

The genus *Artemisia* and its allies: phylogeny of the subtribe Artemisiinae (Asteraceae, Anthemideae) based on nucleotide sequences of nuclear ribosomal DNA internal transcribed spacers (ITS)

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Running head: ITS phylogeny of subtribe Artemisiinae.

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Abstract: Sequences of the internal transcribed spacers (ITS1 and ITS2) of nuclear ribosomal DNA were analysed for 44 *Artemisia* species (46 populations) representing all the five classical subgenera and the geographical range of the genus, 11 species from 10 genera closely related to *Artemisia*, and six outgroup species from five other genera of the Anthemideae. The results definitely support the monophyly of the genus *Artemisia* in its broadest sense (including some

taxa segregated as independent genera, like *Oligosporus* and *Seriphidium*). Eight main clades are established in this molecular phylogeny within *Artemisia*; they agree in part with the classical subdivision of the genus, but they also suggest that some infrageneric groups must be redefined, especially the subgenus *Artemisia*. The subgenera *Tridentatae* and *Seriphidium* are independent from each other. Some of the satellite genera are clearly placed within *Artemisia* (*Artemisiastrum*, *Filifolium*, *Mausolea*, *Picrothamnus*, *Sphaeromeria*, *Turaniphytum*), whereas some others fall outside the large clade formed by this genus (*Brachanthemum*, *Elachanthemum*, *Hippolytia*, *Kaschgaria*). Our results, correlated to other data, such as pollen morphology, allow us to conclude that the subtribe Artemisiinae as currently defined is a very heterogeneous group. Affinities of the largest genus of the subtribe and tribe, *Artemisia*, and of other genera of the subtribe to some genera from other subtribes of the Anthemideae strongly suggest that subtribe Artemisiinae needs a deep revision and redefinition. Phylogenetic utility of region *trnL-F* of the plastid DNA in the genus *Artemisia* and allies was also evaluated; sequences of the *trnL-F* region in *Artemisia* do not provide phylogenetic information.

Introduction

Artemisia L. is the largest genus in the tribe Anthemideae and one of the largest genera in Asteraceae, with more than 500 taxa (the number varies depending on the authors: McArthur, 1979^[43]; Mabberley, 1990^[39]; Ling, 1982^[33], 1991a^[34], 1991b^[35], 1994^[36], 1995a^[37], 1995b^[38]; Bremer and Humphries, 1993^[14]; Oberprieler, 2001^[53]; Vallès and McArthur, 2001^[80]). It is widely distributed in the Northern hemisphere, with two of its main speciation centres in West and Central Asia, and only a few representatives grow in the southern hemisphere. Some species dominate the landscape in arid lands in different regions of the world. Most species in the genus are perennial; only approximately 10 species are annual or biennial. *Artemisia* has two basic chromosome numbers, with ploidy levels ranging from diploid to dodecaploid for $x=9$ and from diploid to hexaploid for $x=8$ (Vallès and McArthur, 2001^[80]). Many species of this genus have a high economic value as medicines, food, forage, ornamentals or soil stabilizers in disturbed habitats; some taxa are toxic or allergenic, and some others are invasive weeds which can adversely affect harvests (Pareto, 1985^[55]; Tan et al., 1998^[74]).

Because of the high number of taxa and the ecological and economic importance of many of them, the genus *Artemisia* has been object of many diversely focused studies. Since Tournefort (1700^[78]), many attempts at infrageneric classification have been made; the comparative analysis of these groups (see Table 1 in Torrell et al., 1999^[75], and in Vallès and McArthur, 2001^[80]) shows that five major divisions, treated as sections or subgenera (*Artemisia*, *Absinthium*, *Dracunculus*, *Seriphidium* and *Tridentatae*) are rather constant from the classical works of Besser (1829^[8], 1832^[9], 1834^[10], 1835^[11]) and Candolle (1837^[15]), with a more recent slight modification concerning the latter group, a North American endemic (Rydberg, 1916^[61]);

McArthur et al., 1981^[46]). Apart from the consideration of the main infrageneric groups at different taxonomic levels and with different circumscription, many authors proposed splitting the large genus *Artemisia*. This started very early with Cassini (1817^[16]), who segregated subgenus *Dracunculus* as an independent genus, *Oligosporus* Cass., which has not been retained by modern authors. More recently, Ling (1991a^[34], 1991b^[35], 1994^[36], 1995a^[37], 1995b^[38]) proposed the consideration of one of the largest subgenera of *Artemisia* as a genus, *Seriphidium* (Besser ex Hook.) Fourr., which has been accepted by Bremer and Humphries (1993^[14]) and Bremer (1994^[13]) in their cladistic revisions of Anthemideae and Asteraceae, respectively. In addition, the genus *Tanacetum* L. has had limit conflicts with *Artemisia*. Apart from the above stated large groups, many other small, often monotypic genera have been segregated from *Artemisia*, or have inherited species formerly belonging to *Artemisia*: *Ajania* Poljakov, *Artemisiastrum* Rydb., *Artemisiella* A. Ghafoor, *Crossostephium* Less., *Elachanthemum* Y. Ling & Y. R. Ling, *Filifolium* Kitam., *Hippolytia* Poljakov, *Kaschgaria* Poljakov, *Lepidolopsis* Poljakov, *Mausolea* Poljakov, *Neopallasia* Poljakov, *Picrothamnus* Nutt., *Stilpnolepis* H. Kraschen., and *Turaniphytum* Poljakov. Finally, there are some genera very closely related to, but never included in *Artemisia*, such as *Sphaeromeria* Nutt. In the pool constituted by the above cited genera, the taxonomy of some species has been quite controversial: they have been either described in *Artemisia* and combined in other genera (such as *Hippolytia megacephala* (Rupr.) Poljakov, described as *Artemisia megacephala* Rupr.), or described in other genera and combined in *Artemisia* (such as *Artemisia incana* (L.) Druce, described as *Tanacetum incanum* L.), or even combined in more than one genus (such as *Kaschgaria brachanthemoides* (C. Winkl.) Poljakov, described as *Artemisia brachanthemoides* C. Winkl., and also combined as *Tanacetum brachanthemoides* (C. Winkl.) H. Krashen.). The genus *Artemisia* and most of its segregated and/or closely related genera constitute the subtribe Artemisiinae Less. emend.

