Generic boundaries and evolution of characters in the *Arctium* group: a nuclear and chloroplast DNA analysis

A. SUSANNA, N. GARCIA–JACAS, R. VILATERSANA & T. GARNATJE

RESUMEN

SUSANNA, A., N. GARCIA–JACAS, R. VILATERSANA & T. GARNATJE (2003). Generic boundaries and evolution of characters in the *Arctium* group: a nuclear and chloroplast DNA analysis. *Collect. Bot. (Barcelona)* **26**: 101-118.

Definir los límites genéricos en el grupo *Arctium* (*Compositae, Cardueae-Carduinae*), formado por los géneros *Arctium, Cousinia, Hypacanthium* y *Schmalhausenia*, ha resultado ser una tarea muy complicada. Concretamente, la separación precisa de *Arctium* y *Cousinia* es muy difícil de establecer. En consecuencia, hemos analizado las secuencias del DNA de dos regiones, el gen cloroplástico *mat*K y los espaciadores ITS 1 y 2 del DNA ribosómico-nuclear, de una amplia representación de todos los géneros del grupo; en el caso de *Cousinia*, centrándonos en las especies más obviamente relacionadas con *Arctium*. Hemos encontrado una correlación exacta entre filogenia molecular y dos caracteres fundamentales, el tipo de polen y el número cromosómico: todas las especies estudiadas que tienen el tipo de polen *Arctiastrum* y el número x= 18, característicos de *Arctium* sensu stricto, forman un clado monofilético, hermano de otro clado monofilético formado por las especies de *Cousinia* sensu stricto. Sin embargo, el clado "Arctioide" no se puede definir con caracteres morfológicos macroscópicos: el carácter que separa *Arctium* y *Cousinia* son las hojas espinoso-pinnatífidas o pinnatisectas de *Cousinia*, que es adaptativo y tiene poca relevancia taxonómica. Según nuestros resultados, las espinas habrían aparecido en dos linajes diferentes: en *Cousinia*, por una parte, y en *Hypacanthium* y *Schmalhausenia*, que son espinosos y morfológicamente más próximos a *Cousinia*, pero que están sin duda más relacionados con *Arctium*, inerme. Evaluamos también las implicaciones de esta incongruencia entre morfología, por un lado, y datos moleculares, palinológicos y cariológicos, por otro. Proponemos algunas soluciones, pero ninguna es totalmente satisfactoria: se necesitan más estudios que incluyan más especies de *Cousinia* subg. *Hypacanthodes*.

Palabras clave: Compositae, *Arctium, Cousinia, Hypacanthium, Schmalhausenia*, evolución, filogenia molecular.

Abstract

SUSANNA, A., N. GARCIA-JACAS, R. VILATERSANA & T. GARNATJE (2003). Generic boundaries and evolution of characters in the *Arctium* group: a nuclear and chloroplast DNA analysis. *Collect. Bot. (Barcelona)* **26**: 101-118.

Generic delineation within the *Arctium* group (*Compositae, Cardueae-Carduinae*), formed by the genera *Arctium, Cousinia, Hypacanthium* and *Schmalhausenia*, has proven a complicated task. In particular, the precise limits between *Arctium* and *Cousinia* are very difficult to establish. Therefore, we have carried out a molecular survey of DNA sequences of two regions, the chloroplast gene *mat*K and the nuclear-ribosomal spacers ITS 1 and 2, of a representation of all the genera of the group (in the case of *Cousinia*, centred in the species more obviously related to *Arctium*). Our results show a precise correlation between molecular phylogeny and

---

Alfonso Susanna, Núria Garcia–Jacas, Roser Vilatersana & Teresa Garnatje, Botanical Institute of Barcelona (C.S.I.C.–Ajuntament de Barcelona), Pg. del Migdia, s/n., E-08038 Barcelona (Spain). Author for correspondence: A. Susanna (asusanna@ibb.csic.es)

two very important characters, pollen type and chromosome numbers: all the investigated species with the *Arctiastrum* pollen type and x= 18, characteristics of *Arctium* sensu stricto, form a monophyletic clade, sister to another monophyletic clade formed by all the investigated species of *Cousinia* sensu stricto. However, the resulting "Arctioid" clade cannot be defined on macroscopic morphologic characters, because the main trait for segregating *Arctium* and *Cousinia*, the spiny pinnatifid-pinnatisect leaves of *Cousinia*, is adaptative and of scarce systematic relevance. In fact, our results suggest that spines have appeared at least in two different lineages: the genera *Hypacanthium* and *Schmalhausenia*, spiny and thus morphologically closer to *Cousinia*, are unambiguously related to the unarmed genus *Arctium*. A hypothesis on the evolution of morphology, pollen and chromosome numbers in the group is formulated. The systematic implications of this incongruence between molecular, pollen and karyology, on the one hand, and morphology, on the other hand, are evaluated. Some possible solutions are proposed, but none of them is totally satisfactory: more studies are necessary with the inclusion of new species of *Cousinia* subgenus *Hypacanthodes*.

Key words: *Compositae*, *Arctium*, *Cousinia*, *Hypacanthium*, *Schmalhausenia*, evolution, molecular phylogeny.

INTRODUCTION

The tribe *Cardueae* has always been a conflicting group, and both tribal and subtribal delineation has been much disputed (CASSINI, 1819; BENTHAM, 1873; HOFFMANN, 1894; WAGENITZ, 1976; DITTRICH, 1977; PETIT et al., 1996; PETIT, 1997; BREMER, 1994). However, all surveys on the basis of DNA analyses support the monophyly of the tribe: cpDNA restriction site data (JANSEN et al., 1990, 1991), cpDNA *rbc*L gene (KIM et al., 1992), cpDNA *mat*K gene (GARCIA-JACAS et al., 2002) and nrDNA spacers ITS1 and 2 (SUSANNA et al., 1995; GARCIA-JACAS et al., 2002). The subtribal limits are more complicated to establish, but the traditional classification in four subtribes (*Carlininae*, *Echinopsidinae*, *Carduinae* and *Centaureinae*) remains the only practical approach (SUSANNA & GARCIA-JACAS, in press) and is generally accepted.

Having acknowledged the tribal and subtribal limits, the next step was to clarify the delimitation of some of the major genera in the tribe. Now, among the most interesting and vexing problems of genus rank that persist in the Cardueae is the marking of the generic boundaries in the *Arctium* group.

The *Arctium* group

The *Arctium* group comprises the genera *Arctium* L., *Cousinia* Cass., *Hypacanthium* Juz. and *Schmalhausenia* C. Winkl. (HÄFFNER, 2000; SUSANNA & GARCIA-JACAS, in press). HÄFFNER (2000) adds the genera *Lipskyella* Juz. and *Tiarocarpus* Rech. f., merged into *Cousinia* by SUSANNA & GARCIA-JACAS (in press).

Three important characters never found in combination elsewhere in the tribe characterize basically the *Arctium* group. First, the receptacle has strongly twisted scales. Second, the achenes are always tigrine (with a pattern of wavy fringes), very often winged, and without a nectary. Third, the pappus is formed by free deciduous bristles (SUSANNA & GARCIA-JACAS, 2003). Both on morphological and molecular grounds, the genera *Arctium* and *Cousinia* belong to the subtribe *Carduinae*. More precisely, the *Arctium* group is part of a large clade including also the subtribe *Centaureinae* and the genera *Saussurea* DC. and *Jurinea* Cass. (GARCIA-JACAS et al., 2002).

Arctium comprises 11 species, according to the latest classification of the genus (DUISTERMAAT, 1996), most of them with a subcosmopolitan distribution. DUISTERMAAT

(1996) recognized four sections: *Arctium* sect. *Arctium* (with all the species classically included in *Arctium*), and three more sections described as sections of the genus *Cousinia* by TSCHERNEVA (1988b, 1988c): sections *Lappaceum*, *Nanarctium* and *Pseudarctium*.

The large genus *Cousinia* is formed of ca. 600 species (MABBERLEY, 1990) located in the Iranian and Turkestanian mountain regions, with an astonishing number of endemics. Both the limits and the sectional classification of *Cousinia* have widely changed. Proposals by BUNGE (1865), BOISSIER (1875), KUNTZE (1891), WINKLER (1892) and BORNMÜLLER (1916) were superseded by TSCHERNEVA (1962) in her treatment for the Flora of the USSR. Later on, this treatment was again deeply modified with the proposal of two new subgenera and many new sections and series (TSCHERNEVA, 1988a). The latest revision classified *Cousinia* in three subgenera and 50 sections (TSCHERNEVA, 1988b, 1988c).

