouping with *A. nesiotica* (discussed later). Therefore, the name "*Nebulosae*" should be conserved for the section keeping the type species (W. Greuter, Botanical Garden and Botanical Museum, Berlin, personal communication), and in consequence, a new section is proposed, based in the unranked infraspecific taxon *Filifoliae* Rydb. (with type species *A. filifolia* and including also *A. pedatifida*), covering the clade D composition. The diagnostic criteria for this section are the same as stated by Rydberg (1916) when describing *Filifoliae*. The circumscription of sections *Nebulosae* and *Tridentatae* and the description of section *Filifoliae*, as

TABLE 4. Genome size and other karyological data of the species of *Sphaeromeria* assessed. In all cases, the standard used for flow cytometry assays was *Petunia hybrida* 'PxPc6'.

Species	P. L.	2C (pg)	S. D.	1C*x* (pg)	2C (Mbp)	HPCV sample	HPCV standard
Sphaeromeria argentea	2	9.26	0.21	4.63	9,056.3	1.02	3.01
Sphaeromeria cana	2	9.2	0.47	4.60	8,997.6	0.73	4.62
Sphaeromeria capitata	2*	6.21	0.07	3.11	6,073.4	1.60	3.37
Sphaeromeria compacta	2	9.28	0.16	4.64	9,075.8	0.36	2.70
Sphaeromeria diversifolia	2	8.2	0.28	4.10	8,019.6	0.49	2.90
Sphaeromeria potentilloides	4*	15.12	0.21	3.78	14,787.4	0.25	6.06
Sphaeromeria ruthiae	2	6.2	0.25	3.10	6,063.6	0.73	3.27
Sphaeromeria simplex	2*	7.15	0.28	3.58	6,992.7	0.51	2.53

Note: P. L. = Ploidy level; 2C (pg) = nuclear DNA content in pg; S. D. = standard deviation; 1C*x* (pg) = monoploid genome size (nuclear DNA content per haploid chromosome set); 2C (Mbp) = nuclear DNA content in Mbp (1 pg = 978 Mbp; Doležel et al., 2003); HPCV = half peak coefficient of variation.

*Ploidy levels inferred from genome size data.

well as the combination of some *Sphaeromeria* species, have been presented in a separate taxonomic note (Garcia et al., in press).

The composition of section *Filifoliae* is diverse, including (1) species previously considered to be members of section *Tridentatae* (*A. bigelovii* and *A. rigida*); (2) some *Artemisia* (*A. pedatifida* Nutt., *A. porteri* Cronquist, and *A. filifolia*) and the monotypic genus *Picrothamnus*; all these were formerly placed in subgenus *Dracunculus* on the basis of their heterogamous capitula with functionally staminate disc florets; and finally, (3) most species from *Sphaeromeria*. From the morphological point of view, most of these species have in common a shrubby habit (though several *Sphaeromeria* are short herbaceous perennials), the presence interxylary cork, and heterogamous capitula (excepting *A. rigida*, with homogamous flower heads).

The third differentiated group in clade B is clade E (PP = 1.0, BS = 100%), including the species *A. californica* and *A. nesiotica*, formerly considered members of subgenus *Artemisia* (Shultz, 2006a). This clade is also strongly supported by the chloroplast reconstruction (Fig. S2, see Supplemental Data with the online version of this article; PP = 1.0). These species are separated from the other North American endemic members of subgenus *Artemisia* (*A. ludoviciana* and *A. palmeri*). They are also distinct from the remainder geographically, as they exclusively inhabit coastal or island regions of the California shore. Shultz (2009) placed them in the new section *Nebulosae* (characterized by heterogamous capitula) together with *A. filifolia*; however, the present data do not support this grouping, as previously discussed. Given the close affinity between both California species (indeed, *A. nesiotica* has been described previously as a subspecific taxon of *A. californica*, *A. californica* Less. var. *insularis* Munz) and their mild distinctiveness from other *Artemisia* taxa (shrubby habit, wandlike stems), section *Nebulosae* should be kept for the two California species, *A. californica and A. nesiotica*, but excluding *A. filifolia*.

Finally, four species (*A. argilosa*, *A. pygmaea*, *S. cana* [D. C. Eaton] A. Heller, and *S. diversifolia* [D. C. Eaton] Rydb.), though clearly members of subgenus *Tridentatae*, are not included in any section, as they do not reside in any strongly supported clade. The position of A. *argilosa* and A. *pygmaea* as a potential sister group to section *Tridentatae* was suggested previously (Garcia, 2007; Garcia et al., 2007), but the present analysis does not fully support this suggestion. The placement of these two *Sphaeromeria* species is additional evidence for the artificial nature of this genus, as discussed later. By and large, the splits graph analysis (Fig. 3) may be illustrative of species relations in this group, in which species from section *Tridentatae* appear closely interrelated, species from section *Filifoliae* are more loosely clustered and much more divergent, and species of section *Nebulosae* are clearly apart from all other species, with the unranked taxa clustering somewhere in between. These groupings also suggest an assemblage in which hybridization might be a common evolutionary phenomenon.

