Evolució, filogènia i sistemàtica del complex *Arctium-Cousinia*

TESI DOCTORAL

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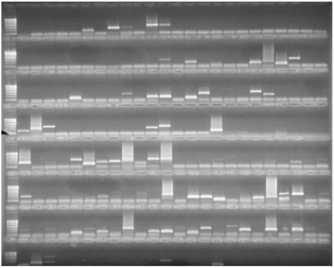
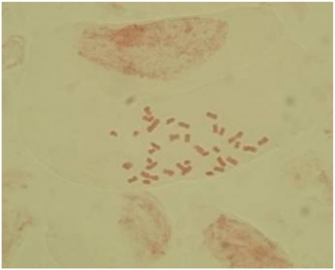




Гербарій Переселенческаго Управленія.

Б. А. Федченко: Самаркандско-Бухарская экспедиція 1913 г.

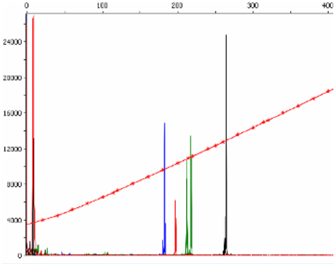
№ 2103

Cousinia anomala Franchet

Самаркандская обл., Самаркандскій уѣздъ

Собр. А. И. Михельсонъ.

B. A. FEDTSCHENKO: Iter Samarcandense Bucharicum 1913.



UNIVERSITAT DE BARCELONA
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EVOLUCIÓ, FILOGÈNIA I SISTEMÀTICA DEL COMPLEX *ARCTIUM-COUSINIA*

Memòria presentada per Sara López Viñallonga per a optar al títol de Doctor per la Universitat de Barcelona

Amb el vist-i-plau dels directors de tesi:

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Dr. Alfonso Susanna de la Serna

i del tutor de tesi:

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Sara López Viñallonga
Barcelona, 2009

Penseu que el mirall de la veritat s'esmicolà a l'origen en fragments petitíssims, i cada un dels trossos recull tanmateix una engruna d'autèntica llum.

Salvador Espriu

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1. Introducció general

1.1. Context taxonòmic del complex *Arctium-Cousinia*

El complex *Arctium-Cousinia* (Susanna et al., 2003; Susanna & Garcia-Jacas, 2007) és un grup natural pertanyent a la tribu Cardueae. Tot i que diversos autors han proposat diferents classificacions d'aquesta tribu, la més àmpliament acceptada estableix una divisió en quatre subtribus: Carlininae, Echinopsinae, Carduinae (inclou el complex *Arctium-Cousinia*) i Centaurinae (Bentham, 1873; Hoffmann, 1894; Bremer, 1994). Recentment s'hi ha incorporat la subtribu Cardopatiinae (Susanna & Garcia-Jacas, 2007).

La tribu Cardueae inclou plantes herbàcies o arbusts perennes, biennals o, més rarament, anuals amb fulles alternes, bràctees involucrals multiseriades, receptacles amb diversos tipus de pàlees, sovint setós, i flòsculs tubulars i actinomorfs amb anteres de base caudada. El seu pol·len és tricolporat, oblat, esfèric o més o menys prolat, espinós, verrucós, escàbrid o gairebé llis (Susanna & Garcia-Jacas, 2007).

Diversos caràcters no trobats en combinació a cap altre grup de la tribu Cardueae caracteritzen el complex *Arctium-Cousinia*: pàlees del receptacle retorçades, aquenis tigrinis (amb un dibuix de línies ondulades), sense nectari, sovint alats, i un papus format per setes decídues individualment. Està format per quatre gèneres: *Arctium* L., *Cousinia* Cass., *Hypacanthium* Juz. i *Schmalhausenia* C. Winkl. Els gèneres *Anura* (Kult.) Tscherneva, *Lipskyella* Juz. i *Tiarocarpus* Rech. f. han estat inclosos al gènere *Cousinia* per Susanna & Garcia-Jacas (2007). Els límits entre aquests gèneres, principalment entre *Arctium* i *Cousinia*, són difícils de distingir i encara no han estat clarament establerts.

El gènere *Arctium*, segons la darrera monografia publicada (Duistermaat, 1996), està format per 11 espècies. Es caracteritza per ser biennal monocàrpic i tenir fulles inermes, cordades i de gran mida i bràctees involucrals amb gloquidis terminals que permeten una eficient dispersió del capítol per exozoocòria. La seva distribució biogeogràfica és subcosmopolita i el seu centre de distribució és Europa (Duistermaat, 1996; Häffner, 2000).

Fig. 1. *A. minus* (Hill.) Bernh.: hàbit, capítols, detall de bràctees involucrals amb gloquidis terminals.

Cousinia és el tercer gènere més nombrós de la família de les Compostes (després de *Senecio* L. i *Vernonia* Schreb.) i el més nombrós de la tribu Cardueae (Rechinger, 1986; Bremer, 1994; Petit, 1997; Susanna & Garcia-Jacas, 2007) donat que conté entre 500 i 700 espècies, depenent dels autors. Es troba exclusivament a l'Àsia Central i Sudoccidental, concretament a les regions muntanyoses del Turkestan (Tien Shan i Pamir-Alaj) i a la regió Irano-Turània (Rechinger, 1986; Knapp, 1987). Segons Rechinger (1986), el gènere *Cousinia* és únic pel que fa al seu gran nombre d'espècies en una àrea geogràfica tan reduïda i probablement també ho sigui en el grau de diversificació que presenta. Està format per tres subgèneres: subg. *Cousinia*, subg. *Cynaroides* Tscherneva i subg. *Hypacanthodes* Tscherneva (Tscherneva, 1988b).

Fig. 2. *Cousinia libanotica* DC, hàbit. *C. pterocaulos* (C. A. Mey.) Rech. f., capítol. *C. hermonis* Boiss., detall de les bràctees involucrals amb espina terminal recta.

El gènere *Hypacanthium* és endèmic de l'oest del Tien Shan i està format per dues espècies perennes, de fulles armades.

Fig. 3. *Hypacanthium echinopifolium* (Bornm.) Juz., hàbit, capítol i detall de les bràctees involucrals amb espina terminal recta.

Schmalhausenia és un gènere monotípic endèmic de les zones subalpina i alpina del nord del Tien Shan. Es caracteritza per tenir fulles profundament dividides amb punxes nombroses i robustes i pel seus capítols sèssils envoltats d'un densíssim toment llanós.

Fig. 4. *Schmalhausenia nidulans* (Regel) Petrak, hàbit, capítol i detall de les bràctees involucrals amb espina terminal recta.

Considerant criteris moleculars, cariològics i palinològics Susanna et al. (2003) subdivideixen el complex en dos clades sense entitat taxonòmica i amb algunes incongruències morfològiques. El primer grup s'anomena Arctioide i comprèn els gèneres *Arctium* (11 espècies), *Hypacanthium* (dues espècies) i *Schmalhausenia* (una espècie) juntament amb *Cousinia* subgèneres *Cynaroides* (15 espècies) i *Hypacanthodes* (10 espècies). Està caracteritzat per un nombre cromosòmic bàsic $x = 18$ i per un pol·len orbicular i espinós de tipus *Arctiastrum* (Schtepa, 1966, 1976; Kuprianova & Tscherneva, 1982). El segon grup s'anomena Cousinioide i està format exclusivament per *Cousinia* subgènere *Cousinia* (*Cousinia* s.str.), molt més ampli pel que fa a nombre d'espècies (unes 500). Cariològicament està caracteritzat per una sèrie disploide de $x =$ 9, 10, 11, 12 i 13 i té un pol·len oblong i llis de tipus *Cousinia* (Schtepa, 1966, 1976; Kuprianova & Tscherneva, 1982).

1.2. Estudis previs sobre el complex *Arctium-Cousinia*

1.2.1. Taxonomia

El gènere *Arctium* va ser descrit per Linné l'any 1753 i el gènere *Cousinia* va ser descrit el 1827 per Cassini basant-se en *Carduus orientalis* Adams, i el cert és que molts autors han discutit la delimitació genèrica d'aquests dos taxons. En canvi, els gèneres *Hypacantium*, descrit per Juzepczuk el 1937, i *Schmalhausenia*, descrit per Winkler el 1892, no han generat aquesta mena de controvèrsies.

Hi ha nombrosos treballs sobre la taxonomia del complex com el que recull el *Prodromus* de De Candolle (1838) que inclou 34 espècies dividides en tres grups establerts segons la morfologia del capítol. El primer intent ambiciós de classificar el complex *Arctium-Cousinia* va ser el 1865 quan Bunge va agrupar 126 espècies en 23 seccions basant-se en l'hàbit, la morfologia del capítol, la textura de les pàlees del receptacle, la forma de les bràctees del receptacle, el color de la corol·la i la presència o absència de pèls al tub de l'antera. Molts tractaments taxonòmics posteriors es basarien en aquesta monografia (Boissier, 1875, 1888; Rechinger, 1953, 1972; Tscherneva, 1962, 1988a). La *Flora Orientalis* de Boissier (1875 i el suplement de 1888) recollia 141 espècies de *Cousinia* agrupades en 14 seccions i 4 espècies del gènere *Arctium* (com molts altres autors dels segles XVIII i XIX, Boissier usa el sinònim il·legítim prelinneà *Lappa*). Més tard, amb un criteri més sintètic, Kuntze (1891) va proposar la transferència de les 187 espècies de *Cousinia* descrites fins llavors al gènere *Arctium*.

Posteriorment, Winkler (1892, 1897) va publicar una altra proposta de classificació del gènere *Cousinia* usant la morfologia del capítol com a principal caràcter taxonòmic per aquest gènere. Va agrupar 267 espècies en tres subgèneres: els subgèneres monotípics *Oligochaete* C. Winkl. i *Dichacantha* Lipsky i el nombrós subgènere *Eucousinia* que englobava les 265 espècies restants alhora agrupades en 19 seccions. Més tard, en una sèrie de publicacions aparegudes entre 1896 i 1941 (llistades a Rechinger, 1953), Bornmüller va introduir unes 30 espècies més. El darrer tractament infragenèric de *Cousinia* va ser publicat el 1988 per Tscherneva. Basant-se en evidències morfològiques, cariològiques i palinològiques va dividir *Cousinia* en els tres subgèneres acceptats actualment: subg. *Cousinia*, subg. *Hypacanthodes* i subg. *Cynaroides*.

Més recentment, Duistermaat (1996, 1997) analitza amb metodologia cladista dades morfològiques, anatòmiques, cariològiques i pol·líniques i estableix la classificació seccional d'*Arctium* que passa a tenir 11 espècies donat que hi inclou sis espècies de *Cousinia* subgènere *Cynaroides*. L'adscripció d'aquest subgènere, però queda irresolta ja que l'autora insinua la possibilitat de transferir-ne totes les espècies a *Arctium*.

A més de les monografies esmentades, trobem tot un seguit de tractaments parcials del complex *Arctium-Cousinia* tenint en compte només les espècies presents a determinades àrees geogràfiques. Destaquem en primer lloc la Flora de la URSS (Tscherneva, 1962, 1988a) on s'estableixen 50 seccions que agrupen 260 espècies presents als estats d'Àsia Central de l'ex-Unió Soviètica i el Càucas i inclou els gèneres *Arctium*, *Cousinia*, *Schmalhausenia*, *Hypacanthium*, *Anura*, *Lipskyella* i *Tiarocarpus*. En segon lloc, destaquem la Flora d'Iran (Rechinger, 1972 i 1979) on s'estableixen 58 seccions que agrupen 350 espècies del gènere *Cousinia* s.str. presents a Iran, Turkmenistan, Afganistan i les regions muntanyoses de Pakistan. Altres tractaments remarcables són els treballs sobre les espècies caucàsiques de *Cousinia* de Takhtajan (1938) i Tamanian (1999) i el tractament de Davis (1975) per a la Flora de Turquia que inclou també el gènere *Arctium*.

Finalment, cal esmentar treballs sobre taxa superiors que aporten informació rellevant sobre el complex *Arctium-Cousinia* com la revisió sistemàtica de la tribu Cardueae ("Cynareae") de Dittrich (1977), els treballs de Petit et al. (1996), Petit (1997), Häffner & Hellwig (1999), Susanna et al. (2006) i Susanna & Garcia-Jacas (2007) sobre la tribu Cardueae i la filogènia de la subtribu Carduinae de Häffner (2000).

1.2.2. Cariologia

Tot i que els recomptes cromosòmics del complex *Arctium-Cousinia* són nombrosos, si tenim en compte el seu elevat nombre d'espècies, es posa de manifest que manca aquest tipus d'informació pel 75% d'elles. Els nombres cromosòmics trobats fins ara són $x = 9$, 10, 11, 12, 13 i 18 (Poddubnaja-Arnoldi, 1931; Koul, 1964; Fedorov, 1969; Podlech & Dieterle, 1969; Podlech & Bader, 1974; Aryavand, 1975, 1976; Ghaffari, 1984; Tscherneva, 1985; Susanna et al., 2003; Ghaffari et al., 2000, 2006). La majoria d'aquests recomptes són consultables *on line* a la base de dades *Index to chromosome numbers in Asteraceae* (http://www.lib.kobe-u.ac.jp/infolib/meta_pub/G0000003asteraceae_e) implementada per Watanabe (2002).

És important remarcar que x = 18 només es dóna a les espècies del grup Arctioide (Tscherneva, 1985; Susanna et al., 2003). Aquest nombre és el més alt de la tribu Cardueae i suggereix que el grup Arctioide seria un paleoploide (Tscherneva, 1985), és a dir, que s'hauria originat per antics esdeveniments de poliploïdia.

En contrast, el grup Cousinioide mostra la sèrie disploide x = 9, 10, 11, 12 i 13. Tal i com succeeix a altres grups de la tribu Cardueae, probablement aquesta disploïdia sigui descendent. En aquest sentit, Frankton & Moore (1961), Fernández Casas & Fernández Morales (1979), Siljak-Yakolev (1986) i Garcia Jacas & Susanna (1992) assenyalen que els nombres cromosòmics bàsics alts s'haurien de considerar com a més primitius que els baixos, fet que s'hauria de considerar com una tendència general segons Stebbins (1950, 1971) i Grant (1981).

1.2.3. Palinologia

Ikuse (1956) va ser el primer en descriure el pol·len d'*Arctium*, basant-se en *A. lappa* L. Posteriorment Schtepa (1966), mitjançant l'estudi de 160 espècies de *Cousinia*, es va adonar que algunes tenien el mateix tipus de pol·len que *Arctium*. En un estudi més exhaustiu, va demostrar que eren les espècies dels subgèneres *Cynaroides* i *Hypacanthodes* les que tenien el pol·len similar a *Arctium* –i va suggerir transferir-les a aquest gènere- mentre *Cousinia* s.str. tenia un tipus de pol·len diferent (Schtepa, 1976). Més tard, Kuprianova & Tscherneva (1982) van confirmar l'existència d'aquests dos tipus de pol·len i els van anomenar *Arctiastrum* i *Cousinia* respectivament. Qaid (1990) aporta més dades amb l'estudi de tres espècies d'*Arctium* i set de *Cousinia*.

El pol·len tipus *Arctiastrum* és equinat i prolat esferoïdal a subprolat (P/E: 1.0-1.2). Les dimensions de l'eix polar són 34-(47)-60 µm i el diàmetre equatorial fa 29-(37)-44 µm. Les costelles equatorials són poc aparents i interrompudes, essent absents sota el mesocolpus. L'exina és lleugerament més prima als pols. El tipus pol·línic *Cousinia* és prolat (P/E: 1.06-1.78), escàbrid o microequinat amb una evident costella equatorial. Les dimensions de l'eix polar són 33-(53)-70 µm i el diàmetre equatorial fa 26-(36)-53 µm. L'exina és conspícuament més prima als pols que a l'equador. Les perforacions del tectum són menys freqüents i estretes que al tipus *Arctiastrum*.

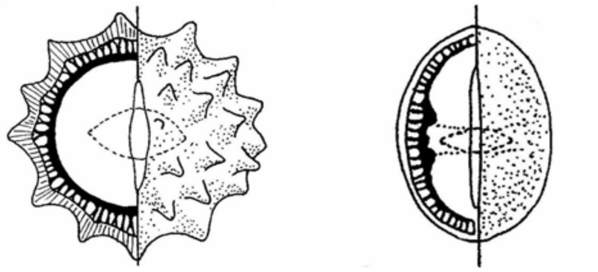

Fig. 5. Tipus pol·línics *Arctiastrum* i *Cousinia*.

El 1996 Duistermaat, mitjançant l'estudi microscòpic de 84 mostres pol·líniques de 73 espècies del complex *Arctium-Cousinia*, arriba a les mateixes conclusions que Schtepa, Kuprianova i Tscherneva i descriu detalladament les característiques dels seus dos tipus pol·línics, el tipus *Arctiastrum* i el tipus *Cousinia*. Malgrat aquesta clara distinció pol·línica, Duistermaat manifesta que el fet que *Cousinia* subgèneres *Cynaroides* i *Hypacanthodes* tinguin el tipus pol·línic *Arctiastrum* no justifica la inclusió de les espècies del dos subgèneres a *Arctium,* donat que a la tribu Cardueae és freqüent trobar el mateix tipus de pol·len a diferents gèneres. Ara bé, alhora suggereix que s'usin les característiques pol·líniques en la delimitació del gènere *Arctium*.

Finalment, l'evidència de que *Schmalhausenia* (Susanna et al., 2003) i *Hypacanthium* (observació feta durant el desenvolupament d'aquesta tesi) tenen el pol·len similar a *Arctium* confirma que el grup Cousinioide té un pol·len de tipus *Cousinia* i el grup Arctioide de tipus *Arctiastrum*. Segons Tscherneva (com. pers.) el pol·len *Arctiastrum* és més primitiu que el de tipus *Cousinia* i reflecteix la natura mesofílica de les espècies que tenen aquest tipus pol·línic, el clade Arctioide.

1.2.4. Biogeografia

Segons Häffner (2000), un dels factors més importants que cal prendre en consideració a l'hora de reconstruir filogènies és la distribució geogràfica dels taxa considerats. Però aquestes dades cal tractar-les amb precaució donat que l'àrea de distribució en cada moment depèn d'esdeveniments històrics i climàtics i altres factors ambientals com els edàfics, tant presents com passats, que sovint són impossibles de reconstruir en detall (Häffner, 2000). Les dades corològiques són la base per a establir teories sobre la localització dels centres d'origen dels taxons sota estudi i per esbrinar com han evolucionat paral·lelament al seu desenvolupament geogràfic (Meusel, 1965).

A la monografia sobre *Arctium* de Duistermaat (1996), les espècies d'*Arctium* s.str. (secció *Arctium*) es defineixen com a subcosmopolites, mentre la resta tenen una distribució centre asiàtica, igual que algunes espècies de *Cousinia*. El centre de distribució d'*Arctium* és Europa (Häffner, 2000) però hi ha espècies naturalitzades pràcticament a tot l'hemisferi nord, on fins i tot algunes d'elles es consideren males herbes, i a alguns països de l'hemisferi sud com Algèria, Egipte, Marroc, Argentina o Austràlia.

El gènere *Cousinia* s. l. té una àrea de distribució extremadament restringida en comparació amb altres gèneres del mateix nombre d'espècies (Rechinger, 1986). Es troba al sud-oest i centre d'Àsia, àrea que es correspon amb la regió biogeogràfica Irano-Turkestània segons Rechinger (1986) o Irano-Turània segons Knapp (1987). Aquesta àrea es considera un centre important de cara a la conservació donat que hi ha una extensa flora paleoxeromòrfica rica en endemismes de rang genèric, específic i subespecífic (Rechinger, 1986).

L'àrea de distribució de *Cousinia* s. l. es subdivideix en dues regions, una occidental i una oriental. La regió occidental conté més de 250 espècies i s'estén des de les terres baixes d'Iraq i l'est de Turquia fins les muntanyes del Kopet Dagh i el Balutxistan iraní i inclou els centres de diversitat 1 a 4 (veure més avall). La regió oriental, amb més de 340 espècies, s'estén des de l'Hindu Kush a través del Pamir-Alaj fins al nord del Tien-Shan i els deserts del nord de Kazakhstan i comprèn els centres de diversitat 5 a 8 (veure més avall). A grans trets cal dir que *Cousinia* subgèneres *Hypacanthodes* i *Cynaroides*, *Schmalhausenia* i *Hypacantium* només creixen a la regió oriental; els dos darrers gèneres són endèmics del Tien-Shan, de la part nord i oest respectivament. *Cousinia* s.str. es troba a ambdues regions.

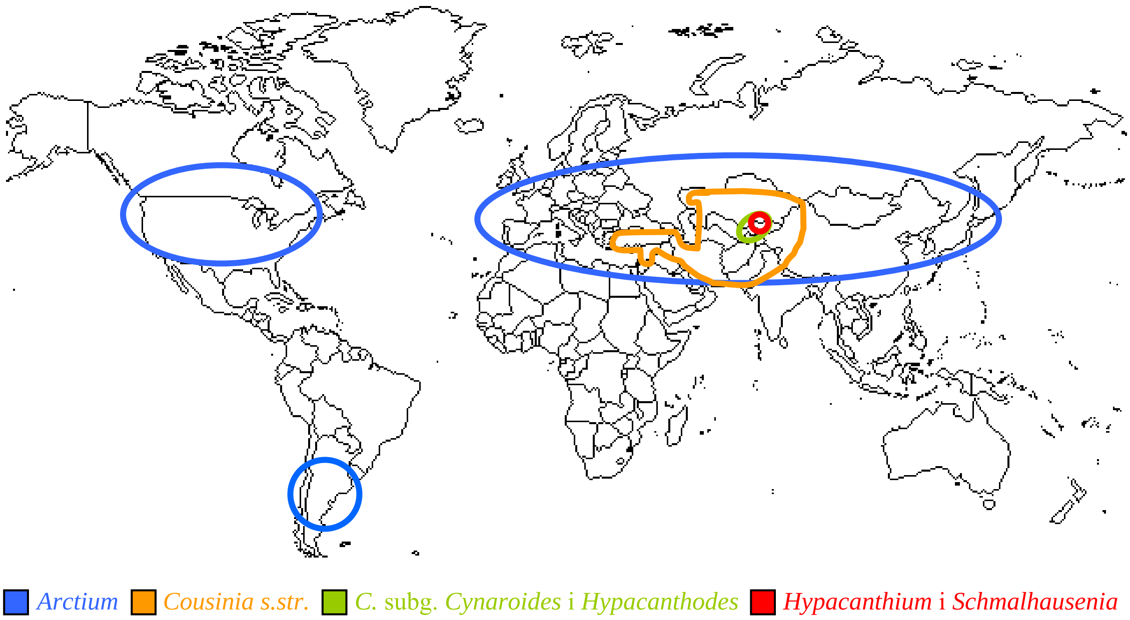

Fig. 6. Àrees de distribució dels diferents taxons del complex *Arctium-Cousinia*.

Segons Tscherneva (1962, 1974), Rechinger (1986) i Knapp (1987) aquests són els vuit centres principals d'especiació de *Cousinia* s. l.:

1) SE d'Anatòlia i N dels Monts Zagros al SE de Turquia i NW d'Iran, amb unes 40 espècies principalment concentrades a l'Azerbaidjan.

2) Monts Zagros a Iran (principalment a les províncies d'Hamadan, Markazi i Luristan) amb més de 40 espècies.

3) Muntanyes d'Elburz, a Iran, amb unes 70 espècies.

4) Serralada del Kopet Dagh, amb unes 60 espècies a l'Iran i més de 30 al Turkmenistan.

5) Hindu Kush occidental, al centre i W d'Afganistan, incloent les províncies de Ghorat, Ghazni i Gardez, amb més de 40 espècies.

6) Hindu Kush oriental, al NE d'Afganistan, incloent les províncies de Bamian, Kabul, Parvan i l'est de Kataghan, amb unes 80 espècies.

7) Pamir-Alaj, a l'Àsia central, és el centre de diversitat més important. A aquesta regió es troben 36 seccions amb 169 espècies, de les quals 130 són endèmiques.
8) Tien-Shan a l'Àsia central, amb més de 60 espècies, de les que 41 són endèmiques.

En aquest context, algunes seccions de *Cousinia* s. l. tenen una àrea de distribució àmplia, com per exemple la secció *Cynaroideae* Bunge (tot i que les seves espècies són endemismes molt restringits), mentre altres seccions són endèmiques de regions relativament reduïdes, com la secció monotípica *Winklerianae* Rech. f. que només es troba a l'oest d'Afganistan (Rechinger, 1972). Segons Tscherneva (1974) la vall del riu Hari Rud (al Turkmenistan) en direcció sud-nord i el meridià de Sistan (61º E) formen una clara frontera dins de l'àrea de distribució de *Cousinia* s. l. i la seva riquesa específica i seccional disminueix dràsticament cap als marges d'aquestes zones.

Com és ben sabut, la capacitat de dispersió de les espècies condiciona la seva àrea de distribució. A tot el complex *Arctium-Cousinia* el papus és extremadament fràgil, cosa que desafavoreix la disseminació dels aquenis, i segons Rechinger (1986) podria explicar l'àrea tan limitada d'algunes espècies. Häffner (2000) postula que en aquest grup el papus tindria una funció únicament de protecció. I encara trobem altres adaptacions morfològiques que retenen els aquenis dins del capítol com ara les pàlees del receptacle molt ben desenvolupades, rígides i sovint escàbrides que no generen moviments xerocàstics d'obertura del capítol (Häffner, 2000).

Dins del complex hi ha tres tendències generals pel que fa la dispersió de les diàspores. Una possibilitat és l'atelecòria, és a dir que en madurar, els aquenis caiguin del capítol dipositant-se al costat de la planta mare, mecanisme freqüent a hàbitats adversos (Häffner, 2000). Un altre mecanisme és la disseminació del capítol sencer que és altament eficient i permet assolir grans distàncies. Un cas especial dins d'aquest mecanisme, present a les espècies amb bràctees involucrals amb gloquidis (algunes espècies del grup Arctioide com ara *Arctium* s.str.), és l'exozoocòria en què el capítol queda adherit al pèl dels animals transportant-lo lluny de la planta mare. La resta d'espècies es dispersen com a estepicursors, és a dir, que la tija es trenca arran de terra i és tota la planta, empesa pel vent, la que actua com a diàspora escampant les llavors. Com a cas extrem, es troben casos de sinaptospèrmia, a algunes espècies amb capítols molt petits, en que les llavors no s'alliberen de l'involucre en madurar o ho fan molt tard.

1.2.5. Filogènia molecular

El treball de Susanna et al. (2003) va ser el primer en usar eines moleculars per establir la filogènia del complex *Arctium-Cousinia*. Es va emprar el gen cloroplàstic *matK* i els espaiadors ITS1 i ITS2 del DNA ribosòmic-nuclear per a descobrir una precisa correlació entre filogènia molecular, palinologia i cariologia dins del complex i es va veure que es podia subdividir clarament en dos clades monofilètics. El primer llinatge, anomenat Arctioide, es caracteritza per un nombre cromosòmic bàsic de $x = 18$ i pol·len de tipus *Arctiastrum* i engloba els gèneres *Arctium*, *Hypacanthium* i *Schmalhausenia* així com els subgèneres *Cynaroides* i *Hypacanthodes* de *Cousinia*. El segon clade, anomenat *Cousinioide* i format exclusivament per *Cousinia* s.str., es caracteritza cariològicament per una sèrie disploide $x = 9$, 10, 11, 12 i 13 i per un pol·len de tipus *Cousinia*. També es va posar de manifest la manca de criteris morfològics que recolzessin aquesta subdivisió. Com a exemple, s'esmenta que el caràcter que separaria *Arctium* i *Cousinia* serien les fulles espinoses i pinnatífides a pinnatisectes de *Cousinia* però aquest caràcter és adaptatiu i té poca rellevància taxonòmica. Segons aquests autors, les espines haurien aparegut a dos llinatges diferents; d'una banda a *Cousinia* i d'altra a *Hypacanthium* i *Schmalhausenia* que són morfològicament més propers a *Cousinia* però estan més relacionats amb *Arctium* pel que fa a la filogènia, la palinologia i la cariologia.

Existeixen treballs sobre taxa superiors que aporten informació important sobre el complex *Arctium-Cousinia* basant-se en tècniques moleculars. Garcia-Jacas et al. (2002) proposen una reconstrucció filogenètica de la tribu Cardueae així com la seva delimitació tribal i subtribal basant-se en el marcador cloroplàstic *matK* i el nuclear ITS. Estableixen que el clade germana de la subtribu *Centaureinae* conté els gèneres *Arctium* i *Cousinia*, conjuntament amb *Jurinea* Cass. (inclosos *Outreya* Jaub. & Spach, *Hyalochaete* Dittrich & Rech. f. i *Jurinella* Jaub. & Spach) i *Saussurea* DC. A aquest treball, el gènere *Cousinia* conforma un clade amb alt suport, indicant que es tracta d'un grup robust i conclouen que és un taxó més recent que *Cirsium* Mill. Finalment, discuteixen la problemàtica en la circumscripció d'alguns gèneres i proposen moure totes les espècies Arctioides de *Cousinia* al gènere *Arctium* com havia proposat anteriorment Duistermaat (1996). Posteriorment, Susanna et al. (2006) revisen les conclusions del treball de Garcia-Jacas et al. (2002), afegint la regió cloroplàstica *trnL-trnF*, però els límits entre aquests gèneres tan conflictius segueixen sense poder ser resolts. Fins i tot se suggereix la possibilitat de transferir les espècies dels quatre gèneres del complex a *Arctium*, tal i com ja havia proposat Kuntze (1891). Pel que fa a *Anura* i *Tiarocarpus*, les anàlisis d'aquest treball recolzen la seva inclusió a *Cousinia*, agrupació coherent amb evidències morfològiques.

1.2.6. Altres

- Quimiotaxonomia

Hi ha molt pocs estudis d'aquest tipus al complex *Arctium-Cousinia*. Un exemple és el treball de Marco et al. (1993) en què, a partir de l'estudi de lactones sesquiterpèniques i altres compostos, els autors conclouen que hi hauria una relació taxonòmica estreta entre *Cousinia* i els gèneres *Centaurea* L. i *Ptilostemon* Iljin. Un altre apunt quimiotaxonòmic relacionat amb aquest complex és que el compost químic anomenat arctiina, present a les subtribus *Carduinae* (inclou el complex *Arctium-Cousinia*) i *Centaureinae* i no a cap altre grup de les Compostes (Hänsel et al., 1964), recolza segons Häfner & Hellwig (1999) la monofília d'aquestes dues subtribus considerades conjuntament.

- Hibridació

Al complex *Arctium-Cousinia* s'han descrit 36 híbrids segons criteris morfològics, ecològics i geogràfics, 30 d'ells pertanyents a *Cousinia* s.str. (Tscherneva, 1962; Rechinger, 1972, 1979; Kamelin, 1973) i 6 a *Arctium* sensu Duistermaat (1996), és a dir, *Arctium* s.str. juntament amb algunes espècies de *Couisnia* subgènere *Cynaroides*. No s'han descrit híbrids a cap dels altres gèneres del complex. Mehregan & Kadereit (en premsa) han complementat aquestes dades amb anàlisis de l'additivitat de seqüències de la regió intergènica nuclear-ribosòmica ITS. Aquests autors han observat que la hibridació és possible en els rangs inter i intraseccional així com dins un mateix clade i entre clades diferents obtinguts al seu estudi filogenètic, i postulen que el 10.7%

de les espècies de *Cousinia* s.str. deuen haver estat implicades en fenòmens d'hibridació interespecífica. Donat aquest baix percentatge, els autors conclouen que la hibridació interespecífica deu haver estat poc important a les espècies del grup Cousinioide. En canvi, no han trobat híbrids entre espècies anuals i perennes de *Cousinia* s.str. ni entre espècies de *Cousinia* s.str. i altres clades del complex *Arctium-Cousinia*. Pel que fa a *Arctium*, el treball de Repplinger et al. (2007) analitza evidències morfològiques i moleculars de tres espècies centreuropees, *A. lappa*, *A. tomentosum* Mill. i *A. minus* i conclou que hi ha d'haver mecanismes d'aïllament pre-zigòtics, ja que els creuaments entre la F_1 són extremadament rars, i post-zigòtics, donada la pràctica absència d'introgressió entre híbrids de generacions més avançades.

- **Datació**

Tot i que no hi ha cap estudi específic de datació del complex *Arctium-Cousinia*, segons Tamanian (1999), l'origen del gènere *Cousinia* s. l. estaria lligat a l'augment general en l'aridesa del clima que va acompanyar l'assecament del mar Tetis al Miocè tardà, fa aproximadament uns 13 milions d'anys. L'autora considera que les formes ancestrals de *Cousinia* serien antics representants d'*Arctium*.

- **Importància econòmica**

Al gènere *Arctium* s'han descrit moltes propietats medicinals: antiinflamatòries (Knipping et al., 2008), antioxidants (Erdemoğlu et al., 2009), anticanceroses (Matsumoto et al., 2006), antisèptiques (Gentil et al., 2006), coagulants (Chen et al., 2004), hepatoprotectives (Lin et al., 2002) i estimulants de la síntesi de col·lagen (Knott et al., 2008). És usat per a tractar reuma, diabetis, nefrosi, al·lèrgies, regeneració dèrmica, en tractaments per a perdre pes, per alleujar la febre i els símptomes d'insolació, per a prevenir l'osteoporosi, com a antitussiu, protector gàstric, diürètic, depuratiu, estimulant digestiu, etc. Els seus principis actius s'extreuen principalment de l'arrel i l'aqueni. Hi ha un gran nombre de medicaments patentats relacionats amb compostos d'*Arctium* i moltes espècies d'aquest gènere han estat usades en la medicina popular de múltiples països des de l'antiguitat. Hi ha molts menys estudis d'aquest tipus sobre el gènere *Cousinia* però també s'hi han descrit propietats anticanceroses (Shahverdi, 2007). A banda de la medicina, trobem altres aplicacions remarcables. *Arctium* és considerada una planta mel·lífera, els seus brots, fulles i tiges joves tenen valor culinari i els seus capítols gloquidiats van servir d'inspiració a l'inventor del Velcro®. A certes regions com Estats Units d'Amèrica o Canadà, però, es considera mala herba. D'algunes espècies de *Cousinia* s'extreu cautxú, d'altres reïna i altres es consideren plantes farratgeres.