The rest of the genera of the *Arctium* group recognized by SUSANNA &GARCIA-JACAS (in press) are very small: *Schmalhausenia* is monotypic, and *Hypacanthium* has only three species. Both genera are narrow endemics of the mountains of Central Asia (Tien-Shan and Pamir).

The extremely conflicting relationships between the genera of the *Arctium* group are reflected in the many reclassifications suggested in the group that imply changes of genus adscription. We shall shortly comment on some especially illustrative examples.

The protologue of the genus *Hypacanthium* placed it near *Schmalhausenia*. Previously, it had been described as *Cousinia echinopifolia* Bornm. and combined as *Schmalhausenia echinopifolia* (Bornm.) Juz. According to TSCHERNEVA (1983), *Hypacanthium* was also connected to *Cousinia* subgenus *Hypacanthodes* Tscherneva sect. *Lacerae*, and related to genus *Schmalhausenia*.

In its turn, *Schmalhausenia* was first described as *Cousinia eriophora* Regel & Schmalh. As we have seen above, it was also related to *Hypacanthium*, and it was even combined as *Arctium eriophorum* (Regel & Schmalh.) Kuntze.

These entangling cases are illustrative enough, but the best example of the problems of generic range in the group is the case of *Arctium* and *Cousinia*.

Relationships between the genera *Arctium* and *Cousinia*

A close connection between both genera has been signalled from old (BOISSIER, 1875; KUNTZE, 1891). All recent studies have confirmed this connection: morphological surveys by DITTRICH, 1977; DUISTERMAAT, 1996, 1997; PETIT et al., 1996; PETIT, 1997; HÄFFNER, 2000; or molecular analyses by HÄFFNER & HELLWIG, 1999; GARCIA-JACAS et al., 2002, are all coincident.

Species of *Arctium* and *Cousinia* have moved between genera very often: Boissier (1875) combined *Lappa* (=*Arctium* !) *amplissima* Boiss. as *Cousinia amplissima*; and KUNTZE (1891) proposed the classification of all the species of *Cousinia* in the genus *Arctium*, in view of the impossibility of establishing the precise limits between both genera.

The main obstacle for the precise delineation between *Arctium* and *Cousinia* is the presence of a group of *Cousinia* species (the "Arctioid" group for DUISTERMAAT, 1996) with many characters shared with *Arctium*: above all, the presence of glochidiate spines in the appendages of the bracts and the big unarmed leaves. The most recent proposal was the solution suggested by DUISTERMAAT (1996) and followed by SUSANNA & GARCIA-JACAS (in press): to classify the more obviously "Arctioid" *Cousinia* species (5 species from different sections) into *Arctium*, while exporting TSCHERNEVA'S (1988 b, 1988 c) sectional classification of these species of *Cousinia* to *Arctium*. Soon after, DUISTERMAAT (1997) suggested a rectification of her own views, and pointed out vaguely that all the species of *Cousinia* subg. *Cynaroides* Tscherneva could be placed in *Arctium*.

We shall shortly explain the main traits that have been used in the delimitation of the genera of the *Arctium* group: morphology, karyology, pollen and geography:

Morphological characters: leaves, bracts and heads

Arctium leaves are always unarmed, often very big (to 80 cm) and usally cordate, both characters being shared by *Cousinia* subg. *Cynaroides* and *Hypacanthodes*. In contrast, *Cousinia* subg. *Cousinia* leaves are small, usually lanceolate and with few exceptions very spiny.

Involucral bracts are a key character. In *Arctium* sensu stricto and part of *Cousinia* subgenus *Cynaroides* (the "Arctioid" species of *Cousinia*), involucral bracts always end in a recurved hook. Instead, in most of the species of *Cousinia*, involucral bracts end in a spine without hook.

Outer florets in *Arctium* often have longer and brighter coloured anther-tubes, mimicking outer radiant ligules. Instead, many *Cousinia* species often have bright-coloured appendages in the innermost bracts, recalling those of *Carlina* L.

As to floral morphology, DUISTERMAAT (1996) concluded that the stigma of *Cousinia* subg. *Cousinia* was very different from the stigma of *Arctium* and the "Arctioid" species. For DUISTERMAAT (1996), *Cousinia* subg. *Cousinia* lacked the hairy thickening below the branches that is a key character for the tribe Cardueae. HÄFFNER (2000) noted that the sweeping ring of hairs was also absent in *Schmalhausenia*.

Habit

According to DUISTERMAAT (1996), monocarpic habit is a key character for *Arctium*. Instead, *Cousinia* species are, with only a few exceptions, perennials. Some "Arctioid" species of *Cousinia* are monocarpic, but most of them are perennials (TSCHERNEVA, 1962).

Dispersal is also different. Both genera share a feature infrequent in the tribe: achenes are dispersed together with the heads. In *Arctium* and most of the "Arctioid" group, propagules are usually the whole heads, dispersed by exozoochory by means of the hooks of the involucral bracts. On the contrary, most species of *Cousinia* are tumble weeds, like many plants from the steppe: the whole plant is dispersed by the wind.

Chromosome numbers

According to literature (revised in DUISTERMAAT, 1996), *Arctium* always has $x = 18$ and $2n = 36$. The same number is shared by all the studied species of *Cousinia* from the subgenera *Cynaroides* and *Hypacanthodes* (TSCHERNEVA, 1985). The genus *Schmalhausenia* also has $2n = 36$ (SUSANNA et al., 2003). This high number, the highest in all the *Cardueae*, suggest that *Arctium*, *Cousinia* subg. *Cynaroides* and *Hypacanthodes*, and *Schmalhausenia*, constitute old polyploid complexes (TSCHERNEVA, 1988 c).

Cousinia subgenus *Cousinia* has $2n = 18$?, 20? [doubtful to us], 22, 24 and 26 (MOORE, 1973, 1977; GOLDBLATT, 1981, 1988; GOLDBLATT & JOHNSON, 1990, 1991; GHAFFARI & DJAVADI, 1998; GHAFFARI et al., 2000; SUSANNA et al., 2003). This long and complex disploid series is a proof that both groups have evolved in different ways.

Pollen

SCHTEPA (1966) first noted that some species of *Cousinia* have the same pollen type as *Arctium*. Later, she discovered that *Cousinia* subgenera *Cynaroides* and *Hypacanthodes* have pollen similar to *Arctium*, while *Cousinia* subg. *Cousinia* has a different pollen type

(SCHTEPA, 1976). KUPRIANOVA & TSCHERNEVA (1982) confirmed the two pollen types and named them *Arctiastrum* (circumscribed to *Arctium* and *Cousinia* subg. *Cynaroides* and *Hypacanthodes*) and *Cousinia* (limited to *Cousinia* subg. *Cousinia*). DUISTERMAAT (1996) arrived at the same conclusions of SCHTEPA (1976) and KUPRIANOVA & TSCHERNEVA (1982). Nevertheless, she did not favour the inclusion of all the species of the *Cousinia* subgenera with *Arctiastrum* pollen type in *Arctium*, because pollen types in the tribe are usually found in more than one genus. According to our observations, *Schmalhausenia* has *Arctiastrum* pollen type, and the pollen type of *Hypacanthium* is unknown.

Geographic distribution

The genus *Arctium* has a peculiar distribution, with two groups with marked differences in this respect. In the definition by DUISTERMAAT (1996), the species of *Arctium* sensu stricto (sect. *Arctium*) are subcosmopolitan. The rest of the species (i.e., all the species previously classified in the genus *Cousinia*) have a central Asian distribution, as the genus *Cousinia*.

On the other hand, distribution of the genus *Cousinia* is characteristic of the mountain flora of the Irano-Turanian Region, coincident with the "Oriental-Turanian Flora Region" defined by MEUSEL et al. (1965); see KNAPP (1987). There are two main centres of speciation: the western region with 251 species, and the eastern region with 340 species (TSCHERNEVA, 1974). Most of the species of *Cousinia* subg. *Cynaroides* and *Hypacanthodes* grow only in the eastern region, as do the genera *Schmalhausenia* and *Hypacanthium*. Only three out of seven sections of subg. *Cynaroides* are present in the western region with only one species endemic to Iran (RECHINGER, 1972, 1979). The other species are present in both regions. Regarding *Cousinia* subgenus *Hypacanthodes*, there are no representatives in the western region.

Our objectives

The combined analysis of the nrDNA region ITS and the chloroplast DNA gene *mat*K has proven an excellent tool for unravelling problems at this level in the *Cardueae* (GARCIA-JACAS et al., 2001, 2002). Thereafter, we have carried out a molecular survey of both regions in a sample of all the genera of the *Arctium* group with the following goals:

1) To verify the monophyly of the *Arctium* group
2) To establish the generic boundaries of *Arctium* and *Cousinia*
3) To compare nuclear ribosomal and chloroplast DNA phylogeny and the evolution of the pollen types, basic chromosome numbers and morphology in the group
4) To contribute with a preliminary study to the infrageneric classification of the genus *Cousinia*.