Genome size comparisons have been performed between sections *Tridentatae* and *Filifoliae* (but not section *Nebulosae* because of its reduced sample size), resulting in a statistically significant difference between sections ($P = 0.0065$); section *Tridentatae* has the largest mean genome size (1C*x*). This is consistent with a larger mean genome size value reported for subgenus *Tridentatae* with respect to the other *Artemisia* subgenera (Garcia et al., 2004, 2008). Moreover, as seen in the bar diagram of Fig. 1, genome size values are much more heterogeneous for section *Filifoliae* than for section *Tridentatae*, consistent with the heterogeneous composition of section *Filifoliae* (Figs. 1 and 3).

Interspecific relations within sections—Many of the groupings within the different supported clades suggest associations between unrelated species. These anomalous groupings may be a response to the geographical proximity of populations that facilitated hybridization and/or introgression processes (reticulation) that complicate the understanding of phylogenetic relations. Although our approach, as others' (Kornkven et al., 1998, 1999; Watson et al., 2002; Shultz, 2006a; Riggins, 2008), fails to discriminate any groupings, previous taxonomic treatments (Ward, 1953; Beetle, 1960; Shultz, 1983) have suggested the existence of distinctive sagebrush lineages (Table 1), in which the species *A. tridentata* Nutt., *A. arbuscula*, and *A. nova* A. Nelson appear closely related, as they do in clades c1 and c2 (Fig. 1). The close relation between these three species has been foreshadowed by several nomenclatural rearrangements, as *A. nova* has been described as a subspecies of *A. tridentata* and also as a subspecies of *A. arbuscula*, which, likewise, has been described as a subspecies of *A. tridentata*.

Genera* Sphaeromeria *and* Picrothamnus: *the need for a nomenclatural revision*—Sphaeromeria*** and *Picrothamnus* are the only genera with an exclusive North American distribution within Artemisiinae (Bremer and Humphries, 1993; Oberprieler et al., 2007). Previous phylogenetic approaches (Watson et al., 2002; Vallès et al., 2003; Lowrey and Shultz, 2006; Shultz, 2006b;

Riggins, 2008; Sanz et al., 2008) did not fully resolve their actual placement, but all noted their close relation to *Artemisia*, given that in most of these authors' phylogenies, *Sphaeromeria* and *Picrothamnus* constituted part of the *Artemisia* clades. From the composition of section *Filifoliae* in the nuclear analysis (Fig. 1, D), that of clade B in the chloroplast one (Fig. S2, see Supplemental Data with the online version of this article), and their placement in the global reconstruction (Fig. 2), it seems clear that neither *Sphaeromeria* nor *Picrothamnus* should be considered independent genera. Moreover, these species appear embedded in the subgenus *Tridentatae* clade. Additionally, in the case of *Sphaeromeria*, species are spread in segregated clades within *Tridentatae*, an indication, as pointed previously, that *Sphaeromeria* is an artificial, polyphyletic genus.

The presence of mostly corymbiform inflorescences rather than the paniculiform, racemiform, or spiciform arrays more typical of *Artemisia* is probably the most visible trait distinguishing *Sphaeromeria* from most *Artemisia* (Fig. 1, photographs), particularly from the North American endemics, though corymbs may have evolved multiple times in different lineages of the genus and among its Eurasian allies. Because of their morphological appearance, many species of *Sphaeromeria* had been classified previously as members of *Tanacetum* L. (see Shultz, 2006a), though some of them had been segregated in the monotypic genera *Chamartemisia* Rydb. (*Sphaeromeria compacta* [H. M. Hall] A. H. Holmgren, L. M. Shultz et Lowrey) and *Vesicarpa* Rydb. (*Sphaeromeria potentilloides* [A. Gray] A. Heller). However, the work of Holmgren et al. (1976) treated *Sphaeromeria* closer to *Artemisia* than to *Tanacetum* on the basis of morphological traits besides inflorescence types; results consistent with these observations were obtained with RAPD analysis (McArthur et al., 1998b) and later were confirmed by other molecular work (Watson et al., 2002; Riggins, 2008). Genome size data for *Sphaeromeria* species are quite heterogeneous, even at the same ploidy level (Fig. 1 and Table 4), which is additional evidence of the heterogeneity of the species constituting this genus and its likely artificial nature. On a larger taxonomic scale, however, the values at diploid and tetraploid levels are consistent with subtribe Artemisiinae genome size data (Garcia et al., 2004, 2008; Pellicer et al., 2010a).