Bremer & Humphries, although other genera close to *Artemisia* are included in different subtribes of Anthemideae, such as Tanacetinae and Handeliinae.

DNA sequencing is one of the most powerful present tools in phylogenetic analysis, to such an extent that Crawford (2000^[17]) stated that currently “plant systematics is in the sequencing phase”. In particular, the internal transcribed spacer region (ITS) of nuclear ribosomal DNA has proved to be useful in inferring phylogenetic relationships at the generic and infrageneric levels in angiosperms (Baldwin et al., 1995^[6]), and has been studied in many groups of Asteraceae (Baldwin, 1992^[4], 1993^[3]; Urbatsch and Baldwin, 1993^[79]; Kim and Jansen, 1994^[26]; Bain and Jansen, 1995^[3]; Susanna et al., 1995^[69], 1999^[70]; Bayer et al., 1996^[7]; Garcia–Jacas et al., 2000^[23]; Vilatersana et al., 2000^[82]; Francisco–Ortega et al., 2001^[22]). There are some recent molecular studies of *Artemisia*, dealing with all the infrageneric groups (Torrell et al., 1999^[75]) or focused on the North American subgenus *Tridentatae* (Kornkven et al., 1998^[28], 1999^[29]; McArthur et al., 1998a^[49], 1998b^[44]); all of these support the monophyly of the genus *Artemisia* in its broad sense. Apart from these taxonomically–oriented papers on large sets of *Artemisia* species, a few other works on DNA in this genus have been published (McArthur et al., 1992^[47]; Watson, 1996^[83]; Francisco–Ortega et al., 1997^[21]; Kornkven and Watson, 1997^[27]; Watson et al., 1998^[84], 2000^[85]; Linder et al., 2000^[32]; Oberprieler and Vogt, 2000^[54]), either concerning a very reduced number of taxa, or without systematic and evolutionary purposes, or only in form of abstract.

In a previous paper using ITS sequencing data of 31 *Artemisia* species (Torrell et al., 1999^[75]) we demonstrated the monophyly of the genus, including the subgenera *Seriphidium* and *Dracunculus* (genus *Oligosporus*), the independence of *Tridentatae* from *Seriphidium*, and the

high polyphyly of the subgenus *Artemisia*. In the present paper, we study the relationships between *Artemisia* and 10 of its closely related genera, as a step towards a redefinition of Artemisiinae. In addition, we perform a deeper analysis of the systematics and the phylogeny of the genus *Artemisia* itself through the consideration of a higher number of taxa.

Materials and Methods

Plant material

We studied 44 *Artemisia* species (46 populations), representing all the five classical subgenera and the geographical range of the genus, and 11 species belonging to 10 genera closely related to *Artemisia*. Voucher specimens were deposited in the Herbarium BCF (Laboratori de Botànica, Facultat de Farmàcia, Universitat de Barcelona), recently merged with BCC (Laboratori de Botànica, Facultat de Biologia, Universitat de Barcelona) in the new Herbarium BCN (Centre de Documentació de Biodiversitat Vegetal de la Universitat de Barcelona); we maintain the BCF numbers (Table 1), with which most of the specimens have already been reported in previous papers. As outgroups, we used six species from five other Anthemideae genera: *Ajania fastigiata*, *Arctanthemum arcticum* and *Chrysanthemum coreanum* (sequences from Francisco–Ortega et al., 1997); and *Ajania pacifica*, *Lepidolopsis turkestanica* and *Tanacetum parthenium*. Outgroups were chosen according to Bremer and Humphries (1993^[14]) and also following Francisco–Ortega et al. (1997^[21]). The 63 accessions used are listed in Table 1. Apart from the five main groups in *Artemisia* mentioned in the introduction, often treated as subgenera, some authors have proposed sections, subsections and series (Rydberg, 1916^[61]; Poljakov, 1961a^[58], 1961b^[59]; Korobkov, 1981^[30]; Ling, 1991a^[34], 1991b^[35], 1995a^[37], 1991b^[38]), but a global treatment of the entire

genus at these levels has not yet been achieved. In the light of this situation, the taxa in Table 1 are grouped by the classical subdivisions. *Brachanthemum*, *Elachanthemum*, *Hippolytia*, *Kaschgaria*, *Lepidolopsis*, *Mausolea*, *Picrothamnus*, *Sphaeromeria* and *Turaniphytum* are presented as separate genera, as presently accepted by most of authors (Poljakov, 1961a^[58], 1961b^[59]; Pavlov, 1966^[56]; Adylov and Zuckerwanik, 1993^[2]; Bremer and Humphries, 1993^[14]; Bremer, 1994^[13]; Abdulina, 1999^[1]). *Artemisiastrum* and *Picrothamnus* are also treated as separate genera, as recognised by Rydberg (1916^[61]). The species synonymy is provided in order to give an idea of the complexity of the taxonomical classification of many of the taxa studied. The analysis of ITS sequences used published sequences along with new sequences. Source of published sequences, voucher data, and GenBank sequence accession numbers for the newly studied species are given in Table 1.

DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted following the CTAB method of Doyle and Doyle (1987^[19]) as modified by Soltis et al. (1991^[67]) from silica gel dried leaves collected in the field, or fresh leaves of plants cultivated in the Botanic Institute of Barcelona or in the Faculty of Pharmacy of the University of Barcelona. In some cases, herbarium material was used.

Double stranded DNA of the ITS region was amplified with the 1406 F primer (Nickrent et al., 1994^[52]) and ITS4 (White et al., 1990^[86]). In some cases, we used ITS1 (White et al., 1990^[86]) and 17 SE (Sun et al., 1994^[68]) as forward primers and 26 SE (Sun et al., 1994^[68]) as reverse primer following the protocol described in Soltis and Kuzoff (1993^[66]) and thermostable DNA polymerase (Ecotaq, Ecogen S. R. L.).