MATERIALS AND METHODS

Plant material

Sampling was based on the sectional classification of the genus *Cousinia* by TSCHERNEVA (1988b, 1988c). We have included 27 taxa of the genus *Cousinia* from 19 sections, three species of the genus *Arctium*, one species of the genus *Hypacanthium* and the only species of the monospecific genus *Schmalhausenia*. The five outgroup species were chosen in the genera *Jurinea* and *Saussurea* according to previous sequence analysis (SUSANNA et al., 1995; GARCIA-JACAS et al., 2002). Voucher data, source and GenBank sequence accession numbers of the ITS and *mat*K sequences for the 37 studied species are given in Table 1.

The analysis sequences used published sequences along with new sequences. ITS sequences of *Arctium lappa* L., *Arctium minus* Bernh., *Cousinia canescens* DC., *C. esfandiarii* Rech. f. & Aellen (both ITS and *mat*K) and *C. onopordioides* Ledeb. were from a previous study (GARCIA-JACAS et al., 2002). Sequence of *Cousinia pterocaulos* (C. A. Mey.) Rech. f. was from HÄFFNER & HELLWIG (1999).

DNA Extraction, Amplification and Sequencing

Total genomic DNA was extracted following the CTAB method of DOYLE and DOYLE (1987) as modified by SOLTIS et al. (1991) and CULLINGS (1992) from silica gel dried leaves collected in the field, or fresh leaves of plants cultivated in the Botanic Institute of Barcelona. In some cases, herbarium material was used.

cpDNA *mat*K gene strategies

Double-stranded DNAs of *mat*K were amplified by PCR with *trn*K-710F (JOHNSON & SOLTIS, 1995) and AST-1R (GARCIA-JACAS et al., 2002) as PCR primers. Sometimes this combination failed to generate double-stranded products for some taxa and we used *mat*K-1848R (JOHNSON & SOLTIS, 1995) primer, as a substitute of primer AST-1R. 40 cycles of amplification were carried out under the following conditions: 94°C for 1 minute 30 seconds, 48°C for 2 minutes and 72°C for 3 minutes, with an additional extension step of 15 minutes at 72°C.

The double-stranded PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen Inc.) and sequenced. Sequencing primers *trn*K-710F, *mat*K-1848R and AST-1R were used. Direct sequencing of the amplified DNA segments was performed using a BigDye Terminator Cycle Sequencing v2.0 (PE Biosystems), following the protocol recommended by the manufacturer. The nucleotide sequencing was performed at the "Serveis Científico-Tècnics" of the University of Barcelona on an ABI PRISM 3700 DNA Analyzed (PE Biosystems). Nucleotide sequences of *mat*K were edited using Chromas 1.56 (Technelysium Pty Ltd) and easily aligned by hand.

nrDNA ITS region strategies

Double-stranded DNA of the ITS region was amplified using the 17SE as forward primer and the 26SE as reverse primer (SUN et al., 1994). The profile used for amplification included a warm start at 94°C for 2 minutes, followed by 80°C for 5 minutes, during which the polymerase (Ecotaq, Ecogen S. R. L.) was added. 30 cycles of amplification were carried out under the following conditions: 94°C for 1 minute 30 seconds, 57°C for 2 minutes and 72°C for 3 minutes, with an additional extension step of 15 minutes at 72°C. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc.). Both strands were sequenced with the sequencing primers 17SE as forward primer, and 26SE as reverse. Direct sequencing of the amplified DNA segments was performed as for the *mat*K region.

Phylogenetic analysis

DNA sequences were aligned visually by sequential pairwise comparison (SWOFFORD & OLSEN, 1990). Data matrices are available on request from the correspondence author. Parsimony analysis involved heuristic searches conducted with PAUP version 4.0b4a

Table 1.- Origin of the materials and herbaria where the vouchers are deposited.

Species	Voucher	Accession	
		ITS	*mat*K
	Arctium		
Arctium lappa L.	Garcia–Jacas et al. (2002)	AF319048 AF319102	AY013520
Arctium leiospermum Juz. & C. Serg.	Kazakhstan, *Susanna 2154 et al.* (BC)	AY373720 AY373687	
Arctium minus Bernh.	Garcia–Jacas et al. (2002)	AF319049 AF319103	AY013521
	Cousinia* subg. *Cousinia		
	Cousinia sect. *Alpinae*		
Cousinia chrysantha Kult.	Kazakhstan, *Susanna 2198 et al.* (BC)	AY373725 AY373692	AY373660
	Cousinia sect. *Badghysia*		
Cousinia piptocephala Bunge	Iran, *K. H. Rechinger 46736* (B)	AY373736 AY373703	
	Cousinia sect. *Carduncellus*		
Cousinia tianshanica Kult.	Kazakhstan, *Susanna 2191 et al.* (BC)	AY373743 AY373710	AY373671
	Cousinia sect. *Chrysoptera*		
Cousinia dissecta Kar. & Kir.	Kazakhstan, *Susanna 2137 et al.* (BC)	AY373728 AY373695	
	Cousinia sect. *Coronophora*		
Cousinia coronata Franch.	Uzbekistan, *Susanna 2039 et al.* (BC)	AY373727 AY373694	AY373662
	Cousinia sect. *Cousinia*		
Cousinia congesta Bunge	Uzbekistan, *Susanna 2059 et al.* (BC)	AY373726 AY373693	AY373664
Cousinia minkwitziae Bornm.	Kazakhstan, *Susanna 2183 et al.* (BC)	AY373735 AY373702	AY373661
Cousinia polycephala Rupr.	Kazakhstan, *Susanna 2161 et al.* (BC)	AY373738 AY373705	AY373668
Cousinia syrdariensis Kult.	Kazakhstan, *Susanna 2159 et al.* (BC)	AY373741 AY373708	AY373669
	Cousinia sect. *Cynaroideae*		
Cousinia canescens DC	Garcia–Jacas et al. (2002)	AF319068 AF319122	
Cousinia onopordioides Ledeb.	Garcia–Jacas et al. (2002)	AF319070 AF319124	
Cousinia purpurea C. A. Mey	Armenia, *K. Tamanian* (ERE)	AY373739 AY373706	AY373663
	Cousinia sect. *Eriocousinia*		
Cousinia caespitosa C. Winkl.	Kazakhstan, *Susanna 2170 et al.* (BC)	AY373724 AY373691	AY373673
	Cousinia sect. *Lachnosphaerae*		
Cousinia eriobasis Bunge	Iran, *K. H. Rechinger 47012* (B)	AY373729 AY373696	
	Cousinia sect. *Leiocaules*		
Cousinia astracanica (Spreng.) Tamamsch.	Kazakhstan, *Susanna 2104 et al.* (BC)	AY373723 AY373690	AY373670

Cousinia sect. *Microcarpae*			
Cousinia arachnoidea Fisch. & C. A. Mey	Kazakhstan, *Susanna 2140 et al.* (BC)	AY373722 AY373689	AY373666
Cousinia microcarpa Boiss.	Kazakhstan, *Susanna 2160 et al.* (BC)	AY373734 AY373701	AY373667
Cousinia platylepis Schrenk	Kazakhstan, *Susanna 2158 et al.* (BC)	AY373737 AY373704	AY373665
Cousinia sewerzowii Regel	Kazakhstan, *Susanna 2178 et al.* (BC)	AY373740 AY373707	AY373674
Cousinia sect. *Stenocephalae*			
Cousinia esfandiarii Rech. f. & Aellen	GARCIA-JACAS et al. (2002)	AF319069 AF319123	AY013537
Cousinia sect. *Xiphiolepides*			
Cousinia pterocaulos (C. A. Mey.) Rech. f.	HÄFFNER & HELLWIG (1999)		
Cousinia* subg. *Cynaroides			
Cousinia sect. *Chrysis*			
Cousinia karatavica Regel & Schmalh.	Kazakhstan, *Susanna 2162 et al.* (BC)	AY373732 AY373699	AY373678
Cousinia sect. *Lappaceae*			
Cousinia lappacea Bunge	Kazakhstan, *Susanna 2150 et al.* (BC)	AY373733 AY373700	AY373677
Cousinia sect. *Oligantha*			
Cousinia triflora Schrenk	Kazakhstan, *Susanna 2157 et al.* (BC)	AY373744 AY373711	AY373675
Cousinia sect. *Pectinatae*			
Cousinia alberti Regel & Schmalh.	Kazakhstan, *Susanna 2206 et al.* (BC)	AY373721 AY373688	AY373680
Cousinia sect. *Pseudarctium*			
Cousinia umbrosa Bunge	Kazakhstan, *Susanna 2100 et al.* (BC)	AY373745 AY373712	AY373676
Cousinia* subg. *Hypacanthodes			
Cousinia sect. *Amberbopsis*			
Cousinia grandifolia Kult.	Kazakhstan, *Susanna 2181 et al.* (BC)	AY373730 AY373697	AY373679
Hypacanthium			
Hypacanthium echinopifolium (Bornm.) Juz.	Kirguizistan, *Iljin* (LE)	AY373746 AY373713	
Schmalhausenia			
Schmalhausenia nidulans (Regel) Petr.	Kazakhstan, *Susanna 2088 et al.* (BC)	AY373752 AY373719	AY373681
Outgroup			
Jurinea albicaulis Bunge	Greece, *Susanna 1957 et al.* (BC)	AY373747 AY373714	AY373684
Jurinea lanipes Rupr.	Kazakhstan, *Susanna 2136 et al.* (BC)	AY373748 AY373715	AY373686
Jurinea robusta Schrenk	Kazakhstan, *Susanna 2103 et al.* (BC)	AY373749 AY373716	AY373685
Saussurea elegans Ledeb.	Kazakhstan, *Susanna 2179 et al.* (BC)	AY373750 AY373717	AY373683
Saussurea maximowiczii Herder	Ofuna Botanical Garden (Japan)	AY373751 AY373718	AY373682