Morphological similarity of *Picrothamnus desertorum* with *Artemisia* members is clearer. This taxon was first described by Nuttall (1841) but later placed in *Artemisia* as *A. spinescens* D. C. Eaton (1871), in section *Dracunculus*. Hall and Clements (1923) also considered it a member of *Dracunculus* because of its functionally male central florets with reduced ovaries and fused style-branches, and they stated that features such as the villous pubescence of achenes and corollas or the characteristic spiny habit could not be contemplated at generic or even sectional value. Later, however, Bremer and Humphries (1993) regarded these traits as autapomorphies of the species; although considered a member of subgenus *Dracunculus* on the basis of floral morphology, its phylogenetic position (in this study and previous ones) within an expanded subgenus *Tridentatae* is clear. This point brings into serious question the validity of floral morphology as a defining subgeneric character; apparently homogamous flower heads have appeared several times during the evolution of *Artemisia* (Watson et al., 2002; Sanz et al., 2008).

The results of this study coupled with previous work recommend a nomenclatural redefinition of these genera, subsuming their species within *Artemisia* subgenus *Tridentatae* section *Filifoliae* or unranked within the subgenus. *Picrothamnus desertorum* should be best recognized as *Artemisia spinescens*, a name that, in addition, constitutes an appropriate description of the species. Indeed, this treatment was yet followed by some authors (L. M. Shultz, personal communication). In the case of *Sphaeromeria*, we are proposing the new names in a taxonomic revision of sections of the subgenus (Garcia et al., in press).

Excluded species from sect. **Tridentatae**—The three species (*Artemisia bigelovii*, *A. pygmaea*, *A. rigida*) excluded from section *Tridentatae* (or the *Tridentatae* core) consist of three interesting cases. Their phylogenetic position in the subgenus has been questioned by several authors on the basis of morphological and molecular data. Previous treatment of these species is illustrative of the taxonomic problems accompanying the subgenus that gave impetus to this study.

First is the case of *Artemisia bigelovii* and its anomalous floral morphology (the only *Tridentatae* taxon with heterogamous capitula; Hall and Clements, 1923; Ward, 1953; Shultz, 1983; Ling, 1991, 1995a). Molecular phylogenetic data (Kornkven et al., 1998), essential oil composition (Holbo and Mozingo, 1965; Geissman and Irwin, 1974), and our own results on molecular cytogenetics and genome size (Garcia et al., 2007, 2008) clearly differentiate it from the *Tridentatae* core. This species generally has been treated as a member of *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 for the segregation of *A. bigelovii* from section *Tridentatae*, though retained in the subgenus *Tridentatae.*

The second case, *Artemisia rigida*, is a species well adapted to particular habitats and displays specialized morphological and anatomical modifications to extreme conditions of aridity (Hall and Clements, 1923; Shultz, 1983; McArthur and Stevens, 2004). Its distinctiveness gave rise to its separate placement in another section within subgenus *Seriphidium*, sect. *Rigidae* Rydb. (Rydberg, 1916). Holbo and Mozingo's (1965) chromatographic characterization also pointed to its exclusion from the *Tridentatae* core, as do our findings with in situ hybridization, genome size studies (Garcia, 2007; Garcia et al., 2008), and the present results, which place it apart from the core of the sagebrushes and suggest a tight relation with *A. bigelovii* (PP = 1.0, BS = 79%, Fig. 1). In this line, Kornkven et al. (1999) stated that *A. rigida* may have diverged early in the evolution of the subgenus, on the basis of its sister position to the core *Tridentatae* species in their phylogeny, in addition to its morphological specialization. Shultz's (2009) analysis on pollen morphology suggested an alliance with *A. cana* and *A. tripartita* (sharing elongated pollen grains, the three of them), which may represent a xeromorphic specialization.