1.3. Justificació del present treball

La present tesi doctoral se centra en el complex *Arctium-Cousinia* (Cardueae, Compositae) i s'ha plantejat en dos nivells: macroevolutiu (establiment de la filogènia i sistemàtica del complex com a eina per inferir la història evolutiva del grup) i microevolutiu (anàlisi de la variabilitat genètica poblacional com a peça clau per a conèixer la dinàmica evolutiva de les espècies).

Nivell macroevolutiu

El fet de ser el complex *Arctium-Cousinia* un dels grups de major nombre d'espècies del regne vegetal (més de 500), unit a que la seva àrea biogeogràfica és de difícil accés (degut tant a la orografia com a la geopolítica de la zona), fa que el seu coneixement sigui incomplet. Paral·lelament, no existeixen gaires estudis centrats en aquest complex i els que hi ha, tot i ser altament meritoris i rigorosos, no han aconseguit establir satisfactòriament la sistemàtica del grup i han derivat en certes controvèrsies. És per tot això que es fa necessària una nova revisió, que prengui com a punt de partida aquests treballs previs, i els complementi amb la nova informació derivada de les darreres tècniques moleculars.

Una primera aproximació a la filogènia del complex *Arctium-Cousinia* es va presentar al congrés *The International Compositae Alliance* celebrat a Barcelona el juny de 2006 sota el títol "Molecular phylogeny of the *Arctium-Cousinia* group: combined analysis of plastid and nuclear DNA sequences". A aquest treball es van analitzar, mitjançant Inferència Bayesiana, les regions ITS i *rpS4-trnT* de 116 espècies del complex amb l'objectiu d'esbrinar les seves relacions inter i intragenèriques. Per tal d'afinar aquests resultats preliminars, es va ampliar el mostreig a 218 espècies que representaven tots els gèneres i subgèneres del complex i un 86% de les seves seccions. Es van analitzar els marcadors ITS (218 espècies) i *rpS4-trnT-trnL* (147 espècies) per separat i conjuntament (138 espècies), mitjançant els mètodes d'Inferència Bayesiana, Parsimònia i Màxima Versemblança, per a datar els principals llinatges del complex i per a determinar tant les seves relacions filogenètiques com l'idoneïtat dels marcadors triats per a l'estudi. Aquest treball ha estat publicat a la revista científica SCI *Taxon* amb el títol "Phylogeny and evolution of the *Arctium-Cousinia* complex (Compositae, Cardueae-Carduinae)".

Paral·lelament, s'ha dut a terme un estudi cariològic d'algunes espècies del complex *Arctium-Cousinia* de les quals no existia cap recompte cromosòmic o bé els que hi havia eren dubtosos. Aquestes dades ens resultaven interessants per a complementar la filogènia que havíem establert anteriorment i per a intentar deduir la història evolutiva del complex. També s'han aportat recomptes del grup *Onopordon* que presenta paral·lelismes amb el complex *Arctium-Cousinia* en alguns aspectes. Aquest treball, titulat "Chromosome counts in the genera *Cousinia*, *Olgaea* and *Syreitschikovia* (Compositae)", ha estat formalment acceptat a la revista científica SCI *Folia Geobotanica*.

Una tercera aproximació a la sistemàtica del complex *Arctium-Cousinia* ha estat morfològica. Durant una estada d'un mes al *Komarov Botanical Institute* s'han pres mesures biomètriques i fotografies de totes les espècies del grup Arctioide dipositades a l'herbari LE que és on hi ha la col·lecció més gran d'aquest grup. També s'ha estudiat la morfologia a partir de plecs d'altres herbaris i recol·leccions pròpies. Així, en total, s'han estudiat més de 300 plecs. Aquestes dades ens han permès conèixer més a fons les espècies d'aquest llinatge tan controvertit per tal d'establir més acuradament la seva delimitació taxonòmica de nivell genèric i subgenèric. Hem complementat aquesta informació amb la seqüenciació de la regió cloroplàstica *rpl32-trnL* que juntament amb els marcadors ITS i *rpS4-trnT-trnL* ens han servit per elaborar una filogènia més acurada del grup Arctioide i proposar una nova classificació taxonòmica. L'article resultant d'aquest estudi s'ha titulat "The *Arctium-Cousinia* complex: disentangling *Arctium* and *Cousinia* (Cardueae, Carduinae)" i es pretén enviar a la revista científica SCI *Taxon*.

Nivell microevolutiu

No existeix cap estudi previ de genètica poblacional per a cap de les espècies d'aquest complex, de manera que es desconeix per complet quina és la seva dinàmica i la seva estructura genètica. En aquest context, els microsatèl·lits són una eina molt eficaç per estudiar diferents aspectes poblacionals i és per això que s'han usat a la present tesi. Es tracta de repeticions de seqüències de dos a sis nucleòtids del DNA genòmic, generalment localitzats a zones no codificants, que es transmeten a la descendència seguint una herència codominant. Presenten una elevada taxa de mutació, cosa que els fa molt polimòrfics inter i intrapoblacionalment, permetent així una caracterització molt fina de les poblacions estudiades i les seves dinàmiques.

S'ha triat *Arctium minus* com a espècie potencialment més interessant per tenir una distribució subcosmopolita; la més àmplia de tot el complex *Arctium-Cousinia*. El material processat correspon a 14 poblacions procedents d'Argentina, Canadà, Croàcia, Eslovàquia, Espanya (2 poblacions), Estats Units d'Amèrica, França, Itàlia (2 poblacions), Marroc, Noruega, Polònia i Turquia. La primera part d'aquest estudi s'ha dut a terme durant una estada de tres mesos al "Laboratory of Molecular Systematics and Evolutionary Genetics" del "Florida Museum of Natural History" on s'han capturat i optimitzat 18 microsatèl·lits. El nostre objectiu ha estat detectar la potencialitat i utilitat de cadascun d'aquests microsatèl·lits per un posterior estudi centrat en la dinàmica de les poblacions d'*Arctium minus*. Així, s'ha fet una caracterització d'aquests microsatèl·lits i d'algunes de les poblacions esmentades per a determinar diferents paràmetres com la presència d'al·lels nuls, si es troben en equilibri Hardy-Weinberg, si hi ha desequilibri en el lligament, quin nivell de variabilitat presenten, etc. Aquest estudi ha generat un article titulat "Isolation and characterization of novel microsatellite markers for *Arctium minus* (Hill) Bernh. (Compositae)" acceptat a la revista científica SCI *American Journal of Botany (Notes).*

Partint dels resultats preliminars que s'acaben de descriure, dels 18 microsatèl·lits analitzats s'han escollit els 8 millors i s'han usat per a genotipar 30 individus de cadascuna de les 14 poblacions esmentades (excepte per les de Canadà, Estats Units d'Amèrica, Marroc i Noruega per a les que només es disposava de 16, 14, 17 i 8 individus respectivament). Amb aquest estudi s'ha pretès aprofundir més en la genètica poblacional d'*Arctium minus* i proporcionar eines per a investigar la colonització del continent Americà per aquesta espècie així com esbrinar si la seva distribució s'ha modificat com a adaptació als canvis climàtics de les darreres glaciacions, patint esdeveniments de colls d'ampolla i efectes fundadors. Aquest treball s'ha titulat "Genetic structure and diversity in *Arctium minus* (Compositae): effects of historical climate change and life history" i es pretén enviar a la revista científica SCI *Molecular Ecology*.

2. Objectius generals

Nivell macroevolutiu

- Determinar la utilitat de les regions ITS, *rpS4*, *trnT*, *trnL* i *rpl32* per a resoldre problemes sistemàtics del complex *Arctium-Cousinia*.

- Comprovar la monofília i estudiar la circumscripció dels gèneres inclosos a l'esmentat complex i estimar-ne les relacions filogenètiques subgenèriques i seccionals, mitjançant l'aportació de dades moleculars i cariològiques.

- Comparar les dades filogenètiques moleculars obtingudes amb caràcters morfològics i cariològics per tal de cercar patrons evolutius al complex *Arctium-Cousinia*.

- Estimar l'edat del complex *Arctium-Cousinia* i el temps de divergència dels seus diferents llinatges mitjançant el rellotge molecular.

- Comprovar la monofília del llinatge Arctioide i estimar-ne les relacions filogenètiques de rang genèric, subgenèric i seccional així com aclarir-ne la taxonomia basant-nos en dades morfològiques i moleculars.

Nivell microevolutiu

- Cercar i capturar microsatèl·lits polimòrfics específics per *Arctium minus*.
- Analitzar l'estructura genètica de les poblacions d'*Arctium minus* en una mostra representativa de la seva àrea de distribució, la més àmplia del complex *Arctium-Cousinia*.
- Detectar possibles efectes de les glaciacions en l'estructura poblacional, la variabilitat genètica i la distribució d'*Arctium minus*.
- Estudiar els processos de colonització del continent americà per part d'*Arctium minus*.

Informe dels directors de la Tesi Doctoral referent al factor d'impacte i a la contribució de la doctoranda a cadascun dels articles publicats

Núria Garcia Jacas i Alfonso Susanna de la Serna, investigadors de l'Instritut Botànic de Barcelona, directors de la Tesi Doctoral elaborada per Sara López Viñallonga, amb el títol "Evolució, filogènia i sistemàtica del complex *Arctium-Cousinia*",

INFORMEN

Que els treballs de recerca duts a terme per Sara López Viñallonga com a part de la seva formació predoctoral i inclosos a la seva Tesi Doctoral han donat lloc a 1 publicació i 4 manuscrits (2 enviats a revisió i 2 més pendents d'enviar en el moment del dipòsit de la tesi). A continuació es detalla la llista d'articles així com els índexs d'impacte (segons el SCI de la ISI web of Knowledge) de les corresponents revistes.

1- López-Vinyallonga S., I. Mehregan, N. Garcia-Jacas, O. Tscherneva, A. Susanna & J. W. Kadereit (2009). Phylogeny and evolution of the *Arctium–Cousinia* complex (Compositae, Cardueae–Carduinae). *Taxon* 58: 153–171.
L'índex d'impacte de la revista *Taxon* és en l'actualitat 2.524. Aquesta revista està inclosa a la categoria "Plant Sciences". Tenint en compte l'índex d'impacte, *Taxon* ocupa el 25è lloc de la seva categoria, que inclou 152 revistes. La responsabilitat de la doctoranda López Vinyallonga en aquest treball ha estat la seqüenciació de tres regions cloroplàstiques de les 150 espècies incloses a l'estudi, la correalització de les anàlisis filogenètiques i la corredacció final. Aquest treball ha estat també part de la Tesi Doctoral d'Iraj Mehregan, centrada a un dels dos gèneres del complex, *Cousinia*; la Tesi Doctoral de la nostra doctoranda ha estat centrada en el gènere *Arctium*.

2- López-Vinyallonga, S., A. Susanna & N. Garcia-Jacas (submitted). Chromosome counts in the genera *Cousinia*, *Olgaea* and *Syreitschikovia* (Compositae). *Folia Geobotanica*.

L'índex d'impacte de la revista *Folia Geobotanica* és en l'actualitat 1.133. Aquesta revista està inclosa a la categoria "Plant Sciences". Tenint en compte l'índex d'impacte, *Folia Geobotanica* ocupa el lloc 51è de la seva categoria, que inclou 152 revistes.

3- López-Vinyallonga, S., A. Susanna & N. Garcia-Jacas (in prep.). The *Arctium-Cousinia* complex: disentangling *Arctium* and *Cousinia* (Cardueae, Carduinae). *Taxon*.

Es preveu enviar aquest article a la revista *Taxon* amb índex d'impacte de 2.524 en l'actualitat. Tenint en compte l'índex d'impacte, *Taxon* ocupa el 25è lloc de la seva categoria, que inclou 152 revistes.

4. - López-Vinyallonga S., Arakaki M., Garcia-Jacas N., Susanna A., Gitzendanner M.A., Soltis D.E. and Soltis P.S. (submitted). Isolation and characterization of novel microsatellite markers for *Arctium minus* L. (Compositae). *American Journal of Botany (Notes)*.

L'índex d'impacte de la revista *American Journal of Botany* és en l'actualitat 2.512. Tenint en compte l'índex d'impacte, ocupa el 26è lloc de la seva categoria, que inclou

152 revistes. Aquesta nota serà una de les notes inaugurals d'una nova sèrie dedicada als resultats d'aïllament de microsatèl·lits a aquesta revista.

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5. López-Vinyallonga, S., P. S. Soltis, A. Susanna & N. Garcia-Jacas (in prep.). Genetic structure and diversity in *Arctium minus* (Compositae): effects of historical climate change and life history (in prep.). *Molecular Ecology*.

Es preveu enviar aquest article a la revista *Molecular Ecology* amb índex d'impacte de 5.169 en l'actualitat. Tenint en compte l'índex d'impacte, aquesta revista ocupa el 6è lloc de la seva categoria, que compren 116 revistes.

A més, CERTIFIQUEN:

Que Sara López Viñallonga ha participat activament en el desenvolupament del treball de recerca associat a cadascun dels articles, així com en la seva elaboració. En concret, la seva participació a cadascuna de les tasques ha estat la següent:

- Plantejament inicial dels objectius de cadascun dels treballs.
- Selecció dels materials d'herbari destinats a la seqüenciació (amb una estada a l'Institut Komarov de Sant Petersburg, Rússia).
- Realització de les seqüències de DNA i les anàlisis filogenètiques.
- Desenvolupament i posada a punt dels microsatèl·lits (amb una estada breu al Soltis Laboratory, Florida Museum of Natural History, Gainesville, Florida, USA).
- Càlcul de resultats i anàlisi de dades.
- Redacció dels articles i seguiment del procés de revisió dels mateixos.

Atentament,

Barcelona, 18 de Juny de 2009

Núria Garcia Jacas

Alfonso Susanna

4. Resum global i discussió dels resultats obtinguts

4.1. Nivell macroevolutiu

4.1.1. Filogènia

4.1.1.1. El complex *Arctium-Cousinia*

El present estudi ha confirmat la monofília del complex *Arctium-Cousinia* alhora que ha mostrat que aquest complex es divideix en dos clades, grups germana un de l'altre, que ja havien estat establerts anteriorment sobre criteris morfològics, cariològics i pol·línics. D'una banda el grup Arctioide format pels gèneres *Arctium*, *Hypacanthium* i *Schmalhausenia* i *Cousinia* subgèneres *Hypacanthodes* i *Cynaroides* i d'altra banda el grup Cousinioide constituït per *Cousinia* subgènere *Cousinia*, molt més ampli pel que fa a nombre d'espècies. Cal destacar que hi ha un tercer clade format únicament per *Cousinia tenella* Fisch. & C. A. Mey., la interpretació del qual s'exposa més endavant.

S'ha detectat que el nivell de resolució filogenètica dins del clade Arctioide és major que al Cousinioide. Donat que són grups germans i que l'edat aproximada dels dos llinatges és similar (veure més avall), aquest fenomen seria explicable per la gran diferència en el nombre d'espècies de cadascun dels clades, idea recolzada pel treball de Susanna et al. (2003) en que incloent un nombre similar d'espècies dels dos llinatges s'obté una resolució filogenètica similar a ambdós.

També s'ha constatat que els caràcters apomòrfics i plesiomòrfics no estan distribuïts de manera uniforme als dos llinatges. El clade Arctioide presenta, com *Jurinea*, *Saussurea* (Punt & Hoen, 2009) i alguns grups basals de la subtribu Centaureineae (Garcia-Jacas et al., 2001; Vilatersana et al., 2001), pol·len espinós, estigma llarg i un nombre cromosòmic alt. En canvi, el clade Cousinioide presenta pol·len llis, estigma curt i uns nombres cromosòmics més baixos, com alguns grups derivats de la subtribu (Garcia-Jacas et al., 2001). Així, doncs, interpretem els caràcters arctioides com a plesiomòrfics i els cousinioides com a apomòrfics i pensem que al complex *Arctium-Cousinia* es pot haver donat la tendència evolutiva d'un tipus de pol·len espinós a llis i una reducció del nombre cromosòmic, tendències reconegudes a les Centaureineae.

Pel que fa als caràcters morfològics tradicionalment més usats per a subdividir aquest gran complex d'espècies, principalment la morfologia de les bràctees involucrals i l'armadura de les fulles, amb aquest treball ha quedat palès que hi han evolucionat múltiples vegades en paral·lel.

Les anàlisis de datació molecular, basades en les taxes de substitució de la regió ITS i en la troballa d'un aqueni fòssil d'*Arctium*, permeten concloure que la separació dels dos llinatges principals del complex es va donar fa 14,8 milions d'anys. També s'ha constatat que la diversificació del clade Arctioide es va produir fa 9,7 milions d'anys i la del clade Cousinioide, lleugerament més recent, 8,7 milions d'anys enrere.

A continuació es comenten les característiques principals dels tres clades obtinguts en les anàlisis dutes a terme.

A. El clade de *Cousinia tenella*

La posició de *C. tenella* en la filogènia molecular presentada aquí suggereix la segregació d'aquesta espècie de *Cousinia* subgènere *Cousinia* però les seves característiques cariològiques ($2n = 26$), pol·líniques (pol·len tipus *Cousinia*) i morfològiques (zona apical i branques de l'estil coberts de pèls llargs) la vinculen clarament al clade Cousinioide. La posició d'aquesta espècie com a grup germana de la resta del complex *Arctium*-*Cousinia* és probablement un artefacte derivat de la seva condició d'anual i autògama. Diferents autors (Bousquet & al., 1992; Laroche & al., 1997; Andreasen & Baldwin, 2001; Soria-Hernanz & al., 2008) han constatat que les plantes anuals acostumen a tenir una taxa d'evolució molecular més elevada que les espècies perennes, fet que s'explica per diferències en el temps de generació, la mida poblacional i la taxa d'especiació. Aquest fenomen de posició incoherent d'espècies anuals s'ha descrit a altres gèneres de la tribu *Cardueae* com *Echinops* L. i *Acantholepis* Less. (Garnatje et al., 2005), *Ptilostemon* Cass. (Vilatersana et al., sotmès) o *Schischkinia* Iljin i *Stizolophus* Cass. (Susanna et al., 2006).

En contrast, la resta d'espècies anuals incloses a aquest estudi s'agrupen en un mateix clade que pertany clarament al llinatge Cousinioide, de manera coherent amb les evidències morfològiques.

B. El clade Cousinioide

La informació que s'aporta sobre el llinatge Cousinioide està condicionada per la pobre resolució filogenètica que resulta de les anàlisis. Aquest fet s'ha constatat comprovant que part dels clades amb un recolzament estadístic superior al 95% no coincideixen amb la taxonomia tradicionalment acceptada. S'ha detectat que les dades analitzades contenen informació filogenètica principalment a les branques distals i que la distribució geogràfica de les espècies sembla un criteri més fiable per a reconèixer relacions filogenètiques entre elles que la taxonomia tradicional, tal i com s'ha evidenciat a altres estudis (González Albaladejo et al., 2005).

Es postula que aquesta pobre resolució filogenètica dins de *Cousinia* s.str. està causada per la suma de múltiples factors. D'una banda, a les anàlisis dutes a terme s'ha obtingut un nombre de caràcters informatius relativament baix en relació al nombre d'espècies del clade Cousinioide, el qual podria ser suficient per a establir-ne la filogènia si no fos perquè els caràcters mostren una homoplàsia considerable. L'explicació més plausible per aquesta limitació és la curta edat del clade en combinació amb el seu elevat nombre d'espècies, ja que hem datat el grup corona d'aquest llinatge en 8,7 milions d'anys i la seva gran diversificació va començar fa només 7 milions d'anys.

Un altre factor a tenir en compte és que moltes espècies de *Cousinia* s. s estan estretament emparentades, són simpàtriques i no se n'ha descrit cap mecanisme d'aïllament reproductiu. Per tant s'han pogut donar processos d'hibridació, que poden haver derivat en especiació, dificultant la reconstrucció filogenètica d'aquest llinatge (Nieto Feliner et al., 2001; Font et al., 2002; Vriesendorp & Bakker, 2005, Suárez-Santiago et al., 2007).

Al clade Cousinioide també es troba un elevat nombre d'espècies alopàtriques i parapàtriques restringides a àrees geogràfiques molt reduïdes i separades entre sí per barreres topogràfiques. Aquest fet implica que el procés d'especiació predominant en aquest llinatge ha degut ser l'especiació geogràfica. Complementàriament, cal dir que la comparació de diferents estudis de vegetació (Zohary, 1973; Frenzel et al., 1992) suggereix que la distribució geogràfica de *Cousinia* s.str. al darrer màxim glacial estava restringida a la zona nord de la seva àrea actual, cosa que implica que ha hagut de patir canvis substancials durant el Quaternari. Ara bé, tenint en compte que hem datat el grup corona d'aquest llinatge en 8,7 milions d'anys, les oscil·lacions del Quaternari no semblen haver-hi provocat extenses extincions. El que no podem aventurar és si aquestes oscil·lacions climàtiques van representar un estímul per a l'especiació del grup, a través de canvis en la seva distribució geogràfica, com ha passat per exemple a *Primula* L. sec. *Auricula* i *Epimedium* L. (Kadereit et al., 2004; Zhang et al., 2007).

Finalment, cal dir que l'esmentada pobresa de resolució filogenètica ha impedit l'establiment d'una subdivisió plausible d'aquest clade. Malgrat això, s'han detectat algunes agrupacions d'espècies que representen llinatges monofilètics i tenen sentit taxonòmic i/o geogràfic. Un exemple il·lustratiu és l'evolució de l'anualitat, característica extremadament poc freqüent a *Cousinia* s.str., però que segons la nostra filogènia s'hi ha originat dues vegades. Un llinatge d'anuals comprèn les espècies *C. bungeana* Regel & Schmalh., *C. polytimetica* Tscherneva, *C. pusilla* C. Winkl. i *C. prolifera* Jaub. & Spach que a gairebé totes les anàlisis que hem dut a terme s'agrupen amb un suport estadístic notablement alt i pertanyen sense cap dubte al clade Cousinioide. L'altre llinatge està format per *C. tenella* i *C. pygmaea* C. Winkl. i presenta alguns problemes d'adscripció filogenètica donat que apareix com a grup germana de la resta del complex *Arctium*-*Cousinia*, com s'ha comentat anteriorment.

C. El clade Arctioide

Tot i que la filogènia presentada a aquesta tesi recolza algunes categories subgenèriques del llinatge Arctioide, s'han detectat notables incongruències respecte la classificació reconeguda tradicionalment. Tots els arbres filogenètics resultants de les anàlisis dutes a terme confirmen la monofília del clade Arctioide però alhora constaten que els subgèneres *Cynaroides* i *Hypacanthodes* pertanyents a *Cousinia*, en la seva circumscripció actual, no són monofilètics. També es posa de manifest, dins del gènere

Cousinia, la manca de monofília de les seccions *Abolinia* Tscherneva i *Lacerae* C. Winkl.

A la llum d'aquests resultats es proposa una redefinició del gènere *Arctium* al qual s'han transferit totes les espècies del llinatge Arctioide pertanyents als gèneres *Anura*, *Cousinia*, *Hypacanthium* i *Schmalhausenia*. S'aporten els canvis nomenclaturals pertinents derivats d'aquesta redefinició taxonòmica, alguns d'ells prèviament establerts per autors com Kuntze (1891) i Duistermaat (1996).

La nova classificació del gènere *Arctium* que es presenta a aquesta tesi s'estableix sobre la base de la filogènia molecular obtinguda i amb el recolzament d'evidències morfològiques sempre que ha estat possible. Així, el gènere *Arctium* s'ha subdividit en dos subgèneres anomenats *Arctium* S. López, Susanna & N. Garcia, *comb. nov.* i *Cynaroides* (Tscherneva) S. López, Susanna & N. Garcia, *comb. nov.* Alhora s'ha redefinit el subgènere *Cynaroides* i s'han descartat els taxons no monofilètics, és a dir, el subgènere *Hypacanthodes* i les seccions *Abolinia* i *Lacerae*. També s'han eliminat les seccions monotípiques *Ctenarctium* Juz., *Nanarctium* Juz. ex Tscherneva i *Oligantha* Juz. ja que queden incloses dins altres seccions amb gran suport i finalment el gènere monotípic *Anura* ja que no té entitat taxonòmica ni morfològica.

La resta de seccions tradicionalment incloses a *Arctium* i *Cousinia* es transfereixen íntegrament al nou gènere *Arctium*: sect. *Amberbopsis* (Tscherneva) S. López, Susanna & N. Garcia, *comb. nov.*, *Lappaceum* (Bunge) Duist., *Pseudarctium* (Juz.) Duist. i *Serratulopsis* (Tscherneva) S. López, Susanna & N. Garcia, *comb. nov.* Només se suggereixen lleugers reajustaments a la sect. *Chrysis* (Juz.) S. López, Susanna & N. Garcia, *comb. nov.*, que incorpora la única espècie de la sect. *Ctenarctium* que ha estat eliminada i a la sect. *Pectinatae* (C. Winkl.) S. López, Susanna & N. Garcia, *comb. nov.*, que incorpora la única espècie de la sect. *Oligantha* també descartada. Alhora es proposen les noves seccions *Anura* S. López, Susanna & N. Garcia, *comb. et stat. nov.*, *Hypacanthodes* S. López, Susanna & N. Garcia, *comb. et stat. nov.* i *Schmalhausenia* S. López, Susanna & N. Garcia, *comb. et stat. nov.*

Tant la nova classificació proposada pel gènere *Arctium* com els canvis nomenclaturals associats es resumeixen a la taula següent:

Espècie	Secció	Subgènere
Arctium arctioides (Schrenk.) Kuntze	*Arctium* L.	*Arctium*
Arctium atlanticum (Pomel) H. Lindb.		
Arctium nemorosum Lej.		
Arctium minus (Hill) Bernh.		
Arctium lappa L.		
Arctium palladini (Marcow.) R.E.Fr. & E.S. Söderb.		
Arctium tomentosum Mill.		
Arctium leiospermum Juz. & Ye. V. Serg.		
Arctium eriophorum (Regel & Schmalh.) Kuntze	*Schmalhausenia* S. López, Susanna & N. Garcia	
Arctium evidens (Tscherneva) S. López, Susanna & N. Garcia		
Arctium echinopifolium (Juz.) S. López, Susanna & N. Garcia		
Arctium grandifolium (Kult.) S. López, Susanna & N. Garcia	*Amberbopsis* (Tscherneva) S. López, Susanna & N. Garcia	
Arctium vavilovii (Kult.) S. López, Susanna & N. Garcia	*Serratulopsis* (Tscherneva) S. López, Susanna & N. Garcia	
Arctium macilentum (C. Winkl.) S. López, Susanna & N. Garcia	*Hypacanthodes* (Tscherneva) S. López, Susanna & N. Garcia	*Cynaroides* (Tscherneva) S. López, Susanna & N. Garcia
Arctium abolinii (Kult. ex Tscherneva) S. López, Susanna & N. Garcia		
Arctium dolichophyllum (Kult.) S. López, Susanna & N. Garcia		
Arctium egregium (Juz.) S. López, Susanna & N. Garcia		
Arctium fedtschenkoanum (Bornm.) S. López, Susanna & N. Garcia		
Arctium korshinskyi (C. Winkl.) S. López, Susanna & N. Garcia		
Arctium pterolepidum (Kult.) S. López, Susanna & N. Garcia		
Arctium ugamicum (Karmysch.) S. López, Susanna & N. Garcia		

Arctium lappaceum (Schrenk.) Kuntze	*Lappaceum* (Bunge) Duist.	
Arctium pallidivirens (Kult.) S. López, Susanna & N. Garcia	*Anura* (Juz) S. López, Susanna & N. Garcia	
Arctium umbrosum (Bunge) Kuntze	*Pseudarctium* (Juz.) Duist.	
Arctium amplissimum (Boiss.) Kuntze		
Arctium pseudarctium (Bornm.) Duist.		
Arctium tomentellum (C. Winkl.) Kuntze		
Arctium triflorum (Schrenk.) Kuntze	*Pectinatae* (C. Winkl.) S. López, Susanna & N. Garcia	
Arctium albertii (Regel & Schmalh.) S. López, Susanna & N. Garcia		
Arctium horrescens (Juz.) S. López, Susanna & N. Garcia		
Arctium pentacanthum (Regel & Schmalh.) Kuntze		
Arctium pentacanthoides (Juz. ex Tscherneva) S. López, Susanna & N. Garcia		
Arctium karatavicum (Regel & Schmalh.) Kuntze	*Chrysis* (Juz.) S. López, Susanna & N. Garcia	
Arctium korolkowii (Regel & Schmalh.) Kuntze		
Arctium chloranthum (Kult.) S. López, Susanna & N. Garcia		
Arctium refractum (Bornm.) S. López, Susanna & N. Garcia		
Arctium schmalhausenii (C. Winkl.) Kuntze		
Arctium aureum (C. Winkl.) Kuntze		
Arctium anomalum (Franch.) Kuntze		
Arctium medians (Juz.) S. López, Susanna & N. Garcia		
Arctium haesitabundum (Juz.) S. López, Susanna & N. Garcia		

Conscients de la manca de caràcters morfològics prou adients per a recolzar la classificació subgenèrica i seccional del gènere *Arctium* presentada a aquesta tesi, es posa de manifest la necessitat d’un treball més exhaustiu.

4.1.2. Cariologia

Nota: a aquest apartat es manté la terminologia prèvia a la redefinició del gènere *Arctium* i el conseqüent canvi nomenclatural proposat per al llinatge Arctioide perquè és com apareix al manuscrit enviat a la revista *Folia Geobotanica*.

En quant a la cariologia, aquesta tesi confirma que les espècies del grup Arctioide tenen un nombre cromosòmic bàsic de $x = 18$ mentre el grup Cousinioide presenta la següent sèrie disploide: $x = 9$, 10, 11, 12 i 13. La disploïdia de *Cousinia* s.str. també s'ha posat de manifest a escala seccional a les seccions *Alpinae* Bunge, *Cousinia*, *Eriocousinia* Tscherneva i *Microcarpae* Bunge.

Tot i que no s'ha pogut establir el sentit de la disploïdia del subgènere *Cousinia* sospitem que serà descendent donat que existeixen altres exemples a la mateixa tribu Cardueae en que es dóna aquest cas (Garcia-Jacas et al., 2001). També hi ha altres casos que recolzen la hipòtesi de la disploïdia descendent com els treballs de Frankton i Moore (1961), Fernández Casas & Fernández Morales (1979), Siljak-Yakolev (1986) o Garcia-Jacas & Susanna (1992) on s'exposa la idea que els nombres cromosòmics bàsics alts s'han de considerar més primitius que no pas els baixos, cosa que ha estat postulada com una tendència general per Stebbins (1950, 1971) i Grant (1981). Un altre argument a favor de la disploïdia descendent és de caire ecològic donat que s'ha especulat que aquesta estaria relacionada amb l'adaptació a ambients extrems (Selvi & Bigazzi, 2002; Watanabe et al., 1999) i les espècies del grup Cousinioide es troben principalment a zones àrides.

Paral·lelament s'ha posat de manifest que els sis nombres cromosòmics bàsics presents al complex *Arctium-Cousinia* no tenen igual ocurrència sinó que $x = 12$, 13 i 18 són els més comuns amb abundàncies relatives del 38.9%, 40.9% i 16.1% respectivament mentre $x = 9$, 10 i 11 només es troben en un 4.7%, 1.3% i 4.0% respectivament.

En superposar els nombres cromosòmics de les espècies incloses al nostre arbre filogenètic Bayesià per les que hi ha recomptes publicats (incloent els nostres propis), s'ha detectat una baixa correspondència entre filogènia i cariologia. Només les espècies del grup Arctioide, amb $2n = 36$, s'agrupen al mateix clade. En canvi, les espècies del grup Cousinioide, que ja no apareixen agrupades en funció de la classificació seccional tradicional, tampoc s'agrupen en funció dels seus nombres cromosòmics.

Pel que fa a la poliploïdia, no n'hem trobat cap cas a tot el complex, cosa infreqüent a la tribu Cardueae donat que és un grup amb moltes espècies poliploides pioneres i colonitzadores. Malgrat això, cal comentar que el nombre cromosòmic bàsic x = 18 de les espècies del clade Arctioide és un dels més alts de la tribu Cardueae, cosa que ens ha portat a hipotetitzar que aquest clade possiblement s'hauria originat per antics esdeveniments de poliploïdia a partir d'un ancestre x = 9, tot i que actualment aquestes espècies estan completament diploiditzades.

Cal afegir que només s'han descrit 26 híbrids i 11 formes intermèdies en aquest gran complex format per més de 500 espècies tot i que és possible que la hibridació sigui més freqüent del que aquesta dada indica però no s'hagi detectat per la manca d'un coneixement taxonòmic prou aprofundit i per l'existència d'espècies molt similars morfològicament. Ara bé, l'absència de nombres cromosòmics poliploides ja esmentada implica que l'especiació per hibridació poliploide probablement no hagi jugat cap paper en l'evolució del complex *Arctium-Cousinia*. En canvi, no hi ha dades que ens permetin deduir el significat evolutiu que hagin pogut tenir els híbrids homoploides, malgrat que pensem que deuen existir, principalment al subgènere *Cousinia*, donada l'existència d'un gran nombre d'espècies estretament relacionades i sovint simpàtriques sense mecanismes evidents d'aïllament reproductiu. Cal tenir en compte, però, que els híbrids homoploides són difícils de detectar donada la manca de caràcters diagnòstics clars com podria ser un canvi en el nombre cromosòmic.