(SWOFFORD, 1999) using TBR branch swapping with character states specified as unordered and unweighted. The indels were coded as fifth base. All most-parsimonious trees (MPTs) were saved. To locate other potential islands of most-parsimonious trees (MADDISON, 1991), we performed 1000 replications with random taxon addition, also with TBR branch swapping. Bootstrap analyses (BS) were performed (FELSENSTEIN, 1985) with 1000 replicates, and decay indices (DI) were calculated (BREMER, 1988; DONOGHUE et al., 1992) to obtain estimates of support for each monophyletic group. All the decay analyses were conducted using the clade-constraint approach as discussed in MORGAN (1997). Three parsimony analyses were performed, with three different data sets: the ITS data, the *mat*K data and the combined ITS and *mat*K data.

The nrDNA ITS and cpDNA *mat*K data sets were tested for congruence using the partition homogeneity test (FARRIS et al., 1995) as implemented in PAUP 4.0b4a, before combining the data sets. The partition homogeneity test was conducted with 1000 replicates, heuristic search option with simple addition sequence, TBR, and MULPARS.

RESULTS

Nuclear ribosomal DNA ITS

The ITS1 and ITS 2 alignment of 37 taxa consisted of 484 positions and contained 131 phylogenetically informative substitutions and 7 phylogenetically informative indels. Mean pairwise distances (as calculated by PAUP) within ingroup varied from 0% (between *Cousinia congesta* Bunge and *C. caespitosa* C. Winkl.; *C. syrdariensis* Kult. and *C. polycephala* Rupr.) to 11.8% (between *C. karatavica* Regel & Schmalh. and *C. pterocaulos*). Mean pairwise distances between ingroup and outgroup varied from 10.9% (between *Saussurea elegans* Ledeb. and *Hypacanthium echinopifolium* Juz.) to 16.8% (between *Jurinea albicaulis* Bunge and *Cousinia syrdariensis*).

The parsimony analysis yielded 91 MPTs of 346 steps in one island. The strict consensus of all the trees is shown in Fig. 1; the consistency index (CI) excluding uninformative characters was 0.4780; the retention index (RI) was 0.7570; and the homoplasy index (HI) was 0.5220.

The strict consensus of the 91 MPTs produced from the ITS analysis (Fig. 1) support monophyly of the *Arctium* group: *Arctium*, *Cousinia*, *Hypacanthium* and *Schmalhausenia* (BS = 93%, DI = 7). Within this clade, there were two branches: a first one, which we will name the "Arctioid" clade, comprised the genera *Arctium*, *Hypacanthium* and *Schmalhausenia* and the representatives of *Cousinia* subg. *Cynaroides* and *Hypacanthodes* (BS = 76%, DI = 2); and a second one formed by the representatives of *Cousinia* subgenus *Cousinia* (BS = 86%, DI = 2). Within the "Arctioid" clade, there were two clades; one was formed by the genera *Arctium*, *Hypacanthium* and *Schmalhausenia* and *Cousinia grandifolia* Kult. from subgenus *Hypacanthodes* (BS = 94%, DI = 3); the other clade was formed by the representatives of *Cousinia* subgenus *Cynaroides*, with very strong support (BS = 100%, DI = 11).

Chloroplast DNA *mat*K

The *mat*K alignment of 27 taxa consisted of 985 positions and contained 21 phylogenetically informative substitutions. Mean pairwise distances (as calculated by PAUP) within ingroup varied from 0% (between *Cousinia polycephala, C. syrdarienis* and *C. minkwitziae* Bornm.; *C. caespitosa* and *C. astracanica* (Spreng.) Tamamsch.; *C. grandifolia* and *C. karatavica*) to 1.3% (between *Schmalhausenia nidulans* (Regel) Petr. and *Cousinia esfandiarii*). Mean pairwise distances between ingroup and outgroup varied from 0.3% (between *Jurinea robusta* Schrenk and *Cousinia caespitosa*) to 1.3% (between *Jurinea lanipes* Rupr. and *Schmalhausenia nidulans*).

The parsimony analysis yielded 11431 MPTs of 25 steps in one island. The strict consensus of all the trees is shown in Fig. 2; the consistency index excluding uninformative characters (CI) was 0.6333; the retention index (RI) was 0.8000; and the homoplasy index (HI) was 0.3667.

The strict consensus of the 11431 MPTs produced from the *mat*K analysis (Fig. 2) supports monophyly of the *Arctium* group: *Arctium*, *Cousinia* and *Schmalhausenia* (BS = 78%, DI = 2). Within this group, the only well defined clade was formed by *Cousinia lappacea* Bunge as sister (BS = 71%, DI = 1) of a clade formed by the genera *Arctium*, *Schmalhausenia* and the representatives of *Cousinia* subgenera *Cynaroides* and *Hypacanthodes* (BS = 92%, DI = 2). The rest was unresolved as a politomy.

Combined nrDNA ITS and cpDNA *mat*K

The P-value resulting from the partition homogeneity test (P = 0.0570) indicates that data partitions are random, and there is congruence between nrDNA ITS and cpDNA *mat*K data sets, at a significance threeshold of P = 0.05 (FARRIS et al., 1995).

The combined ITS-*mat*K alignment of 29 taxa consisted of 1469 positions with 149 phylogenetically informative substitutions and 6 phylogenetically informative indels. Mean pairwise distances (as calculated by PAUP) within ingroup varied from 0% (between *Cousinia syrdariensis* and *C. polycephala*) to 3.9% (between *Cousinia congesta* and *Arctium minus*). Mean pairwise distances between ingroup and outgroup varied from 4.1% (between *Saussurea elegans* and *Cousinia microcarpa* Boiss.) to 6% (between *Jurinea albicaulis* and *Schmalhausenia nidulans*).

The parsimony analysis yielded 8 MPTs of 329 steps in one island. The strict consensus of all the trees is shown in Fig. 3; the consistency index (CI) excluding uninformative characters was 0.5650; the retention index (RI) was 0.7895; and the homoplasy index (HI) was 0.4350.

Like the ITS or *mat*K alone, the strict consensus of the 8 MPTs obtained from the combined analysis (Fig. 3) strongly supports (BS = 93%, DI = 6) the monophyly of the *Arctium* group (*Arctium*, *Cousinia* and *Schmalhausenia*). We have found again the two clades of the ITS analysis. One clade, the "Arctioid" clade, was formed by the genera *Arctium*, *Schmalhausenia* and the representatives of *Cousinia* subgenera *Cynaroides* and *Hypacanthodes*, with strong support (BS = 97%, DI = 5). Within the "Arctioid" clade, there were two branches, one with the genera *Arctium* and *Schmalhausenia*, and *Cousinia grandifolia* from subgenus *Hypacanthodes* (BS = 89%, DI = 3), and a second branch formed by *Cousinia* subgenus *Cynaroides* (BS = 100%, DI =12). The second clade, the *Cousinia* clade, was formed by all the representatives of *Cousinia* subgenus *Cousinia* (BS = 89%, DI = 4). Relationships within the *Cousinia* clade are unclear because of the limited scope of our sampling.