The profile used for amplification included a hot start at 94°C for 2 min, followed by 80°C for 5 min, during which the polymerase was added. Thirty cycles of amplification were carried out under the following conditions: 94°C for 1 min 30 sec, 55°C for 2 min, and 72°C for 3 min, with an additional extension step of 15 min at 72°C.

The double stranded PCR products were cleaned using the QIAquick PCR Purification Kit (QIAGEN Inc.). Direct sequencing of the amplified DNA segments was performed using a Thermo Sequenase II Dye Terminator Cycle sequencing kit (Amersham), following the manufacturer's protocol. The nucleotide sequencing was performed at the "Serveis Científicotècnics" of the University of Barcelona on an ABI 377 Automated DNA Sequencer (PE Biosystems). Both strands were sequenced with the sequencing primers 1406F, ITS1 and 17SE as forward primers and ITS4 and 26 SE as reverse primers.

The *trnL-F* region was amplified using the primers "c" and "f" (Taberlet et al., 1991^[72]) which amplified the intron, the 3' exon, and the intergenic spacer. The double stranded PCR products were produced with 30 cycles of denaturation (94°C for 1 min), primer annealing (48°C for 1 min), and extension (72°C for 2 min), followed by a final extension step (72°C for 7 min). Direct sequencing was performed in both directions using one of the two amplification primers.

Phylogenetic analysis

The nucleotide sequences were edited using Chromas 1.56 (Technelysium Pty Ltd) and were easily aligned visually by sequential pairwise comparison (Swofford and Olsen, 1990^[72]). Data

matrices are available on request from the first author. Parsimony analysis involved heuristic searches conducted with PAUP version 4.0b8 (Swofford, 1999^[71]) using TBR branch swapping with character states specified as unordered and unweighted. As in previous ITS analyses (Vilatersana et al., 2000^[82]; Garcia–Jacas et al., 2000^[23]), indels were coded as a fifth base, with the goal of saving the potential phylogenetic information of shared indels (Bain and Jansen, 1995^[3]; Samuel et al., 1998^[62]). All most–parsimonious trees (MPT) were saved. To locate islands of most–parsimonious trees (Maddison, 1991^[40]), we performed 100 replications with random taxon addition, also with TBR branch swapping.

Bootstrap (BS) analysis was performed (Felsenstein, 1985^[20]), and decay index (DI) was calculated (Bremer, 1988^[12]; Donoghue et al., 1992^[18]) to obtain estimates of support for ITS data. Due to the practical impossibility of performing bootstrap analyses in the direct way with PAUP 4.0b8, we used the approach by Lidén et al. (1997^[31]) using 1000 replicates, random taxon addition with 10 replicates per replicate and no branch swapping. Results obtained with this method are very similar to other approaches (Mort et al., 2000^[51]).

Regarding decay analysis, the search of trees only two steps longer than the shortest trees was impossible. Therefore, these decay analyses were conducted using the clade–constraint approach as discussed in Morgan (1997^[50]). Even with this approach, we had to abort most of the heuristic searches after saving 200,000 trees. For this reason, decay indices could be slightly over–estimated in some cases.

Results

The size and composition of the ITS1 and ITS2 regions, and the size of the *trnL-F* region, are summarized in Table 2. For the *trnL-F* region, the number of informative characters was extremely low: only one base change plus three informative indels. Thereafter, we discarded it as useless to our purposes. For ITS, the results of the heuristic search are shown in Table 2, and the strict consensus tree of the equally most parsimonious trees resulting from the heuristic search is showed in Fig. 1.

The genus *Artemisia* in its classical circumscription forms a statistically well-supported clade (bootstrap –BS– 81%, decay index –DI– of 3), including some of the small closely related genera. This clade is united without support with the related genera *Kaschgaria*, *Hippolytia* and *Brachanthemum*. In its turn, *Artemisia* s. l. plus *Kaschgaria*, *Hippolytia* and *Brachanthemum* is united to two of the genera of the outgroup, *Lepidolopsis* and *Tanacetum*, forming a clade with very high support (BS=100%, DI=11). The position of the genus *Elachanthemum* as sister of all the above-cited complex is also strongly supported (BS=95%, DI=3) (Fig. 1).

Within the *Artemisia* clade, eight multispecific clades (numbered from 1 to 8 in Fig. 1), all of them statistically well supported, can be distinguished: 1) members of subgenera *Artemisia* and *Absinthium* (BS=87%, DI=4) forming two subclades, 2) the *A. absinthium* group (BS=89%, DI=2), of subgenus *Absinthium*, 3) subgenus *Seriphidium* (BS=84%, DI=3), together with *A. annua* (subgenus *Artemisia*) as sister, 4) subgenus *Tridentatae* (BS=96%, DI=3), 5) genus *Sphaeromeria* (BS=97%, DI=5), 6) the *Artemisia vulgaris* group (BS=91%, DI=3), belonging to subgenus *Artemisia*, 7) genus *Artemisiastrum*, grouped in a well supported (BS=100%, DI=13) clade with *Artemisia mexicana* from subgenus *Artemisia*, and 8) subgenus *Dracunculus*, including the genera *Mausolea* and *Turaniphytum*, also with high support (BS=98%, DI=6). In

addition, one species of subgenus *Seriphidium*, two of the subgenus *Absinthium*, five of the subgenus *Artemisia* and the genera *Picrothamnus* and *Filifolium* are not included in any clade.