Hem buscat a les Carduinae un cas d'evolució assimilable al complex *Arctium-Cousinia* i hem trobat un clar paral·lelisme amb el grup *Onopordum*. El gènere *Onopordum* L., biennal i colonitzador amb nombre cromosòmic bàsic de x = 17 és comparable a *Arctium* pel que fa al cicle vital i al nombre cromosòmic. D'altra banda, les espècies dels gèneres perennes d'Àsia Central *Alfredia* Cass., *Ancathia* DC, *Olgaea* Iljin, *Synurus* Iljin i *Syreitschikovia* Pavlov tenen un nombre cromosòmic bàsic de x = 13 i x = 12 i són comparables al gènere *Cousinia* s.str.

4.2. Nivell microevolutiu

Pel que fa a la genètica poblacional, els nou microsatèl·lits polimòrfics dissenyats per *Arctium minus* han mostrat poca variabilitat. Aquesta característica es posa de manifest a partir de diferents paràmetres com el reduït nombre d'al·lels per locus (de dos a 10), valors baixos d'heterozigositat esperada (entre 0.015 i 0.487) i observada (entre 0.016 i 0.694), i un valor relativament alt de consanguinitat (0.316). Paral·lelament, sis loci han mostrat una desviació estadísticament significativa de l'equilibri Hardy-Weinberg i nou de 36 comparacions entre parelles de loci han mostrat un significatiu desequilibri en el lligament. S'ha detectat la presència d'al·lels nuls, amb freqüències baixes o moderades, als loci Am31 i Am34 per cinc i tres poblacions respectivament. Els resultats obtinguts s'expliquen per un dèficit d'heterozigots, que pot ser degut a la consanguinitat detectada o un artefacte associat a la presència d'al·lels nuls, i pel fet que *A. minus* és autògam facultatiu.

A partir d'aquesta caracterització dels nou loci microsatèl·lits esmentats a l'anterior paràgraf i dos més que es van acabar d'optimitzar, s'han escollit els vuit millors i s'han usat per a l'estudi de l'estrucutra de la variabilitat genètica d'*A. minus*. Els patrons de variació genètica detectats són consistents amb l'estil de vida d'*A. minus*.

Pel que fa a la component intrapoblacional, diversos paràmetres ens han mostrat una baixa diversitat genètica per *A. minus* i s'ha vist que les poblacions més polimòrfiques són les procedents d'Eslovàquia (SZI) i nord-oest d'Espanya (ELE) i les menys polimòrfiques han resultat provenir de Turquia (TMA), Marroc (MTA) i nord d'Itàlia (ITR). Paral·lelament s'han obtingut alts valors d'endogàmia i un excés d'homozigots estadísticament significatiu. Tots aquests resultats són explicables per la capacitat facultativa d'autofecundació d'*A. minus* i la curta durada del seu cicle de vida.

En quant a la component interpoblacional, s'ha obtingut que la major part de la variabilitat genètica de l'espècie es troba entre poblacions i no dins de les poblacions degut a un baix flux gènic entre elles juntament amb la capacitat d'autofecundació de l'espècie. Tot i que *A. minus* té un mecanisme de dispersió de llavors força eficient, la seva distribució disjunta i la dinàmica d'aparició/desaparició d'algunes de les seves poblacions dificulten l’intercanvi genètic entre elles. Paral·lelament, esdeveniments esporàdics de dispersió de llavors a llarga distància semblen l’explicació més plausible per l’absència d’aïllament per distància, detectada tant dins del rang natural d’*A. minus* com amb les poblacions del límit de la seva àrea de distribució.

No s’ha detectat el patró esperat per una espècie que ha patit els canvis climàtics derivats de l’alternança de períodes glacials i interglacials esdevinguts a Europa durant el Pleistocè. Hi ha evidències de que moltes espècies europees van patir colls d’ampolla durant les èpoques glacials, quan es van refugiar al sud del continent (Palmé & Vendramin, 2002; Rendell & Ennos, 2002; Grivet & Petit, 2003; Sharbel et al., 2006). S’han descrit refugis a la Península Ibèrica, la Península Itàlica i als Monts Carpats, principalment (Hewitt, 1996; Petit et al., 2003). A les èpoques interglacials, les espècies haurien recolonitzat Europa des d’aquests refugis donant lloc a un patró de menor diversitat a mesura que ens allunyem dels refugis. A *A. minus* no hem trobat indicis d’aquest patró ja que no hi ha un gradient de major diversitat al sud a menor diversitat al nord ni més diferències entre les suposades àrees de refugi que entre les poblacions del nord, com tampoc entre refugis i les poblacions del nord del continent. Malgrat això, indicis com la presència d’al·lels privats a les poblacions del sud d’Itàlia i Marroc ens fan pensar que *A. minus* sí que va patir les oscil·lacions climàtiques d’aquest període, refugiant-se al sud i recolonitzant la resta del continent amb la retirada dels gels, però el seu reflex a l’estructura genètica de l’espècie ha quedat emmascarat per esdeveniments de dispersió a llarga distància i la influència humana, principalment.

La manca de diferenciació entre les poblacions europees i les americanes confirmen l’acció humana recent com un dels mecanismes que influeix més decisivament en l’estructura genètica i biogeogràfica d’*A. minus*, tal com ha estat descrit per a d’altres espècies pioneres com *Arabidopsis thaliana* (L.) Heynh. (Sharbel et al., 2006).

5. Conclusions finals

1. Es confirma la monofília del complex *Arctium-Cousinia* així com la seva subdivisió en dos clades també monofilètics anomenats Arctioide i Cousinioide.

2. En la seva circumscripció actual, s'ha posat de manifest la parafília dels gèneres *Arctium* i *Cousinia* i de *Cousinia* subgèneres *Cynaroides* i *Hypacanthodes* en que s'ha dividit tradicionalment el llinatge Arctioide.

3. Les regions ITS, *rpS4, trnT, trnL* i *rpl32* proporcionen informació valuosa per a resoldre problemes sistemàtics del complex *Arctium-Cousinia* tot i que al clade Cousinioide aquesta informació no és suficient per a establir una filogènia sòlida del subgènere *Cousinia*.

4. El nivell de resolució filogenètica dins del clade Cousinioide és menor que a l'Arctioide degut a un alt nivell d'homoplàsia, resultant de la curta edat del clade en combinació amb el seu elevat nombre d'espècies, juntament amb la probable existència d'híbrids homoploides no detectats.

5. S'interpreta que els caràcters arctioides serien plesiomòrfics i els cousinioides apomòrfics, detectant una possible tendència evolutiva d'un tipus de pol·len espinós a llis i una reducció del nombre cromosòmic. En canvi, la morfologia de les bràctees involucrals i l'armadura de les fulles, caràcters tradicionalment usats per a subdividir el complex, així com la condició d'anualitat hi han evolucionat múltiples vegades en paral·lel.

6. La separació dels llinatges Arctioide i Cousinioide es va donar fa 14,8 milions d'anys i la seva diversificació s'ha datat en 9,7 i 8,7 milions d'anys, respectivament.

7. S'ha detectat que dins del llinatge Cousinioide les dades analitzades contenen informació filogenètica principalment a les branques distals i que la distribució

geogràfica de les espècies es mostra com un criteri més fiable per a reconèixer relacions filogenètiques entre elles que la taxonomia tradicional.

8. Es postula que el procés d'especiació predominant al clade Cousinioide ha degut ser l'especiació geogràfica donat el seu elevat nombre d'espècies al·lopàtriques i parapàtriques restringides a àrees geogràfiques molt reduïdes i separades entre sí per barreres topogràfiques.

9. Es proposa una redefinició del gènere *Arctium* al qual s'han transferit totes les espècies del llinatge Arctioide pertanyents als gèneres *Anura*, *Cousinia*, *Hypacantium* i *Schmalhausenia* amb la proposta dels canvis nomenclaturals pertinents.

10. El gènere *Arctium* s'ha subdividit en dos nous subgèneres anomenats *Arctium* i *Cynaroides*, s'ha eliminat el subgènere *Hypacanthodes* i s'ha redefinit el subgènere *Cynaroides*, que pertanyien a *Cousinia*, perquè no eren monofilètics.

11. A grans trets, la classificació seccional tradicional d'*Arctium* i *Cousinia* es transfereix al nou gènere *Arctium*. Desapareixen les seccions *Abolinia, Ctenarctium, Lacerae, Nanarctium* i *Oligantha* de *Cousinia* i s'incorporen les seccions *Anura*, *Hypacanthodes* i *Schmalhausenia*.

12. Conscients de la manca de caràcters morfològics prou adients per a recolzar la classificació subgenèrica i seccional del gènere *Arctium* presentada a aquesta tesi, es posa de manifest la necessitat d'un treball més exhaustiu.

13. Els recomptes cromosòmics realitzats confirmen que les espècies del grup Arctioide tenen un nombre cromosòmic bàsic x = 18 mentre el grup Cousinioide presenta la següent sèrie disploide, que s'hipotetitza que és descendent: x = 9, 10, 11, 12 i 13, mostrant disploïdia també a escala seccional.

14. S'ha detectat una baixa correspondència entre filogènia i cariologia; només les espècies del grup Arctioide, amb $2n$ = 36, s'agrupen al mateix clade.

15. La inexistència de poliploïdia al complex *Arctium-Cousinia* implica que l'especiació per hibridació poliploide no deu haver estat significativa en la seva evolució. Malgrat això, el nombre cromosòmic bàsic $x = 18$ de les espècies del clade Arctioide ens ha portat a hipotetitzar que podria ser un paleopoliploide originat a partir d'un ancestre $x = 9$.

16. Es posa de manifest el clar paral·lelisme entre el complex *Arctium-Cousinia* i el grup *Onopordum*, definint-se dues línies clarament separades en funció de la forma vital, l'amplitud biogeogràfica i el nombre cromosòmic, en que *Onopordum* seria comparable a *Arctium* i els gèneres *Alfredia, Ancathia, Olgaea, Synurus* i *Syreitschikovia* serien comparables al gènere *Cousinia* s.str.

17. Els microsatèl·lits dissenyats específicament per *Arctium minus* han resultat poc polimòrfics, reflectint la reduïda variabilitat existent a les seves poblacions.

18. Els patrons de variació genètica detectats a *Arctium minus* són consistents amb les seves característiques biològiques. Així la baixa diversitat genètica, els alts valors d'endogàmia i l'excés d'homozigots detectats s'expliquen per la seva capacitat d'autofecundació i el seu curt cicle de vida.

19. La major part de la variabilitat genètica d'*Arctium minus* es troba entre poblacions degut a un baix flux gènic entre elles juntament amb la seva capacitat d'autofecundació.

20. Certs indicis recolzen la idea que *Arctium minus* s'hauria refugiat al sud de la seva àrea de distribució actual durant les glaciacions del Pleistocè i posteriorment hauria recolonitzat Europa. Aquests patrons s'haurien vist emmascarats per influència recent de l'activitat humana i per esdeveniments de dispersió de llavors a llarga distància com s'exemplifica a la colonització del continent Americà.

7. Compendi de publicacions

7. 1. Phylogeny and evolution of the *Arctium-Cousinia* complex (Compositae, Cardueae-Carduinae)

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RESUM. A aquest article s'investiguen la filogènia i l'evolució del complex *Arctium-Cousinia* que inclou els gèneres *Arctium*, *Cousinia*, *Hypacanthium* i *Schmalhausenia* i té la seva màxima diversitat a la regió Irano-Turania i a les muntanyes d'Àsia Central. Per aquest treball s'han generat seqüències dels marcadors ITS, i *rpS4-trnT-trnL* per 138 espècies, incloses 129 espècies de les aproximadament 600 que té el gènere *Cousinia*. Tal i com ja s'havia vist a anàlisis prèvies, *Cousinia* no és monofilètic donat que els subgèneres *Cynaroides* i *Hypacanthodes*, que sumen unes 30 espècies, són més propers a *Arctium*, *Hypacanthium* i *Schmalhausenia* (conjunt que s'ha anomenat clade Arctioide) que a *Cousinia* s.str. (o clade Cousinioide). La distinció entre els clades Arctioide i Cousinioide està recolzada per la morfologia del pol·len i el nombre cromosòmic tal i com havien posat de manifest autors anteriors. Pel que fa a la delimitació genèrica del clade Arctioide, la distribució dels caràcters morfològics tradicionalment considerats més determinants com la forma i armadura de la fulla i la morfologia de les bràctees involucrals, és en part incongruent amb les relacions filogenètiques derivades de les dades moleculars. Amb aquest treball no hem pogut aportar cap solució taxonòmica per aquest conflicte i interpretem que els esmentats caràcters són homoplàsics. Malgrat que la resolució filogenètica del clade Cousinioide és reduïda, es posa de manifest que els marcadors ITS i *rpS4-trnT-trnL* contenen una informació filogenètica limitada però valuosa. Un exemple és que quatre de les cinc espècies anuals de *Cousinia* s.str. incloses a les anàlisis cauen al mateix grup tot i pertànyer a dues seccions diferents. Aquesta pobresa de resolució filogenètica del clade Cousinioide probablement sigui conseqüència del reduït nombre de caràcters informatius combinat amb l'elevat nombre d'espècies d'aquest jove llinatge (uns 8.7 milions d'anys). S'hipotetitza que el mecanisme d'especiació d'aquest llinatge és eminentment al·lopàtric.

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Phylogeny and evolution of the *Arctium-Cousinia* complex (Compositae, Cardueae-Carduinae)

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The phylogeny and evolution of the *Arctium*-*Cousinia* complex, including *Arctium*, *Cousinia* as one of the largest genera of Asteraceae, *Hypacanthium* and *Schmalhausenia*, is investigated. This group of genera has its highest diversity in the Irano-Turanian region and the mountains of Central Asia. We generated ITS and *rpS4*-*trnT*-*trnL* sequences for altogether 138 species, including 129 (of ca. 600) species of *Cousinia*. As found in previous analyses, *Cousinia* is not monophyletic. Instead, *Cousinia* subgg. *Cynaroides* and *Hypacanthodes* with together ca. 30 species are more closely related to *Arctium*, *Hypacanthium* and *Schmalhausenia* (Arctioid clade) than to subg. *Cousinia* (Cousinioid clade). The Arctioid and Cousiniod clades are also supported by pollen morphology and chromosome number as reported earlier. In the Arctioid clade, the distribution of morphological characters important for generic delimitation, mainly leaf shape and armature and morphology of involucral bracts, are highly incongruent with phylogenetic relationships as implied by the molecular data. No taxonomic solution for this conflict can be offered, and the characters named are interpreted as homoplasious. Although phylogenetic resolution in the Cousinioid clade is poor, our ITS and *rpS4*-*trnT*-*trnL* sequences contain some phylogenetic information. For example, the six annual species of the Cousinioid clade fall into two groups. Poor phylogenetic resolution probably results from lack of characters and the high number of taxa in this species-rich and comparatively young (ca. 8.7 mya) lineage. We hypothesize that speciation in the Cousinioid clade was mainly allopatric.

KEYWORDS: *Arctium*, *Cousinia*, Irano-Turanian region, ITS, molecular clock, Pamir-Alay, *rpS4*-*trnT*-*trnL*, speciation, Tien Shan

INTRODUCTION

The *Arctium*-*Cousinia* complex (Compositae, Cardueae-Carduinae) in its most recent circumscription (Susanna & Garcia-Jacas, 2007) is a natural group composed of four genera. The largest genus of the group, one of the largest of the Compositae and among the 50 largest of flowering plants (Frodin, 2004), is *Cousinia* Cass. with more than 600 species (Mabberley, 2008). The other genera of the complex are *Arctium* L. (incl. part of *Cousinia*, see below) with 27 species, *Hypacanthium* Juz. with two species (Tscherneva, 1982) and the monotypic *Schmalhausenia* C. Winkl. *Lipskyella* Juz. and *Tiarocarpus* Rech. f., recognized as separate genera by Häffner (2000), were included in *Cousinia* by Susanna & Garcia-Jacas (2007).

Three characters never found in combination elsewhere in the tribe characterize this complex according to Susanna & al. (2003a). The receptacle has strongly twisted scales, the achenes are always streaky (with wavy fringes), very often winged and without a nectary, and the pappus is formed by free deciduous bristles.

According to Rechinger (1986) and Knapp (1987), the vast majority of species of the *Arctium*-*Cousinia* complex is distributed in the Turkestan mountain region (Tien Shan and Pamir-Alay) and the Irano-Turanian region (Fig. 1; the "Oriental-Turanian Floristic Region" of Meusel & al., 1965). Within this range, following Knapp (1987), most species of *Cousinia* subgg. *Cynaroides* and *Hypacanthodes* grow only in the mountainous terrain of the Pamir-Alay range and in the western Tien Shan in Central Asia. The two species of *Hypacanthium* are endemic to the western Tien Shan, and the monotypic *Schmalhausenia* is endemic to the subalpine and alpine zone in the northern Tien Shan. *Arctium* s.str. is Eurosiberian in distribution. Those species of *Cousinia* included in *Arctium* by Duistermaat (1996) and Susanna & Garcia-Jacas (2007; see below) are, like the remainder of *Cousinia*, distributed in Central Asia.

Both morphological (Boissier, 1875; Kuntze, 1891; Dittrich, 1977; Tscherneva, 1988a–c; Duistermaat, 1996, 1997; Petit & al., 1996; Petit, 1997; Häffner, 2000) and molecular (Häffner & Hellwig, 1999; Garcia-Jacas & al., 2002; Susanna & al., 2003a, 2006) analyses of the *Arctium-Cousinia* complex concluded that the limits between the biennial *Arctium* and the perennial, biennial and only rarely annual *Cousinia* are unclear. This resulted in many reclassifications with many species changing generic affiliation. As one extreme, Kuntze (1891) proposed the classification of all species of *Cousinia* in *Arctium*.

The difficulties in generic delimitation arise from the incongruent distribution of several morphological, pollen, karyological and molecular characters.

Arctium has leaves which are always unarmed, often large (to 80 cm), and usually cordate. Such leaves are also found in *Cousinia* subg. *Cynaroides* (with the exception of *Cousinia korolkovii* Regel & Schmalh., *C. haesitabunda* Juz. and *C. chlorantha* Kult. with lanceolate leaves) and *C. grandifolia* Kult. of *Cousinia* subg. *Hypacanthodes*. This group of *Cousinia* species was referred to as the "Arctioid" group by Duistermaat (1996). In contrast, *Cousinia* subg. *Cousinia*, nine species of subg. *Hypacanthodes*, all species of *Hypacanthium* and the monotypic *Schmalhausenia* have smaller leaves which usually are lanceolate and often very spiny. In *Arctium* and part of *Cousinia* subg. *Cynaroides* involucral bracts always end in a recurved hook whereas in most other species of *Cousinia* involucral bracts end in an unhooked spine. Whereas in species with hooked involucral bracts usually the entire capitulum is dispersed as a burr adhering to passing animals, most species of *Cousinia* release their achenes and/or disperse as tumbleweeds. As regards floral morphology, Duistermaat (1996) observed that the stigma of *Cousinia* subg. *Cousinia* is very different from that of *Arctium* and the "Arctioid" species of *Cousinia*. Whereas in the former the apical part of the style and the stylar branches are covered with long hairs, and a ring of sweeping hairs at the articulation of the base of the stylar apex is absent, the style is glabrous and cylindrical in the latter, and a ring of sweeping hairs is present at the thickened articulation. Häffner (2000) noted that a ring of sweeping hairs is also absent in *Schmalhausenia*. Outer florets in *Arctium* often have long and brightly coloured anther-tubes. Many species of *Cousinia* have brightly coloured appendages in the innermost involucral bracts, recalling those of *Carlina* L.

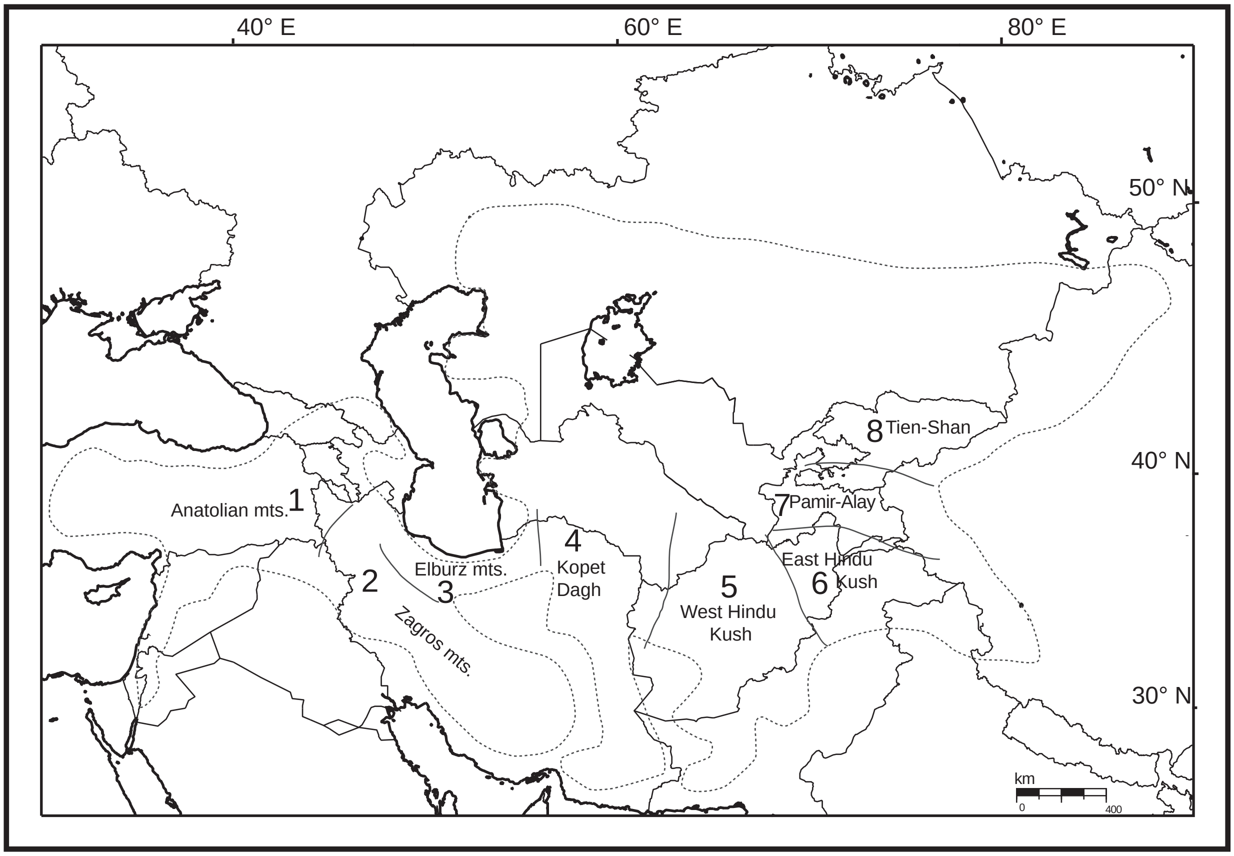


Fig. 1. Geographical distribution of the *Arctium-Cousinia* complex (excl. *Arctium* s.str.). The eight main centres of diversity recognized are based on Rechinger (1986) and Knapp (1987).

This distribution of characters (leaf, involucral bract, style morphology) led Duistermaat (1996) to the conclusion that the obviously "Arctioid" species of *Cousinia* should be transferred to *Arctium* within which their sectional classification by Tscherneva (1988a–c) should be retained. However, later Duistermaat (1997) vaguely suggested that all species of *Cousinia* subg. *Cynaroides* could be placed in *Arctium*. This latter proposal was followed by Susanna & Garcia-Jacas (2007) but does not solve the problematic placement of *Hypacanthium*, *Schmalhausenia* and *Cousinia* subg. *Hypacanthodes*.

Schtepa (1966, 1976) and Kuprianova & Tscherneva (1982) found that *Cousinia* subgg. *Cynaroides* and *Hypacanthodes* have orbicular and spiny pollen grains similar to those of *Arctium*, which they called the *Arctiastrum* pollen type, whereas *Cousinia* subg. *Cousinia* has oblong and smooth pollen grains which they called the *Cousinia* pollen type. *Schmalhausenia* (Susanna & al., 2003a) as well as *Hypacanthium* (S. López-Vinyallonga, unpub. data) have the *Arctiastrum* pollen type.

According to Duistermaat (1996), *Arctium* always has $x = 18$ and $2n = 36$ chromosomes. This number is also found in all species studied of *Cousinia* subgg. *Cynaroides* and *Hypacanthodes* (Tscherneva, 1985) and in *Schmalhausenia* according to Susanna & al. (2003b), and is perfectly correlated with the *Arctiastrum* pollen type. In contrast, *Cousinia* subg. *Cousinia*, with the *Cousinia* pollen type, has $2n = 18$, 20, 22, 24 and 26 chromosomes according to Ghaffari & al. (2006) and earlier authors. Finally, a DNA sequence analysis by Susanna & al. (2003a) grouped all species with $2n = 36$ chromosomes and the *Arctiastrum* pollen type in a monophyletic clade, the Arctioid clade, and species with $2n = 22$, 24 and 26 and the *Cousinia* pollen type in a second monophyletic clade, the Cousinioid clade.

In summary, pollen morphology, chromosome number and DNA data imply a subdivision of the *Arctium-Cousinia* complex into two major lineages which are not congruent with overall morphology particularly because parts of *Cousinia* subgg. *Cynaroides* and *Hypacanthodes* (Arctioid clade) are more similar to subg. *Cousinia* (Cousinioid clade), and other parts (the Arctioid species) are more similar to *Arctium*.

Of the genera and lineages introduced above, *Cousinia* subg. *Cousinia* is most remarkable by containing a high number of species in a comparatively small geographical area. After establishment of *Cousinia* by Cassini (1827), Candolle (1838) recognized 34 species, Bunge (1865) 126 species, Boissier (1875, 1888) 141 species (in the *Flora Orientalis* area), Winkler (1892, 1897) 267 species, and an additional 30 species were added in several publications by Bornmüller (1896–1941; listed by Rechinger, 1953). In her treatment of *Cousinia* for the *Flora of the USSR*, Tscherneva (1962) recognized about 262 species in about 50 sections, and in his treatment of the genus for *Flora Iranica*, Rechinger (1972) placed more than 350 species in 58 sections. A careful comparison of the systems of Rechinger (1953, 1972, 1979) and Tscherneva (1962, 1988a, b) reveals an approximate number of ca. 630 species in 70 sections and three subgenera. Of these 630 species, ca. 600 belong into subg. *Cousinia*.

The molecular analysis of the *Arctium-Cousinia* complex by Susanna & al. (2003a) included 21 species of *Cousinia* subg. *Cousinia*, 1 species of subg. *Hypacanthodes* and 5 species of subg. *Cynaroides*. By increasing the sample of *Cousinia* subg. *Cousinia* to 106 species (of ca. 600), of subg. *Hypacanthodes* to 6 species (of 10) and of subg. *Cynaroides* to 14 species (of 20), where the sample included represents the large majority of subgeneric units recognized by Tscherneva (1962, 1988c) and Rechinger (1953, 1972, 1979), we pursue several aims. First, we want to investigate whether the subdivision of the *Arctium-Cousinia* complex into two lineages, the Arctioid clade with *Arctium* s.str., *Cousinia* subgg. *Hypacanthodes* and *Cynaroides*, *Hypacanthium* and *Schmalhausenia*, and the Cousinioid clade with *Cousinia* subg. *Cousinia* as found by Susanna & al. (2003a) and as supported by pollen morphology, chromosome number and DNA sequence data can be verified with a strongly enlarged species sample. Second, we aim at obtaining a better understanding of phylogenetic relationships within the Arctioid clade in order to resolve the character incongruences discussed above and to arrive at a new generic classification of this clade. Third, we want to investigate phylogenetic relationships within the large subg. *Cousinia*. The last aims both at examining the validity of the sectional (and subsectional) classification of this group as proposed by Tscherneva (1962, 1988a, b) and Rechinger (1953, 1972, 1979), and at providing a basis for discussing possible evolutionary mechanisms underlying the species-richness of subg. *Cousinia*.

MATERIAL AND METHODS

Plant material. — Samples for analysis were obtained either from silica-gel dried leaves of specimens collected in the wild, from fresh plants cultivated at the Botanic Institute of Barcelona, or from herbarium specimens deposited mainly in B, BC, JE, KAS, LE, M, and MJG (Appendix 1). The data described and discussed here are based on a sample of 138 species for which both ITS and *rpS4-trnT-trnL* could be obtained. These 138 species represent 60 sections of *Cousinia* (out of 70 described by Rechinger, 1972, 1979, and Tscherneva, 1988a–c), three species of *Arctium* and one each of *Hypacanthium* and *Schmalhausenia*. Four outgroup taxa were selected on the basis of previous analyses of the group (Garcia-Jacas

& al., 2002; Susanna & al., 2006). These were *Saussurea elegans* Ledeb., *S. maximowiczii* Herder, *Jurinea albicaulis* Bunge and *J. humilis* (Desf.) DC. Eighty additional species were sequenced for ITS and 19 for *rpS4-trnT-trnL*. Results obtained for this additional sample are not presented and discussed only briefly. Voucher data and sources of material of the above 138 species are given in Appendix 1. Some of the ITS sequences were taken from previous studies but all *rpS4-trnT-trnL* sequences were newly generated.

DNA extraction, amplification and sequencing. — Total genomic DNA was extracted either following the protocol of the CTAB method of Doyle & Doyle (1987) and Culling (1992), or following the manufacturer's protocols of the NucleoSpin® Plant Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) or QIAprep® Miniprep (Qiagen Inc., Valencia, California, U.S.A.).

nrDNA ITS. – Double-stranded DNA of the internal transcribed spacer region (ITS1, 5.8S, ITS2) was amplified using 18S as forward primer and 28S as reverse primer (Muir & Schlötterer, 1999). Reactions were performed in 25.0 µl volumes with 10% 10× Biotherm buffer, 5% 50 mM $MgCl_2$ (GeneCraft, Münster, Germany), 2% of 20 mM dNTPs mix, 2% of each primer at 25 pmol/µl conc., 1.0 unit (0.2 µl) BioTherm™ polymerase (GeneCraft) and 1.0 µl of template DNA (30–60 ng/µl). This was filled up to 25.0 µl with distilled sterilized water. In some cases 1.0 µl DMSO (dimethyl sulfoxide) per reaction tube was added to improve PCR amplifications (Frackman & al., 1998). The profile used for amplifications consisted of a preheat for 60 s at 94°C, followed by 35 cycles of 94°C for 18 s, 52°C for 30 s, and 72°C for 60 s and a post-treatment of 78 s at 52°C plus 8 min at 72°C. PCR products were purified using the NucleoSpin® Extract Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol.

Cycle sequencing reactions were conducted using 0.5–2.5 µl of the purified PCR product and ABI-PRISM® BigDye™ Terminators v3.0 Cycle Sequencing Reagents (BD 3.0 in 10 ml reactions) for 30 cycles for 10 s at 96°C and 4 min at 55°C. Sequences were detected on automated sequencers (ABI 373 or 377).

For sequence analysis, ITS fragments were edited and assembled with the aid of Sequencher 4.1.2 (Gene Codes Corporation, Ann Arbor, Michigan, U.S.A.). Edited sequences were aligned with MacClade 4.1 (Maddison & Maddison, 2000).

cpDNA rpS4-trnT-trnL. – The double-stranded DNA intergenic spacer *rpS4-trnT-trnL* was amplified by PCR with rpS4R2 (Shaw & al., 2005), trnL-b (Taberlet & al., 1991), trnA2 (Cronn & al., 2002) and trnA2R (5′-AGG TTA GAG CAT CGC ATT TG-3′ designed for this study) as PCR primers. When the rpS4R2 (forward)/trnL-b (reverse) primer combination failed to generate double-stranded products, two separate PCR reactions were performed with the following primer combinations: rpS4R2/trnA2R (reverse) and trnA2(forward)/trnL-b. Reactions were performed in 25.0 µl volumes with 10% 10× AmpliTaq buffer, 10% 50 mM $MgCl_2$, 10% of 20 mM dNTPs mix, 2% of each primer at 25 pmol/µl conc., 1.0 unit (0.2 µl) AmpliTaq DNA polymerase (Applied Biosystems, Foster City, California, U.S.A.), and 5.0 µl of template DNA (30–60 ng/µl). The volume was filled up to 25.0 µl with distilled sterilized water. The profile used for amplification consisted of a preheat for 1 min 35 s at 95°C, followed by 35 cycles of 95°C for 1 min 30 s, 52°C for 1 min 30 s and 72°C for 2 min and a post-treatment of 10 min at 72°C.

Double-stranded PCR products were purified with either the QIAquick® Purification Kit (Qiagen Inc., Valencia, California, U.S.A.) or DNA Clean & Concentrator-5 (Zymo Research, Orange, California, U.S.A.) following the manufacturer's protocols. Direct sequencing of the amplified DNA segments was performed using 0.5–1.0 µl of the purified PCR product with a "Big Dye® Terminator v3.1 kit" (Applied Biosystems, Foster City, California, U.S.A.). The profile used consisted of a hot start at 96°C for 1 min followed by 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. Nucleotide sequencing was performed at the Serveis Científico-Tècnics of the University of Barcelona on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, California, U.S.A.). Nucleotide sequences were edited with Chromas 2.0 (Technelysium Pty. Ltd., Tewantin, Australia) and aligned visually by sequential pairwise comparison (Swofford & Olsen, 1990).

Data matrices are available on request from the corresponding author.