DISCUSSION

Monophyly of the *Arctium* group

The use of a combination of *mat*K and ITS data has proven again a good choice for our study: the resulting tree from the analysis of the combined data shows excellent resolution at all taxonomical levels (Fig. 3). The *mat*K alone is not suitable for our purposes (Fig. 2)

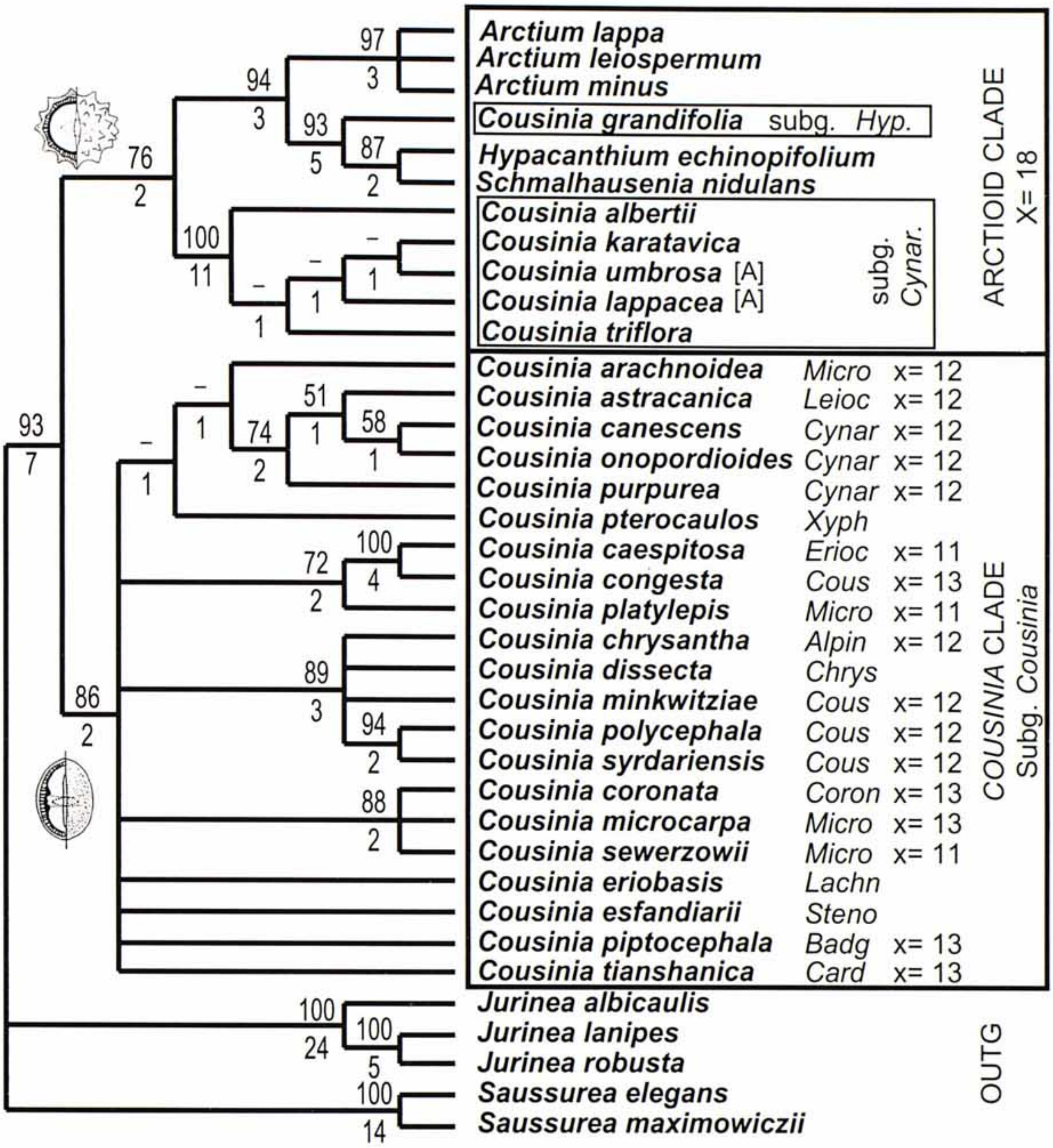

Fig. 1. Strict consensus tree of the most parsimonious trees generated by the ITS region matrix. Subgenera: *Cynar.= Cynaroides*; *Hyp.= Hypacanthodes*. Sections: *Alpin= Alpinae*; *Badg= Badghysia*; *Card= Carduncellus*; *Chrys= Chrysoptera*; *Coron= Coronophora*; *Cous= Cousinia*; *Cynar= Cynaroideae*; *Erioc= Eriocousinia*; *Lachn= Lachnosphaerae*; *Leioc= Leiocaules*; *Micro= Microcarpae*; *Steno= Stenocephalae*; *Xyph= Xiphiolepides*. [A] = Arctioid species. OUTG= Outgroup.


Arctiastrum pollen type


Cousinia pollen type

as it even fails in supporting monophyly of the group (BS = 78% and DI = 2 only). However, it is worth remarking that the *mat*K analysis reflects the cut between the "Arctioid" and the *Cousinia* clades, even with poor support (BS = 71%, DI = 1). This demonstrates that the separation of both groups is very old, as could be expected from the different pollen types and chromosome numbers.

As already suggested on the basis of morphology, the *Arctium* group form a strongly supported monophyletic clade, both in the ITS (BS = 93%, DI = 7) and combined (BS = 93%, DI = 6) analyses (Figs. 1, 3 respectively). This result confirms the importance of achene characters in the *Cardueae*, as this group was defined mainly on the basis of shared achene apomorphies: wavy or tigrine pattern in the pericarp and pappus setae individually deciduous (SUSANNA & GARCIA-JACAS, in press).

Character evolution in the *Arctium* group

Habit and morphology

Our results suggest that the spiny habit is a derived character in the group. We can recall that GARCIA-JACAS et al. (2002) placed the *Arctium* group in a clade formed by the subtribe *Centaureinae* and its potential sister groups, the genera *Jurinea* and *Saussurea*. Within this group, unarmed habit is overwhelmingly dominant: all the species of *Jurinea* and *Saussurea* are unarmed, and spines are a very rare feature among the *Centaureinae*: the only spiny genera in the subtribe are those of the *Carthamus* complex, which are a derived group (GARCIA-JACAS et al., 2001), a fact that reinforces the hypothesis of the derived character of spines. Ancestors of the *Arctium* group were probably unarmed plants that developed spines, like the genera of the *Carthamus* complex, as a secondary adaptation to the defence against herbivores. In the case of *Cousinia*, spines have appeared twice in different lineages (Figs. 1, 3): one in *Cousinia* subgenus *Cousinia* and a second one in *Hypacanthium* and *Schmalhausenia*.

Pollen and chromosomes

We shall discuss together both characters as they appear to be correlated. As has been repeatedly demonstrated in the closely related *Centaureinae*, pollen and karyology are the most reliable characters for phylogenetic reconstruction and usually show also a narrow correlation to molecular phylogenies (SUSANNA et al., 1995; GARCIA-JACAS et al., 2001). In the *Arctium* group, the correlation is also very narrow and they are the only ones that define truly monophyletic groups (Figs. 1, 3).

Pollen evolution also follows the pattern suggested by WAGENITZ (1955) for the *Centaureinae*, widely confirmed by molecular tools (GARCIA-JACAS et al., 2000, 2001). *Arctiastrum* pollen type of the "Arctioid" clade (*Arctium*, *Schmalhausenia* and *Cousinia* subg. *Cynaroides* and *Hypacanthodes*) is ancestral as compared to the *Cousinia* pollen type: *Arctiastrum* is spiny, very similar to the ancestral *Serratula* pollen type of the *Centaureinae*, while *Cousinia* pollen type is smooth, much akin to the *Dealbata*, *Cyanus* and *Montana* derived pollen types of the *Centaureinae* (VILATERSANA et al., 2001).

As to chromosomal evolution, the "Arctioid" clade is also ancestral to the *Cousinia* clade in this character. It is generally accepted that higher chromosome numbers are ancestral as compared to lower ones, and this trend has been demonstrated in groups closely related to the *Arctium* group like subtribe *Centaureinae* (GARCIA-JACAS et al., 2001). In our case, the base chromosome number x = 18 of the "Arctioid" clade is ancestral as compared to the x = 11, 12 or 13 of the *Cousinia* clade (Figs. 1, 3).