Discussion

Delimitation of the genus Artemisia

The present results confirm the monophyly of the genus *Artemisia* in its broadest sense, clearly including *Seriphidium*. This agrees with preceding papers addressing this question (Kornkven et al., 1998^[28], 1999^[29]; McArthur et al., 1998a^[49], 1998b^[44]; Torrell et al., 1999^[75]). Nevertheless, Watson et al. (2000^[85]) continued using *Seriphidium* as a genus distinct from *Artemisia*, although one of these authors postulated its inclusion in the latter in the above cited papers (Kornkven et al., 1998^[28], 1999^[29]). In addition, Watson et al. (2000^[85]) used *Artemisia tridentata* (cited as *Seriphidium tridentatum*) as a member of the putative genus *Seriphidium*. A first attempt at an ITS-based phylogeny of *Artemisia* (Torrell et al., 1999^[75]) already demonstrated that subgenus *Tridentatae* is independent from *Seriphidium*, and the present data support this statement. Section *Tridentatae* was originally created as a section within subgenus *Seriphidium* (Rydberg, 1916^[61]), but many authors, using different approaches (morphological, chemical, molecular) suggested convergent evolution for both groups from members of *Artemisia* or *Dracunculus* (McArthur and Plummer, 1978^[45]; McArthur, 1979^[43]; McArthur et al., 1981^[46]; Seaman, 1982^[63]; Shultz, 1986^[65]; McArthur et al., 1998b^[44]; Torrell et al., 1999^[75]). Jeffrey (1995^[25]) stated that, if *Seriphidium* is to be segregated from *Artemisia*, section *Tridentatae* would be better retained in the latter than transferred to the former.

Dracunculus forms a very well delimited clade in the ITS phylogeny. It appears, together with genus *Filifolium*, as one of the possible sister groups of the rest of *Artemisia*, so that if one of the subgenera of *Artemisia* should be erected as an independent genus, *Dracunculus* would be the best candidate. This idea was early proposed by Cassini (1817^[16]), who described the genus *Oligosporus* Cass. (with a different name because, at the generic level, *Dracunculus* was already occupied by a genus of the family Araceae), but has not been supported by other authors. Its affinities in many fields make us believe that *Dracunculus* is better placed within the genus *Artemisia*, where it could represent a rather primitive stock (Torrell and Vallès, unpubl. res.).

Infrageneric circumscriptions in Artemisia

According to the present data, several well-defined groups appear in the genus *Artemisia*. They are not fully coincident with the classic subgenera, but some of them agree totally or partially with the currently used infrageneric classification. The best defined group that exactly represents one or the classical infrageneric taxa is the *Dracunculus* clade (number 8 in Fig. 1), discussed above, which is perfectly coincident with the subgenus *Dracunculus*. Within this clade, the *Artemisia campestris* complex forms a subclade, which is consistent with cytogenetic data (Torrell et al., 2001^[76], and unpubl. res.). Another subclade is constituted by two very closely related species, *A. dracunculus* and *A. dracunculoides*; they are members of a polyploid series (Rousi, 1969^[60]; Vallès et al., 2001^[81]), and the latter is often considered as a synonym of the former. Finally, the genera *Mausolea* and *Turaniphytum* are also included in the *Dracunculus* clade in the ITS phylogeny; their position will be further discussed, together with that of other genera.

All the studied taxa of subgenus *Seriphidium* except one are grouped in a clade (number 3 in Fig. 1), with the addition, as sister, of *A. annua* from subgenus *Artemisia*. This is an annual taxon, apparently without morphological, ecological or chemical affinities with the members of *Seriphidium*, but data on molecular cytogenetics showed that its genome structure is quite similar to that of the subgenus *Seriphidium* (Torrell et al., 2003^[77]). The only species of *Seriphidium* not included in the *Seriphidium* clade in the ITS phylogeny is *A. leucodes*, one of the extremely rare annual taxa in this subgenus. The unexpected placement of annuals in ITS sequence analyses of groups with predominance of perennials is a curious but frequent distortion in another tribe of the Asteraceae (Cardueae, Garcia-Jacas, unpubl. res.).

Subgenus *Tridentatae* appears as a very well-supported clade (number 4 in Fig. 1) distinct from *Seriphidium*, as above discussed. Another independent group is formed by the two *Sphaeromeria* species studied (clade number 5 in Fig. 1). The genus *Sphaeromeria* was classically treated as a section of *Tanacetum*. Holmgren et al. (1976^[24]), on the basis of morphological and anatomical evidence, considered it closer to *Artemisia* than to *Tanacetum*. Our pollen data supported this position, because *Sphaeromeria* has *Artemisia* pollen type (Martín et al., 2001^[41]). Present molecular evidence reinforces this idea, and is consistent with the results of McArthur et al. (1998a^[49]), who showed a phenogram based on randomly amplified polymorphic DNA (RAPD) with the *Sphaeromeria* species grouped with *Artemisia*, and separated from *Tanacetum*.

The *Artemisia vulgaris* complex is clearly defined within the genus from a morphological point of view. It appears split in two clades (numbers 6 and 7 in Fig. 1) in the present ITS phylogeny, one of them including the monotypic genus *Artemisiastrum*, erected on the basis of an *Artemisia* species of the *A. vulgaris* group. This large pool of species belongs to subgenus *Artemisia*, the

most heterogeneous in the genus on the basis of morphological, chemical, ecological and karyological characters. The analysis of the ITS sequences already revealed this situation (Torrell et al., 1999^[75]), as confirmed by the present data, based on a significantly higher number of species. The fact that five of the 10 taxa not included in any clade belong to the subgenus *Artemisia* (the five remaining being one from subgenus *Seriphidium*, two from subgenus *Absinthium*, and the genera *Filifolium* and *Picrothamnus*) is consistent with the heterogeneity of this subgenus. The placement of members the subgenus *Artemisia* in four of the eight clades also illustrates this situation, confirming the idea, already stated by Persson (1974^[57]) on the basis of morphological characters, that present infrageneric subdivisions do not represent natural groups.