Analyses. — *Bayesian Inference*. – Bayesian analyses of the ITS1 + ITS2, *rpS4-trnT-trnL* and combined ITS1 + ITS2 + *rpS4-trnT-trnL* datasets were performed with MrBayes software package 3.0b4 (Huelsenbeck & Ronquist, 2001). Bayesian analyses consist of maximum likelihood (ML) comparisons of trees where tree topology and ML parameters are permuted using a Markov Chain Monte Carlo method and sampled periodically. The sample trees are drawn from a posterior probability distribution (Huelsenbeck & Ronquist, 2001). In order to select the best-fit model of substitution, the program Modeltest 3.5 was used (Posada & Crandall, 1998, 2001).

Following the results for DNA evolution of our data as indicated by ModelTest, the ML parameters in MrBayes were set as follows: Nst = 6, Rmat = for ITS dataset: A-C = 0.7720, A-G = 2.7242, A-T = 1.2057, C-G = 0.3372, C-T = 9.8048, G-T = 1.0000 (Proportion of invariable sites [I] = 0.1915; Gamma distribution shape parameter = 0.6024; Model selected: SYM + I + G); for *rpS4-trnT-trnL* dataset: A-C = 0.4777, A-G = 0.6338, A-T = 0.0968, C-G = 0.6238, C-T = 1.1010, G-T = 1.0000 (Proportion of invariable sites [I] = 0.5280; Gamma distribution shape parameter = 0.9553; Model selected: GTR + I + G); for ITS + *rpS4-trnT-trnL* combined dataset: A-C = 0.5621, A-G = 1.2123, A-T = 0.3214, C-G = 0.3810, C-T = 5.0583, G-T = 1.0000 (Proportion of invariable sites [I] = 0.5978; Gamma distribution shape parameter = 0.5788; Model selected: GTR + I + G) and Rates = gamma.

The Markov Chain Monte Carlo process was set so that four chains ran simultaneously for 2,000,000 generations, with trees being sampled every 100 generations for a total of 20,000 trees in the initial sample. As ‘‘stationarity’’ was achieved by the 3,000th tree, the first 3,000 trees were discarded and the posterior probability of the phylogeny and its branches was determined from the remaining 17,000 trees. Internodes with posterior probabilities ≥ 95% were considered to be well-supported.

Parsimony and Maximum Likelihood. – For the combined dataset (138 taxa), a parsimony analysis and a maximum likelihood analysis (ML) were performed with PAUP Version 4.0b10 (Swofford, 2002). For the parsimony analysis, due to the impracticability of performing standard analyses on our large dataset, we used the PAUPRat approach (Nixon, 1999; Sikes & Lewis, 2001), a tool for implementing Parsimony Ratchet searches using PAUP. Rather than performing many thorough and independent heuristic searches, the Ratchet performs what amounts to a single long search comprising a series of short heuristic searches. We have performed 200 iterations with TBR branch swapping. All most parsimonious trees (MPT) were saved and PAUP was used for computing a strict consensus tree. For the strict consensus tree, consistency index (CI), retention index (RI) and homoplasy index (HI), all excluding uninformative characters, have been calculated.

A bootstrap analysis (BS) (Felsenstein, 1985) was done to obtain support estimates for the nodes in the strict consensus tree. The analysis was performed using the approach by Lidén & al. (1997) using 1,000 replicates, random taxon addition with 10 replicates per replicate and no branch swapping.

For the ML analysis, we started with a tree generated by Neighbor Joining (NJ) and used the SYM + G (Zharkikh, 1994) evolutionary model which best fits the data according to MrModeltest 2.2 (Nylander, 2004). Because of the large size of our dataset we accelerated the analysis using a ratchet approach according to the following parameters suggested by Morrison (2007): SPR branch swapping, re-weight 25% of the characters, 10 iterations of re-weighting, ApproxLim no more than 2% which indicates that approximate scores within 2% of the optimum will be evaluated fully, and MULTREES inactivated.

Partition homogeneity test. – In order to investigate whether the ITS and *rpS4-trnT-trnL* datasets can be combined, a partition homogeneity test (Farris & al., 1994; implemented in PAUP* 4.0b10; Swofford, 2002) with 10 homogeneity replicates, 10 random addition sequences, tree-bisection-reconnection (TBR) branch swapping on best only and MULTREES on was performed. In order to explore the congruency of the two datasets further, the congruency of individual clades with ≥ 0.95 PP found in the ITS and *rpS4-trnT-trnL* datasets was compared.

Molecular clock analysis. – A likelihood ratio test (Felsenstein, 1988) was performed on the tree resulting from the ML analysis (1,000 random addition sequence, TBR and MULTREES on, after Zhang & al., 2007) of the ITS dataset. Modeltest 3.5 (Posada & Crandall, 1998) was used to perform the likelihood ratio test comparing the log-likelihood scores of the tree with and without enforcing a molecular clock. Degrees of freedom were equal to s – 2 where s equals the number of taxa in the analysis. A significant difference at the 0.01 level between two scores was observed. Following this, PAUP* 4.0b10 (Swofford, 2002) was used to calculate genetic distances among taxa. After eliminating all taxa with large genetic distances to *Saussurea elegans*, a clock-like tree with 112 taxa was obtained.

As we could not identify appropriate fossils of the study group to calibrate our molecular clock tree (but see below), published ITS substitution rates were used for calibration. According to Kay & al. (2006), ITS substitution rates in herbaceous angiosperms vary between 1.72×10^{-9} to 8.34×10^{-9} (mean = 4.13×10^{-9}) substitutions/site/year. The approximate age of seven nodes of our molecular clock tree was determined using the above three rates.

RESULTS

Partition homogeneity test. — No significant incongruence (P = 0.01) between the two datasets was detected in the partition homogeneity test.

The clades with ≥ 0.95 PP found in the ITS and *rpS4-trnT-trnL* trees, respectively, in the majority of cases are not congruent among the trees (Figs. 2, 3; Appendix 2). Thus, of the seven clades (or nested subclades) found in the *rpS4-trnT-trnL* tree, only one (clade 1) is found in the ITS tree (clade 8, containing one additional species). The remaining six clades are not present in the ITS tree, and the constituent species are separated by at least one branch with ≥ 0.95 PP. Equally, of the 28 clades found in the ITS tree, only three clades (clades 2, 9, 17) plus two species of one clade (clade 8) are recovered as clades with ≥ 0.95 PP by the *rpS4-trnT-trnL* data. The third species of clade 8 of the ITS tree, *C. gmelinii* C. Winkl., is separated from the other two species by several branches in the *rpS4-trnT-trnL* tree although none of these has ≥ 0.95 PP. The remaining 24 ITS clades are not present in the *rpS4-trnT-trnL* tree, and in 13 of these clades the species constituting the ITS clades are separated by at least one branch with ≥ 0.95 PP in the *rpS4-trnT-trnL* topology. Considering the results of this clade-by-clade comparison, the finding of congruency between the two datasets by the partition homogeneity test is somewhat surprising.

Phylogenetic analysis. — The numerical results of the analyses of the ITS, *rpS4-trnT-trnL* and combined ITS + *rpS4-trnT-trnL* datasets are given in Table 1. The individual datasets were analyzed with Bayesian inference (BI), and the combined dataset with Maximum Parsimony (MP), Bayesian inference (BI) and Maximum Likelihood (ML). The results of the analyses of all three datasets with BI are shown in Figs. 2–4, the result of the MP analysis is partially shown in Fig. 6 (only the Arctioid clade) and the results of the ML analysis of the combined dataset are not shown.

All analyses confirmed the monophyly of the *Arctium*-*Cousinia* complex. Support for this was strong in the MP and BI analyses (BS = 89%, PP = 1.00). Except for the BI analysis of *rpS4-trnT-trnL* alone, all analyses also show a clear subdivision of the complex into three groups: an Arctioid clade moderately supported by BS but well

Table 1. Numerical results of the analyses of the ITS, *rpS4-trnT-trnL* and combined ITS + *rpS4-trnT-trnL* datasets.

	ITS				***rpS4-trnT-trnL***				**Combined ITS + *rpS4-trnT-trnL***			
Total characters	489				1,109				1,598			
	tree	**–out.**	**Cous.**	**Arct.**	**tree**	**–out.**	**Cous.**	**Arct.**	**tree**	**–out.**	**Cous.**	**Arct.**
Inf.	172	143	143	51	28	21	21	7	216	171	140	58
Tree length	799	680	546	85	78	55	38	11	980	836	679	105
CI	0.308	0.290	0.300	0.647	0.603	0.545	0.579	0.636	0.299	0.272	0.274	0.590
RI	0.721	0.728	0.624	0.886	0.922	0.931	0.909	0.810	0.711	0.715	0.586	0.849
HI	0.692	0.710	0.700	0.353	0.397	0.455	0.421	0.364	0.701	0.728	0.726	0.410

Tree length, CI, RI and HI were calculated for entire trees, for trees without outgroups and for the Arctioid and the Cousinioid clades. Abbreviations: Arct., Arctioid clade; CI, consistency index; Cous., Cousinioid clade; HI, homoplasy index; Inf., phylogenetically informative characters; –out., outgroups excluded; RI, retention index; tree, the entire tree including outgroups.

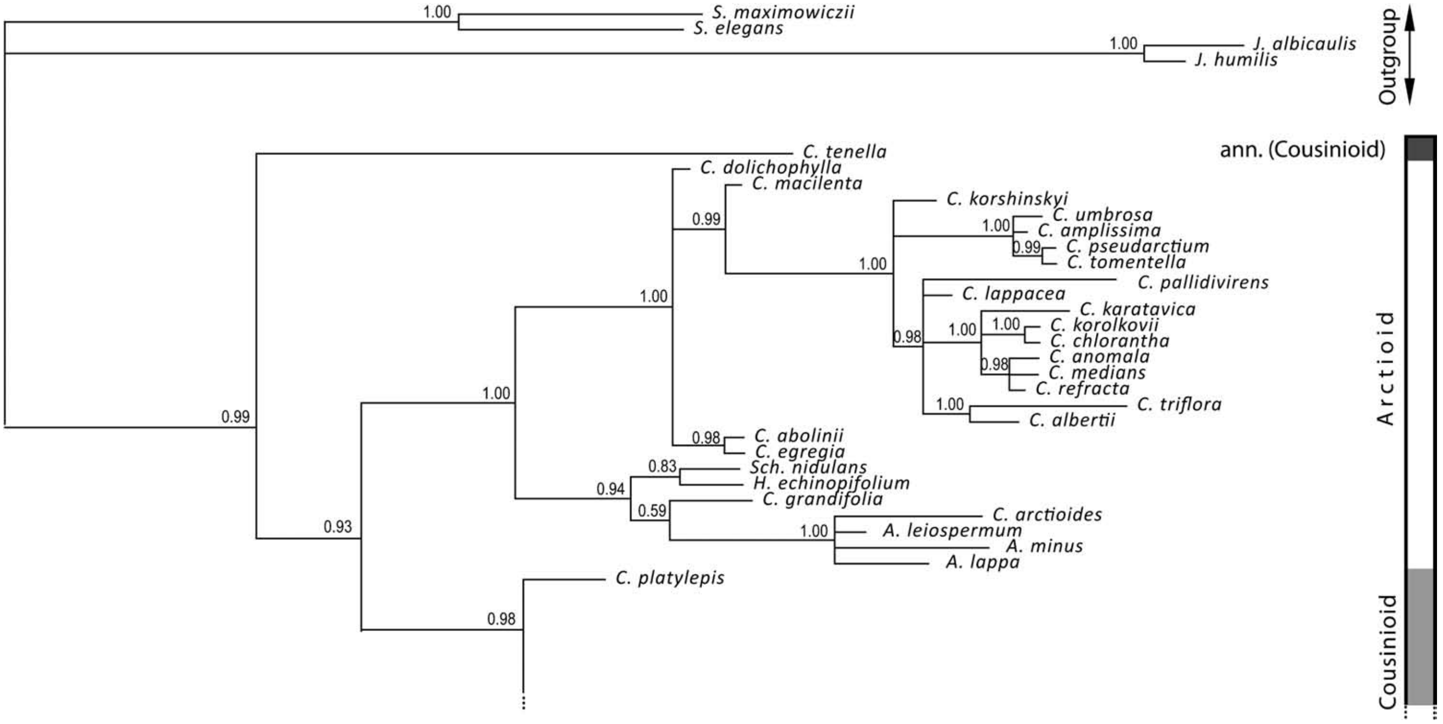
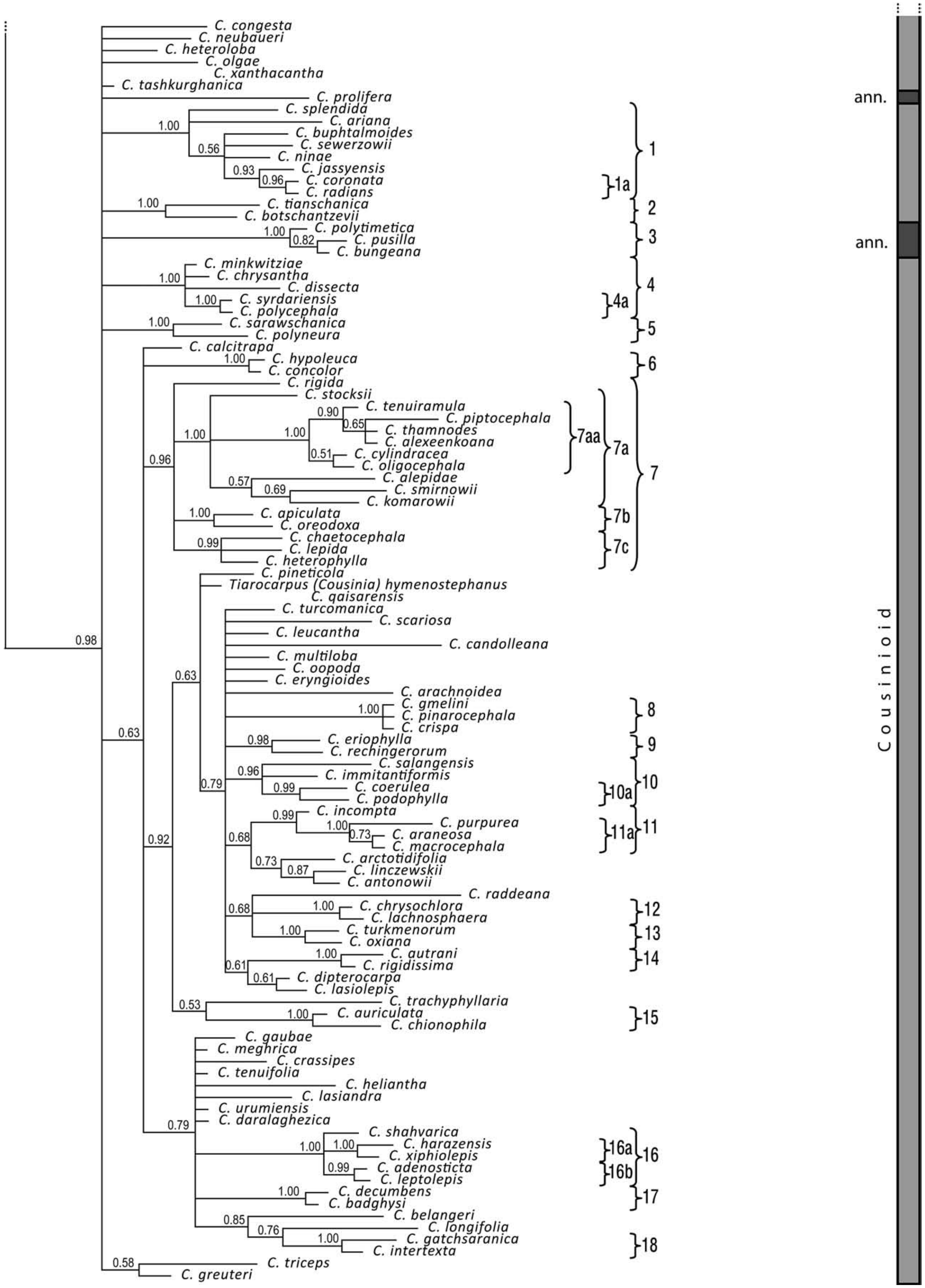


Fig. 2. 50% majority-rule consensus tree obtained from the Bayesian analysis of the ITS dataset. Numbers on branches are posterior probabilities. ann. = annual species. Numbers at curly brackets indicate clades with >0.95 PP discussed in the text.


C. congesta
C. neubaueri
C. heteroloba
C. olgae
C. xanthacantha
C. tashkurghanica
C. prolifera
C. splendida
1.00
C. ariana
C. buphtalmoides
0.56
C. sewerzowii
C. ninae
0.93
C. jassyensis
0.96
C. coronata
C. radians
1.00
C. tianschanica
C. botschantzevii
1.00
C. polytimetica
0.82
C. pusilla
C. bungeana
C. minkwitziae
1.00
C. chrysantha
C. dissecta
1.00
C. syrdariensis
C. polycephala
1.00
C. sarawschanica
C. polyneura
C. calcitrapa
1.00
C. hypoleuca
C. concolor
C. rigida
C. stocksii
0.90
C. tenuiramula
C. piptocephala
0.65
C. thamnodes
1.00
1.00
C. alexeenkoana
0.96
0.51
C. cylindracea
C. oligocephala
0.57
C. alepidae
0.69
C. smirnowii
C. komarowii
1.00
C. apiculata
C. oreodoxa
0.99
C. chaetocephala
C. lepida
C. heterophylla
C. pineticola
Tiarocarpus (Cousinia) hymenostephanus
C. qaisarensis
C. turcomanica
C. scariosa
C. leucantha
0.98
C. candolleana
C. multiloba
C. oopoda
0.63
C. eryngioides
C. arachnoidea
1.00
C. gmelini
C. pinarocephala
0.63
C. crispa
0.98
C. eriophylla
C. rechingerorum
C. salangensis
0.96
C. immitantiformis
0.79
0.99
C. coerulea
C. podophylla
0.99
C. incompta
1.00
C. purpurea
0.92
0.68
0.73
C. araneosa
C. macrocephala
0.73
C. arctotidifolia
0.87
C. linczewskii
C. antonowii
C. raddeana
0.68
1.00
C. chrysochlora
C. lachnosphaera
1.00
C. turkmenorum
C. oxiana
1.00
C. autrani
0.61
C. rigidissima
0.61
C. dipterocarpa
C. lasiolepis
0.53
C. trachyphyllaria
1.00
C. auriculata
C. chionophila
C. gaubae
C. meghrica
C. crassipes
C. tenuifolia
C. heliantha
C. lasiandra
C. urumiensis
C. daralaghezica
0.79
C. shahvarica
1.00
1.00
C. harazensis
C. xiphiolepis
0.99
C. adenosticta
C. leptolepis
1.00
C. decumbens
C. badghysi
0.85
C. belangeri
0.76
C. longifolia
1.00
C. gatchsaranica
C. intertexta
0.58
C. triceps
C. greuteri
1
1a
2
3
4
4a
5
6
7aa
7a
7
7b
7c
8
9
10
10a
11
11a
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14
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Cousinioid

supported by PP (BS = 72%, PP = 1.00) which contains *Arctium*, *Cousinia* subgg. *Cynaroides* and *Hypacanthodes*, *Hypacanthium* and *Schmalhausenia*, a Cousinioid clade with BS < 50% but with strong Bayesian support (PP = 1.00) which contains most species of *Cousinia* subg. *Cousinia*, and *C. tenella* Fisch. & C.A. Mey. as sister to the above two clades (BS = 54%, PP = 0.89). In the BI analysis of *rpS4-trnT-trnL*, *C. tenella* falls into the Arctioid clade with only low PP.

The Arctioid clade. – The description of the Arctioid clade is based on the tree obtained from the MP analysis of the combined dataset (Fig. 6). This tree is essentially congruent with the BI tree but better suited for character mapping. Within the Arctioid clade relationships among species are reasonably well resolved and this clade is subdivided into two subclades (Fig. 6). The first subclade, with moderate BS (80%) but strong PP support (1.00) is formed by most species of *Cousinia* subgg. *Cynaroides* and *Hypacanthodes*. This subclade consists of an unresolved polytomy containing four species of subg. *Hypacanthodes* (*C. egregia* Juz., *C. dolichophylla* Kult., *C. abolinii* Kult. ex Tscherneva, *C. macilenta* Winkl.) in two lineages plus a well-supported clade (BS = 90%, PP = 1.00) formed by most of species of subg. *Cynaroides* which in turn fall into two unsupported subgroups. The second subclade also has good support (BS = 81%, PP = 1.00) and is formed by *Arctium* together with *C. arctioides* Schrenk of subg. *Cynaroides* (BS = 92%, PP = 1.00), *Cousinia grandifolia* Kult. of subg. *Hypacanthodes*, *Schmalhausenia* and *Hypacanthium*. Relationships in the Arctioid clade as described above are essentially similar in the BI analyses of the ITS and combined dataset and in the ML analysis of the combined dataset.

The Cousinioid clade. – Relationships among species in the Cousinioid clade are essentially unresolved in the MP analysis of the combined dataset. Resolution is better in the BI analyses of the ITS and combined datasets and will be further discussed below (see Discussion).

Molecular clock analysis. — The results of our molecular clock analysis are shown in Table 2 and Fig. 5. Mai (2001) reported fossil *Arctium* achenes from a German Miocene locality. The age of this fossil can be dated

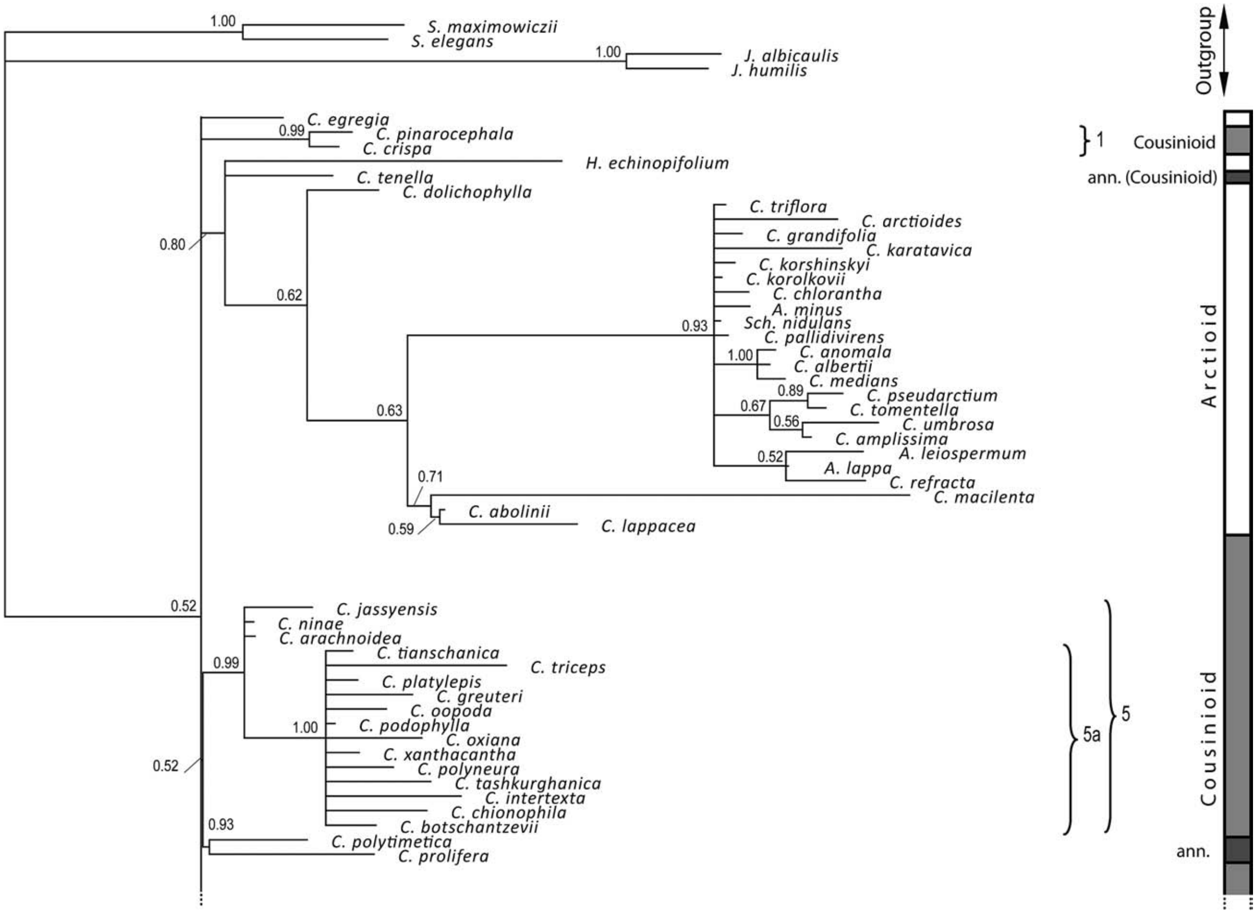


Fig. 3. 50% majority-rule consensus tree obtained from the Bayesian analysis of the *rpS4-trnT-trnL* dataset. Numbers on branches are posterior probabilities. ann. = annual species. Numbers at curly brackets indicate clades with >0.95 PP discussed in the text.

to 7–9 mya (V. Wähnert, pers. comm.). Considering that the achenes of *Arctium* can not be reliably distinguished from those of the other genera of the *Arctium-Cousinia* complex, this fossil essentially is compatible with the ages calculated for the complex with all three ITS substitution rates. When considering the occurrence of this achene in Germany, and assuming that the geographical distribution of the complex in the past was similar to its distribution today, with *Arctium* being the only genus occurring in Europe, the fossil probably is best assigned to *Arctium*. This would imply that of the three ITS substitution rates used for the calibration of the molecular clock, the mean rate fits best. With this rate, the clade containing *Arctium* (node F in Fig. 5) was dated to ca. 6.3 my (crown group age). Based on the above considerations, we will refer mainly to ages obtained when using the mean ITS substitution rate in the following discussion, and these ages are also indicated in Fig. 5.

DISCUSSION

The *Arctium-Cousinia* complex. — As already found by previous authors (Susanna & al., 2003a; Susanna & Garcia-Jacas, 2007), the *Arctium-Cousinia* complex clearly is monophyletic with good support in the MP and BI analyses (BS = 89%, PP = 1.00). In all analyses except the BI analysis of *rpS4-trnT-trnL* a division into three groups, the Arctioid clade, the Cousinioid clade and *Cousinia tenella* was found.

Table 2. Absolute ages in million years (my) obtained from the molecular clock analysis.

	Rate 1 (my)	Mean rate (my)	Rate 2 (my)
Node A	45.930	19.128	9.472
Node B	35.465	14.770	7.314
Node C	23.256	9.685	4.796
Node D	20.930	8.717	4.317
Node E	16.860	7.022	3.477
Node F	15.116	6.295	3.118
Node G	10.465	4.358	2.158

Rate 1 = 1.72 × 10^{-9} substitutions/site/year; mean rate = 4.13 × 10^{-9} substitutions/site/year; rate 2 = 8.34 × 10^{-9} substitutions/site/year (Kay & al., 2006).

When comparing the Arctioid and Cousinioid clades, two major differences can be observed. First, whereas intraclade relationships are reasonably well-resolved in the Arctioid clade, very little resolution was obtained in the Cousinioid clade. Considering that the two clades are sister to each other, and that crown group diversification of the two clades started at approximately the same time (mean rate: Arctioid clade: ca. 9.7 mya, Cousinioid clade: ca. 8.7 mya; Table 2), differential age of the two groups can not be advocated as explanation for the difference in phylogenetic resolution. Instead, we believe that the striking difference in species number is responsible for the difference

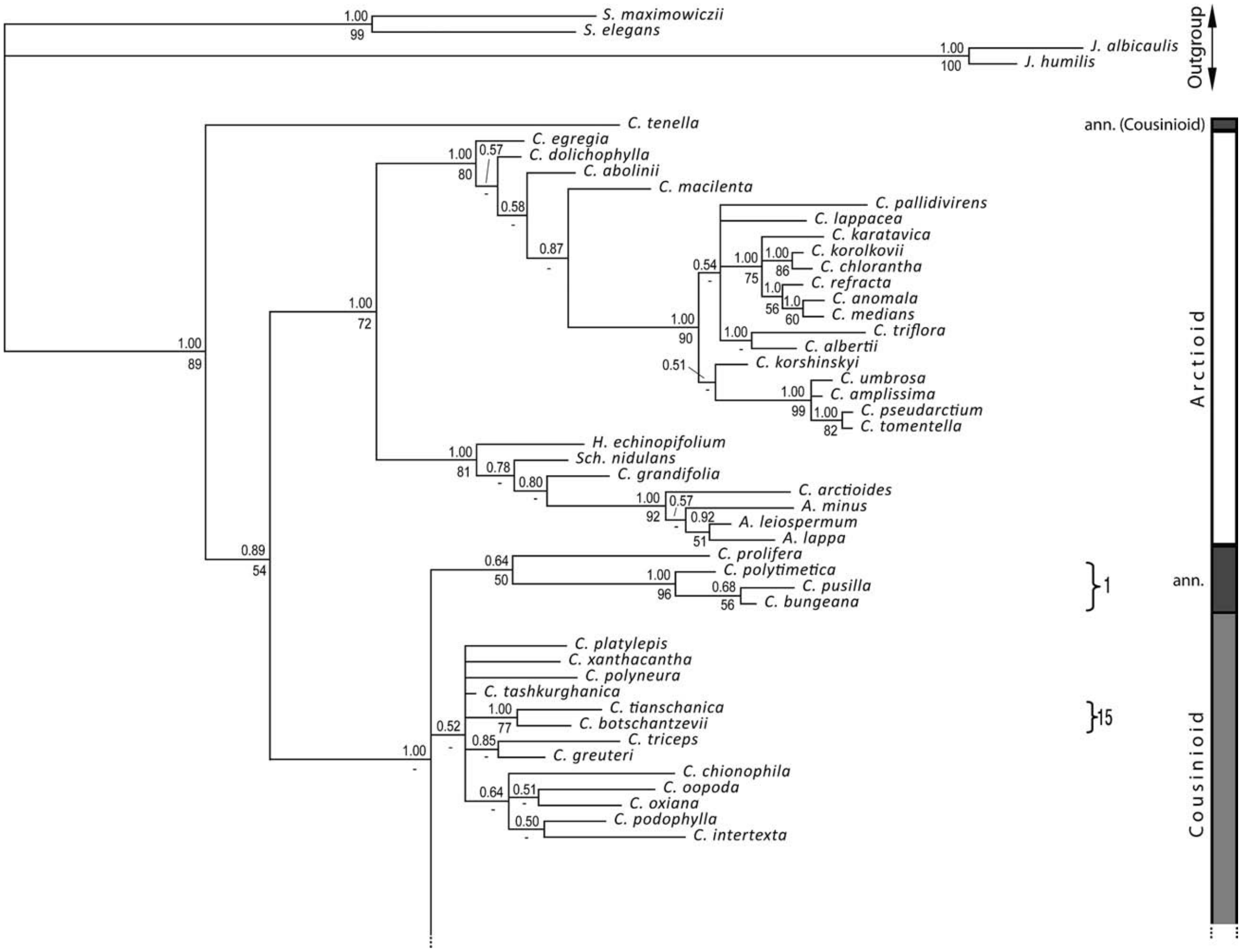
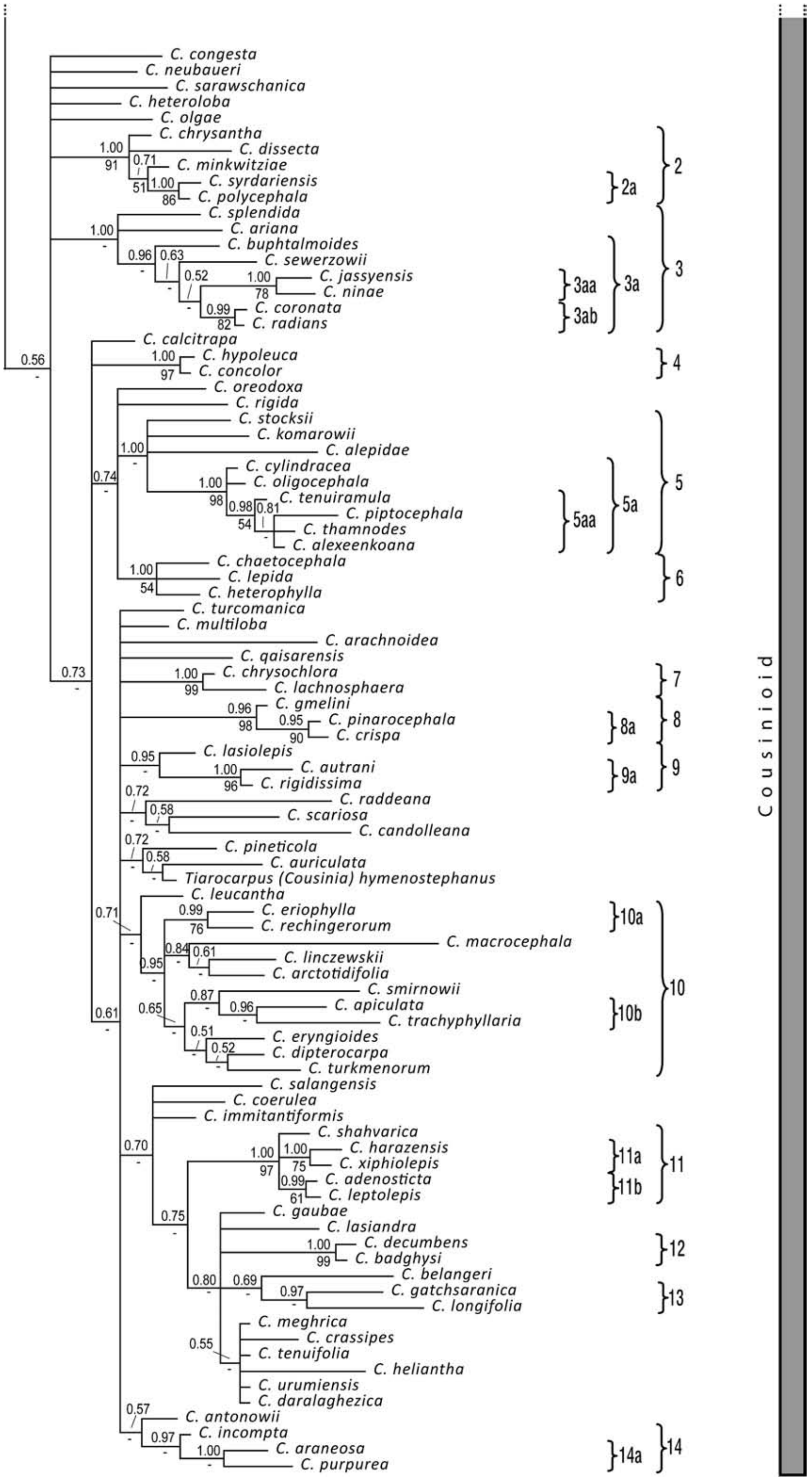


Fig. 4. 50% majority-rule consensus tree obtained from the Bayesian analysis of the combined ITS and *rpS4-trnT-trnL* dataset. Numbers above branches are posterior probabilities and numbers below branches are bootstrap percentages. ann. = annual species. Numbers at curly brackets indicate clades with >0.95 PP discussed in the text.

in phylogenetic resolution. If the Cousinioid clade were to be reduced to a random sample of species equal in number to that of the Arctioid clade, phylogenetic resolution probably would be greatly improved. Indeed, this prediction is to some extent confirmed by the data of Susanna & al. (2003a), who obtained some phylogenetic structure among the 21 species of the Cousinioid clade included in their analysis.