The last case is pygmy sagebrush, *Artemisia pygmaea*, a dwarf shrub with different leaf morphology and larger seeds compared with the other *Tridentatae* and considered the most xerophytic taxon in the subgenus (Cronquist, 1994; McArthur and Stevens, 2004). It is a relatively uncommon species, limited to limestone soils in the desert areas of central and western Utah, eastern Nevada, and northern Arizona (Ward, 1953). On the basis of specialized ecologically adaptive features, Rydberg (1916) placed *A. pygmaea* in a separate section (sect. *Pygmaea* Rydb.). Essential oil composition (Holbo and Mozingo, 1965; Geissman and Irwin, 1974), some differences in karyotype morphology (Garcia et al., 2007), and a significantly larger genome size (Garcia et al., 2008) also differentiate it from most members of section

Tridentatae. Previous molecular biology studies have placed this species as sister to the other *Tridentatae* (Kornkven et al., 1998; Watson et al., 2002), and our data also place it as a sister to the core *Tridentatae*, along with two *Sphaeromeria* species.

Each of the three species, *Artemisia bigelovii*, *A. pygmaea*, *and A. rigida*, while not placed in section *Tridentatae*, are still included in subgenus *Tridentatae*.

A broader approach: circumscription of the New World* Artemisia *species in the framework of the genus—With the purpose of not only obtaining an adequate phylogenetic delimitation of the North American subgenus *Tridentatae* but also of other New World endemic *Artemisia*, we conducted a larger phylogenetic analysis of the genus. In this analysis (global data set, Fig. 2) the western North American and other New World endemic *Artemisia* are resolved exclusively in clade A (PP = 1.0, BS = 71%). Within this clade, clade B (supported by a PP = 0.95, BS = 54%) is constituted by species from subgenera *Artemisia* and *Absinthium*, including some North American; clade C (PP = 1.0 and BS = 85%) contains the majority of New World endemics. Given that American species appear in two different clades, our data indicate that New World endemics are of multiple origins, supporting the hypothesis by Tkach et al. (2008) of several migrations from Asia to North America. Clade D (PP = 1.0 and BS = 90%) within the large clade A, is constituted by subgenus *Seriphidium* species that also have a close relation with subgenus *Artemisia*, suggesting that both *Tridentatae* and *Seriphidium* could have their ancestry in species from subgenus *Artemisia*.

In clade B, the North American endemics from subgenus *Artemisia* (both *A. ludoviciana* subspecies, *A. mexicana* Willd. *A. michauxiana* Besser, and *A. palmeri*) appear in a highly supported clade (b_1, PP = 1.0, BS = 100%) together with *A. hultenii* Maximova and *A. tilesii* Ledeb. (also called *A. hultenii* subsp. *tilesii*), also from this subgenus. These latter species are native to Siberia (the type is described from Kamchatka) but are widely distributed in northern North America. It is likely that, given their geographic distribution and phylogenetic position, these species are involved in the origin of *A. ludoviciana* and relatives (assigned to subgenus *Artemisia* with their disciform, heterogamous heads and glabrous receptacles, among other defining characteristics). It is also possible that hybridization between Asiatic and American species explains the position of Siberian *Artemisia*. The sister clade to this grouping (b_2, PP = 1.0, BS = 88%) constitutes the *A. vulgaris* complex, a group of species from subgenus *Artemisia* previously pointed out by others (Sanz et al., 2008; Tkach et al., 2008; Pellicer et al., 2010b). Clade B is finally completed with a group (b_3) constituted by subarctic species (from Siberia and Alaska) currently classified in subgenus *Absinthium*.

It is evident, not only from this clade but also from the rest of the tree, that subgenera *Artemisia* and *Absinthium* are interrelated and their taxonomic separation is probably artificial. Comprehensive studies of the whole genus have provided results intermixing subgenera *Artemisia* and *Absinthium* and placing species within phylogenetic trees irrespective of putative *Artemisia/Absinthium* classical lineages (Torrell et al., 1999; Vallès et al., 2003; Riggins, 2008; Sanz et al., 2008; Tkach et al., 2008), another sign that both should be placed in a unified subgenus *Artemisia*. Nevertheless, it seems that most subgenus *Absinthium* members of this sampling do form clade E in the global analysis, with only some subgenus *Artemisia* members intermixed.