Genera closely related to Artemisia

Six of the 10 studied genera are placed within *Artemisia* in the ITS phylogeny. *Filifolium* and *Picrothamnus* occupy isolated positions, at the same level as the main clades. *Turaniphytum* and *Mausolea* are included in subgenus *Dracunculus*. This is not surprising, since on the basis of morphological characters, they were already classified in this subgenus in classical treatments of the genus *Artemisia*, before those two genera were segregated. *Artemisiastrum* is grouped with members of the *Artemisia vulgaris* complex. Morphological evidence and classical classification support this. Finally, *Sphaeromeria* is independently placed (both *Sphaeromeria* species appearing together in a small clade). We already stated that *Sphaeromeria* was considered closer to *Artemisia* than to *Tanacetum* (Holmgren et al., 1976^[24]; McArthur et al., 1998a^[49]). In the latter work, the only species of the genus *Picrothamnus* (*P. desertorum*, treated as *Artemisia spinescens*) was also grouped (together with a *Sphaeromeria* species) with *Artemisia* and not with *Tanacetum*, as it occurs in the present ITS phylogeny. Five of these six genera included in

the *Artemisia* clade in the ITS sequence analysis were segregated from *Artemisia*. Only *Filifolium* was not originally described in *Artemisia*, but in *Tanacetum*, although it has been also combined under *Artemisia*. All these six genera (*Sphaeromeria*, *Picrothamnus*, *Artemisiastrum*, *Mausolea*, *Turaniphytum*, *Filifolium*) share a morphological character that we consider a good taxonomic marker in subtribe Artemisiinae: pollen grain exine ornamentation. The genera belonging to subtribe Artemisiinae (Bremer and Humphries, 1993^[14]) can be separated in two groups: one with *Artemisia*-type exine ornamentation, with small spinules, and the other one with *Anthemis*-type exine ornamentation, with long spines; the above six genera all have *Artemisia* pollen type surface (Martín et al., 2001^[41], 2003^[42]). The genus *Kaschgaria* also has this pollen type, whereas *Hippolytia* and *Brachanthemum*, placed in the same position in the strict consensus tree (*Kaschgaria* and *Hippolytia* are united in a weakly supported clade), have *Anthemis*-type pollen, cf. Martín et al., 2001^[41]). *Elachanthemum*, a monotypic genus originally described in *Artemisia* which has *Artemisia*-type pollen, appears as sister of the clade formed by the big *Artemisia* group and several other genera.

Considerations regarding subtribe Artemisiinae

Bremer and Humphries (1993^[14]), in their cladistic revision of tribe Anthemideae, considered subtribe Artemisiinae Less. emend. Bremer and Humphries as formed by two main groups: first, a pool of genera closely related to *Artemisia*; second, a group of genera more related to *Chrysanthemum sensu lato* from a different subtribe, Chrysantheminae Less. emend. Bremer and Humphries. On the other hand, some of the genera with species formerly classified in *Artemisia* are included in other subtribes: in subtribe Tanacetinae Bremer and Humphries (*Hippolytia* Poljakov includes species originally described in *Tanacetum* and others combined in

Chrysanthemum, cf. Shih, 1979^[64]); and in subtribe Handeliinae Bremer and Humphries (*Lepidolopsis* Poljakov was described as *Artemisia*). Pollen morphology shows that exine ornamentation is a good marker that distinguishes these two groups of taxa (Martín et al., 2001^[41], 2003^[42]). The present ITS phylogeny (Fig. 1) basically agrees with this separation, and suggests five levels of affinity with *Artemisia*:

1) The genera included in the *Artemisia* clade, which have the strongest relationship with this genus; all of them have the *Artemisia* pollen type, and most of them are often included in the genus *Artemisia*.

2) Three genera placed independently at the same level as the *Artemisia* clade. One of them (*Kaschgaria*) has the *Artemisia* pollen type and the other two (*Brachanthemum*, *Hippolytia*) have the *Anthemis* pollen type. These genera are most often considered distinct from *Artemisia*, although two of them (*Kaschgaria*, *Hippolytia*) have been segregated from the larger genus, and one (*Hippolytia*) is not even included in subtribe Artemisiinae.

3) The clade including *Lepidolopsis* (a monotypic genus from subtribe Handeliinae, originally described as *Crossostephium* and combined in *Artemisia* and in *Tanacetum*), and *Tanacetum*; it is strongly supported (BS of 100%), implying either a close relationship between their subtribes, Handeliinae and Tanacetinae respectively, or a subtribal missplacement of *Lepidolopsis*. Conversely, it is worth mentioning that *Hippolytia*, from Tanacetinae, is not included in this clade formed by *Lepidolopsis* and *Tanacetum*.

4) The genus *Elachanthemum*, the only exception to the consistency between ITS phylogeny and

pollen data; it is clearly placed far from *Artemisia* in the ITS analysis, in spite of having *Artemisia* pollen type (Martín et al., 2003^[42]).

5) The genera placed in the outgroup clades, the less closely related to *Artemisia*. We can point out that the two species of *Ajania* included in this study do not form a clade. *Ajania* is a heterogeneous genus, with some taxa originally described in different other genera. Bremer and Humpries (1993^[14]) stated that “possibly *Ajania* or part of the genus is the sister group of *Artemisia* and the other genera with smooth or short–spined pollen”. Our results (Fig. 1) show that this assertion is not exactly true, and emphasize the complexity of subtribe Artemisiinae as currently considered.

Concluding remarks

On the basis of data coming from different techniques, several authors have already stated that the infrageneric taxa classically recognised in *Artemisia* do not represent natural groups (Persson, 1974^[57]; Torrell et al., 1999^[75]; Vallès and McArthur, 2001^[80]). Pollen morphology also supports the idea that subtribe Artemisiinae needs to be reviewed (Martín et al., 2001^[41], 2003^[42]). In their revision of tribe Anthemideae, Bremer and Humphries (1993^[14]) admitted that some of the proposed subtribes, Artemisiinae among them, required changes in their delimitation. The present ITS sequence analysis clearly supports these idea, showing that the structuration *Artemisia* and that of Artemisiinae are two not satisfactorily solved questions. This notwithstanding, our previous paper on ITS phylogeny, dealing with 31 *Artemisia* species, and the present one, including 44 *Artemisia* species and 11 species of related genera, represent a first approach to the solution of these systematic and phylogenetic problems. On the one hand, some

groups clearly established within *Artemisia* may point towards the proposal of an infrageneric classification. On the other hand, different levels are defined in the relationships between *Artemisia* and its segregate and/or related genera included in Artemisiinae or in other subtribes of Anthemideae, which mean a first step in the redefinition of the subtribe.