Second, presumably plesiomorphic and apomorphic characters are distributed unevenly across the two clades.



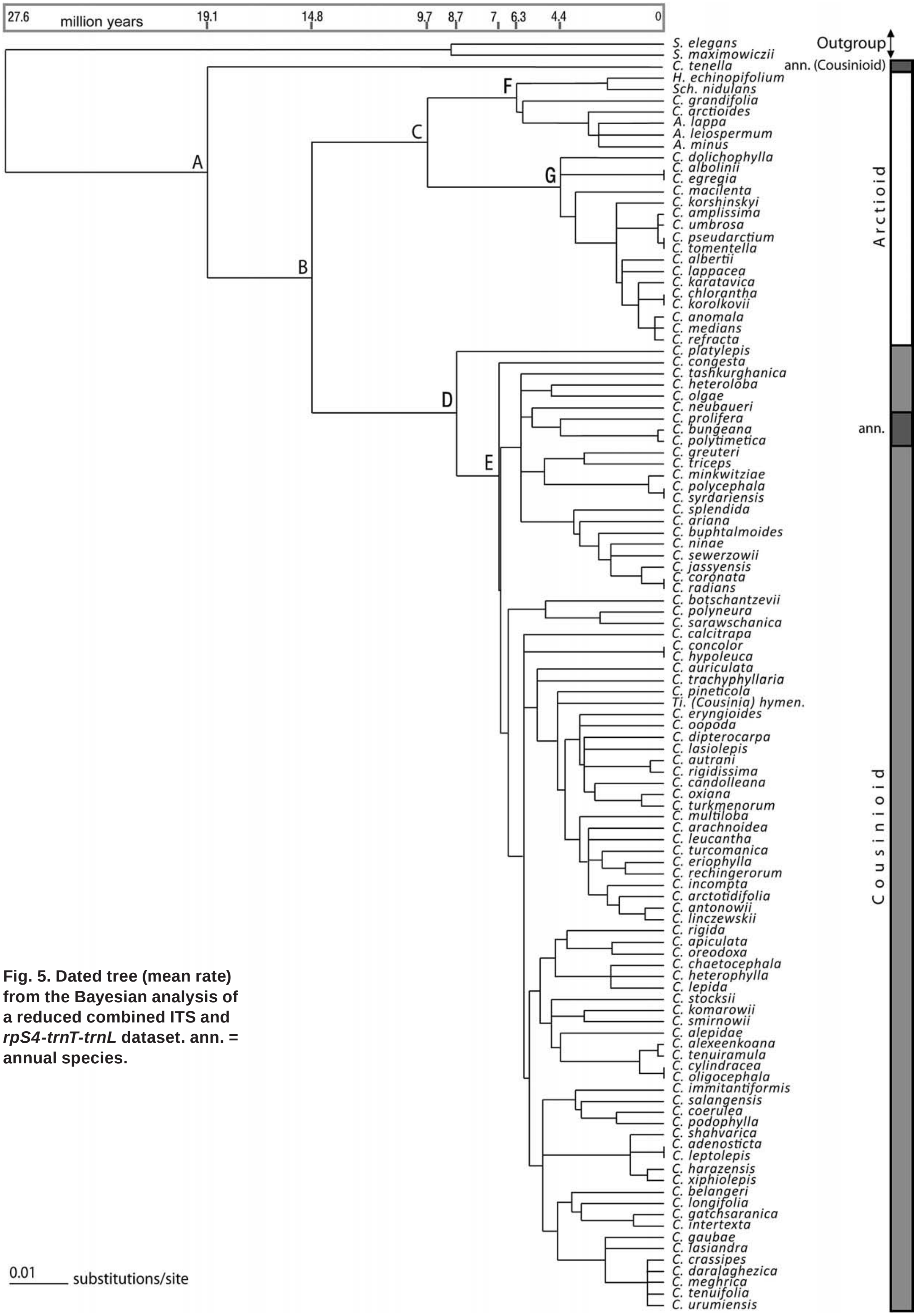


Fig. 5. Dated tree (mean rate) from the Bayesian analysis of a reduced combined ITS and *rpS4-trnT-trnL* dataset. ann. = annual species.

Whereas the spiny pollen (and possibly also long stigmata) of the Arctioid clade can also be found in *Jurinea* and *Saussurea* (Punt & Hoen, 2009) as closest relatives of the *Arctium*-*Cousinia* complex, the short stigmata and smooth pollen of the Cousinioid clade appear to be apomorphic. In other groups of Cardueae, pollen morphology is associated with the pollen presentation mechanism. In Centaureinae (Garcia-Jacas & al., 2001; Vilatersana & al., 2001), basal groups have spiny pollen and long, usually dry stigmata and lack a touch-sensitive pollen presentation mechanism. More derived groups have very short, sticky stigmata and smooth pollen, and this is associated with touch-sensitive pollen presentation. We hypothesize that a similar transition of characters has taken place in the *Arctium*-*Cousinia* complex. As regards chromosome numbers, the Arctioid clade is rather stable with a base chromosome number of $x = 18$. Much in contrast to this, considerable descending dysploidy ($x = 9, 10, 11, 12, 13$) is found in the Cousinioid clade. Of the closest relatives of the complex, *Jurinea* has a base chromosome number of $x = 17$ and *Saussurea* of $x = 13$. In general, basal Carduinae have higher chromosome numbers. The distribution of chromosome numbers in comparison of the Arctioid and Cousinioid clades again is paralleled in subtribe Centaureinae where an evolutionary trend from high to low chromosome numbers has been reported (Garcia-Jacas & al., 2001). Finally, it could be argued that the Arctioid and Cousinioid clades differ in habitat diversity. Whereas species of the Arctioid clade, with the obvious exception of the widespread colonizers mainly of *Arctium*, are more or less confined to high mountain habitats, species of the Cousinioid clade have diversified and grow in widely different habitats including semidesert areas, open hilly areas, alpine ecosystems, high altitude areas transitional between humid and semiarid ecosystems, oak forest floors and rarely in lowlands. Possibly the differential species richness of the Arctioid and Cousinioid clades on the one hand and the unequal distribution of plesiomorphic vs. apomorphic characters among the two clades are not only correlated but causally linked. However, whether transition to apomorphic characters in the Cousinioid clade is cause or effect of its higher species number can not be easily decided with the data available.

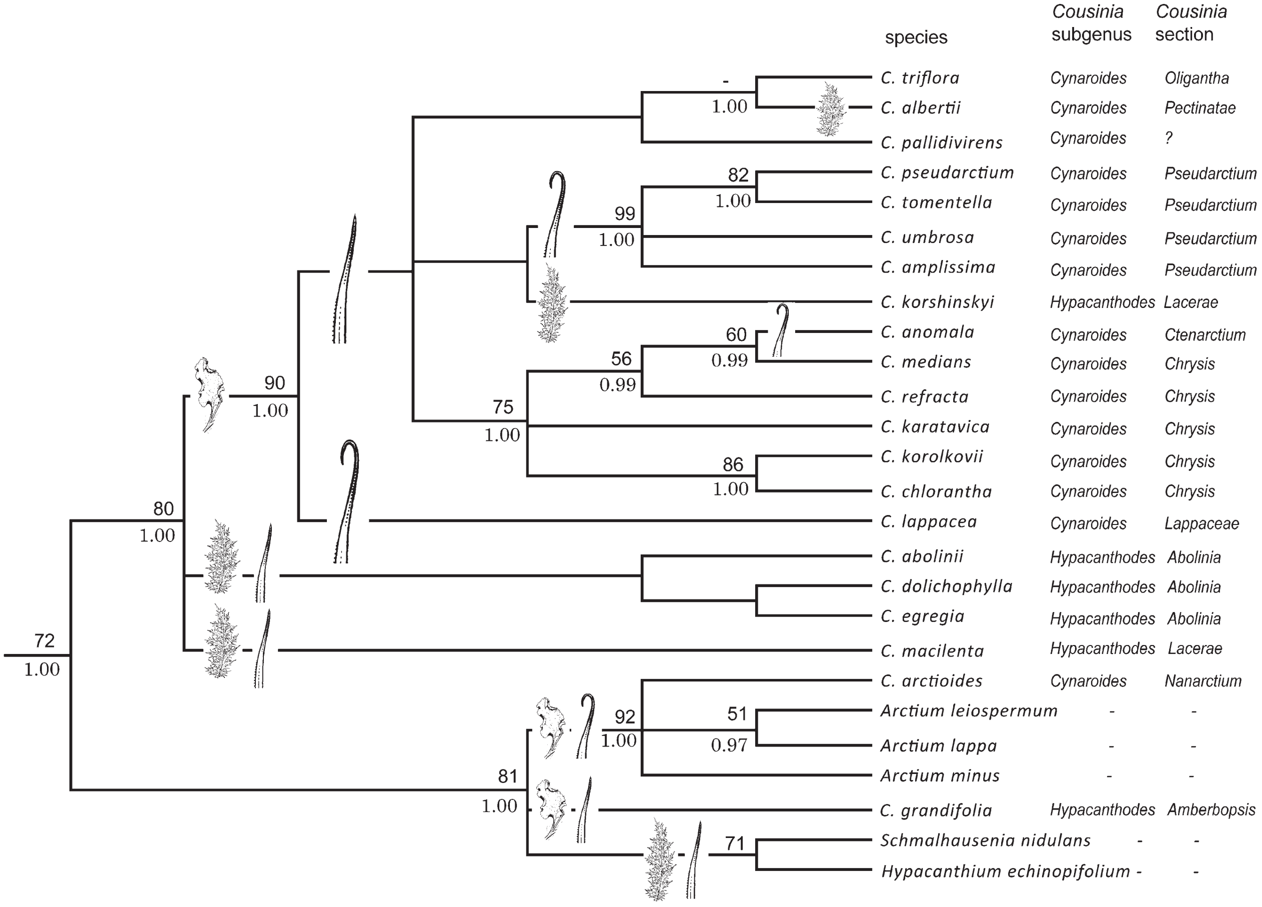


Fig. 6. Distribution of leaf and involucral bract morphology among species of the Arctioid clade mapped on an MP tree of the combined ITS and *rpS4-trnT-trnL* dataset.

The Arctioid clade. — As described in the introduction, the Arctioid clade can not be characterized morphologically beyond pollen and style morphology (neither chromosome number). Part of it (*Arctium* and four species of *Cousinia* subg. *Cynaroides*) have unarmed, large cordate leaves and involucral bracts ending in a recurved hook. Of the remainder of subg. *Cynaroides*, six species have leaves which are not cordate but involucral bracts ending in a hooked spine, and twelve species have involucral bracts ending in a straight spine. The distribution of these two characters precludes the morphological definition of a monophyletic *Arctium*. In subg. *Hypacanthodes* no species has either cordate leaves or hooked involucral bracts. The distribution of spiny leaves also runs across established generic and subgeneric boundaries. All species of *Arctium* and almost all species of *Cousinia* subg. *Cynaroides* have unarmed leaves, and only *C. alberti* Regel & Schmalh. and *C. horrescens* Juz. are spiny. In contrast to this, *Schmalhausenia*, *Hypacanthium* and *Cousinia* subg. *Hypacanthodes* (except *C. grandifolia*) have strongly spiny leaves.

A logical solution to the above character distribution would be to establish a broadly redefined *Schmalhausenia* including *Hypacanthium* and the spiny species of *Cousinia* subg. *Hypacanthodes*, and a broadly redefined *Arctium* including subg. *Cynaroides* and the unarmed *C. grandifolia* from *Cousinia* subg. *Hypacanthodes*. However, this solution clearly is not compatible with molecular evidence presented here. In fact, the tree topology (Fig. 6) suggests the recognition of two to four groups: *(1) Schmalhausenia* plus *Hypacanthium*, *(2) Cousinia grandifolia*, *(3) Arctium* incl. *Cousinia arctioides* and *(4)* the remaining species of *Cousinia* subgg. *Cynaroides* and *Hypacanthodes*. However, this solution is not compatible with morphological evidence. In conclusion, our declared aim to revise generic limits in the Arctioid clade on the basis of a larger taxon sample could not be achieved because of the molecular placement and morphology of the species of *Cousinia* subgg. *Cynaroides* and *Hypacanthodes*. When considering the distribution of leaf and involucral bract morphology across the phylogeny of the entire complex (Fig. 6), it is obvious that either cordate and unarmed or spiny leaves and apically hooked or straight involucral bracts—these two characters are not always correlated—evolved several times in parallel. In view of the uncertainties in our phylogeny we did not try to formally optimize the evolution of these characters.

Interestingly, the only colonizing species of the *Arctium-Cousinia* group, i.e., most species of *Arctium*, have capitula with hooked involucral bracts. Such capitula are dispersed epizoochorously and may be direct cause of the colonizing success of the genus. However, some species with hooked involucral bracts are geographically limited to the Tien-Shan. It seems possible that the biennial habit of *Arctium*—most other species of the Arctioid clade are perennial—contributed to this colonizing success as already suspected by Duistermaat (1996).

The sectional classification of *Cousinia* subg. *Cynaroides* and subg. *Hypacanthodes* is only partly supported by our results, and neither subgenus is monophyletic as presently circumscribed (Fig. 6). One species of subg. *Cynaroides*, *C. arctioides*, is placed within the *Arctium* clade with good parsimony and Bayesian support (BS = 92%, PP = 1.00). This position is fully congruent with morphology as *C. arctioides* has unarmed leaves as well as involucral bracts ending in a recurved hook. We propose to follow the taxonomical criterion of Kuntze (1891) and Duistermaat (1996) which consider this species as *Arctium arctioides* (Schrenk) Kuntze.

Apart from this, our phylogeny is largely congruent with the current sectional classification of subg. *Cynaroides*. The species of sect. *Chrysis* (*C. medians* Juz. through *C. chlorantha*) form a clade with low BS (75%) but high PP (1.0) support. However, this clade also includes *C. anomala* Franch. of sect. *Ctenarctium*. This grouping is not supported by morphological characters as *C. anomala* has involucral bracts ending in a hook and oblong-lanceolate leaves. Section *Pseudarctium* (*C. pseudarctium* Bornm. through *C. amplissima* Boiss.) is a strongly supported clade (BS = 99%, PP = 1.00) containing species with involucral bracts with hooked spines and large, soft and slightly pubescent leaves. Sections *Oligantha* and *Pectinatae* group together with BS < 50% but strong BI support (PP = 1.00). This grouping is not supported by any morphological characters and may result from the poor representation of sect. *Pectinatae* in our sample.

Two species of subg. *Hypacanthodes* were resolved in unexpected position. Thus, *C. korshinskyi* Winkl. of sect. *Lacerae* is part of the subg. *Cynaroides* clade discussed above and separated from the other species of sect. *Lacerae* included in the analysis (*C. macilenta*). This grouping is incongruent with morphology because *C. korshinskyi* is more similar to the rest of subg. *Hypacanthodes* than to subg. *Cynaroides*. *Cousinia grandifolia* is part of the clade containing *Arctium*, *Schmalhausenia* and *Hypacanthium*. In this case, this placement is well correlated with morphology. *Cousinia grandifolia* shares the possession of large, unarmed, oblong-ovate leaves with *Arctium*, and involucral bracts ending in a spine without hook with *Schmalhausenia* and *Hypacanthium*. With these characters *C. grandifolia* is somewhat intermediate between *Arctium* on the one hand and *Schmalhausenia* and *Hypacanthium* on the other hand.

The sectional classification of subg. *Hypacanthodes* is not confirmed by our data. Although the species of sect. *Abolinia* form a clade which is homogeneous morphologically, this clade has no statistical support. *Cousinia macilenta* and *C. korshinskyi* of sect. *Lacerae* are placed in two different and strongly supported clades (see above).

They had been placed in separate series of sect. *Lacerae*, *Macilentae* and *Pterolepideae*, by Tscherneva (1962) and differ in pubescence, height and branching of the stem, leaf size, capitulum pubescence, shape and texture of the involucral bracts and achene ornamentation.

The Cousinioid clade. — As evident from our Bayesian analyses of the ITS, *rpS4-trnT-trnL* and combined datasets (Figs. 2–4), and also from the MP and ML analyses of the combined dataset (not shown), very little phylogenetic resolution can be obtained within *Cousinia* subg. *Cousinia*. Although a varying number of mostly small species groups with high support is recognizable in the different datasets, relationships among species groups are essentially unresolved.

In the following discussion, we first will explore in detail the phylogenetic information contents of our data. This we will do by examining whether clades with ≥ 0.95 PP are plausible in terms of traditional taxonomic assignment (as a proxy for morphological similarity) and/or geographical distribution by growing in the same general area. For this purpose, the following eight major centres of species diversity were defined (Fig. 1): Anatolian mountains, Zagros mountains, Elburz mountains, Kopet Dagh and mountains to the south, West Hindu Kush (C & W Afghanistan), East Hindu Kush (NE Afghanistan), Pamir-Alay and Tien-Shan (Rechinger, 1986; Knapp, 1987). Second, we will investigate possible causes for the very limited amount of phylogenetic resolution obtained.

Phylogenetic information in the rpS4-trnT-trnL, ITS and combined datasets. – In the *rpS4-trnT-trnL* dataset, seven clades (or nested subclades) with ≥ 0.95 PP were obtained (Appendix 2). Of these, clades 3 and 4 are not plausible in terms of traditional taxonomic assignment or geographical distribution. Clade 1 is plausible in both terms, and clades 2–2a and 5–5a, with few exceptions (2–2a: *C. araneosa* DC., *C. macrocephala* C.A. Mey., *C. gatchsaranica* Mehregan, Assadi & Attar; 5–5a: *C. oxiana* Tscherneva, *C. intertexta* Freyn & Sint.), contain species from the Kopet Dagh area (S Turkmenistan, NE & E Iran and NW Afghanistan) and Central Asia, respectively, but of different sections of *Cousinia* subg. *Cousinia*. In the ITS dataset, 28 clades (or nested subclades) with ≥ 0.95 PP were obtained (Appendix 2). Of these, 16 are plausible both in terms of taxonomic assignment and geographical distribution, 2 are plausible in terms of taxonomic assignment only, 4 are plausible in terms of geographical distribution only, and the remaining 6 clades are not plausible in either terms. In the combined dataset, 28 clades (or nested subclades) with ≥ 0.95 PP were obtained (Appendix 2). Of these, 13 are plausible both in terms of taxonomic assignment and geographical distribution, 4 are is plausible in terms of taxonomic assignment only, 4 in terms of geographical distribution only, and the remaining 7 clades are not plausible in either terms.

To summarize the above considerations, our datasets do appear to contain some plausible phylogenetic information mainly in their distal branches. When considering terms of clade plausibility, it also appears that geographical distribution is a more reliable criterion for recognizing phylogenetic relatedness than traditional taxonomic assignment as also found in other studies (e.g., González-Albaladejo & al., 2005).

Most interestingly, and in support of the above conclusion, the annual species (except *C. pygmaea* C. Winkl.—which does not appear in the trees shown—and *C. tenella* which groups outside *Cousinia* subg. *Cousinia* in all our analyses, for discussion see below) group in one clade in the majority of our analyses. In the ITS dataset (Fig. 2), *C. bungeana*, *C. polytimetica* and *C. pusilla* form one clade with 1.0 PP which is part of a polytomy also containing *C. prolifera*. In the *rpS4-trnT-trnL* dataset (Fig. 3), *C. prolifera* Jaub. & Spach and *C. polytimetica* Tscherneva form one clade (0.93 PP) widely separated (although by no branch with ≥ 0.95 PP) from *C. pusilla* C. Winkl. and *C. bungeana* Regel & Schmalh. which are part of a large polytomy. In the Bayesian combined dataset (Fig. 4) these four annual species form one clade with 0.64 PP, of which *C. bungeana*, *C. polytimetica* and *C. pusilla* form a subclade with 1.0 PP. These four annual species are also recovered as a monophyletic group in the MP analysis of the combined dataset (50% BS; *C. bungeana*, *C. polytimetica*, *C. pusilla*: 96% BS; not shown), and *C. bungeana*, *C. polytimetica* and *C. pusilla* are recovered in the ML analysis of the combined dataset (not shown). The monophyly of the annual species (except *C. pygmaea* and *C. tenella*) in the combined dataset even more than the above considerations implies that our ITS and *rpS4-trnT-trnL* sequence data do contain limited but valuable phylogenetic information.

Possible causes for lack of phylogenetic resolution in Cousinia subg. Cousinia. – The first obvious reason for the lack of phylogenetic resolution obtained in the analyses of the ITS, *rpS4-trnT-trnL* and combined datasets is the small number of characters available in relation to the number of taxa analysed. For the 106 species included of *Cousinia* subg. *Cousinia*, the *rpS4-trnT-trnL* dataset yielded 21, the ITS dataset 143 and the combined dataset 140 parsimony informative characters (Table 1). Although in theory the number of parsimony informative characters in the ITS and combined datasets would be sufficient to resolve a phylogeny of 106 species, they are not, because the characters show considerable homoplasy. Although the RI for the *rpS4-trnT-trnL* dataset is high (0.922), it is fairly low for the ITS (0.721) and combined datasets (0.711). The most likely reason for the lack of characters in *Cousinia* subg. *Cousinia* is its comparatively young age (Table 2, Fig. 5) in combination with its high species number. Thus, using the average substitution rate for ITS for herbaceous perennials (Kay & al., 2006) as to some extend justified by the age of the fossil *Arctium* achene by Mai (2001; see above), a crown group age of ca. 8.7 my (node D) was found for *Cousinia* subg. *Cousinia*, and its major diversification started only ca. 7 mya (node E).

Considering the existence of a large number of closely related and often sympatric species with no obvious mechanism of reproductive isolation in *Cousinia* subg. *Cousinia*, hybridization and hybrid speciation seems possible and clearly would confound the reconstruction of phylogeny (Nieto-Feliner & al., 2001; Font & al., 2002; Vriesendorp & Bakker, 2005; Suárez-Santiago & al., 2007). Based on the examination of literature reports of hybrids and intermediate forms and the morphological and molecular examination of some suspected hybrids, Mehregan & Kadereit (in press) concluded that although hybridization does occur in *Cousinia*, it seems to be comparatively rare in the genus. Thus, only 26 hybrids and 12 intermediate forms in *Cousinia* subg. *Cousinia* have been published (Tscherneva 1962; Rechinger, 1972, 1979). These hybrids and intermediate forms involve ca. 11% of the currently recognized species of *Cousinia* subg. *Cousinia*. The two hybrids examined in detail (*C. shahvarica* × *C. decumbens*, *C. eryngioides* × *C. chrysochlora*), confirmed by additivity of their ITS sequences, showed reduced pollen fertility. It is possible that hybridization is much more frequent but undetected because of lack of sufficiently detailed taxonomic knowledge and the existence of several morphologically very similar species. However, the complete absence of polyploid chromosome numbers in *Cousinia* subg. *Cousinia* among the 148 species analyzed (http://www-asteraceae.cla.kobe-u.ac.jp/search.html) implies that polyploid hybrid speciation played no role in the evolution of *Cousinia* subg. *Cousinia*. Nothing can be said about the evolutionary significance of homoploid hybrid speciation in this group.

In summary, there is little evidence that hybridization and/or hybrid speciation are important factors hindering the reconstruction of the phylogeny of *Cousinia* subg. *Cousinia*.

The phylogeny and evolution of Cousinia subg. Cousinia. – As evident from the above discussion, very little can be said about the phylogeny of *Cousinia* subg. *Cousinia*. Although a number of species groups can be recognized which may well represent monophyletic lineages as judged from their taxonomic assignment and/or geographical distribution, relationships among species groups are entirely unclear and no major subdivision of *Cousinia* subg. *Cousinia* is evident from our data. This is most likely the result of paucity of phylogenetic informative characters contained in our ITS and *rpS4*-*trnT*-*trnL* datasets in relation to species number. This in turn is the result of the comparatively young geological age of the group in combination with its species richness. Apart from the recovery of a number of probably monophyletic species groups, our data contain valuable information about the evolution of annuality, a very rare condition in *Cousinia* subg. *Cousinia*. It appears that annuality originated twice in *Cousinia* subg. *Cousinia*. The first lineage of annuals, comprising *C. bungeana*, *C. polytimetica*, *C. pusilla* and *C. prolifera* has been discussed above and is well-nested within *Cousinia* subg. *Cousinia*. The second lineage comprises *C. tenella* and *C. pygmaea* and will be discussed further below.

With its comparatively young age and high species number *Cousinia* subg. *Cousinia* represents a massive radiation which is quite unusual because it took place in a comparatively small geographical area. Based on the results of an ongoing taxonomic revision of part of *Cousinia* subg. *Cousinia* (sect. *Cynaroideae*; Mehregan & Kadereit, 2008), mainly two conclusions can be drawn about the evolution of this group. First, it seems very likely that the number of species described (ca. 600) vastly overstates the number of existing species. Thus, our revision reduced the number of species in sect. *Cynaroideae* from 110 to 31. If reduction in species number by two-thirds eventually will also take place in other parts of *Cousinia* subg. *Cousinia*, it would still remain a large genus with about 200 species. Second, of the ca. 30 species recognized by Mehregan & Kadereit (2008), only two are geographically widespread, namely *C. calocephala* Jaub. & Spach and *C. onopordioides* Ledeb. The remaining species have rather narrow and, most importantly, allo- or parapatric geographical ranges where species boundaries are marked by prominent topographical barriers. This may imply that the predominant speciation process in *Cousinia* subg. *Cousinia* may be geographical speciation. Today, the group is found mainly (but not exclusively) in vegetation types classified as 'Kurdo-Zagrosian and other steppe-forest climaxes' by Zohary (1973). When this type of vegetation is considered essentially identical with 'Mediterranean to sub-Mediterranean *Artemisia*-*Ephedra*-*Juniperus*-Compositae steppe of mountainous regions' as defined by Frenzel & al. (1992), the reconstruction of vegetation during the last glacial maximum (20.000 to 18.000 years ago) by Frenzel & al. (1992) suggests that the geographical range of *Cousinia* subg. *Cousinia* in the last glacial maximum was compressed in only the northern parts of its extant range. This in turn implies substantial changes in geographical distribution of species during the Quaternary. Considering the age of the crown group diversification of *Cousinia* subg. *Cousinia* (ca. 8.7 mya; Table 2) and the age of species which in most cases is older than the onset of the Quaternary (Fig. 5), the climatic oscillations of the Quaternary do not appear to have resulted in widespread extinction of the group. Whether these climatic oscillations instead were a stimulus for speciation (through changes in geographical distribution), as found for, e.g., *Primula* L. sect. *Auricula* and *Epimedium* L. (Kadereit & al., 2004; Zhang & al., 2007), we can not say without further analysis.

***Cousinia tenella.* —** In all our analyses except the BI analysis of the *rpS4*-*trnT*-*trnL* dataset alone, *C. tenella* is sister to the Arctioid and Cousinioid clades. Support for the sister group relationship between the Arctioid and Cousinioid clades increases (BS = 94%, PP = 1.00; tree not shown) when *C. tenella* is removed from the data matrix.

Although only *C. tenella* was included in the datasets shown here, an enlarged ITS dataset of 216 species (not shown) shows *C. pygmaea* to be sister to *C. tenella* with 0.84 PP. Such relationship is plausible considering the great similarity between the two species which differ only in flower colour, relative length of corolla tube and limb and achene shape (Rechinger, 1972). The placement of *C. tenella* in most of our analyses might suggest segregation of *C. tenella* and *C. pygmaea* from *Cousinia* subg. *Cousinia*. On the basis of karyology, pollen type and stigma morphology, however, *C. tenella* (and probably *C. pygmaea*) clearly falls into the Cousinioid clade. *Cousinia tenella* has $2n = 26$ chromosomes according to Aryavand (1975) and Susanna & al. (2003b), the Cousinioid pollen type and stigma morphology (Susanna, pers. comm.). The fact that this species is resolved as sister to the rest of the *Arctium*-*Cousinia* complex probably is due to its annuality, autogamy and probably increased mutation rate. There exist other examples for unexpected placements of annuals in tribe Cardueae. In *Echinops* L., annual species together with the annual *Acantholepis* Less. are sister to the rest of the genus (Garnatje, 2005); in *Ptilostemon* Cass., the only annual species, *Ptilostemon stellatus* (L.) Greuter, is sister to the rest of the genus (Vilatersana & al., in prep.); and in subtribe *Centaureinae* the annual *Schischkinia* Iljin and *Stizolophus* Cass. are successive sisters to the rest of the subtribe (Garcia-Jacas & al., 2006). In all these cases, the position of annuals as sister to the remainder of their respective groups is not congruent with morphology. Several authors have pointed out that annual plants often have an elevated rate of molecular evolution compared with perennials, explained by generation time, population size, and speciation rate (Bousquet & al., 1992; Laroche & al., 1997; Andreasen & Baldwin, 2001). If this indeed should be the explanation for the unexpected placement of *C. tenella*, it is interesting to observe that this problem does not appear to exist for the equally annual *C. bungeana*/*C. polytimetica*/*C. pusilla*/*C. prolifera* lineage which clearly falls into the Cousinioid clade as expected from morphology.

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Appendix 1. Species included in the molecular analysis with voucher information and GenBank accession numbers (ITS1; ITS2; *rpS4-trnT-trnL*). For all species of *Cousinia* the subgeneric classification is given. An asterisk (*) indicates sequences previously published.