Clade C (PP = 1.0, BS = 85%) includes all the species previously described in subgenus *Tridentatae* with the difference, with respect to the former analysis (reduced data set), that section *Tridentatae* is not monophyletic. Both *Sphaeromeria* and *Picrothamnus* appear again subsumed in the same clade, thus supporting their placement in subgenus *Tridentatae*. It is likely that (1) the inclusion of many other nonendemic *Artemisia*, (2) the exclusion of subspecific and presumed hybrid entities, and (3) the shorter length of the ETS region used for this analysis with respect to the previous analysis blur relations within this subgenus. But on the other hand, the inclusion of additional nonendemic *Artemisia* may also constitute a clue about the possible ancestors of *Tridentatae* and other New World relatives. It has been suggested that subgenus *Seriphidium* was the closest relative to *Tridentatae* because of a synapomorphy of both subgenera, the homogamous capitula (Ward, 1953; Beetle, 1960; Ling, 1991, 1995a, b). An alternative scenario was proposed by McArthur and Plummer (1978) and McArthur et al. (1981), in which subgenus *Artemisia* could be basal to *Tridentatae*, as supported by our analysis. The most closely related group to clade C (New World *Tridentatae* and other endemics) is clade B, consisting in major part of species from subgenus *Artemisia.* In addition, some of the non-*Tridentatae* species in clade C also belong to subgenus *Artemisia*: *A. comata* Rydb., *A. flava* Jurtsev, *A. furcata* M. Bieb., and *A. hyperborea* Rydb. are Beringian (*A. furcata* being the only one with an amphi-Beringian range with extensions further south in Eurasia and North America), which fits with a likely biogeographic history of the New World species deriving from an ancestral *Artemisia* (subgenus) stock (map in Fig. 2).

Another clade of species consists of the South American endemics (c_1, PP = 1.0, BS = 100%). These are interesting, since the genus is not particularly abundant in the southern Hemisphere (Bremer and Humphries, 1993). Because these species display the typical capitula traits related to subgenus *Artemisia*, they have been considered as such, though previously one of them had been classified as a member of *Seriphidium* (de Candolle, 1837; Bremer and Humphries, 1993). *Artemisia echegarayi* Hieron, *A. mendozana* DC., and *A. mendozana* DC. var. *paramilloensis* F. A. Roig et J. A. Ambrosetti are endemic to Argentina; *A. copa* Phil. occurs in Argentina, Chile, the West Indies, and Mexico (Ling, 1995a). Given that some *Tridentatae* species are also present in Mexico and considering the position of the South American endemics in the phylogeny, it is likely that sagebrushes may be involved in the origin of this group, with *A. copa* being the sister species (PP = 1.0, BS = 100%) to the other South American endemics (except *A. magellanica* Sch. Bip., as indicated later). Although there is no clear sister-group relation with any other specific taxon in this clade, the position of the South American *Artemisia* within subgenus *Tridentatae* is clear, supporting a recent hypothesis about the origin of these species (Pellicer et al., 2010b). There is another Patagonian species, *A. magellanica*, of a clearly different phylogenetic origin than the c_1 group. This species is closely related to *A. biennis* Willd. (PP = 1.0, BS = 100%) and to species of subgenus *Dracunculus* (PP = 1.0, BS = 84%), suggesting that both in North and South America, the various endemic *Artemisia* species have their origins in different subgenera.

Conclusions—The results provided by this phylogenetic study call for a redefinition of subgenus *Tridentatae* to include

other western North America endemics. An expanded circumscription of the subgenus is proposed, dividing it into three sections: *Tridentatae*, *Filifoliae*, and *Nebulosae*, the last two erected to accommodate species and other genera that have been considered closely related, but in undefined ways, to the core sagebrushes. These conclusions have taxonomic–nomenclatural consequences. The genera *Sphaeromeria* and *Picrothamnus* should be treated as *Artemisia* species, and new nomenclatural combinations have been proposed (Garcia et al., in press) in this sense.

Relationships between species within the different sections are, however, difficult to interpret. Because of absence of reproductive barriers, reticulate events involving different kinds of hybridization among taxa (allopolyploidy, homoploid hybrid formation, introgression) may be abundant, giving birth to multiple and recurrent combinations that have surely contributed to the blurring of relations among taxa and have enhanced the well-deserved reputation of this group of being taxonomically difficult, even though these processes also may contribute to its evolutionary success, current species richness, and diversity. A different approach, probably involving studies at the population level, would be useful in elucidating particular interspecific relations in this group.

The phylogenetic position of the Beringian species from subgenus *Artemisia* within the *Tridentatae* clade and that of the other North American endemics in another clade of subgenus *Artemisia* suggest that the ancestors of the New World species should be found in subgenus *Artemisia* and that there have been at least two colonization events from Asia to America, giving rise to the North American endemics (map in Fig. 2).

For the South American (*A. copa*, *A. echegarayi*, *A. mendozana* var. *mendozana*, and *A. mendozana* var. *paramilloensis*) and the Beringian (*A. comata*, *A. flava*, *A. furcata*, and *A. hyperborea*) species, we question their transfer to subgenus *Tridentatae*. A more complete study should be performed on them, including morphological and ecological aspects as well as analysis of more DNA regions. In this sense, we prefer keeping the concept of subgenus *Tridentatae* sensu Shultz (2009): a New World alliance of shrubby species endemic to western North America.

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