With our previous and present results, we have reached the limits of the ITS resolution power in the phylogeny of Artemisiinae. The high level of homoplasy and the low number of informative characters provided by the sequences of the ITS region (Table 2) point out that other regions should be explored for a better understanding of the limits and phylogeny of the genus *Artemisia*; and it is also obvious from our research that chloroplast DNA *trnL-F* spacer is not useful at this level. We need to look for new genome regions for future research, and we need to include representatives of all the remaining genera in the subtribe in future studies, in order to clarify a subtribal classification that our molecular analyses have weakened. The results will surely allow us to present more specific and well founded proposals for a natural classification of genus *Artemisia* and subtribe Artemisiinae.

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Table 1 Origin, herbarium vouchers, and GenBank accession numbers of the populations analyzed.

TAXON	ORIGIN OF MATERIAL AND VOUCHER	GENBANK ACCESSION NUMBER
Genus <i>Artemisia</i> L.		
Subgenus <i>Artemisia</i>		
<i>A. annua</i> L.	Torrell et al. (1999 ^[75]), BCF 43820	AF045383, AF079935
<i>A. tournefortiana</i> Reichb.	Torrell et al. (1999 ^[75]), BCF 43812	AF045384, AF079936
<i>A. vulgaris</i> L.	Torrell et al. (1999 ^[75]), BCF 43822	AF045385, AF079937
<i>A. vulgaris</i> L.	Torrell et al. (1999 ^[75]), BCF 43922	AF045386, AF079938
<i>A. verlotiorum</i> Lamotte	Torrell et al. (1999 ^[75]), BCF 33102	AF045387, AF079939
<i>A. chamaemelifolia</i> Vill.	Torrell et al. (1999 ^[75]), BCF 43666	AF045388, AF079940
<i>A. molinieri</i> Quézel, Barbero & Loisel	Torrell et al. (1999 ^[75]), BCF 41791	AF045389, AF079941
<i>A. lucentica</i> O.Bolòs, Vallès & Vigo	Torrell et al. (1999 ^[75]), BCF 43814	AF045390, AF079943
<i>A. reptans</i> C.Sm. in Buch	Torrell et al. (1999 ^[75]), BCF 37108 ^[75]	AF045391, AF079944
<i>A. mexicana</i> Willd.	Torrell et al. (1999 ^[75]), BCF 43699	AF045414, AF079966
<i>A. afra</i> Jacq. ex Willd.	Torrell et al. (1999 ^[75]), BCF 43697	AF045392, AF140484
<i>A. haussknechtii</i> Boiss.	Iran, Hamadan, Kabud Rahan: Daghdagh–abad, Aholiabad mountains, rocky places. Garcia–Jacas, Mozaffarian, Susanna 1718 and Vallès, 11 Aug. 1996 (BCF 45578).	AF504146, AF504173
<i>A. incana</i> (L.) Druce (<i>Tanacetum incanum</i> L.)	Armenia, Krasnoselsk: north shores of lake Sevan, between villages of Shorza and Artanish, 2000m. Fajvush, Gabrielian, Garcia–Jacas, Guara, Hovhannisian, Susanna 1521B, Tamanian and Vallès, 16 Aug. 1995 (BCF 45608).	AF504147, AF504174
<i>A. judaica</i> L.	Egypt, Es Sina': 5 km N of Saint Katherine, dry stony soils in Wadi Sheik. Badr, Guara, Kamel and Vallès, 8 Apr. 1995 (BCF 45613).	AF504148, AF504175
<i>A. santolinifolia</i> Turcz. ex H. Kraschen.	Kazakhstan, Almatynskaya oblast: road from Almaty to the Astronomical Observatory, 11 km from the Observatory, road edges, 1500 m. Ivaschenko, Susanna 2099 and Vallès, 21 Aug. 2000 (BCF 51231).	AF504155, AF504182
Subgenus <i>Absinthium</i> (Mill.) Less.		
<i>A. arborescens</i> L.	Torrell et al. (1999 ^[75]), BCF 43819	AF045393, AF079945
<i>A. absinthium</i> L.	Torrell et al. (1999 ^[75]), BCF 43821	AF045394, AF079946
<i>A. thuscula</i> Cav. (<i>A. canariensis</i> Less.)	Francisco–Ortega et al. (1997 ^[21]), ORT	L77740
<i>A. umbelliformis</i> Lam. subsp. <i>umbelliformis</i>	Torrell et al. (1999 ^[75]), BCF 43811	AF045395, AF079947
<i>A. granatensis</i> Boiss.	Torrell et al. (1999 ^[75]), BCF 43777	AF045397, AF079949
<i>A. splendens</i> Willd.	Torrell et al. (1999 ^[75]), BCF 43926	AF045396, AF079948
<i>A. aschurbajewii</i> C. Winkler	Kazakhstan, Almatynskaya oblast: Northern Alatau mountains, rocky meadows near the Astronomical Observatory, 2700 m. Ivaschenko, Susanna 2089 and Vallès, 21 Aug. 2000 (BCF 50695).	AF504143, AF504170