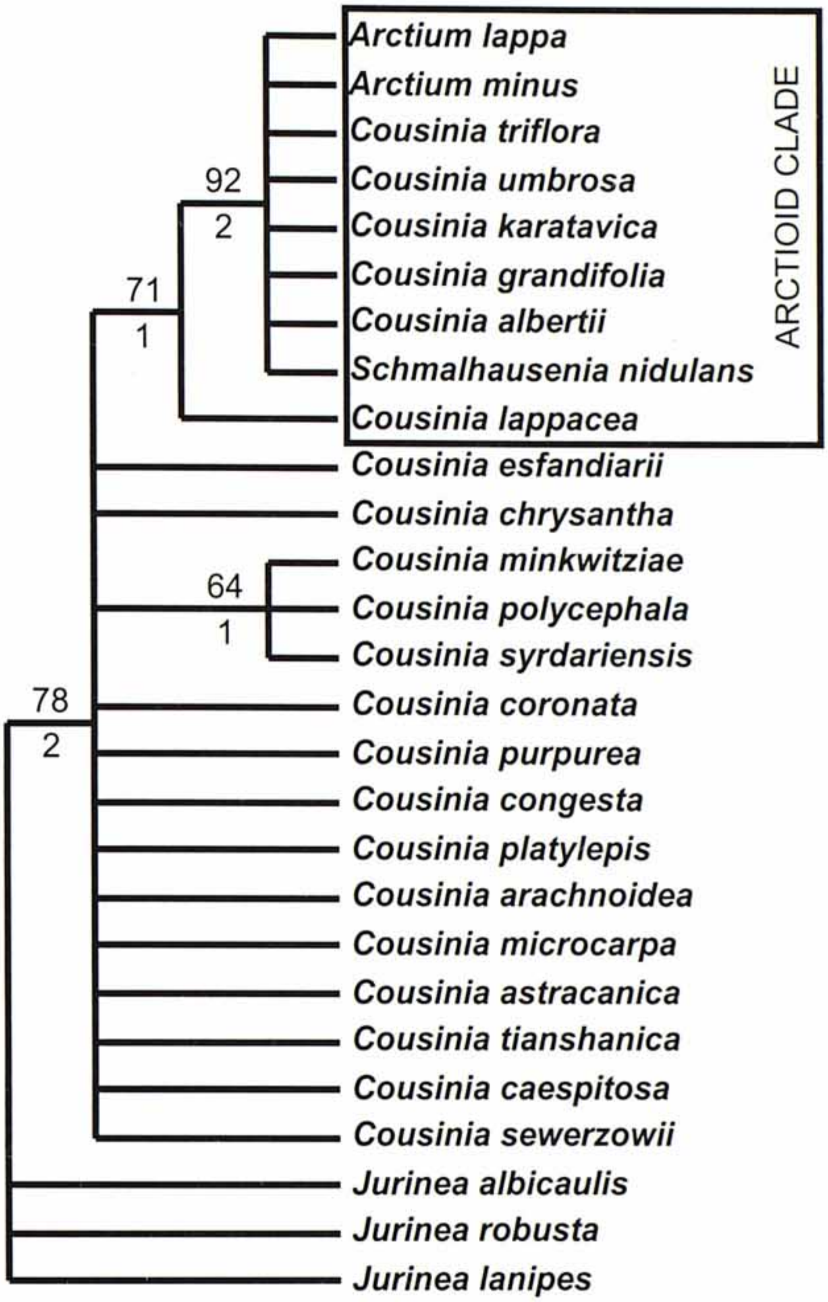

Fig. 2.- Strict consensus tree of the most parsimonious trees generated by the *mat*K matrix.

Generic implications

The delineation of *Arctium* and *Cousinia*

Our results pose a very interesting but vexing problem on the generic delineation of *Arctium* and *Cousinia*: how to conjugate conflicting morphological, on the one hand, and molecular, pollen and karyological data, on the other hand.

Molecular analyses suggest a clear separation (and a very ancient one, as reflected by the *mat*K data) between the "Arctioid" clade and *Cousinia* sensu stricto, strongly supported by pollen and karyological data (Figs. 1, 2, 3). The problem is, then, what to do with *Hypacanthium* and *Schmalhausenia*. On strict molecular, pollen and karyological grounds, they belong without any doubt to the "Arctioid" clade... whose homogeneity they destroy on morphological grounds! As stated in the introduction of this paper, the only useful characters for segregating *Arctium* and *Cousinia* are the leaves and, partly, the appendages of the bracts. Pollen types and karyology are excellent markers, but they are not macroscopic and hence of little practical utility. Morphology of the stigmas could be useful (it should be fully explored in *Cousinia* s. str.), but it is very difficult to observe too. Our own observations suggest that there are some differences in length and distribution of the collector hairs: they are long and sparsely distributed along the whole length of the stigma in *Cousinia* s. str., and short and concentrated on the basal thickening in *Arctium*. *Schmalhausenia* is intermediate between both models, but closer to *Arctium*. However, from our point of view, differences are not so substantive as claimed by DUISTERMAAT (1996).

We have to resort to the leaves and, if we follow this morphological definition, *Hypacanthium* and *Schmalhausenia* (probably a single genus) would be classified in *Cousinia* sensu stricto, contradicting molecular, karyological and pollen evidence. There are three possible solutions to this incongruence.

The first one should be to keep present classification in four genera with a different delimitation: *Arctium* (including *Cousinia* subgenera *Cynaroides* and *Hypacanthodes*), *Cousinia*, *Hypacanthium* and *Schmalhausenia*, disregarding the fact of the polyphyly of the resulting genus *Arctium* (Figs. 1, 3). This solution does not seem adequate, if our goal is to achieve a natural classification.

A second alternative would be to elevate the subgenera of *Cousinia* with *Arctiastrum* pollen type to the genus rank. The resulting *Arctium* group would encompass six genera: *Arctium* sensu str., *Cousinia* s. str., *Cynaroides*, *Hypacanthium*, *Hypacanthodes* and *Schmalhausenia*. The possible genus *Cynaroides* stands as a very well defined group on molecular grounds (BS = 100%, DI = 11 in the ITS tree, Fig. 1; BS = 100%, DI = 12 in the combined tree, Fig. 3), and our sampling is complete enough as to draw conclusions (five out of seven sections of the subgenus, according to TSCHERNEVA'S (1988b, 1988c) classification). In contrast, our sampling of sect. *Hypacanthodes* is too short (one out of four sections) and the only included species forms a well-supported clade with *Hypacanthium* and *Schmalhausenia* (BS = 93%, DI = 5 in the ITS tree, Fig. 1; BS = 90%, DI = 5 in the combined tree [without *Hypacanthium*], Fig. 3), a grouping that needs verification. However, we can anticipate that the problem of making up these six genera is, again, morphological. The boundaries between *Hypacanthium* and *Schmalhausenia*, on the one hand, and *Cousinia* sensu str., on the other hand, are unclear: pollen type and karyology are the only reliable characters that separate them. The stigma characters could be of help, but further studies are needed in *Cousinia*. Besides, the limits between *Arctium* and a new genus *Cynaroides* would also be uncertain on strict morphological grounds because subg. *Cynaroides* includes the arctioid species of *Cousinia*.

Finally, there is the solution suggested by KUNTZE (1891): to lump together all the genera described in the *Arctium* group in a single genus *Arctium* (as this genus would be nomenclaturally prioritary). We are conscious of the nomenclatural consequences of this solution: some 420 species of *Cousinia* will have to be changed to *Arctium* -KUNTZE (1891) already proposed ca. 180 new combinations-. Nevertheless, if new studies in the group (especially with the inclusion of more species of *Cousinia* subgenus *Hypacanthodes*) confirm our results, it will probably be the only solution.