***Hypacanthium echinopifolium* (Bornm.) Juz.**, Kyrgyzstan: Sine loc., *Iljin s.n.* (LE), AY373746*; AY373713*; EU661125. ***Jurinea albicaulis* Bunge**, Greece: Makedonia, Halkidiki, between Arnea and Paleokastro, *Susanna 1957 & al.* (BC), AY373747*; AY373714*; EU661092. ***Jurinea humilis* DC.**, Spain: Huesca, Plan, La Barana, *P. Montserrat s.n.* (JACA), L35868*; EU661093. ***Saussurea elegans* Ledeb.**, Kazakhstan: Aksu-Dzabagly reservation, 1,800 m, *Susanna 2179 & al.* (BC), AY373750*; AY373717*; EU661090. ***Saussurea maximowiczii* Herder**, Japan: Ofuna Botanical Garden, AY373751*; AY373718*; EU661091. ***Schmalhausenia nidulans* (Regel) Petrak**, Kazakhstan: Almatinskaya oblast, Alatau mt., above Almaty, *Susanna 2088 & al.* (BC), AY373752*; AY373719*; EU661124. ***Tiarocarpus hymenostephanus* Rech. f.**, Afghanistan: W, Herat, Band-e Farsi, 10 SW Farsi, 3,200 m, *Freitag 6841* (KAS), EU923831; EU923945; EU661139. ***Tiarocarpus neubaueri* (Rech. f.) Rech. f.**, Afghanistan: C, Bamian, Band-i Amir, Hochfächen, 3,000 m, *Dieterle 896* (M), EU923775; EU923889; EU661140. ***Arctium lappa* L.**, Iran: NNE, Khorassan: 25 SW Bodjnourd, 1,500 m, *Mehregan 140* (MJG), EU923773; EU923887; EU661123. ***Arctium leiospermum* Juz. & C. Serg.**, Kazakhstan: Zambylskaya oblast, Kurdai pass, 900 m, *Susanna 2154 & al.* (BC), AY373720*; AY373687*; EU661121. ***Arctium minus* (Hill.) Bernh.**, Belgium: Lovaina Botanical Garden, AF19049*; AF19103*; EU661122. ***Cousinia oopoda* Juz.** (subg. *Cousinia* sect. *Actinia* Bunge), Tajikistan: South Tajikistan, west mountainside, Khodzha-Kazjyan range, Koy-Pjez-Tau mt., limestone outcrops, 1,240 m, *Kinzikaeva 3585* (LE), EU923839; EU923953; EU661232. ***Cousinia tashkurghanica* Rech. f.** (subg. *Cousinia* sect. *Actinia* Bunge), Afghanistan: N, Mazar Sharif, Tang-e Sayyad, S of Tashkurghan, 600 m, *Freitag 3905* (KAS), EU923777; EU923891; EU661234. ***Cousinia oligocephala* Boiss.** (subg. *Cousinia* sect. *Albidae* Rech. f.), Iran: Fars, Inter Shiraz and Sepidan, Shoul pass, 2,000 m, *Mehregan 218* (MJG), EU923796; EU923910; EU661208. ***Cousinia chrysantha* Kult.** (subg. *Cousinia* sect. *Alpinae* Bunge), Kazakhstan: Shimkientskaya oblast, Aksu Dzabagly reservation, Darbassa canyon, 1,840 m, *Susanna 2198 & al.* (BC), AY373725*; AY373692*; EU661136. ***Cousinia dipterocarpa* Bornm. & Rech. f.** (subg. *Cousinia* sect. *Alpinae* Bunge), Iran: Khorassan, 27 km from Ghouchan to Bajgiran, Alam Ali pass, 1,820 m, *Mehregan 167* (MJG), EU923862; EU923976; EU661155. ***Cousinia lasiolepis* Boiss.** (subg. *Cousinia* sect. *Alpinae* Bunge), Iran: Yasoudj, Dena mts., Bizhan Pass, 3,300 m, *Mehregan 202* (MJG), EU923863; EU923977; EU661177. ***Cousinia multiloba* DC.** (subg. *Cousinia* sect. *Alpinae* Bunge), Iran: Khorassan, SW Bodjnourd, Salouk mt., 2,200 m, *Mehregan 189* (MJG), EU923835; EU923949; EU661202. ***Cousinia serawschanica* C. Winkl.** (subg. *Cousinia* sect. *Alpinae* Bunge), Tajikistan: Voru, 2,000–2,300 m, *Susanna 2516 & al.* (BC), EU923874; EU923988; EU661192. ***Cousinia splendida* C. Winkl.** (subg. *Cousinia* sect. *Alpinae* Bunge), Tajikistan: Fan mountains, road above Iskandar-Kul, 2,300 m, *Susanna 2507 & al.* (BC), EU923871; EU923985; EU661197. ***Cousinia badghysi* Kult.** (subg. *Cousinia* sect. *Badghysia* Tschern.), Turkmenistan: Badghys, Eroylanduz lake, SE ¨sopki¨ Kazan, *Kamelin 360* (LE), EU923872; EU923986; EU661166. ***Cousinia piptocephala* Bunge** (subg. *Cousinia* sect. *Badghysia* Tschern.), Iran: Isfahan, 5km from Mourcheh Khort towards Natanz, 1,500 m, *Mehregan 188* (MJG), EU923801; EU923915; EU661204. ***Cousinia ariana* Bornm.** (subg. *Cousinia* sect. *Carduncellus* (Juz.) Rech. f.), Afghanistan: E, Kabul, N Salang, 3,150 m, *Freitag 3494* (KAS), EU923788; EU923902; EU661221. ***Cousinia buphthalmoides* Regel** (subg. *Cousinia* sect. *Carduncellus* (Juz.) Rech. f.), Afghanistan: Unai-Pass, E side, 3,050 m, *Freitag 6268* (KAS), EU923789; EU923903; EU661179. ***Cousinia tianschanica* Kult.** (subg. *Cousinia* sect. *Carduncellus* (Juz.) Rech. f.), Kazakhstan: Shimkientskaya oblast, Aksu Dzabagly reservation, Aksu canyon, *Susanna 2191 & al.* (BC), AY373743*; AY373710*; EU661127. ***Cousinia dissecta* Kar. & Kir.** (subg. *Cousinia* sect. *Chrysoptera* Tschern.), Kazakhstan: Almatinskaya oblast, Sugati mts., Kokpekti narrows, *Susanna 2137 & al.* (BC), AY373728*; AY373695*; EU661141. ***Cousinia oxiana* Tschern.** (subg. *Cousinia* sect. *Chrysoptera* Tschern.), Afghanistan: NW, Herat, 34 km N of Shindand, 1,320 m, *Amin 5252* (KAS), EU923854; EU923968; EU661227. ***Cousinia turkmenorum* Bornm.** (subg. *Cousinia* sect. *Chrysoptera* Tschern.), Iran: Khorassan, 20 km from Mashhad to Neishabour, 1,150 m, *Mehregan 197* (MJG), EU923855; EU923969; EU661156. ***Cousinia greuteri* Rech. f.** (subg. *Cousinia* sect. *Congesta* Bunge), Afghanistan: N, Robatak pass between Haibak and Puli-Khumri, 1,400 m, *Tonchev 2200* (KAS), EU923783; EU923897; EU661237. ***Cousinia coronata* Franch.** (subg. *Cousinia* sect. *Coronophora* (Juz.) Rech. f), Uzbekistan: road Tashkent–Samarkand, 10 km from the crossroads to Jizzak, 400 m, *Susanna 2039 & al.* (BC), AY373727*; AY373694*; EU661178. ***Cousinia radians* Bunge** (subg. *Cousinia* sect. *Coronophora* (Juz.) Rech. f.), Iran: Khorassan, 66 km from Mashad to Kalat-Naderi, 1,000 m, *Ghahreman & al. s.n.* (TUH), EU923791; EU923905; EU661217. ***Cousinia congesta* Bunge** (subg. *Cousinia* sect. *Cousinia*), Uzbekistan: between Samarkand and Kitov, Takhta–Karachi pass, 1,600 m, *Susanna 2059 & al.* (BC), AY373726*; AY373693*; EU661131. ***Cousinia daralaghezica* Takht.** (subg. *Cousinia* sect. *Cousinia*), Armenia: Vajots Dzor, Eghegnadzor distr., Eghegnadzor town, *Tamanian s.n.* (ERE), EU923813; EU923927; EU661216. ***Cousinia intertexta* Freyn & Sint.** (subg. *Cousinia* sect. *Cousinia*), Turkey: Sivas, Divrigi-Arapkir, 20 km S Divrigi, 1,410 m, *Nydegger 17154* (M), EU923817; EU923931; EU661235. ***Cousinia meghrica* Takht.** (subg. *Cousinia* sect. *Cousinia*), Armenia: Sjunik, Meghri distr., Agarak village, *Tamanian s.n.* (ERE), EU923814; EU923928; EU661142. ***Cousinia minkwitziae* Bornm.** (subg. *Cousinia* sect. *Cousinia*), Kazakhstan: Zambylskaya oblast, Talaski Alatau, 6 km W from Il Tai, 1,000 m, *Susanna 2183 & al.* (BC), AY373735*; AY373702*; EU661135. ***Cousinia polycephala* Rupr.** (subg. *Cousinia* sect. *Cousinia*), Kazakhstan: Zambylskaya oblast, 30 km from the Kurdai pass, *Susanna 2161 & al.* (BC), AY373738*; AY373705*; EU661138. ***Cousinia syrdariensis* Kult.** (subg. *Cousinia* sect. *Cousinia*), Kazakhstan: Zambylskaya oblast, 30 km from the Kurdai pass, *Susanna 2159 & al.* (BC), AY373741*; AY373708*; EU661133. ***Cousinia tenuifolia* C.A. Mey.** (subg. *Cousinia* sect. *Cousinia*), Iran: Azerbaijan, 10 km from Sarab to Ardebil, 1,400 m, *Mehregan 186* (MJG), EU923812; EU923926; EU661171. ***Cousinia urumiensis* Bornm.** (subg. *Cousinia* sect. *Cousinia*), Iran: Azerbaijan, Urmia, inter Silvana and Razhan, 1,650 m, *Mehregan 201* (MJG), EU923809; EU923923; EU661205. ***Cousinia araneosa* DC.** (subg. *Cousinia* sect. *Cynaroideae* Bunge), Iran: Hamedan, Alvand mt., 10 km from Hamedan to Toiserkan, Ganjnameh Valley, 2,400 m, *Mehregan 219* (MJG), EU923870; EU923984; EU6611678. ***Cousinia macrocephala* C.A. Mey.** (subg. *Cousinia* sect. *Cynaroideae* Bunge), Iran: Azerbaijan, Arasbaran, Molouk pass, 1,500 m, *Mehregan 212* (MJG), EU923852; EU923966; EU661169. ***Cousinia purpurea* C.A. Mey. in DC.** (subg. *Cousinia* sect. *Cynaroideae* Bunge), Armenia: Ararat, near the village of Shahap, roadsides, *Tamanian s.n.* (ERE), AY373739*; AY373706*; EU661137. ***Cousinia decumbens* Rech. f.** (subg. *Cousinia* sect. *Decumbentes* Rech. f.), Iran: Semnan, Shahroud, mt. Shahvar, SW Slopes, 3,600 m,

Appendix 1. Continued.

Mehregan 110 (MJG), EU923821; EU923935; EU661157. ***Cousinia bungeana* Regel & Schmalh.** (subg. *Cousinia* sect. *Dichotomae* Bunge), Sine loc.: *Ovczinnikov 16104* (TAD), EU923786; EU923900; EU661130. ***Cousinia polytimetica* Tschern.** (subg. *Cousinia* sect. *Dichotomae* Bunge), Uzbekistan: Bukharskaya reg., deserted left bank of Zeravshan river, to SE from Uzlishkent vil., *Kryakin s.n.* (LE), EU923785; EU923899; EU661244. ***Cousinia pusilla* C. Winkl.** (subg. *Cousinia* sect. *Dichotomae* Bunge), Tajikistan: S Tajikistan, spackled rock mountains to the S from Besharcha mts. to Babatag range, *Botschantzev 117* (LE), EU923787; EU923901; EU661189. ***Cousinia auriculata* Boiss.** (subg. *Cousinia* sect. *Eriocousinia* Tschern.), Afghanistan: E, Parvan, Ghowrband, Sorkh-Parsa, 2,050 m, *Freitag 1128* (KAS), EU923830; EU923944; EU661191. ***Cousinia chionophila* Rech. & Koie** (subg. *Cousinia* sect. *Eriocousinia* Tschern.), Afghanistan: SE, Ghazni, mts. SE of Dashti Nawor (Sperlu Buli), 3,850 m, *Freitag 1478* (KAS), EU923829; EU923943; EU661247. ***Cousinia gmelini* C. Winkl.** (subg. *Cousinia* sect. *Eriocousinia* Tschern.), Iran: Kandevan to Yoush, *Djavadi s.n.* (IRAN), EU923842; EU923956; EU661207. ***Cousinia polyneura* Rech. f.** (subg. *Cousinia* sect. *Eriocousinia* Tschern.), Afghanistan: E, Kabul, mts. above Paghman, 4,000 m, *Freitag 3555* (KAS), EU923781; EU923895; EU661231. ***Cousinia xanthacantha* Regel** (subg. *Cousinia* sect. *Eriocousinia* Tschern.), Afghanistan: Kabul, in dec. orient. jugi Unai, *Breckle 2286* (M), EU923778; EU923892; EU661230. ***Cousinia harazensis* Rech. f.** (subg. *Cousinia* sect. *Harazensis* Rech. f.), Iran: Mazandaran, Haraz road, Rineh, 1,900 m, *Mehregan 175* (MJG), EU923825; EU923939; EU661146. ***Cousinia gatchsaranica* I. Mehregan, Assadi & Attar** (subg. *Cousinia* sect. *Haussknechtii* Rech. F.), Iran: Gachsaran, N slopes of Khami mt., above Tol Chegah, 2,100 m, *Mehregan 83246* (MJG), EU923816; EU923930; EU661159. ***Cousinia helianthia* Bunge** (subg. *Cousinia* sect. *Helianthae* Bunge), Iran: Khorassan, 30 km from Sabzevar to Esferaien, 1,550 m, *Mehregan 170* (MJG), EU923810; EU923924; EU661172. ***Cousinia coerulea* Kult. ex Tschern.** (subg. *Cousinia* sect. *Homalochaete* C. Winkl.), Tajikistan: Vorzov canyon, Kudratov, *Susanna 2459 & al.* (BC), EU923873; EU923987; EU661144. ***Cousinia crassipes* Kult.** (subg. *Cousinia* sect. *Homalochaete* C. Winkl.), Iran: Khorassan, Kalat-e Naderi, Ghareh-Sou, 1,400 m, *Mehregan 144* (MJG), EU923811; EU923925; EU661143. ***Cousinia podophylla* Tschern.** (subg. *Cousinia* sect. *Homalochaete* C. Winkl.), Sine loc., *Ouchinniku 258* (TAD), EU923847; EU923961; EU661233. ***Cousinia raddeana* C. Winkl.** (subg. *Cousinia* sect. *Hoplophylla* Tschern.), Iran: Khorassan, Mashhad, Kalat-e Naderi road, Sanganeh, Chahchaheh, 1,600 m, *Ghahreman & al. 27315* (TUH), EU923853; EU923967; EU661194. ***Cousinia immitantiformis* Rech.f** (subg. *Cousinia* sect. *Immitantes* Rech. f.), Afghanistan: NW, Sabzak pass, NE Herat, 2,400 m, *Freitag 6784* (KAS), EU923846; EU923960; EU661149. ***Cousinia salangensis* Rech. f.** (subg. *Cousinia* sect. *Immitantes* Rech. f.), Afghanistan: E, Kabul, S Salang valley, marble area, 2,500 m, *Freitag 3490* (KAS), EU923845; EU923959; EU661134. ***Cousinia jassyensis* C. Winkl.** (subg. *Cousinia* sect. *Jurineopsis* (Juz.) Tschern.), Kyrgyzstan: SW, Jalal Abad Oblast, Toktogul Rayoh, 790 m, *Martins 872* (JE), EU923790; EU923904; EU661240. ***Cousinia smirnowii* Trautv.** (subg. *Cousinia* sect. *Kopetdagia* Tschern.), Iran: Khorassan, SW Bodjnourd, Salouk mt., 2,300 m, *Mehregan 152* (MJG), EU923802; EU923916; EU661154. ***Cousinia chrysochlora* Rech. f. & Koeie** (subg. *Cousinia* sect. *Lachnosphaerae* Rech. f.), Iran: Khorassan, 30 km from Sedeh to Asad Abad, Mahousak, 2,000 m, *Mehregan 198* (MJG), EU923856; EU923970; EU661148. ***Cousinia lachnosphaera* Bunge** (subg. *Cousinia* sect. *Lachnosphaerae* Rech. f.), Iran: Khorassan, 40 km from Gonabad to Ferdous, 1,900 m, *Mehregan 145* (MJG), EU923857; EU923971; EU661209. ***Cousinia alepideae* Boiss.** (subg. *Cousinia* sect. *Lasiandrae* Bunge), Afghanistan: SW, Ghazni, Jamroud, 45 km SW of Ghazni, 2,010 m, *Freitag 2008* (KAS), EU923794; EU923908; EU661182. ***Cousinia lasiandra* Bunge** (subg. *Cousinia* sect. *Lasiandrae* Bunge), Iran: Khorassan, 3 km from Doust Abad to Birjand, 1,500 m, *Mehregan 127* (MJG), EU923822; EU923936; EU661185. ***Cousinia triceps* Kult.** (subg. *Cousinia* sect. *Leiacanthos* Tschern.), Uzbekistan: Kughitang range, Tanghydevol canyon, rocky area, 870 m, *Kamelin 870* (LE), EU923784; EU923898; EU661129. ***Cousinia antonowii* C. Winkl.** (subg. *Cousinia* sect. *Leiocaules* Bunge), Iran: Khorassan, 10 km from Tivan to Bajgiran, 1,590 m, *Mehregan 196* (MJG), EU923849; EU923963; EU661200. ***Cousinia arctotidifolia* Bunge** (subg. *Cousinia* sect. *Leiocaules* Bunge), Iran: Khorassan, Shirvan, 10 km from Ziarat to Lojelli, 1,215 m, *Mehregan 120* (MJG), EU923848; EU923962; EU661165. ***Cousinia heterophylla* Boiss.** (subg. *Cousinia* sect. *Lepidae* Bunge), Afghanistan: W, N-Kattawz, between Kotanni-Kotal and Patanna, 2,150 m, *Freitag 3345* (KAS), EU923804; EU923918; EU661184. ***Cousinia lepida* (Bunge ex) Boiss.** (subg. *Cousinia* sect. *Lepidae* Bunge), Iran: Khorassan, Esferaien, 5 km Dahaneh Ojagh to Esferaien, 1,420 m, *Mehregan 114* (MJG), EU923805; EU923919; EU661183. ***Cousinia linczewskii* Juz.** (subg. *Cousinia* sect. *Leucocaulon* Tschern.), Iran: Khorassan, Inter Mashhad and Sarakhs, Mazd-Avand pass, 1,000 m, *Mehregan 138* (MJG), EU923850; EU923964; EU661153. ***Cousinia turcomanica* C. Winkl.** (subg. *Cousinia* sect. *Leucocaulon* Tschern.), Iran: Khorassan, 110 km W of Bojnourd, between Spakhou and Kastan, 2,000 m, *Mehregan 105* (MJG), EU923834; EU923948; EU661174. ***Cousinia rigida* Kult.** (subg. *Cousinia* sect. *Lopholepis* Tschern.), Kyrgyzstan: Talasskaya reg., north mountainside of Talassky Alatau, Kur-Bokair canyon, south rocky slope, *Popova s.n.* (LE), EU923869; EU923983; EU661222. ***Cousinia arachnoidea* Fisch. & C.A. Mey.** (subg. *Cousinia* sect. *Microcarpae* Bunge), Kazakhstan: Almatinskaya oblast, Malai Sary pass, 700 m, *Susanna 2140 & al.* (BC), AY373722*; AY373689*; EU661248. ***Cousinia ninae* Juz.** (subg. *Cousinia* sect. *Microcarpae* Bunge), Kyrgyzstan: Oshskaya, Torgulsky reg., Oitaya area north from Shoporovo vil., speckled soils, *Sultanova s.n.* (LE), EU923868; EU923982; EU661224. ***Cousinia platylepis* Schrenk. ex Fisch. & C.A. Mey.** (subg. *Cousinia* sect. *Microcarpae* Bunge), Kazakhstan: Zambylskaya oblast, 30 km from the Kurdai pass, *Susanna 2158 & al.* (BC), AY373737*; AY373704*; EU661236. ***Cousinia sewertzowii* Regel** (subg. *Cousinia* sect. *Microcarpae* Bunge), Kazakhstan: Aksu-Dzabagly reservation, 1,800 m, *Susanna 2178 & al.* (BC), AY373740*; AY373707*; EU661175. ***Cousinia prolifera* Jaub. & Spach** (subg. *Cousinia* sect. *Microcousinia* Tschern.), Iran: Khorassan, 110 km from Birjand to Ferdous, 1,500 m, *Mehregan 233* (MJG), EU923779; EU923893; EU661245. ***Cousinia heteroloba* Rech. f.** (subg. *Cousinia* sect. *Molles* Schrenk.), Afghanistan: NE, Andarab valley below Khinjan, near Ghazan, 1,000 m, *Freitag 3043* (KAS), EU923776; EU923890; EU661198. ***Cousinia candolleana* Jaub. & Spach.** (subg. *Cousinia* sect. *Myriotemae* Rech. f.), Iran: Ghazvin, 10 km from Abgarm to Avadj, 1,950 m, *Mehregan 239* (MJG), EU923858; EU923972; EU661195. ***Cousinia olgae* Regel. & Schmalh.** (subg. *Cousinia* sect. *Olgaeanthe* Tschern.), Iran: Khorassan, 40 km from Torbat-e Djam to Saleh Abad, 1,550 m, *Mehregan 194* (MJG), EU923780; EU923894; EU661203. ***Cousinia eriophylla* (Kult.) Bornm.** (subg. *Cousinia* sect. *Platyacanthae* Rech. f.), Iran: Khorassan, Inter Ghouchan and Bajgiran, 5 km S of Dorbadam, 1,500 m, *Mehregan 190* (MJG), EU923840; EU923954; EU661160. ***Cousinia rechingerorum* Bornm.** (subg. *Cousinia* sect. *Platyacanthae* Rech. f.), Iran: Khorassan: 30 km W of Torbat-e Djam, E slopes of Bezd

Appendix 1. Continued.

mt., 2,000 m, *Mehregan 163* (MJG), EU923841; EU923955; EU66116. ***Cousinia trachyphyllaria*** **Bornm. & Rech. f.** (subg. *Cousinia* sect. *Platyacanthae* Rech. f.), Iran: Khorassan, Neishabour, Khorw, 2,100 m, *Mehregan 217* (MJG), EU923828; EU923942; EU661163. ***Cousinia apiculata*** **Tschern.** (subg. *Cousinia* sect. *Pseudactinia* Tschern.), Iran: Khorassan, Shirvan, Sarani, 2,300 m, *Ghahreman & Attar s.n.* (MJG), EU923792; EU923906; EU661162. ***Cousinia oreodoxa*** **Bornm. & Sint.** (subg. *Cousinia* sect. *Pseudactinia* Tschern.), Iran: Khorassan, Inter Bodjnourd and Raz, 7 km from Tangeh Torkeman to Ashkhaneh, 1,000 m, *Mehregan 150* (MJG), EU923793; EU923907; EU661151. ***Cousinia belangeri*** **DC.** (subg. *Cousinia* sect. *Pugioniferae* Bunge), Iran: Tehran, 15 km to Karaj, 1,200 m, *Mehregan s.n.* (MJG), EU923819; EU923933; EU661210. ***Cousinia calcitrapa*** **DC.** (subg. *Cousinia* sect. *Pugioniferae* Bunge), Iran: Fars, pass inter Sepidan and Yassoudj, 20 km from Sepidan, 2,400 m, *Mehregan 203* (MJG), EU923820; EU923934; EU661220. ***Cousinia incompta*** **DC.** (subg. *Cousinia* sect. *Pugioniferae* Bunge), Iran: 20 km Borujen to Isfahan, 2,400 m, *Mehregan 128* (MJG), EU923851; EU923965; EU661186. ***Cousinia pineticola*** **Rech. f. & Gilli** (subg. *Cousinia* sect. *Racemosae* Rech. f.), Afghanistan: E, Kabul, Korogh Koh, SW of Kabul, 2,600 m, *Freitag 1568* (KAS), EU923832; EU923946; EU661107. ***Cousinia botschantzevii*** **Juz. ex Tschern.** (subg. *Cousinia* sect. *Regelianae* (Juz.) Tschern.), Uzbekistan: Kuramit mt., Koitash range, S slope above the Koitash village, 1,850 m, *Kamelin 29* (LE), EU923782; EU923896; EU661250. ***Cousinia autranii*** **C. Winkl.** (subg. *Cousinia* sect. *Rigidissimae* Rech. f.), Afghanistan: W, Herat, Ghorat, Bande Frasi, 15 km SSE Farsi, 2,400–2,500 m, *Freitag 6831* (KAS), EU923860; EU923974; EU661105. ***Cousinia rigidissima*** **Rech. f.** (subg. *Cousinia* sect. *Rigidissimae* Rech. f.), Afghanistan: W, Herat, Ghorat, in summo jugo 5 km S Dahan-e Kaftarkhan ad viam inter Tulak et Farsi, 2,350 m, *Podlech 19193* (M), EU923861; EU923975; EU661187. ***Cousinia scariosa*** **Regel** (subg. *Cousinia* sect. *Scariosae* Rech. f.), Afghanistan: N, Sar-e Pol, Sangcharak, 40 km SSW mts. above Damdaran, 1,900–2,500 m, *Freitag 6594* (KAS), EU923838; EU923952; EU661190. ***Cousinia eryngioides*** **Boiss.** (subg. *Cousinia* sect. *Sciadocousinia* Tschern.), Iran: Khorassan, 50 km from Mashhad to Neishabour, 1,500 m, *Mehregan 168* (MJG), EU923836; EU923950; EU661164. ***Cousinia concolor*** **Bunge** (subg. *Cousinia* sect. *Serratuloides* Bunge), Iran: Semnan, Shahroud, S slopes of Shahvar mt., above Nekarmann, 2,500 m, *Mehregan 158* (MJG), EU923808; EU923922; EU661150. ***Cousinia crispa*** **Jaub. & Spach**. (subg. *Cousinia* sect. *Serratuloides* Bunge), Iran: Chalous road, 10 km from Kandavan to Chalous, 2,000 m, *Mehregan 185* (MJG), EU923843; EU923957; EU661241. ***Cousinia hypoleuca*** **Boiss.** (subg. *Cousinia* sect. *Serratuloides* Bunge), Iran: Tehran, Inter Firouzkouh and Polur, 5 km W of Lasem, 2,500 m, *Mehregan 177* (MJG), EU923807; EU923921; EU661176. ***Cousinia pinarocephala*** **Boiss.** (subg. *Cousinia* sect. *Serratuloides* Bunge), Iran: Tehran, Firouzkouh, Gadouk pass, 2,200 m, *Mehregan 192* (MJG), EU923844; EU923958; EU661223. ***Cousinia adenosticta*** **Bornm.** (subg. *Cousinia* sect. *Sphaerocephalae* Bunge), Iran: Tehran, between Shemshak and Dizin, 3,000 m, *Mehregan 165* (MJG), EU923827; EU923941; EU661110. ***Cousinia leptolepis*** **(Bornm. & Gauba) Rech. f.** (subg. *Cousinia* sect. *Sphaerocephalae* Bunge), Iran: Tehran, Karadj, Kuh Dashteh, S slopes, 2,200 m, *Mehregan 111* (MJG), EU923826; EU923940; EU661206. ***Cousinia shahvarica*** **Rech.f.** (subg. *Cousinia* sect. *Sphaerocephalae* Bunge), Iran: Semnan, Shahroud, mt. Shahvar, S slopes, 3,000 m, *Mehregan 117* (MJG), EU923823; EU923937; EU661180. ***Cousinia xiphiolepis*** **Boiss.** (subg. *Cousinia* sect. *Sphaerocephalae* Bunge), Iran: Tehran, inter Firouzkouh and Polur, 5 km W of Lasem, 2,600 m, *Mehregan 179* (MJG), EU923824; EU923938; EU661188. ***Cousinia longifolia*** **C. Winkl. & Bornm.** (subg. *Cousinia* sect. *Spinuliferae* Rech. f.), Iran: Kerman, Laleh zar to Rabor, *Attar 226* (MJG), EU923815; EU923929; EU661158. ***Cousinia qaisarensis*** **Rech. f.** (subg. *Cousinia* sect. *Spinuliferae* Rech.f.), Afghanistan: NW, Fariab, Maimana, Qaisar, 20 km, mts. above Shakh (Gala Shakh), 2,200 m, *Freitag 6745* (KAS), EU923833; EU923947; EU661201. ***Cousinia alexeenkoana*** **Bornm.** (subg. *Cousinia* sect. *Stenocephalae* Bunge), Iran: Isfahan, 10 km from Khaansaar to Boein, 2,500 m, *Mehregan 132* (MJG), EU923799; EU923913; EU661181. ***Cousinia cylinracea*** **Boiss.** (subg. *Cousinia* sect. *Stenocephalae* Bunge), Iran: Fars, inter Shiraz and Sepidan, Shoul pass, 2,000 m, *Mehregan 213* (MJG), EU923797; EU923911; EU661108. ***Cousinia gaubae*** **Bornm.** (subg. *Cousinia* sect. *Stenocephalae* Bunge), Iran: Ghazvin, Karadj, in declivibus lapidosis, 1,400 m, *Rechinger 5504* (M), EU923818; EU923932; EU661109. ***Cousinia tenuiramula*** **Rech. f.** (subg. *Cousinia* sect. *Stenocephalae* Bunge), Iran: Bakhtiari, Rokh pass, 2,200 m, *Mehregan 206* (MJG), EU923798; EU923912; EU661214. ***Cousinia thamnodes*** **Boiss. & Hausskn.** (subg. *Cousinia* sect. *Stenocephalae* Bunge), Iran: Gachsaran, Khami mt. above Tol Chegah, 3,170 m, *Mehregan 237* (MJG), EU923800; EU923914; EU661212. ***Cousinia komarovii*** **(O. Kuntze) C. Winkl.** (subg. *Cousinia* sect. *Stenoloma* Juz.), Iran: Khorassan, 15 km Bajgiran to Ghouchan, 1,700 m, *Mehregan 156* (MJG), EU923803; EU923917; EU661152. ***Cousinia leucantha*** **Bornm. & Sint.** (subg. *Cousinia* sect. *Stenoloma* Juz.), Iran: Golestan Nat. Park, 12 km ENE of Tange Gol, 1,900–2,100 m, *Akhani 11357* (M), EU923837; EU923951; EU661170. ***Cousinia stocksii*** **C.Winkl.** (subg. *Cousinia* sect. *Stocksianae* Rech. f.), Afghanista: W, Farah, Schucht, 1 km nördlich Hakumate Purchaman, 1,880 m, *Podlech 21787* (M), EU923795; EU923909; EU661116. ***Cousinia tenella*** **Fisch. & C.A. Mey.** (subg. *Cousinia* sect. *Tenellae* Bunge), Iran: Golestan Nat. Park, between Sharlegh and Cheshmeh Khan, *Akhani 243* (MJG), EU923774; EU923888; EU661104. ***Cousinia chaetocephala*** **Kult.** (subg. *Cousinia* sect. *unknown*), Iran: Khorassan, Tchenaran, 3km from Meritchegan to Radekan, 1,500 m, *Mehregan 115* (MJG), EU923806; EU923920; EU661145. ***Cousinia pallidivirens*** **Kult.** (subg. *Cynaroides* Tschern. unassigned to any section), Uzbekistan: Sine loc., *Botschantzev s.n.* (LE), EU923768; EU923882; EU661126. ***Cousinia chlorantha*** **Kult.** (subg. *Cynaroides* Tschern. sect. *Chrysis* Juz.), Uzbekistan: Malguzar mt., S slope, by Tashkesken ¨say¨, *Kamelin 199* (LE), EU923864; EU923978; EU661120. ***Cousinia karatavica*** **Regel et Schmalh.** (subg. *Cynaroides* Tschern. sect. *Chrysis* Juz.), Kazakhstan: Zambylskaya oblast, Karatau mt., Kuyuk pass, 1,000 m, *Susanna 2162 & al.* (BC), AY373732*; AY373699*; EU661101. ***Cousinia korolkowii*** **Regel & Schmalh.** (subg. *Cynaroides* Tschern. sect. *Chrysis* Juz.), Uzbekistan: N macro-mountainside, Nuratau range, Sintob kishlak surroundings, rocky slopes, *Botschantzev 427* (LE), EU923865; EU923979; EU661103. ***Cousinia medians*** **Juz.** (subg. *Cynaroides* Tschern. sect. *Chrysis* Juz.), Uzbekistan: S, Supkhandarbinskaya reg., between kishlaks Sajrob and Shurob, 121-122 km of the road from Termez, Speckled rock outcrops, *Botschantzev 240* (LE), EU923769; EU923883; EU661100. ***Cousinia refracta*** **(Bornm.) Juz.** (subg. *Cynaroides* Tschern. sect. *Chrysis* Juz.), Tajikistan: Kondara river canyon, Varzowski Rayon reservation, *Susanna 2456 & al.* (BC), EU923867; EU923981; EU661111. ***Cousinia anomala*** **Franch.** (subg. *Cynaroides* Tschern. sect. *Ctenarctium* Juz.), Tajikistan: Slopes above Voru, about 300 m above Kishlak, 2,200–2,300 m, *Susanna 2521 & al.* (BC), EU923770; EU923884; EU661115. ***Cousinia lappacea*** **Schrenk** (subg. *Cynaroides* Tschern. sect. *Lappaceae* Bunge), Kazakhstan: Zambylskaya oblast, Kurdai pass, 900 m, *Susanna 2150 & al.* (BC), AY373733*;

Appendix 1. Continued.

AY373700*; EU661112. ***Cousinia arctioides*** **Schrenk** (subg. *Cynaroides* Tschern. sect. *Nanarctium* Juz. ex. Tschern.), Kazakhstan: Dzhezkazganskaya reg., Turgajskaya lowland, 49 km to SW from Dzhezkazgana, right bank of Kumula river, Tamarix bushland, *Kamelin 6434* (LE), EU923772; EU923886; EU661118. ***Cousinia triflora*** **Schrenk** (subg. *Cynaroides* Tschern. sect. *Oligantha* Juz.), Iran: Golestan Nat. Park, Yakhbala pass, *Akhani 102* (MJG), EU923771; EU923885; EU661094. ***Cousinia albertii*** **Regel et Schmalh.** (subg. *Cynaroides* Tschern. sect. *Pectinatae* C. Winkl.), Kazakhstan: Shimkientskaya oblast, Mashat canyon, 1,840 m, *Susanna 2206 & al.* (BC), AY373721*; AY373688*; EU661099. ***Cousinia amplissima*** **(Boiss.) Boiss.** (subg. *Cynaroides* Tschern. sect. *Pseudarctium* Juz.), Iran: Dena, 15 km from Meimand to Yassoudj, 2,500 m, *Mehregan 174* (MJG), EU923766; EU923880; EU661098. ***Cousinia pseudarctium*** **Bornm.** (subg. *Cynaroides* Tschern. sect. *Pseudarctium* Juz.), Tajikistan: Vorzov valley, 2 km N kishlak Ziddy, *Susanna 2477 & al.* (BC), EU923876; EU923990; EU661095. ***Cousinia tomentella*** **C. Winkl.** (subg. *Cynaroides* Tschern. sect. *Pseudarctium* Juz.), Tajikistan: S mountainside of Guissar range, left bank of Varzob river, Deamalik kishlak surroundings, 1,800 m, *Tschukavina 10512* (LE), EU923767; EU923881; EU661097. ***Cousinia umbrosa*** **Bunge** (subg. *Cynaroides* Tschern. sect. *Pseudarctium* Juz.), Kazakhstan: Almatinskaya oblast, Alatau mt. above Almaty, 1,200 m, *Susanna 2100 & al.* (BC), AY373745*; AY373712*; EU661096. ***Cousinia abolinii*** **Kult. ex Tscherneva** (subg. *Hypacanthodes* Tschern. sect. *Abolinia* Tschern.), Kyrgyzstan: SW, Jalal Abad Oblast, Kara Saj Tal, Aksy Rayan, 1,030 m, *Lazkov s.n.* (JE), EU923763; EU923877; EU661113. ***Cousinia dolichophylla*** **Kult.** (subg. *Hypacanthodes* Tschern. sect. *Abolinia* Tschern.), Uzbekistan: Ugamsky range above Nanaj, right edge of Pskem valley, "shiblyak", *Kamelin 265* (LE), EU923875; EU923989; EU661117. ***Cousinia egregia*** **Juz.** (subg. *Hypacanthodes* Tschern. sect. *Abolinia* Tschern.), Uzbekistan: Angren valley, rise to Kamchik pass, rubby slope, *Kamelin 420* (LE), EU923866; EU923980; EU661196. ***Cousinia grandifolia*** **Kult.** (subg. *Hypacanthodes* Tschern. sect. *Amberbopsis* Tschern.), Kazakhstan: Zambylskaya oblast, Talaski Alatau, 6 km W from Il Tai, 1,000 m, *Susanna 2181 & al.* (BC), AY373730*; AY373697*; EU661114. ***Cousinia korshinskyi*** **C. Winkl.** (subg. *Hypacanthodes* Tschern. sect. *Lacerae* C. Winkl.), Kyrgyzstan: isolated terrain feature Kanka, upper waters of river Kanka, near snow pot, h. 2,300 m, *E.M. Il'ina s.n.* (LE), EU923765; EU923879; EU661102. ***Cousinia macilenta*** **C. Winkl** (subg. *Hypacanthodes* Tschern. sect. *Lacerae* C. Winkl.), Tajikistan: SW, Jugum Hissaricum (Gissar), divorticum aquarum inter flumina Ljuczob et Unou, 3,000 m, *Zaprojagaev s.n.* (M), EU923764; EU923878; EU661119.