<i>A. austriaca</i> Jacq.	Armenia, Sevan: lake Sevan, peninsula of Sevan, waste fields, 2000 m. Faivush, Gabrielian, Garcia-Jacas, Guara, Hovhannisian, Susanna 1502, Tamanian and Vallès, 15 Aug. 1995 (BCF 45503).	AF504144, AF504171
<i>A. persica</i> Boiss.	Uzbekistan, Western Tian-Shan: Chimgan mountains, Chimgan skiing resort, rocky meadows, 1800 m. Kapustina, Susanna 2067 and Vallès, 9 Nov. 1999 (BCF 51229).	AF504152, AF504179
<i>A. rutifolia</i> Stephan ex Spreng.	Kazakhstan, Almatynskaya oblast: Aktogai, dry slopes near Charin valley. Ivaschenko, Susanna 2120 and Vallès, 23 Aug. 2000 (BCF 51230).	AF504153, AF504180
<i>A. sieversiana</i> Willd.	Uzbekistan, Tashkent: Tashkent NE surroundings, near Botanical Institute Experimental Station, ruderal. Kapustina, Malcev, Susanna 2065 and Vallès, 9 Nov. 1999 (BCF 49153).	AF504156, AF504183
Subgenus <i>Dracunculus</i> (Besser) Rydb.		
<i>A. campestris</i> L.	Torrell et al. (1999 ^[75]), BCF 43816	AF045398, AF079950
<i>A. crithmifolia</i> L.	Torrell et al. (1999 ^[75]), BCF 43815	AF045399, AF079962
<i>A. monosperma</i> Delile	Torrell et al. (1999 ^[75]), BCF 41581	AF045400, AF079951
<i>A. scoparia</i> Waldst. & Kit.	Torrell et al. (1999 ^[75]), BCF 43813	AF045402, AF079953
<i>A. dracunculus</i> L.	Torrell et al. (1999 ^[75]), BCF 42415	AF045401, AF079952
<i>A. dracunculoides</i> Pursh.	United States of America, Arizona: Globe, Pinal Mountain, ruderal in path edges. Peñuelas, 16 Dec. 1995 (BCF 45562).	AF504145, AF504172
<i>A. marshalliana</i> Spreng.	Armenia, Krasnoselsk: northern shore of Sevan lake, between Shorzha and Ardanish, 25 km from Sevan, 2000 m. Faivush, Gabrielian, Garcia-Jacas, Guara, Hovhannisian, Susanna 1522, Tamanian and Vallès, 17 Aug. 1995 (BCF 45615).	AF504150, AF504177
Subgenus <i>Seriphidium</i> Besser		
<i>A. herba-alba</i> Asso	Torrell et al. (1999 ^[75]), BCF 40435	AF045403, AF079954
<i>A. herba-alba</i> Asso	Torrell et al. (1999 ^[75]), BCF 43927	AF045404, AF079955
<i>A. inculta</i> Delile	Torrell et al. (1999 ^[75]), BCF 41110	AF045405, AF079956
<i>A. fragrans</i> Willd.	Torrell et al. (1999 ^[75]), BCF 43924	AF045406, AF079957
<i>A. sieberi</i> Besser	Torrell et al. (1999 ^[75]), BCF 43925	AF045407, AF079958
<i>A. araxina</i> Takht.	Torrell et al. (1999 ^[75]), BCF 43810	AF045408, AF079959
<i>A. caerulea</i> L. subsp. <i>caerulea</i>	Torrell et al. (1999 ^[75]), BCF 43817	AF045409, AF079960
<i>A. barrelieri</i> Besser	Torrell et al. (1999 ^[75]), BCF 43818	AF045410, AF079961
<i>A. leucodes</i> Schrenk	Uzbekistan, Dgizak: near lake Aidarkul, 1 km from Issikkul, semidesert. Kapustina, Khassanov, Susanna 2064 and Vallès, 8 Nov. 1999 (BCF 49160).	AF504149, AF504176
<i>A. santolina</i> Schrenk	Uzbekistan, Bukhara: road from Gazli to Nukus, 70 km from Gazli, sandy desert, 200 m. Kapustina, Khassanov, Susanna 2042 and Vallès, 3 Nov. 1999 (BCF 49162).	AF504154, AF504181
Subgenus <i>Tridentatae</i> (Rydb.) McArthur		
<i>A. tridentata</i> Nutt. subsp. <i>vaseyana</i> (Rydb.) Beetle	Torrell et al. (1999 ^[75]), BCF 43702	AF045411, AF079963
<i>A. nova</i> Nelson	Torrell et al. (1999 ^[75]), BCF 43701	AF045412, AF079964
<i>A. cana</i> Pursh subsp. <i>cana</i>	Torrell et al. (1999 ^[75]), BCF 43703	AF045413, AF079965
Genus <i>Artemisiastrum</i> Rydb.		
<i>Artemisiastrum palmeri</i> (A. Gray) Rydb. (<i>Artemisia palmeri</i> A. Gray)	United States of America, California, San Diego Co.: Poway, beside Sycamore Creek, oak woodland.	AF504151, AF504178