Molecular phylogeny and sectional classification of Cousinia

We are aware of the severe limitations of our sampling regarding *Cousinia* subg. *Cousinia*. However, from our study emerges a general incongruence between sectional classification

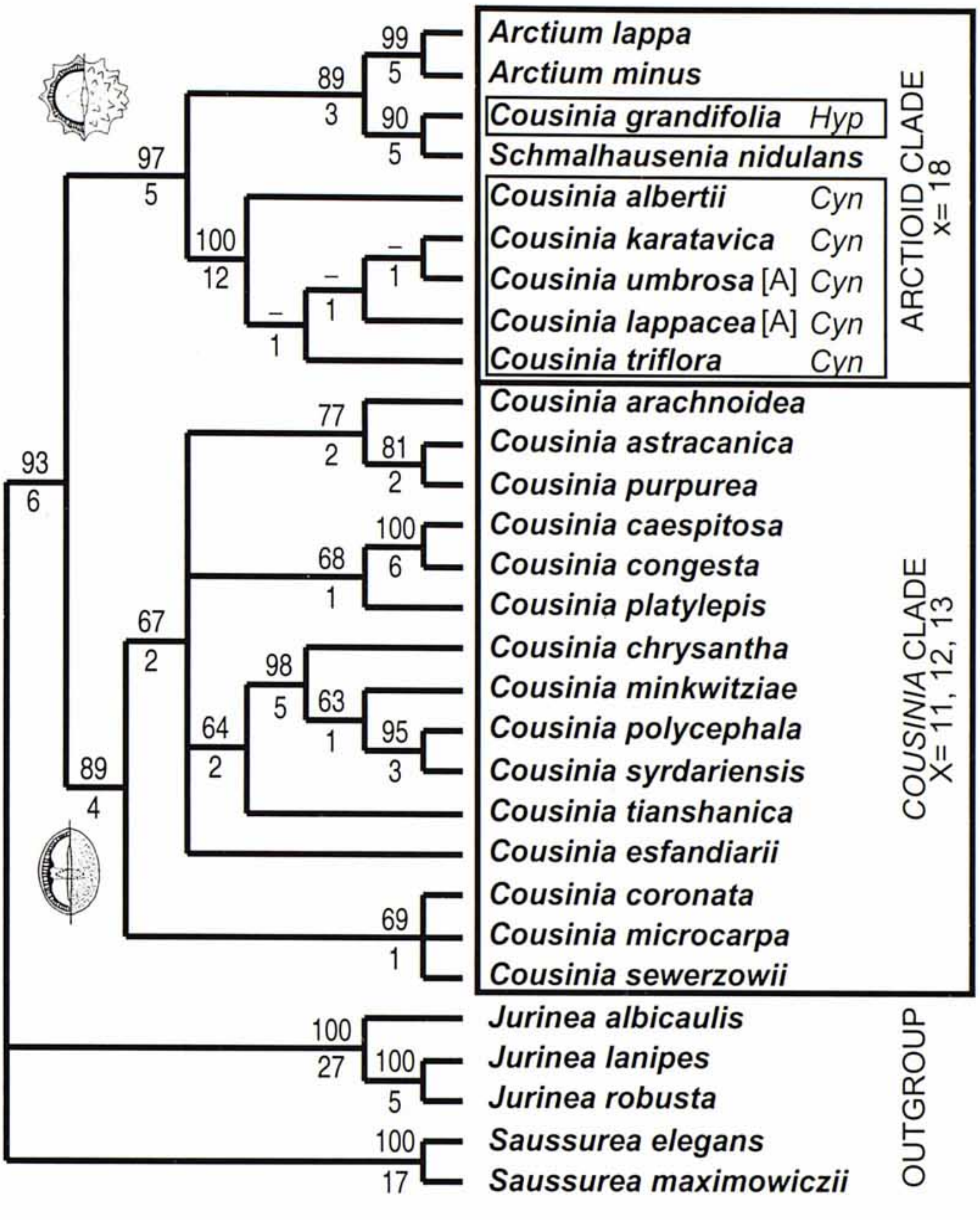

Fig. 3. Strict consensus tree of the most parsimonious trees generated by the combined *mat*K-ITS matrix. Subgenera: *Cynar.*= *Cynaroides*; *Hyp.*= *Hypacanthodes*. [A] = Arctioid species.


Arctiastrum pollen type


Cousinia pollen type

by TSCHERNEVA (1988b, 1988c), mapped in the ITS consensus tree (Fig. 1), and molecular phylogeny. By way of example, the four representatives of sect. *Cousinia* do not form a monophyletic clade. Species of sect. *Cousinia* with x = 12 are placed in the same clade, whilst *Cousinia congesta* with x = 13 is placed in a different clade (Fig. 1). Another case is sect. *Microcarpae*, with four representatives that are placed in three different clades

(Fig. 1). In both cases, the groups seem to be more correlated to chromosome numbers than to present classification. This result opens an interesting way to further studies in the genus *Cousinia*.

ACKNOWLEDGEMENTS

Authors thank A. I. Ivaschenko, L. Kapustina, F. Khassanov and J. Vallès for their help with field collections in Central Asia; J. Vallès again for his help with the Russian literature; R. Vogt, Botanical Museum of Berlin, for authorising the use of herbarium material for our molecular analyses; and the Ofuna Botanical Garden (Japan) for providing seeds of *Saussurea maximowiczii* Herder. This research was supported by the Dirección General de Enseñanza Superior, Spain (project PB 97/1134); the Dirección General de Investigación, Ministerio de Ciencia y Tecnología, Spain (projects BOS2001-3041-C02-02); and the Generalitat de Catalunya (ajuts a grups de recerca consolidats 1999SGR00332 and 2001SGR00125).

REFERENCES

BENTHAM, G. (1873). *Compositae*. In: Bentham, G. & J. D. Hooker (eds.), *Genera Plantarum*. Lovell Reeve & Co., London: 162-533.

BOISSIER, E. (1875). *Flora orientalis* 3. H. Georg, Genève - Basel - Lyon.

BORNMÜLLER, J. (1916). Ein beitrag zur Kenntnis der Gattung *Cousinia*. *Beih. Bot. Centralbl.* 34 (2): 131-203.

BREMER, K. (1988). The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* 42: 795-803.

BREMER, K. (1994). *Asteraceae. Cladistics and classification.* Timber Press, Portland.

BUNGE, A. (1865). Übersichtliche Zusammenstellung der Arten der Gattung *Cousinia* Cass. *Mém. Acad. Imp. Sci. Saint Pétersbourg, sér. VII,* 9 (2): 1-56.

CASSINI, H. (1819). [Different articles]. *Dictionnaire de Sciences Naturelles*. Paris. Cited by King, R. & H. W. Dawson (1975), *Cassini on Compositae*. Oriole, New York.

CULLINGS, K. W. (1992). Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. *Molec. Ecol.* 1: 233-240.

DITTRICH, M. (1977). *Cynareae*-systematic review. In Heywood, V. H., J. B. Harborne & B. L. Turner (eds.), *The Biology and Chemistry of the Compositae*. Academic Press, London - New York - San Francisco: 999-1015.

DONOGHUE, M. J., R. G. OLMSTEAD, J. F. SMITH & J. D. PALMER (1992). Phylogenetic relationships of *Dipsacales* based on *rbc*L sequences. *Ann. Missouri Bot. Garden* 79: 333-345.

DOYLE, J. J. & J. L. DOYLE (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* 19: 11-15.

DUISTERMAAT, H. (1996). Monograph of *Arctium* L. (*Asteraceae*). Generic delimitation (including *Cousinia* Cass. p. p.), revision of the species, pollen morphology and hybrids. *Gorteria* 3: 1-143.

DUISTERMAAT, H. (1997). *Arctium* getting entangled to *Cousinia* (*Asteraceae*: *Cardueae*). *Bocconea* 5: 685-689.

FARRIS, J. S., M. KÄLLERSJÖ, A. G. KLUGE & C. BULT (1995). Testing significance of incongruence. *Cladistics* 10: 315-319.

FELSENSTEIN, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.

GARCIA-JACAS, N., T. GARNATJE, A. SUSANNA & R. VILATERSANA (2002). Tribal and subtribal delimitation and phylogeny of the *Cardueae* (*Asteraceae*): a combined nuclear and chloroplast DNA analysis. *Molec. Phyl. Evol.* 22 (1): 51-64.

GARCIA-JACAS, N., A. SUSANNA, T. GARNATJE & R. VILATERSANA (2001). Generic delimitation and phylogeny of the subtribe *Centaureinae* (*Asteraceae*): a combined nuclear and chloroplast DNA analysis. *Ann. Bot. (London)* 87: 503-515.

GARCIA-JACAS, N., A. SUSANNA, V. MOZAFFARIAN, & R. ILARSLAN (2000). The natural delimitation of *Centaurea* (*Asteraceae: Cardueae*): ITS sequences analysis of the *Jacea* group. *Pl. Syst. Evol.* 223: 185-199.

GHAFFARI, S. M. & S. B. DJAVADI (1998) Chromosome studies and distribution of nine species of *Cousinia* section *Stenocephalae* (*Asteraceae*) in Iran. *Bull. Soc. Neuchâteloise Sci. Nat.* 121: 61-68.

GHAFFARI, S. M., F. ATTAR & A. GHAHREMAN (2000). Distribution and chromosome studies on some species of *Cousinia* Cass. (section *Cynaroideae*) from Iran. *Pakistan J. Bot.* 32 (2): 311-316.

GOLDBLATT, P. (1981). Index to plant chromosome numbers 1975-1978. *Monogr. Syst. Bot. Missouri Bot. Garden* 5.

GOLDBLATT, P. (1988). Index to plant chromosome numbers 1975-1978. *Monogr. Syst. Bot. Missouri Bot. Garden* 23.