Appendix 2. Geographical distribution and taxonomic composition of clades found in the BI analyses of the ITS, *rps4-trnT-trnL* and combined ITS + *rps4-trnT-trnL* datasets. Numbers refer to clades or subclades of the Cousinioid group with PP ≥ 0.95 in Figs. 2–4. Geographical distribution according to the eight main centres of diversity of *Cousinia* as shown in Fig. 1.

rps4-trnT-trnL: CLADE 1: *C. pinarocephala*, sect. *Serratuloides*, Reg. 3; *C. crispa*, sect. *Serratuloides*, Reg. 3. CLADE 2: *C. araneosa*, sect. *Cynaroideae*, Reg. 2; *C. leucantha*, sect. *Stenoloma*, Reg. 4; (NESTED SUBCLADE 2a: *C. smirnowii*, sect. *Kopetdagia*, Reg. 4; *C. komarowii*, sect. *Stenoloma*, Reg. 4; *C. linczewskii*, sect. *Leucocaulon*, Reg. 4; *C. dipterocarpa*, sect. *Alpinae*, Reg. 4; *C. turkmenorum*, sect. *Chrysoptera*, Reg. 4; *C. decumbens*, sect. *Decumbentes*, Reg. 4; *C. eriophylla*, sect. *Platyacanthae*, Reg. 4; *C. rechingerorum*, sect. *Platyacanthae*, Reg. 4; *C. apiculata*, sect. *Pseudactinia*, Reg. 4; *C. trachyphyllaria*, sect. *Platyacanthae*, Reg., 4; *C. gatchsaranica*, sect. *Haussknechtii*, Reg. 2; *C. macrocephala*, sect. *Cynaroideae*, Reg. 1; *C. eryngioides*, sect. *Sciadocousinia*, Reg. 3, 4; *C. arctotidifolia*, sect. *Leiocaules*, Reg. 4; *C. badghysi*, sect. *Badghysia*, Reg. 4; *C. longifolia*, sect. *Spinuliferae*, Reg. 4). CLADE 3: *C. sewertzowii*, sect. *Microcarpae*, Reg. 7; *C. turcomanica*, sect. *Leucocaulon*, Reg. 4. CLADE 4: *C. serawschanica*, sect. *Alpinae*, Reg. 7; *C. raddeana*, sect. *Hoplophylla*, Reg. 4, 5, 7; *C. splendida*, sect. *Alpinae*, Reg. 7; *C. heteroloba*, sect. *Molles*, Reg. 5, 6. CLADE 5: *C. jassyensis*, sect. *Jurineopsis*, Reg. 8; *C. ninae*, sect. *Microcarpae*, Reg. 7, 8; *C. arachnoidea*, sect. *Microcarpae*, Reg. 8; (NESTED SUBCLADE 5a: *C. tianschanica*, sect. *Carduncellus*, Reg. 8; *C. triceps*, sect. *Leiacanthos*, Reg. 7; *C. platylepis*, sect. *Microcarpae*, Reg. 5, 7, 8; *C. greuteri*, sect. *Congesta*, Reg. 5, 6; *C. oopoda*, sect. *Actinia*, Reg. 7; *C. podophylla*, sect. *Homalochaete*, Reg. 7; *C. oxiana*, sect. *Chrysoptera*, Reg. 4; *C. xanthacantha*, sect. *Eriocousinia*, Reg. 6; *C. polyneura*, sect. *Eriocousinia*, Reg. 6; *C. tashkurghanica*, sect. *Actinia*, Reg. 5; *C. intertexta*, sect. *Cousinia*, Reg. 1; *C. chionophila*, sect. *Eriocousinia*, Reg. 5; *C. botschantzevii*, sect. *Regelianae*, Reg. 7). **ITS:** CLADE 1: *C. splendida*, sect. *Alpinae*, Reg. 7; *C. ariana*, sect. *Carduncellus*, Reg. 5, 6; *C. buphthalmoides*, sect. *Carduncellus*, Reg. 5, 6, 7; *C. sewertzowii*, sect. *Microcarpae*, Reg. 7; *C. ninae*, sect. *Microcarpae*, Reg. 8, 7; *C. jassyensis*, sect. *Jurineopsis*, Reg. 8; (NESTED SUBCLADE 1a: *C. coronata*, sect. *Coronophora*, Reg. 7; *C. radians*, sect. *Coronophora*, Reg. 4). CLADE 2: *C. tianschanica*, sect. *Carduncellus*, Reg. 8; *C. botschantzevii*, sect. *Regelianae*, Reg.7. CLADE 3: *C. polytimetica*, sect. *Dichotomae*, Reg. 7; *C. pusilla*, sect. *Dichotomae*, Reg. 7; *C. bungeana*, sect. *Dichotomae*, Reg. 8. CLADE 4: *C. minkwitziae*, sect. *Cousinia*, Reg. 8; *C. chrysantha*, sect. *Alpinae*, Reg. 8; *C. dissecta*, sect. *Chrysoptera*, Reg. 8; (NESTED SUBCLADE 4a: *C. syrdariensis*, sect. *Cousinia*, Reg. 8; *C. polycephala*, sect. *Cousinia*, Reg. 7, 8). CLADE 5: *C. serawschanica*, sect. *Alpinae*, Reg. 7; *C. polyneura*, sect. *Eriocousinia*, Reg. 6. CLADE 6: *C. hypoleuca*, sect. *Serratuloides*, Reg. 3; *C. concolor*, sect. *Serratuloides*, Reg. 3, 4. CLADE 7: *C. rigida*, sect. *Lopholepis*, Reg. 8; (NESTED SUBCLADE 7a: *C. stocksii*, sect. *Stocksianae*, Reg. 4, 5; (NESTED SUBCLADE 7aa: *C. tenuiramula*, sect. *Stenocephalae* s.l, Reg. 2; *C. piptocephala*, sect. *Badghysia*, Reg. 2; *C. thamnodes*, sect. *Stenocephalae* s.l., Reg. 2; *C. alexeenkoana*, sect. *Stenocephalae* s.l., Reg. 2; *C. cylindracea*, sect. *Stenocephalae* s.l., Reg. 2; *C. oligocephala*, sect. *Stenocephalae* s.l., Reg. 2)); (NESTED SUBCLADE 7b: *C. apiculata*, sect. *Pseudactinia*, Reg. 4; *C. oreodoxa*, sect. *Pseudactinia*, Reg. 4); (NESTED SUBCLADE 7c: *C. chaetocephala*, sect. unknown, Reg. 4; *C. lepida*, sect. *Lepidae*, Reg. 4; *C. heterophylla*, sect. *Lepidae*, Reg. 4, 5). CLADE 8: *C. gmelinii*, sect. *Serratuloides*, Reg. 3; *C. pinarocephala*, sect. *Serratuloides*, Reg. 3; *C. crispa*, sect. *Serratuloides*, Reg. 3. CLADE 9: *C. eriophylla*, sect. *Platyacanthae*, Reg. 4; *C. rechingerorum*, sect. *Platyacanthae*, Reg. 4. CLADE 10: *C. salangensis*, sect. *Immitantes*, Reg. 6; *C. immitantiformis*, sect. *Immitantes*, Reg. 5; (NESTED SUBCLADE 10a: *C. coerulea*, sect. *Homalochaete*, Reg. 7; *C. podophylla*,

Appendix 2. Continued.

sect. *Homalochaete*, Reg. 7). CLADE 11: *C. incompta*, sect. *Pugioniferae*, Reg. 2; (NESTED SUBCLADE 11a: *C. purpurea*, sect. *Cynaroideae*, Reg. 1; *C. araneosa*, sect. *Cynaroideae*, Reg. 2; *C. macrocephala*, sect. *Cynaroideae*, Reg. 1). CLADE 12: *C. chrysochlora*, sect. *Lachnosphaerae*, Reg. 4; *C. lachnosphaera*, sect. *Lachnosphaerae*, Reg. 4, 5. CLADE 13: *C. turkmenorum*, sect. *Chrysoptera*, Reg. 4; *C. oxiana*, sect. *Chrysoptera*, Reg. 4. CLADE 14: *C. autranii*, sect. *Rigidissimae*, Reg. 5; *C. rigidissimae*, sect. *Rigidissimae*, Reg. 5. CLADE 15: *C. auriculata*, sect. *Eriocousinia*, Reg. 5, 6, 7; *C. chionophila*, sect. *Eriocousinia*, Reg. 5. CLADE 16: *C. shahvarica*, sect. *Sphaerocephalae*, Reg. 3; (NESTED SUBCLADE 16a: *C. harazensis*, sect. *Sphaerocephalae*, Reg. 3; *C. xiphiolepis*, sect. *Sphaerocephalae*, Reg. 3); (NESTED SUBCLADE 16b: *C. adenosticta*, sect. *Sphaerocephalae*, Reg. 3; *C. leptolepis*, sect. *Sphaerocephalae*, Reg. 3). CLADE 17: *C. decumbens*, sect. *Decumbentes*, Reg.3; *C. badphysi*, sect. *Badghysia*, Reg. 4. CLADE 18: *C. gatchsaranica*, sect. *Haussknechtii*, Reg. 2; *C. intertexta*, sect. *Cousinia*, Reg. 1. **Combined ITS + *rps4-trnT-trnL:*** CLADE 1: *C. polytimetica*, sect. *Dichotomae*, Reg. 7; *C. pusilla*, sect. *Dichotomae*, Reg. 7; *C. bungeana*, sect. *Dichotomae*, Reg. 8. CLADE 2: *C. chrysantha*, sect. *Alpinae*, Reg. 8; *C. dissecta*, sect. *Chrysoptera*, Reg. 8; (NESTED SUBCLADE 2a: *C. minkwitziae*, sect. *Cousinia*, Reg. 8; *C. syrdariensis*, sect. *Cousinia*, Reg. 8; *C. polycephala*, sect. *Cousinia*, Reg. 7, 8). CLADE 3: *C. splendida*, sect. *Alpinae*, Reg. 7; *C. ariana*, sect. *Carduncellus*, Reg. 6, 5; (NESTED SUBCLADE 3a: *C. buphthalmoides*, sect. *Carduncellus*, Reg. 5, 6, 7; *C. sewertzowii*, sect. *Microcarpae*, Reg. 7; (NESTED SUBCLADE 3aa: *C. jassyensis*, sect. *Jurineopsis*, Reg. 8; *C. ninae*, sect. *Microcarpae*, Reg. 7, 8); (NESTED SUBCLADE 3ab: *C. coronata*, sect. *Coronophora*, Reg. 7; *C. radians*, sect. *Coronophora*, Reg. 4)). CLADE 4: *C. hypoleuca*, sect. *Serratuloides*, Reg. 3: *C. concolor*, sect. *Serratuloides*, Reg. 3, 4. CLADE 5: *C. stocksii*, sect. *Stocksianae*, Reg. 4, 5; *C. komarowii*, sect. *Stenoloma*, Reg. 4; *C. alepideae*, sect. *Lasiandrae*, Reg. 5; (NESTED SUBCLADE 5a: *C. cylindracea*, sect. *Stenocephalae* s.l., Reg. 2; *C. oligocephala*, sect. *Stenocephalae* s.l., Reg. 2; (NESTED SUBCLADE 5aa: *C. tenuiramula*, sect. *Stenocephalae* s.l, Reg. 2; *C. piptocephala*, sect. *Badghysia*, Reg. 2; *C. thamnodes*, sect. *Stenocephalae* s.l., Reg. 2; *C. alexeenkoana*, sect. *Stenocephalae* s.l., Reg. 2)). CLADE 6: *C. chaetocephala*, sect. unknown, Reg. 4; *C. lepida*, sect. *Lepidae*, Reg. 4; *C. heterophylla*, sect. *Lepidae*, Reg. 4, 5. CLADE 7: *C. chrysochlora*, sect. *Lachnosphaerae*, Reg. 4; *C. lachnosphaera*, sect. *Lachnosphaerae*, Reg. 4, 5. CLADE 8: *C. gmelinii*, sect. *Serratuloides*, Reg. 3; (NESTED SUBCLADE 8a: *C. pinarocephala*, sect. *Serratuloides*, Reg. 3; *C. crispa*, sect. *Serratuloides*, Reg. 3). CLADE 9: *C. lasiolepis*, sect. *Alpinae*, Reg. 2, 4, 5; (NESTED SUBCLADE 9a: *C. autranii*, sect. *Rigidissimae*, Reg. 5; *C. rigidissimae*, sect. *Rigidissimae*, Reg. 5). CLADE 10: *C. araneosa*, sect. *Cynaroideae*, Reg. 2; *C. linczewskii*, sect. *Leucocaulon*, Reg. 4; *C. arctotidifolia*, sect. *Leiocaules*, Reg. 4; *C. smirnowii*, sect. *Kopetdagia*, Reg. 4; *C. eryngioides*, sect. *Sciadocousinia*, Reg. 3, 4; *C. dipterocarpa*, sect. *Alpinae*, Reg. 4; *C. turkmenorum*, sect. *Chrysoptera*, Reg. 4.; (NESTED SUBCLADE 10a: *C. eriophylla*, sect. *Platyacanthae*, Reg. 4; *C. rechingerorum*, sect. *Platyacanthae*, Reg. 4); (NESTED SUBCLADE 10b: *C. apiculata*, sect. *Pseudactinia*, Reg. 4; *C. trachyphyllaria*, sect. *Platyacanthae*, Reg. 4). CLADE 11: *C. shahvarica*, sect. *Sphaerocephalae*, Reg. 3; (NESTED SUBCLADE 11a: *C. harazensis*, sect. *Sphaerocephalae*, Reg. 3; *C. xiphiolepis*, sect. *Sphaerocephalae*, Reg. 3); (NESTED SUBCLADE 11b: *C. adenosticta*, sect. *Sphaerocephalae*, Reg. 3; *C. leptolepis*, sect. *Sphaerocephalae*, Reg. 3). CLADE 12: *C. decumbens*, sect. *Decumbentes*, Reg. 3; *C. badphysi*, sect. *Badghysia*, Reg. 4. CLADE 13: *C. gatchsaranica*, sect. *Haussknechtii*, Reg. 2; *C. longifolia*, sect. *Spinuliferae*, Reg. 4. CLADE 14: *C. incompta*, sect. *Pugioniferae*, Reg. 2; (NESTED SUBCLADE 14a: *C. araneosa*, sect. *Cynaroideae*, Reg. 2; *C. purpurea*, sect. *Cynaroideae*, Reg. 1). CLADE 15: *C. tianschanica*, sect. *Carduncellus*, Reg. 8; *C. botschantzevii*, sect. *Regelianae*, Reg. 7.

7. 2. The *Arctium-Cousinia* complex: disentangling *Arctium* and *Cousinia* (Cardueae, Carduinae)

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RESUM. A aquest treball s'ha investigat la filogènia del llinatge Arctioide del complex *Arctium-Cousinia* per tal d'intentar resoldre l'antiga controvèrsia de la delimitació entre els gèneres *Arctium* i *Cousinia*. Per fer-ho s'han analitzat dades moleculars d'una regió nuclear (ITS) i dues cloroplàstiques (*rpS4-trnT-trnL* i *rpl32-trnL*) de 37 espècies que s'han complementat amb evidències morfològiques, quan ha estat possible, gràcies a l'estudi de 323 plecs d'herbari. Com a resultat, s'ha proposat un gènere *Arctium* monofilètic i més ampli de manera que ara comprèn, a més de les espècies del gènere *Arctium* tal i com s'entenia fins ara, tota la resta d'espècies del clade Arctioide pertanyents als gèneres *Anura*, *Cousinia* (subg. *Cynaroides* i *Hypacanthodes*), *Hypacanthium* i *Schmalhausenia*. Aquest, alhora s'ha subdividit en dos nous subgèneres anomenats subg. *Arctium* i subg. *Cynaroides*. Aquests canvis han comportat la supressió de *Cousinia* subg. *Hypacanthodes* i la reordenació del subg. *Cynaroides* donat que tal i com estaven definits no eren monofilètics. En canvi, la classificació seccional tradicional s'ha mantingut en la seva major part. S'inclou una proposta de canvi nomenclatural de totes les espècies transferides a *Arctium* des de la resta de gèneres esmentats. Malgrat tot, degut a la manca de caràcters morfològics prou adients per a recolzar l'aproximació presentada aquí, cal un treball més exhaustiu per tal de poder establir una classificació més robusta en els rangs subgenèric i seccional.

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The *Arctium-Cousinia* complex: disentangling *Arctium* and *Cousinia* (Cardueae, Carduinae)

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Abstract

The phylogeny of the Arctioid lineage of the *Arctium-Cousinia* complex is investigated in an attempt to establish the conflictive generic boundaries of *Arctium* and *Cousinia*. This work is based on analyses of one nuclear (ITS) and two chloroplastic DNA regions (*rpS4-trnT-trnL* and *rpl32-trnL*) of 37 species complemented with morphological evidences when possible (323 herbarium sheets are studied). A broadly monophyletic genus *Arctium* is proposed which is divided into two new monophyletic subgenera, subg. *Arctium* and subg. *Cynaroides*. These rearrangements lead to the suppression of subgenus *Hypacanthodes* and the recombination and redefinition of subgenus *Cynaroides* because as presently defined they are not monophyletic. In contrast, the traditional sectional classification is mainly maintained.

INTRODUCTION

The *Arctium-Cousinia* complex (Cardueae, Carduinae) is a large group containing between 500 and 600 species. According to Susanna & al. (2003a) it is a monophyletic complex characterized by a receptacle with strongly twisted scales, pappus formed by free deciduous bristles and achenes streaky (with wavy fringes) and very often winged lacking the apical nectary, typical of the true thistles (Susanna & Garcia-Jacas, 2007). It comprises the genera *Anura* (Juz.) Tscherneva, *Arctium* L., *Cousinia* Cass., *Hypacanthium* Juz. and *Schmalhausenia* C. Winkl. (Table 1). As stated by Rechinger (1986) and Knapp (1987), the vast majority of species of this complex is distributed in the Turkestan mountain region (Tien Shan and Pamir-Alay) and the Irano-Turanian region, except *Arctium* s.str. which is Eurosiberian in distribution.

Table 1. Classification of the Arctioid group according to Tscherneva (1962, 1982, 1988a, b, c) and Duistermaat (1996).

Species	Section	Genus and Subgenus
A. pallidivirens (Kult.) Tscherneva		*Anura* (Juz.) Tscherneva
A. atlanticum (Pomel) H. Lindb.	*Arctium*	*Arctium* L.
A. lappa L.		
A. minus (Hill) Bernh.		
A. nemorosum Lej.		
A. palladini (Marcow.) R. E. Fr. & E. S. Söderb.		
A. tomentosum Mill.		
C. aurea C. Winkl.	*Chrysis* Juz.	*Cousinia* subg. *Cynaroides* Tscherneva
C. chlorantha Kult.		
C. haesitabunda Juz.		
C. karatavica Regel & Schmalh.		
C. korolkowii Regel & Schmalh.		
C. medians Juz.		
C. refracta (Bornm.) Juz.		
C. schmalhausenii C. Winkl.		
C. anomala Franch.	*Ctenarctium* Juz.	
C. lappacea Schrenk	*Lappaceae* Bunge	
C. arctioides Schrenk	*Nanarctium* Juz. ex. Tscherneva	
C. triflora Schrenk	*Oligantha* Juz.	
C. albertii Regel & Schmalh.	*Pectinatae* C. Winkl.	
C. horrescens Juz.		
C. pentacantha Regel & Schmalh.		
C. pentacanthoides Juz. ex Tscherneva		
C. amplissima (Boiss.) Boiss.	*Pseudarctium* Juz.	
C. pseudarctium Bornm.		
C. tomentella C. Winkl.		
C. umbrosa Bunge		
C. abolinii Kult. ex Tscherneva	*Abolinia* Tscherneva	*Cousinia* subg. *Hypacanthodes* Tscherneva
C. dolichophylla Kult.		
C. egregia Juz.		
C. grandifolia Kult.	*Amberbopsis* Tscherneva	
C. pterolepida Kult.	*Lacerae* C. Winkl.	
C. korshinskyi C. Winkl.		
C. macilenta C. Winkl.		
C. fedtschenkoana Bornm.		
C. ugamensis Karmysch.		
C. vavilovii Kult.	*Serratulopsis* Tscherneva	
H. echinopifolium (Bornm.) Juz.		*Hypacanthium* Juz.
H. evidens Tscherneva		
S. nidulans (Regel) Petrak		*Schmalhausenia* C. Winkl.

The genus *Arctium* was first described by Linnaeus (1753) although some authors in the 18th and 19th century kept using the illegitimate prelinnaean synonym *Lappa* Scop. Later, *Cousinia* was described by Cassini (1827) based on *Carduus orientalis* Adam, *Schmalhausenia* was described by Winkler (1892) and finally Juzepczuk (1937) described the genus *Hypacanthium*.

There are a number of works dealing with the *Arctium-Cousinia* complex, most of them based on morphological characters (De Candolle, 1838; Bunge, 1865; Boissier, 1875, 1888; Kuntze, 1891; Winkler, 1892, 1897; Dittrich, 1977; Duistermaat, 1996, 1997; Petit, 1997; Häffner, 2000). Many others also use biogeographical evidences (Takhtajan, 1938; Rechinger, 1953, 1972, 1979, 1986; Tscherneva, 1962, 1974, 1982, 1988a, 1988b, 1988c; Davis, 1975; Knapp, 1987; Tamanian, 1999), palynological data (Schtepa, 1966, 1973, 1976; Kuprianova & Tscherneva, 1982; Qaid, 1990; Petit et al., 1996), karyological information (Poddubnaja-Arnoldi, 1931; Koul, 1964; Fedorov, 1969; Podlech & Dieterle, 1969; Podlech & Bader, 1974; Aryavand, 1975, 1976; Ghaffari, 1984; Tscherneva, 1985; Susanna et al., 2003b; Ghaffari et al., 2000, 2006; López-Vinyallonga et al., in press) or molecular data (Häffner & Hellwig, 1999; Garcia-Jacas et al., 2002; Susanna et al., 2003a, 2006; López-Vinyallonga et al., 2009).

Recent studies (Susanna et al., 2003, 2006; López-Vinyallonga et al., 2009) have shown a clear subdivision of the *Arctium-Cousinia* complex into two monophyletic lineages. The first one is the Arctioid group with *Arctium* (6 species), *Cousinia* subgenera *Cynaroides* Tscherneva (20 species, including the monospecific genus *Anura* merged here by Susanna & al., 2006) and *Hypacanthodes* Tscherneva (10 species), *Hypacanthium* (2 species) and the monotypic *Schmalhausenia*. It is characterized by a chromosome number of $x = 18$, Arctiastrum pollen type (orbicular and spiny) and style glabrous and cylindrical with a ring of sweeping hairs at the thickened articulation. The second one is the Cousinioid group which comprises only *Cousinia* subg. *Cousinia* (c.a. 500 species) and is characterized by a dysploid series of chromosome numbers of $x = 9$, 10, 11, 12 and 13, Cousinioid pollen type (oblong and smooth) and the apical part of the style and the stylar branches covered with long hairs.

Despite all the aforementioned works, the precise limits between *Arctium* and *Cousinia* are unclear and have not been clearly established yet. All the problems for establishing an accurate delimitation of these genera are circumscribed to the Arctioid group, for two main reasons: firstly because a group of *Cousinia* species share some characters with *Arctium* and secondly both *Cousinia* subgenera *Cynaroides* and *Hypacanthodes* are not monophyletic (López-Vinyallonga et al., 2009). These unclear boundaries are reflected in many reclassifications and even changes of genus adscription such as some illustrative examples we shortly comment.

Duistermaat (1996) included five species of *Cousinia* subg. *Cynaroides* in *Arctium* s.l., which according to her analyses remained monophyletic and easily recognizable by the presence of hooked involucral bracts and would contain 11 species. Duistermaat decided not to include *C. anomala* in *Arctium*, as suggested by Schtepa (1971, 1973) based on pollen type and despite having hooked involucral bracts. Following Duistermaat's cladogram this solution would mean that at least the entire subgenus *Cynaroideae* should be included in the genus *Arctium* leading, according to her, to a poorly recognizable genus. In spite of all these considerations, Duistermaat admitted that accepting *Arctium* s.l. as a separate genus would leave *Cousinia* paraphyletic (Duistermaat, 1996). As an extreme, Kuntze (1891) adopted the solution of merging the whole genus *Cousinia* into *Arctium* in view of the impossibility of establishing clear boundaries between these two genera.

Following the latest molecular phylogeny available (López-Vinyallonga et al., 2009), current classification of the Arctioid group (Table 1) does not reflect natural phylogenetic relationships among its species. Therefore, the main goal of the present work is to establish the generic boundaries of *Arctium* and *Cousinia*. Furthermore, we aim to provide a preliminary infrageneric classification of the Arctioid lineage.

MATERIAL AND METHODS

Molecular phylogenetic study

Plant material

The data described and discussed here are based on a sample of 37 species for which sequences of the nuclear ribosomal internal transcribed spacer region ITS1-ITS2, and the chloroplastic intergenic spacers *rpS4-trnT-trnL* and *rpl32-trnL* were obtained. We have included the four sections of subg. *Hypacanthodes*, the seven sections of subg. *Cynaroides*, four species of the two sections of *Arctium*, the two species of *Hypacanthium* and the unique *Schmalhausenia* species. Two outgroup taxa were selected from *Cousinia* subgenus *Cousinia* on the basis of the previous analyses in López-Vinyallonga et al. (2009): *Cousinia meghrica* Takht. and *Cousinia serawschanica* C. Winkl. All the 37 *rpl32-trnL* sequences included in the analyses are new and so are 10 out of 37 ITS and 10 out of 37 *rpS4-trnT-trnL* sequences. Voucher data, sources of material and GenBank accession numbers of the above 37 species are given in Table 2.

Table 2. Voucher data, sources of material and GenBank accession numbers (ITS1; ITS2; *rpS4-trnT-trnL*; *rpl32-trnL*) of the 37 species studied in the present work. Na means non available data.

Species	Locality	GenBank accessions
A. pallidivirens (Kult.) Tscherneva	Uzbekistan: Sine loc., *Botschantzev s.n.* (LE)	EU923768; EU923882; EU661126; Na
A. lappa L.	Iran: NNE, Khorassan: 25 SW Bodjnourd, 1500 m, *Mehregan 140* (MJG)	EU923773; EU923887; EU661123; Na
Arctium leiospermum Juz. & C. Serg.	Kazakhstan: Zambylskaya oblast, Kurdai pass, 900 m, *Susanna 2154 & al.* (BC)	AY373720; AY373687; EU661121; Na
A. minus (Hill) Bernh.	Belgium: Lovaina Botanical Garden	AF19049; AF19103; EU661122; Na
A. palladini (Marcow.) R.E.Fr. & E.S. Söderb.	Caucasus: sine col. *311* (LE)	Na; Na; Na; Na
C. meghrica Takht.	Armenia: Sjunik, Meghri distr., Agarak village, *Tamanian s.n.* (ERE)	EU923814; EU923928; EU661142; Na
C. serawschanica C. Winkl.	Tajikistan: Voru, 2000–2300 m, *Susanna 2516 & al.* (BC)	EU923874; EU923988; EU661192; Na
C. aurea C. Winkl.	Tajikistan: Schtut, road to Penjikent, *Susanna 2514 & al.* (BC)	Na; Na; Na; Na
C. chlorantha Kult.	Uzbekistan: Malguzar mt., S slope, by Tashkesken ¨say¨, *Kamelin 199* (LE)	EU923864; EU923978; EU661120; Na

C. karatavica Regel & Schmalh.	Kazakhstan: Zambylskaya oblast, Karatau mt., Kuyuk pass, 1000 m, *Susanna 2162 & al.* (BC)	AY373732; AY373699; EU661101; Na
C. korolkowii Regel & Schmalh.	Uzbekistan: N macro-mountainside, Nuratau range, Sintob kishlak surroundings, rocky slopes, *Botschantzev 427* (LE)	EU923865; EU923979; EU661103; Na
C. medians Juz.	Uzbekistan: S, Supkhandarbinskaya reg., between kishlaks Sajrob and Shurob, 121-122 km of the road from Termez, speckled rock outcrops, *Botschantzev 240* (LE)	EU923769; EU923883; EU661100; Na
C. refracta (Bornm.) Juz.	Tajikistan: Kondara river canyon, Varzowski Rayon reservation, *Susanna 2456 & al.* (BC)	EU923867; EU923981; EU661111; Na
C. schmalhausenii C. Winkl.	Uzbekistan: *Botschantzev 275* (LE)	Na; Na; Na; Na
C. anomala Franch.	Tajikistan: slopes above Voru, about 300 m above the kishlak, 2200–2300 m, *Susanna 2521 & al.* (BC)	EU923770; EU923884; EU661115; Na
C. lappacea Schrenk	Kazakhstan: Zambylskaya oblast, Kurdai pass, 900 m, *Susanna 2150 & al.* (BC)	AY373733; AY373700; EU661112; Na
C. arctioides Schrenk	Kazakhstan: Dzhezkazganskaya reg., Turgajskaya lowland, 49 km to SW from Dzhezkazgana, right bank of Kumula river, *Tamarix* bushland, *Kamelin 6434* (LE)	EU923772; EU923886; EU661118; Na
C. triflora Schrenk	Iran: Golestan Nat. Park, Yakhbala pass, *Akhani 102* (MJG)	EU923771; EU923885; EU661094; Na
C. albertii Regel & Schmalh.	Kazakhstan: Shimkientskaya oblast, Mashat canyon, 1840 m, *Susanna 2206 & al.* (BC)	AY373721; AY373688; EU661099; Na
C. horrescens Juz.	Kazakhstan: Tien Shan occidentalis, ad declivia saxoso-arcillosa secus canales Bos-su prope pagum Niakbek, *Granitov 478* (LE)	Na; Na; Na; Na
C. pentacantha Regel & Schmalh.	Tajikistan: *Spiridonow* 173 (LE)	Na; Na; Na; Na
C. pentacanthoides Juz. ex Tscherneva	Uzbekistan: sine col. *65* (LE)	Na; Na; Na; Na
C. amplissima (Boiss.) Boiss.	Iran: Dena, 15 km from Meimand to Yassoudj, 2500 m, *Mehregan 174* (MJG)	EU923766; EU923880; EU661098; Na
C. pseudarctium Bornm.	Tajikistan: Vorzov valley, 2 km N kishlak Ziddy, *Susanna 2477 & al.* (BC)	EU923876; EU923990; EU661095; Na
C. tomentella C. Winkl.	Tajikistan: S mountain side of Guissar range, left bank of Varzob river, Deamalik kishlak surroundings, 1800 m, *Tschukavina 10512* (LE)	EU923767; EU923881; EU661097; Na
C. umbrosa Bunge	Kazakhstan: Almatinskaya oblast, Alatau mt. above Almaty, 1200 m, *Susanna 2100 & al.* (BC)	AY373745;AY373712; EU661096; Na
C. abolinii Kult. ex Tscherneva	Kyrgyzstan: SW, Jalal Abad Oblast, Kara Saj Tal, Aksy Rayan, 1030 m, *Lazkov s.n.* (LE)	EU923763; EU923877; EU661113; Na
C. dolichophylla Kult.	Uzbekistan: Ugamsky range above Nanaj, right edge of Pskem valley, “shiblyak”, *Kamelin 265* (LE)	EU923875; EU923989; EU661117; Na
C. egregia Juz.	Uzbekistan: Angren valley, rise to Kamchik pass, rubby slope, *Kamelin 420* (LE)	EU923866; EU923980; EU661196; Na
C. grandifolia Kult.	Kazakhstan: Zambylskaya oblast, Talaski Alatau, 6 km W from Il Tai, 1000 m, *Susanna 2181 & al.* (BC)	AY373730; AY373697; EU661114; Na
C. korshinskyi C. Winkl.	Kyrgyzstan: isolated terrain feature Kanka, upper waters of river Kanka, near snow pot, 2300 m, *E. M. Il'ina s.n.* (LE)	EU923765; EU923879; EU661102; Na

C. macilenta C. Winkl.	Tajikistan: SW, Jugum Hissaricum (Gissar), divorticum aquarum inter flumina Ljuczob et Unou, 3000 m, *Zaprojagaev s.n.* (M)	EU923764; EU923878; EU661119; Na
C. fedtschenkoana Bornm.	Tajikistan: *Romashchenko 632 & Susanna* (BC)	Na; Na; Na; Na
C. vavilovii Kult.	Kazakhstan: prov. Syr-Darja, distr. Aulie-ata. ad declivia saxosa regionis subalpinae in montibus Alexandri prope Utsch-Bulak, *Popov* (B)	Na; Na; Na; Na
H. echinipifolium (Bornm.) Juz.	Kyrgyzstan: sine loc., *Iljin s.n.* (LE)	AY373746; AY373713; EU661125; Na
H. evidens Tscherneva	Uzbekistan: sine loc., sine col. (LE)	Na; Na; Na; Na
S. nidulans (Regel) Petrak	Kazakhstan: Almatinskaya oblast, Alatau mt., above Almaty, *Susanna 2088 & al.* (BC)	AY373752; AY373719; EU661124; Na

DNA extraction, amplification and sequencing strategies

Total genomic DNA was extracted either following the protocol of the CTAB method of Doyle & Doyle (1987) or following the manufacturer's instructions of the NucleoSpin® Plant Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Double-stranded DNA of ITS and *rpS4-trnT-trnL* were amplified by PCR following the protocol given in López-Vinyallonga et al. (2009). The double-stranded *rpl*32-*trn*L DNA region was amplified by PCR using the forward primer *rpl32*F and reverse primer *trnL*(UAG) (Shaw et al., 2007). Reactions were performed in 25.0 µl volumes with 10% 10x AmpliTaq buffer, 10% 50 mM MgCl2, 10% of 20 mM dNTPs mix, 2% of each primer at 25 pmol/µl conc., 1.0 unit (0.2 µl) AmpliTaq DNA polymerase (Applied Biosystems, Foster City, California, U.S.A.), and 5.0 µl of template DNA (30–60 ng/µl). The volume was filled up to 25.0 µl with distilled sterilized water. The profile used for amplification consisted of a preheat for 3 min at 95°C, followed by 34 cycles of 94°C for 40 s, 54°C for 40 s and 72°C for 1 min 40 s and a post-treatment of 10 min at 72°C.