	MacGregor, 24 Oct 2001.	
Genus <i>Brachanthemum</i> DC.		
<i>Brachanthemum titovii</i> H. Kraschen.	Kazakhstan, Almatynskaya oblast: Aktogai, dry slopes near Charin valley. Ivaschenko, Susanna 2116 and Vallès, 22 Aug. 2000 (BCF 50355).	AF504158, AF504185
Genus <i>Elachanthemum</i> Y. Ling & Y. R. Ling		
<i>Elachanthemum intricatum</i> (Franch.) Y. Ling and Y. R. Ling (<i>Artemisia intricata</i> Franch.)	People's Republic of Mongolia, Dzhung Gobi aymak: Ukho–Aloch–Ulya mountain, way from Khayrkhan somon to Altay somon, 12–16 km from the beginning of the canyon, sandy hills. Grubov, Pushashev, Dariymaa, 20 Aug. 1979 (LE, not numbered)	AF504159, AF504186
Genus <i>Filifolium</i> Kitam.		
<i>Filifolium sibiricum</i> (L.) Kitam. (<i>Artemisia sibirica</i> (L.) Maxim., <i>Tanacetum sibiricum</i> L.)	Russia, Primorskii kray, Khorolskii rayon: near Popovk, meadow on sandy soil. Patrievskaya, 1 Sep. 1954 (LE, not numbered).	AF504160, AF504187
Genus <i>Hippolytia</i> Poljakov		
<i>H. megacephala</i> (Rupr.) Poljakov (<i>Artemisia megacephala</i> Rupr.)	Kazakhstan, Zhabul'skaya oblast: Aksu–Zhabagli nature reserve, 15 km from Zhabagli, alpine meadows, 2700 m. Ivaschenko, Susanna 2169 and Vallès, 29 Aug. 2000 (BCF 50686).	AF504161, AF504188
Genus <i>Kaschgaria</i> Poljakov		
<i>K. brachanthemoides</i> (C. Winkl.) Poljakov (<i>Artemisia brachanthemoides</i> C. Winkl., <i>Tanacetum brachanthemoides</i> (C. Winkl.) H. Kraschen.)	Kazakhstan, Almatynskaya oblast: Kurtagai canyon, 2 km from river Charin bridge in the road to Narienkul. Ivaschenko, Susanna 2133 and Vallès, 24 Aug. 2000 (BCF 50457)	AF504162, AF504189
Genus <i>Mausolea</i> Bunge		
<i>M. eriocarpa</i> (Bunge) Poljakov (<i>Artemisia eriocarpa</i> Bunge)	Uzbekistan, Bukhara: road from Gazli to Nukus, 10 km from Gazli, sandy desert, 200 m. Kapustina, Khassanov, Susanna 2041 and Vallès, 3 Nov. 1999 (BCF 49168).	AF504164, AF504191
Genus <i>Picrothamnus</i> Nutt.		
<i>P. desertorum</i> Nutt. (<i>Artemisia spinescens</i> D.C. Eaton)	USA, Utah, Millard county: desert near USDA Desert Experimental Range. Kapustina, McArthur and Vallès, 19 Jun. 2000 (BCF 49501).	AF504157, AF504184
Genus <i>Sphaeromeria</i> Nutt.		
<i>S. diversifolia</i> (D.C. Eaton) Rydb. (<i>Tanacetum diversifolium</i> D.C. Eaton)	USA, Utah, Utah county: Santiaquin Canyon, rocks in a <i>Picea</i> forest. Kapustina, McArthur and Vallès, 16 Jun. 2000 (BCF 49505).	AF504165, AF504192
<i>S. ruthiae</i> Holmgren, Shultz & Lowrey	USA, Utah, Zion National Park: Pine Creek Canyon, rocks, 1585 m. Kapustina, McArthur and Vallès, 16 Jun. 2000 SSLP (McArthur et al., 1989)	AF504166, AF504193
Genus <i>Turaniphytum</i> Poljakov in Komarov		
<i>T. eranthemum</i> (Bunge) Poljakov (<i>Artemisia eranthema</i> Bunge)	Kazakhstan, Almatynskaya oblast: 1 km north to the road Kapchagai–Bakanas, 6 km from Bakanas, sandy desert. Ivaschenko, Susanna 2143 and Vallès, 26 Aug. 2000 (BCF 50684).	AF504168, AF504195
Outgroup		
<i>Ajania fastigiata</i> (C. Winkl.) Poljakov (<i>Artemisia fastigiata</i> C. Winkl.)	Kazakhstan, Almatynskaya oblast: road from Almaty to the Astronomical Observatory, 20 km from the Observatory, loam hills, 1000 m. Ivaschenko, Susanna 2098 and Vallès, 21 Aug. 2000 (BCF 50687).	AF504142, AF504169
<i>Ajania pacifica</i> (Nakai) K. Bremer & Humphries	Francisco–Ortega et al. (1997 ^[21]), NCP–PI479350	L77787

<i>Arctanthemum arcticum</i> (L.) Tzvelev	Francisco–Ortega et al. (1997 ^[21]), NCP–PI50226	L77756
<i>Chrysanthemum coreanum</i> H. Lév. & Vaniot (<i>Dendranthema coreanum</i> (H. Lév. & Vaniot) Vorosch.)	Francisco–Ortega et al. (1997 ^[21]), THM 2430	L77802
<i>Lepidolopsis turkestanica</i> (Regel & Schmalh.) Poljakov (<i>Crossostephium turkestanicum</i> Regel & Schmalh., <i>Artemisia turkestanica</i> (Regel & Schmalh.) Franch., <i>Tanacetum turkestanicum</i> (Regel & Schmalh.) Poljakov)	Kazakhstan, Chimkentskaya oblast: road Almaty– Chimkent, 40 km from Chimkent, near Sostube, dry hills, 500 m. Ivaschenko, Susanna 2209 and Vallès, 1 Sep. 2000 (BCF 50685).	AF504163, AF504190
<i>Tanacetum parthenium</i> (L.) Schultz Bip.	Spain, Catalonia: Mosqueroles, cultivated in a homegarden, Bonet, 17 Jun. 1995 (BCF 47221).	AF504167, AF504194

Table 2 Numeric results of the ITS and *trnL-F* sequence analysis.

Data set	ITS1	ITS2	<i>trnL-F</i>
Length (bp, aligned)	257	227	889
Length (bp, unaligned)	249–255	201–220	–
Informative characters	68	51	1
Informative indels	3	6	5
G+C contents	51.116	53.539	–
Range of divergence, ingroup (%)	0–16	0–14.3	–
Range of divergence, ingroup-outgroup (%)	0.8–17.7	1.4–17	–
Number of MPTs	217		
Number of steps	244		
Consistency index (CI) (excluded uninformative characters)	0.3688		
Homoplasy index (HI) (excluded uninformative characters)	0.6312		
Retention index (RI)	0.7104		

Figure caption:

Fig. 1 Strict consensus tree of the 217 most parsimonious trees showing the hypothetical ITS phylogeny of *Artemisia* and other Artemisiinae genera. Consistency index excluding uninformative characters (CI)=0.3688; retention index (RI)=0.7104; homoplasy index excluding uninformative characters (HI)=0.6312. Numbers above branches are bootstrap percentages; numbers below branches are decay indices. Numbers within circles indicate the eight main clades discussed in the text. *ART*=Subgenus *Artemisia*; *SERIPHIDIUM* and *SER*=Subgenus *Seriphidium*; *ABS*=Subgenus *Absinthium*; *DRACUNC*=Subgenus *Dracunculus*; *TRI*=Subgenus *Tridentatae*.



Artemisia-type pollen.



Anthemis-type pollen.