GOLDBLATT, P. & D. E. JOHNSON (1990). Index to plant chromosome numbers 1975-1978. *Monogr. Syst. Bot. Missouri Bot. Garden* 30.

GOLDBLATT, P. & D. E. JOHNSON (1991). Index to plant chromosome numbers 1975-1978. *Monogr. Syst. Bot. Missouri Bot. Garden* 40.

HÄFFNER, E. (2000). On the phylogeny of the subtribe *Carduinae* (tribe *Cardueae*, *Compositae*). *Englera* 21.

HÄFFNER, E. & F. H. HELLWIG (1999). Phylogeny of the tribe *Cardueae* (*Compositae*) with emphasis on the subtribe *Carduinae*: an analysis based on ITS sequence data. *Willdenowia* 29: 27-39.

HOFFMANN, O. (1894). *Compositae*. In Engler, A. & A. E. K. Prantl (eds.), *Die natürlichen Pflanzenfamilien*. Wilhelm Engelmann, Leipzig: 324-333.

JANSEN, R. K., K. E. HOLSINGER, H. J. MICHAELS & J. D. PALMER (1990). The phylogenetic analysis of chloroplast DNA restriction site data at higher taxonomic levels: an example from the *Asteraceae*. *Evolution* 44: 2089-2105.

JANSEN, R. K., K. E. HOLSINGER & J. D. PALMER (1991). Phylogeny and character evolution in the *Asteraceae* based on chloroplast DNA restriction site mapping. *Syst. Bot.* 16: 98-115.

JOHNSON, L. A. & D. E. SOLTIS (1995). Phylogenetic inference in *Saxifragaceae* sensu stricto and *Gilia* (*Polemoniaceae*) using *mat*K sequences. *Ann. Missouri Bot. Garden* 82: 149-175.

KIM, K.-J., R. K. JANSEN, R. S. WALLACE, H. J. MICHAELS & J. D. PALMER (1992). Phylogenetic implications of *rbc*L sequence variation in the *Asteraceae*. *Ann. Missouri Bot. Garden* 79: 428-445.

KNAPP, H. D. (1987). On the distribution of the genus *Cousinia* (*Compositae*). *Pl. Syst. Evol.* 155: 15-25.

KUNTZE, O. (1891). *Revisio generum plantarum I.* Arthur Felix, Leipzig.

KUPRIANOVA, L. A. & O. V. TSCHERNEVA (1982). Pollen morphology and ultrastructure of palynoderma in the species of the genus *Cousinia* (*Asteraceae*) in relation to the systematics of the genus [in Russian]. *Bot. Žurn. SSSR* 67 (5): 581-589.

MABBERLEY, D. J. (1990). *The plant book.* Cambridge University Press, Cambridge.

MADDISON, D. R. (1991). The discovery and importance of multiple islands of most parsimonious trees. *Syst. Zool.* 40: 315-328.

MEUSEL, H., E. JÄGER & E. WEINERT (1965). *Vergleichende Chorologie der zentraleuropäischen Flora 1.* G. Fischer, Jena.

MOORE, R. J. (1973). Index to plant chromosome numbers 1967/71. *Regnum Vegetabile* 90.

MOORE, R. J. (1977). Index to plant chromosome numbers 1973/74. *Regnum Vegetabile* 96.

MORGAN, D. R. (1997). Decay analysis of large sets of phylogenetic data. *Taxon* 46: 509-517.

PETIT, D. P. (1997). Generic interrelationships of the *Cardueae* (*Compositae*): a cladistic analysis of morphological data. *Pl. Syst. Evol.* 207: 173-203.

PETIT, D. P., J. MATHEZ & A. QAID (1996). Early differentiation of the *Cardueae sensu lato*: morphology and pollen. In Hind, D. J. N. & H. J. Beentje (eds.), *Compositae: Systematics. Proceedings of the International Compositae Conference, Kew, 1994*. Royal Botanical Gardens, Kew: 79-93.

RECHINGER, K. H. (1972). *Cousinia.* In Rechinger, K. H. (ed.), *Flora Iranica 90. Compositae-Cynareae I.* Akademische Druck- und Verlagsanstalt, Graz.

RECHINGER, K. H. (1979). *Cousinia.* In Rechinger, K. H. (ed.), *Flora Iranica 139a. Compositae III-Cynareae*. Akademische Druck- und Verlagsanstalt, Graz: 108-153.

SCHTEPA, I. S. (1966). On the problem of the affinity between the genera *Arctium* L. and *Cousinia* Cass. of the family *Compositae*. The importance of palynological analysis for the stratigraphy and paleofloristic investigation. Akademiya Nauk, Leningrad: 35-36.

SCHTEPA, I. S. (1976). Palynological investigation of *Cousinia* sect. *Eriocousinia* (*Compositae*). *Proceedings of the 4th International Palynological Conference*: 31-33.

SOLTIS, D. E., P. S. SOLTIS, T. G. COLLIER & M. L. EDGERTON (1991). The *Heuchera* group (*Saxifragaceae*): evidence for chloroplast transfer and paraphyly. *Amer. J. Bot.* 78: 1091-1112.

SUN, Y., D. Z. SKINNER, G. H. LIANG & S. H. HULBERT (1994). Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. *Theor. Appl. Genet.* 89: 26-32.

SUSANNA, A. & GARCIA-JACAS, N. (in press). The tribe *Cardueae*. In Kubitzki, J. (ed.), *The Families and Genera of Flowering Plants, Asteraceae* (Kadereit, J., ed.). Springer, Heidelberg.

SUSANNA, A., N. GARCIA-JACAS, D. E. SOLTIS & P. S. SOLTIS (1995). Phylogenetic relationships in tribe *Cardueae* (*Asteraceae*) based on ITS sequences. *Amer. J. Bot.* 82: 1056-1068.

SUSANNA, A., N. GARCIA-Jacas, R. VILATERSANA, T. GARNATJE, J. VALLÈS & S. M. GHAFFARI (2003). New chromosome counts in the genus *Cousinia* and the related genus *Schmalhausenia* (*Asteraceae, Cardueae*). *Bot. J. Linn. Soc.* (in press).

SWOFFORD, D. L. (1999). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0b4a. *Sinauer, Sunderland.*

SWOFFORD, D. L. & OLSEN, G. J. (1990). Phylogeny reconstruction. In Hillis, D. & C. Moritz (eds.), *Molecular systematics*. Sinauer, Sunderland: 411-501.

TSCHERNEVA, O. V. (1962). *Rod 1578. Cousinia* Cass. In Schischkin, B. K. & E. G. Bobrov (eds.), *Flora SSSR 27.* Akademiya Nauk, Moscow - Leningrad: 110-351.

TSCHERNEVA, O. V. (1974). Brief analysis of *Cousinia* Cass. species geographical distribution [in Russian]. *Bot. Žurn. SSSR* 59 (2): 183-191.

TSCHERNEVA, O. V. (1983). The genus *Hypacanthium* (*Asteraceae*) and its representatives in middle Asia [in Russian]. *Bot. Žurn. SSSR* 68 (5): 632-635.

TSCHERNEVA, O. V. (1985). Chromosome numbers in the species of the genus *Cousinia* (*Asteraceae*) in the flora of the USSR [in Russian]. *Bot. Žurn. SSSR* 70 (6): 856-857.

TSCHERNEVA, O. V. (1988a). New supraspecific taxa of the genus *Cousinia* (*Asteraceae*) [in Russian]. *Bot. Žurn. SSSR* 73 (4): 594-597.

TSCHERNEVA, O. V. (1988b). A synopsis of the *Cousinia* (*Asteraceae*) genus system in the flora of the USSR [in Russian]. *Bot. Žurn. SSSR* 73 (6): 870-876.

TSCHERNEVA, O. V. (1988c). *The Cousinia of the SSSR* [in Russian]. Leningrad: Akademiya Nauk.

VILATERSANA, R., J. MARTÍN VILLODRE, A. SUSANNA, N. GARCIA-JACAS & T. GARNATJE (2001). Pollen studies in subtribe *Centaureinae* (*Asteraceae*): the *Carthamus* complex and the genus *Aegialophila* analyzed with electron microscopy. *Pl. Biol.* 3: 607-615.

WAGENITZ, G. (1955). Pollenmorphologie und Systematik in der Gattung *Centaurea* L. s. l. *Flora* 142: 213-279.

WAGENITZ, G. (1976). Systematics and Phylogeny of the *Compositae* (*Asteraceae*). *Pl. Syst. Evol.* 125: 29-46.

WINKLER, C. (1892). *Synopsis specierum generis Cousiniae* Cass. *Trudy Glavn. Bot. Sada* 12 (2): 181-286.