All PCR products were purified with either the QIAquick® Purification Kit (Qiagen Inc., Valencia, CA, USA) or DNA Clean & Concentrator-5 kit (Zymo Research, Orange, CA, USA) following the manufacturer's protocols. Direct sequencing of the amplified DNA segments was performed as explained in López-Vinyallonga et al. (2009) at the "Serveis Científico-Tècnics" of the University of Barcelona on an ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were edited with Chromas 2.0 (Technelysium Pty. Ltd., Tewantin, Australia) and Bioedit 7.0.1 (Hall, 1999) and aligned manually by sequential pairwise comparison

(Swofford & Olsen, 1990). Data matrices are available on request from the corresponding author.

Phylogenetic analyses

ITS, *rpS4-trnT-trnL* and *rpl32-trnL* data sets were analyzed by Bayesian Inference one by one independently as well as combined, so we have built seven matrices to work with. All the analyses were performed with MrBayes software package 3.1.2 (Ronquist & Huelsenbeck, 2003) as explained in López-Vinyallonga et al. (2009) using parameters from the model GTR + G as indicated by ModelTest 3.5 (Posada & Crandall, 1998; 2001) as the best fitting model of molecular evolution for all three markers independently and combined. Congruence in the phylogenetic signal of the cpDNA and nuclear datasets was examined by a visual comparison of tree topologies with branch support ≥ 0.95 PP.

Two simultaneous and independent analyses were performed and for each of them the Markov Chain Monte Carlo process was set so that four chains ran simultaneously for 2.000.000 generations sampling one out of every 200 generations, which resulted in a total of 10,000 sample trees in each run. As stationarity was achieved by the 1.000th tree, the first 999 trees were discarded in order to avoid those trees that might have been sampled prior to convergence of the Markov chains. Therefore the posterior probability of the phylogeny and its branches was determined from the remaining 19.000 trees. Internodes with posterior probabilities ≥ 95% were considered to be well-supported.

Morphological survey

Those analyses based on molecular data were complemented with morphological evidences based on the study of 323 herbarium sheets, including type specimens, from LE, M and W altogether with some specimens collected by the authors and some colleagues, mentioned in the acknowledgements section, deposited in BC after being revised by Dra. Tscherneva.

RESULTS

As a result of the aforementioned analyses, seven trees have been recovered: ITS (Fig. 1; named nuclear from now), *rpS*4-*trnT*-*trnL* (not shown; named *trnT* from now), *rpl32*-*trnL* (not shown; named *rpl32* from now), *rpS*4-*trnT*-*trnL* + *rpl32*-*trnL* (not shown; named chloroplastic from now), ITS + *rpS*4-*trnT*-*trnL* (Fig. 2; named ITS-*trnT* from now), ITS + *rpl32*-*trnL* (not shown; named ITS-*rpl32* from now) and ITS + *rpS*4-*trnT*-*trnL* + *rpl32*-*trnL* (Fig. 3; named combined from now. The subgeneric classification proposed in the present work is shown in this figure).

By tree topology comparison, it seems like there is a little amount of conflictive signal between the two chloroplastic markers although in general, Bayesian inference analyses do not show significant incongruent topologies. Therefore the data from the three markers can be combined in order to improve the phylogenetic signal of the analyses for the Arctioid lineage.

Each chloroplastic marker by itself provides scarce phylogenetic information and consequently both *trnT* and *rpl32* trees consist of unresolved polytomies (trees not shown). In addition, the trees recovered from the ITS-*rpl32* and combined analyses are essentially identical (that's why the first one is not shown), although with the addition of the third marker clades support increases. Further, in the trees resulting from nuclear (Fig. 1), ITS-*trnT* (Fig. 2), ITS-*rpl32* (tree not shown) and combined (Fig. 3) datasets, relationships among species are reasonably well resolved. To sum up, the discussion of the results is based on nuclear (Fig. 1), ITS-*trnT* (Fig. 2) and combined trees (Fig. 3).

All seven analyses confirm the monophyly of the Arctioid clade with the highest support and in most of the analyses a clear subdivision of the Arctioid lineage into two groups is found (PP = 1.00; Figs. 1 - 3). The first subclade formed by all the species of the genera *Arctium*, *Hypacanthium* and the monotypic *Schmalhausenia* together with *Cousinia arctioides*, *C. vavilovii* and *C. grandifolia* (PP = 1.00 in the four analyses mentioned above; Figs. 1 - 3). This clade is subdivided again into one lineage comprising the genus *Arctium* and *C. arctioides* (PP = 1.00 in the same four analyses; Figs. 1 - 3) and a second one merging *Hypacanthium*, *Schmalhausenia*, *C. vavilovii* and

C. grandifolia (PP = 0.99 and PP = 1.00 for ITS-*rpl32* (tree not shown) and combined (Fig. 3) datasets respectively) which have no statistical support in the nuclear (Fig. 1) and ITS-*trnT* (Fig. 2) analyses.

The second subclade with the remaining *Cousinia* species (PP = 1.00 in the four analyses mentioned above; Figs. 1 - 3) which in turn is grouped in two subgroups. The first one contains most species of the paraphyletic subgenus *Hypacanthodes*, although is not statistically supported in any analysis, and the remaining species group together in a second clade with strong support in the four analyses (PP = 0.97 and PP = 1.00 in the nuclear and the rest of analyses respectively; Figs. 1 - 3). In this second clade, the next subdivision leaves *C. lappacea* apart from the rest of the species (PP = 1.00 in all but the nuclear tree; Figs. 1 - 3). After that, *C. korshinsky*i appears isolated from the rest of the species of the polytomy (PP = 1.00 in the nuclear tree (Fig. 1) and without support in the rest of the analyses; Figs. 2, 3). From the remaining species of this second subclade, all trees agree in the monophyly of sect. *Pseudarctium* which is weakly supported in the two independent chloroplastic analyses while is strongly supported in the nuclear (Fig. 1) and the combined (Figs. 2, 3) analyses with PP = 1.00. The nuclear (Fig. 1) and all the combined analyses but the chloroplastic (Figs. 2, 3), also show sect. *Chrysis* to be monophyletic (PP = 1.00) including *C. anomala*, the only species of section *Ctenarctium*. The species of sect. *Pectinatae* group together with *C. triflora*, from the monotypic section *Oligantha*, with strong support in the nuclear and ITS-*trnT* analyses (PP = 0.99; Figs. 1, 2). In the ITS-*rpl32* (not shown) and combined (Fig. 3) trees, A. *palladivirens* merges with the clade of sect. *Pectinatae* leading to a weaker clade while in the nuclear (Fig. 1) and ITS-*trnT* (Fig. 2) analyses appears isolated and then the support for the sect. *Pectinatae* clade reaches PP = 0.99.

DISCUSSION

As found previously (López-Vinyallonga et al., 2009), the Arctioid lineage is clearly monophyletic with strong support and is sharply divided into two monophyletic subclades which leads to two main conclusions regarding generic and subgeneric adscription. First of all, the genus *Arctium*, as established by Duistermaat (1996), is not monophyletic because the species of *Arctium* sect. *Arctium* and sect. *Nanarctium* fall into the first strongly supported subgroup (Figs. 1 - 3 clade A) while the species of sect. *Pseudarctium* group in the other highly supported subclade (Figs. 1 - 3 clade B). In addition, subgenera *Cynaroides* and *Hypacanthodes* as established by Tscherneva (1988c) are not monophyletic either (Figs. 1 - 3). *Cousinia arctioides* belongs to the first subgroup whereas the remaining species of Subg. *Cynaroides* belongs to the second one. A parallel case in found in Subg. *Hypacanthodes* where all the species but *C. vavilovii* and *C. grandifolia* fall in the second subclade (Figs. 1 - 3).

Because the two broad sister subclades resulting from the split of the Arctioid lineage have strong statistical support in most of the analyses performed, we have decided to give them subgeneric taxonomic rank and name them Subg. *Arctium* and Subg. *Cynaroides* (Fig. 3). Subgenus *Arctium* comprises all the species of *Arctium* sect. *Arctium* sensu Duistermaat (1996) included in the analyses altogether with *Cousinia arctioides*, *C. vavilovii*, *C. grandifolia*, *Schmalhausenia nidulans* and the two species of *Hypcacanthium*. In turn, all the species of *Arctium* sect. *Arctium* sensu Duistermaat (1996) (*A. minus*, *A. leiospermum*, *A. palladini* and *A. lappa*) and *Cousinia* sect. *Nanarctium* (*C. arctioides*) form a strong supported clade which we give sectional rank as sect. *Arctium*. This section is characterized by involucral bracts ending in hooked apical appendage and achenes oblong with apical ridge. We shall comment that our results indicate that *A. leiospermum* and *A. lappa* are different species against the idea of the former being a synonym of *A. lappa* (Duistermaat, 1996). The rest of the species of Subg. *Arctium -C. grandifolia, C. vavilovii*, *S. nidulans*, *H. evidens* and *H. echinopifolium-* are merged in a highly supported group in the analysis with the three markers combined (Fig. 3) but weakly supported in the rest of the analyses (Figs. 1, 2). Taking into account the great morphologic differences between these taxa and the traditional classification, we consider this group as a polytomy. Despite this, *S. nidulans*, *H. evidens* and *H. echinopifolium* are really close to each other as well as quite similar morphologically and therefore we propose grouping them into the newly established sect. *Schmalhausenia* (Fig. 3).

Regarding subgenus *Cynaroides,* it includes most of the species of the Arctioid lineage and is again divided into two subgroups (Fig. 3). The first one, weakly supported, comprises most species of the paraphyletic subgenus *Hypacanthodes*, excluding *C.*

grandifolia and *C. vavilovii* merged in the new Subg. *Arctium*, and *C. korshinskyi*, the type species of this paraphyletic subgenus, which appears separated by two branches with PP = 1.00 from the rest of its species. The solution we suggest, waiting for more studies and given its morphological unity, is maintaining this weakly supported group with lower taxonomic rank as sect. *Hypacanthodes*, comprising *C. abolinii*, *C. dolichophylla, C. egregia, C. fedtschenkoana, C. korshinskyi* and *C. macilenta*.

As far as sectional classification is concerned, many species are merged in our analyses congruently with the sectional classification by Tscherneva (1962, 1988a, b, c) and, as pointed out in the results, most of these sections are monophyletic and therefore should be maintained as currently established. All seven trees obtained agree in the monophyly of sect. *Pseudarctium* (Fig. 3) which is well characterized morphologically by apically hooked involucral bracts with marginal glands. Furthermore, our results suggest that *C. amplissima* and *C. pseudarctium* are different species, as Duistermaat (1996) stated, instead of being *C. amplissima* a synonym of *C. pseudarctium* (Tscherneva, 1962).

Most of our analyses also show the sect. *Chrysis* to be monophyletic although *C. anomala*, the only species of sect. *Ctenarctium*, unexpectedly falls into this clade (Fig. 3). The placement of *C. anomala* leads us to suppress sect. *Ctenarctium*. In addition, a subdivision of this lineage, congruent with morphological evidences, is found between *C. anomala, C. aurea, C. medians, C. refracta* and *C. schmalhausenii* (PP = 0.89, PP = 0.97 and PP = 0.99 in the nuclear, ITS-*trnT* and combined analyses respectively) having entire leaves on the one hand and *C. karatavica*, *C. chlorantha* and *C. korolkovii* with pinnatipartite leaves on the other hand.

Besides, our results suggest that sect. *Pectinatae,* including *C. triflora* from the monotypic sect. *Oligantha*, is monophyletic as well (Fig. 3). Although in some analyses *A. palladivirens* merges with this clade reducing its statistical support, we prefer to maintain sect. *Pectinatae* because all its species (including *C. triflora*) have very characteristic involucral bracts with 2 to 6 pairs of spines along margins, not found in any other species of the entire Arctioid lineage. We propose to suppress sect. *Oligantha* in view of the mentioned placement of *C. triflora* supported by morphological evidence.

According to our results (Fig. 3), we suggest maintaining most of the monotypic sections as currently accepted based on morphological evidences (Tscherneva, 1962, 1988a, b, c): sect. *Amberbopsis (C. vavilovii),* sect. *Serratulopsis (C. grandifolia*) and sect. *Lappaceae (C. lappacea*). Finally, we propose to reduce the taxonomic rank of the monotypic genus *Anura* to sectional level as sect. *Anura*, comprising only *A. pallidivirens*.

At this point the main aim of this work, what means to establish the generic boundaries of *Arctium* and *Cousinia*, must be faced. Taking into account that *Arctium* as currently accepted is not monophyletic, it has to be redefined. If we want to keep together the species belonging to *Arctium* according to Duistermaat (1996), the most parsimonious option is to transfer all the Arctioid species to *Arctium*, turning it into a broader and monophyletic genus, which is supported by our analyses. Moreover, the Arctioid lineage is sharply differentiated from the Cousinioid by some important traits, mainly pollen type as well as basic chromosome number, and the molecular phylogeny supports this great differentiation, showing at the same time the monophyly of both clades and the paraphyly of genus *Cousinia*. Therefore the inclusion of the Arctioid species into a broader genus *Arctium* would turn *Cousinia* into a monophyletic genus and would sort out the ancient entanglement between these two genera.

Despite the undeniable monophyly of the Arctioid group and its uniformity regarding chromosome number, pollen type and style morphology, there are no morphological characters suitable enough to establish a strong classification based on our molecular analyses. As ascertained in López-Vinyallonga et al. (2009), the distribution of characters such as spiny *vs*. unarmed leaves or apically hooked *vs*. straight involucral bracts runs across generic and subgeneric boundaries. These characters, among others traditionally used in the definition of taxa of different rank in the *Arctium-Cousinia* complex, appear scattered through all the trees obtained denoting that they evolved several times in parallel. Therefore, we present a redefinition of the Arctioid lineage based on the present molecular phylogeny and supported by morphological evidences when possible. In addition, we provide nomenclatural changes when necessary.

Genus *Arctium* L., Sp. Pl. 2: 816 (1753)
Biennial or perennial with rootstock or taproot, spiny or unarmed suffruticose herbs. Leaves leathery or herbaceous, dentate, lobed, pinnatipartite to pinnatisect or rarely entire. Basal leaves in a rosette, the cauline ones similar to bottom leaves but gradually diminishing to stem apex, the most distal ones usually sessile. Synflorescence paniculate, racemose or corymbose. Capitula homogamous, solitary or in clusters, sessile to long pedunculate with three to more than 100 florets, spherical to ovoid, glabrous to densely arachnoid. Involucral bracts pluriseriate, imbricate, basally appressed, apex hooked, curved or ending in a straight spine. Receptacle densely covered with rough and smooth bristles. Bristles of pappus scabrous, free, deciduous. Florets uniform, hermaphrodite. Corolla tubulose-campanulate, white, yellow or pink to purple, glabrous or with glandular hairs, limb campanulate. Anthers with basal appendages fringed (occasionally entire), apical appendages deltoid, glabrous or dorsally villose. Style glabrous with a ring of sweeping hairs at the thickened articulation. Achenes glabrous, oblong or ovate, sometimes somewhat compressed, smooth, rugose or with longitudinal ridges, brown; often with dark and irregular spots, sometimes with apical rim.

Subg. *Arctium*: Biennial or perennial herbs. Leaves leathery or herbaceous, spiny or unarmed, lobed, two-times pinnatisect or rarely entire. Synflorescence racemose or corymbose. Capitula short to long-pedunculate (0,2 – 8 cm) with more than 20 to more than 100 florets, spherical or subspherical, glabrous, tomentose or lanuginose, sometimes glandular. Involucral bracts apex hooked or ending in a straight spine. Corolla tubulose-campanulate, pink to purple, sometimes whitish or yellow, glabrous or with glandular hairs. Anthers with basal appendages entire to fringed.

Subg. *Cynaroides*: Perennial herbs. Leaves leathery or herbaceous, spiny or unarmed, dentate, lobed, pinnatipartite to pinnatisect or rarely entire. Synflorescence paniculate, racemose or corymbose. Capitula sessile to long-pedunculate (up to 10 cm) with three to more than 50 florets, usually ovoid, sometimes subespherical or oblong, glabrous to densely arachnoid. Involucral bracts apex hooked, curved or ending in a straight spine. Corolla tubulose-campanulate, usually yellow and sometimes white or pink, glabrous. Anthers with basal appendages fringed.

Next we list the nomenclatural changes derived from the present work. We provide synonyms only when necessary for avoiding possible confusions. New proposals are boldfaced.

Genus *Arctium*

Arctium Subgenus *Arctium*

Arctium* Sect. *Amberbopsis (Tscherneva) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia* Sect. *Amberbopsis* Tscherneva in Bot. Mater. Gerb. Inst. Bot. Akad. Nauk Uzbeksk. SSR 17: 83 (1962)
Arctium grandifolium (Kult.) S. López, Susanna & N. Garcia, ***comb. nov***.
Basionym: *Cousinia grandifolia* Kult. in Trudy Sredne-Aziatsk. Gosud. Univ., Ser. 8b, Bot. 6: 9 (1929)

Arctium Sect. *Arctium*
Arctium arctioides (Schrenk) Kuntze, Revis. Gen. Pl. 1: 307 (1891)
Basionym: *Cousinia arctioides* Schrenk in Bull. Phys.-Math. Acad. Petersb. 2: 115 (1844)

Arctium atlanticum (Pomel) H. Lindb. in Acta Soc. Sci. Fenn. n. s. B, 1, 2: 153 (1932)

Arctium lappa L., Sp. Pl. 2: 816 (1753)

Arctium leiospermum Juz. & Ye. V. Serg. in Bot. Mater. Gerb. Bot. Inst. Komarova Akad. Nauk SSSR 18: 299 (1957)

Arctium minus (Hill) Bernh., Syst. Verz. Erfurt: 154 (1800)

Arctium nemorosum Lej. in Mag. Hort. 1: 289 (1833)

Arctium palladini (Marcow.) R. E. Fr. & E. S. Söderb. in Delect. Spor. Sem. Hort. Bot. Berg. (1923)

Arctium tomentosum Mill., Gard. Dict., ed. 8, 3 (1768)

Arctium* Sect. *Schmalhausenia S. López, Susanna & N. Garcia, ***comb.* et *stat. nov.***
Basionym: Gen. *Schmalhausenia* C. Winkl. in Trudy Imp. S.-Peterburgsk. Bot. Sada 12: 281 (1892)

Arctium echinopifolium (Juz.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia echinopifolia* Bornm. in Beih. Bot. Centralbl. 34: 192 (1916).
Synonym: *Hypacanthium echinopifolium* (Bornm.) Juz. in Trudy Bot. Inst. Akad. Nauk SSSR, Ser. 1, Fl. Sist. Vysš. Rast. 3: 324 (1937)

Arctium eriophorum (Regel & Schmalh.) Kuntze, Revis. Gen. Pl. 1: 307 (1891)
Basionym: *Cousinia eriophora* Regel & Schmalh. in Trudy Imp. S.-Peterburgsk. Bot. Sada 6: 313 (1879)
Synonym: *Schmalhausenia nidulans* Petr. in Allg. Bot. Z. Syst. 20: 117 (1914)

Arctium evidens (Tscherneva) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Hypacanthium evidens* Tscherneva in Bot. Žhurn. (Moscow & Leningrad) 68: 634 (1983)

Arctium* Sect. *Serratulopsis (Tscherneva) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia* Sect. *Serratulopsis* Tscherneva in Bot. Mater. Gerb. Inst. Bot. Akad. Nauk Uzbeksk. SSR 17: 83 (1962)

Arctium vavilovii (Kult.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia vavilovii* Kult. in Bjull. Sredne-Aziatsk. Gosud. Univ. 12. Suppl. 15 (1926)

Arctium **Subgenus** ***Cynaroides*** (Tscherneva) S. López, Susanna & N. Garcia, ***comb. nov.***

Basionym: *Cousinia* Subg. *Cynaroides* Tscherneva in Bot. Žhurn. (Moscow & Leningrad) 73: 594 (1988)

Arctium **Sect.** ***Anura*** S. López, Susanna & N. Garcia, ***comb. nov.***

Basionym: *Cousinia* Sect. *Anura* Juz. in Trudy Tadžikistansk. Bazy 8: 512, 546 (1940)

Synonym: Gen. *Anura* (Juz.) Tscherneva in Bot. Mater. Gerb. Inst. Bot. Akad. Nauk Uzbeksk. SSR 17: 107 (1962)

Arctium pallidivirens (Kult.) S. López, Susanna & N. Garcia, ***comb. nov.***

Basionym: *Cousinia pallidivirens* Kult. in Trudy Sredne-Aziatsk. Gosud. Univ., Ser. 8b, Bot. 6: 3 (1929)

Synonym: *Anura pallidivirens* (Kult.) Tscherneva, Fl. URSS 27: 134 (1962)

Arctium **Sect.** ***Chrysis*** (Juz.) S. López, Susanna & N. Garcia, ***comb. nov.***

Basionym: *Cousinia* Sect. *Chrysis* Juz. in Trudy Tadžikistansk. Bazy 8: 515, 548 (1940)

Arctium anomalum (Franch.) Kuntze, Revis. Gen. Pl. 1: 307 (1891)

Basionym: *Cousinia anomala* Franch. in Ann. Sci. Nat. Bot. 6: 316 (1883)

Arctium aureum (C. Winkl.) Kuntze, Revis. Gen. Pl. 1: 307 (1891)

Basionym: *Cousinia aurea* C. Winkl. in Trudy Imp. S.-Peterburgsk. Bot. Sada 10: 475 (1887)

Arctium chloranthum (Kult.) S. López, Susanna & N. Garcia, ***comb. nov.***

Basionym: *Cousinia chlorantha* Kult. in Trudy Sredne-Aziatsk. Gosud. Univ., Ser. 8b, Bot. 6: 4 (1929)

Arctium haesitabundum (Juz.) S. López, Susanna & N. Garcia, ***comb. nov.***

Basionym: *Cousinia haesitabunda* Juz. in Trudy Tadžikistansk. Bazy 8: 516, 549 (1940)

Arctium karatavicum (Regel & Schmalh.) Kuntze, Revis. Gen. Pl. 1: 308 (1891)
Basionym: *Cousinia karatavica* Regel & Schmalh. in Trudy Imp. S.-Peterburgsk. Bot. Sada 6: 317 (1879)

Arctium korolkowii (Regel & Schmalh.) Kuntze, Revis. Gen. Pl. 1: 308 (1891)
Basionym: *Cousinia korolkovii* Regel & Schmalh. in Trudy Imp. S.-Peterburgsk. Bot. Sada 6: 315 (1879)

Arctium medians (Juz.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia medians* Juz. in Trudy Bot. Inst. Akad. Nauk SSSR, Ser. 1, Fl. Sist. Vysš. Rast. 3: 297 (1936)

Arctium refractum (Bornm.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia aurea β refracta* Bornm. in Beih. z. Bot. Centralbl., Bd. 34: 138 (1917)
Synonym: *Cousinia refracta* (Bornm.) Juz. in Trudy Bot. Inst. Akad. Nauk SSSR, Ser. 1, Fl. Sist. Vysš. Rast. 3: 297 (1937)

Arctium schmalhausenii (C. Winkl.) Kuntze, Revis. Gen. Pl. 1: 308 (1891)
Basionym: *Cousinia schmalhausenii* C. Winkl. in Trudy Imp. S.-Peterburgsk. Bot. Sada 10: 474 (1887)

Arctium* Sect. *Hypacanthodes S. López, Susanna & N. Garcia, ***comb. et stat. nov.***
Basionym: *Cousinia* Subg. *Hypacanthodes* Tscherneva in Bot. Žhurn. (Moscow & Leningrad) 73: 594 (1988)

Arctium abolinii (Kult. ex Tscherneva) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia abolinii* Kult. ex Tscherneva in Bot. Mater. Gerb. Inst. Bot. Akad. Nauk Uzbeksk. SSR 17: 88 (1962)

Arctium dolichophyllum (Kult.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia dolichophylla* Kult., Trudy Turkestansk. Naučn. Obšč. 1: 112 (1923)

Arctium egregium (Juz.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia egregia* Juz. in Bot. Mater. Gerb. Glavn. Bot. Sada RSFSR 5: 110 (1924)

Arctium fedtschenkoanum (Bornm.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia fedtschenkoana* Bornm. in Beih. Bot. Centralbl. 34: 199 (1916)

Arctium korshinskyi (C. Winkl.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia korshinskyi* C. Winkl. in Trudy Imp. S.-Peterburgsk. Bot. Sada 14: 236 (1897)

Arctium macilentum (C. Winkl.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia macilenta* C. Winkl. in Trudy Imp. S.-Peterburgsk. Bot. Sada 14: 222 (1897)

Arctium pterolepidum (Kult.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia pterolepida* Kult. in Trudy Sredne-Aziatsk. Gosud. Univ., Ser. 8b, Bot. 6: 27 (1929)

Arctium ugamicum (Karmysch.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia ugamica* Karmysch., Fl. Kazakhst. 9: 228, 568 (1966)

Arctium Sect. *Lappaceum* (Bunge) Duist.

Arctium lappaceum (Schrenk) Kuntze, Revis. Gen. Pl. 1: 308 (1891)
Basionym: *Cousinia lappacea* Schrenk, Enum. Pl. Nov. 1: 42 (1841)

Arctium* Sect. *Pectinatae (C. Winkl.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia* Sect. *Pectinatae* C. Winkl. in Trudy Imp. S.-Peterburgsk. Bot. Sada 12: 263 (1892)
Arctium alberti (Regel & Schmalh.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia alberti* Regel & Schmalh. in Trudy Imp. S.-Peterburgsk. Bot. Sada 6: 315 (1879)

Arctium horrescens (Juz.) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia horrescens* Juz. in Trudy Bot. Inst. Akad. Nauk SSSR, Ser. 1, Fl. Sist. Vysš. Rast. 3: 322 (1937)

Arctium pentacanthoides (Juz. ex Tscherneva) S. López, Susanna & N. Garcia, ***comb. nov.***
Basionym: *Cousinia pentacanthoides* Juz. ex Tscherneva in Bot. Mater. Gerb. Inst. Bot. Akad. Nauk Uzbeksk. SSR 17: 102 (1962)

Arctium pentacanthum (Regel & Schmalh.) Kuntze, Revis. Gen. Pl. 1: 308 (1891)
Basionym: *Cousinia pentacantha* Regel & Schmalh. in Trudy Imp. S.-Peterburgsk. Bot. Sada 6: 315 (1879)

Arctium triflorum (Schrenk) Kuntze, Revis. Gen. Pl. 1: 308 (1891)
Basionym: *Cousinia triflora* Schrenk in Bull. Cl. Phys.-Math. Acad. Imp. Sci. Saint-Pétersbourg 3: 108 (1845)

Arctium Sect. *Pseudarctium* (Juz.) Duist. in Gorteria 3: 112 (1996)
Arctium amplissimum (Boiss.) Kuntze, Revis. Gen. Pl. 1: 307 (1891)
Basionym: *Cousinia amplissima* Boiss., Fl. Orient. [Boissier] 3: 462 (1875)

Arctium pseudarctium (Bornm.) Duist. in Gorteria 3: 114 (1996)
Basionym: *Cousinia pseudarctium* Bornm. in Beih. Bot. Centralbl. 34: 135 (1916)

Arctium tomentellum (C. Winkl.) Kuntze, Revis. Gen. Pl. 1: 308 (1891)

Basionym: *Cousinia tomentella* C. Winkl. in Trudy Imp. S.-Peterburgsk. Bot. Sada 10: 469 (1887)

Arctium umbrosum (Bunge) Kuntze, Revis. Gen. Pl. 1: 308 (1891)
Basionym: *Cousinia umbrosa* Bunge, Gatt. Cous. 10

Table 3. Classification of the Arctioid group suggested according with the present results and based on Tscherneva (1962, 1982, 1988a, b, c) and Duistermaat (1996).

Species	Section	Subgenus
Arctium grandifolium (Kult.) S. López, Susanna & N. Garcia	*Amberbopsis* (Tscherneva) S. López, Susanna & N. Garcia	*Arctium* S. López, Susanna & N. Garcia
Arctium arctioides (Schrenk.) Kuntze	*Arctium* L.	
Arctium atlanticum (Pomel) H. Lindb.		
Arctium lappa L.		
Arctium leiospermum Juz. & Ye. V. Serg.		
Arctium minus (Hill) Bernh.		
Arctium nemorosum Lej.		
Arctium palladini (Marcow.) R.E.Fr. et E.S. Söderb.		
Arctium tomentosum Mill.		
Arctium vavilovii (Kult.) S. López, Susanna & N. Garcia	*Serratulopsis* (Tscherneva) S. López, Susanna & N. Garcia	
Arctium echinopifolium (Juz.) S. López, Susanna & N. Garcia	*Schmalhausenia* S. López, Susanna & N. Garcia	
Arctium eriophorum (Regel & Schmalh.) Kuntze		
Arctium evidens (Tscherneva) S. López, Susanna & N. Garcia		
Arctium pallidivirens (Kult.) S. López, Susanna & N. Garcia	*Anura* S. López, Susanna & N. Garcia	*Cynaroides* (Tscherneva) S. López, Susanna & N. Garcia
Arctium anomalum (Franch.) Kuntze	*Chrysis* (Juz.) S. López, Susanna & N. Garcia	
Arctium aureum (C. Winkl.) Kuntze		
Arctium chloranthum (Kult.) S. López, Susanna & N. Garcia		
Arctium haesitabundum (Juz.) S. López, Susanna & N. Garcia		
Arctium karatavicum (Regel & Schmalh.) Kuntze		
Arctium korolkowii (Regel & Schmalh.) Kuntze		
Arctium medians (Juz.) S. López, Susanna & N. Garcia		
Arctium refractum (Bornm.) S.		

López, Susanna & N. Garcia		
Arctium schmalhausenii (C. Winkl.) Kuntze		
Arctium abolinii (Kult. ex Tscherneva) S. López, Susanna & N. Garcia	*Hypacanthodes* S. López, Susanna & N. Garcia	
Arctium dolichophyllum (Kult.) S. López, Susanna & N. Garcia		
Arctium egregium (Juz.) S. López, Susanna & N. Garcia		
Arctium fedtschenkoanum (Bornm.) S. López, Susanna & N. Garcia		
Arctium korshinskyi (C. Winkl.) S. López, Susanna & N. Garcia		
Arctium macilentum (C. Winkl.) S. López, Susanna & N. Garcia		
Arctium pterolepidum (Kult.) S. López, Susanna & N. Garcia		
Arctium ugamicum (Karmysch.) S. López, Susanna & N. Garcia		
Arctium lappaceum (Schrenk.) Kuntze	*Lappaceum* (Bunge) Duist.	
Arctium albertii (Regel & Schmalh.) S. López, Susanna & N. Garcia	*Pectinatae* (C. Winkl.) S. López, Susanna & N. Garcia	
Arctium horrescens (Juz.) S. López, Susanna & N. Garcia		
Arctium pentacanthoides (Juz. ex Tscherneva) S. López, Susanna & N. Garcia		
Arctium pentacanthum (Regel & Schmalh.) Kuntze		
Arctium triflorum (Schrenk.) Kuntze		
Arctium amplissimum (Boiss.) Kuntze	*Pseudarctium* (Juz.) Duist.	
Arctium pseudarctium (Bornm.) Duist.		
Arctium tomentellum (C. Winkl.) Kuntze		
Arctium umbrosum (Bunge) Kuntze		

CONCLUDING REMARKS

Based on the present molecular analyses a broadly monophyletic genus *Arctium* is proposed which is divided into two new monophyletic subgenera, Subg. *Arctium* and Subg. *Cynaroides*. These rearrangements lead to the suppression of subgenus *Hypacanthodes* and the recombination and redefinition of subgenus *Cynaroides* because as previously defined they were paraphyletic. In contrast, the traditional sectional classification is mainly maintained. Further work is needed in order to establish a definitive and robust subgeneric and sectional classification of the Arctioid lineage.

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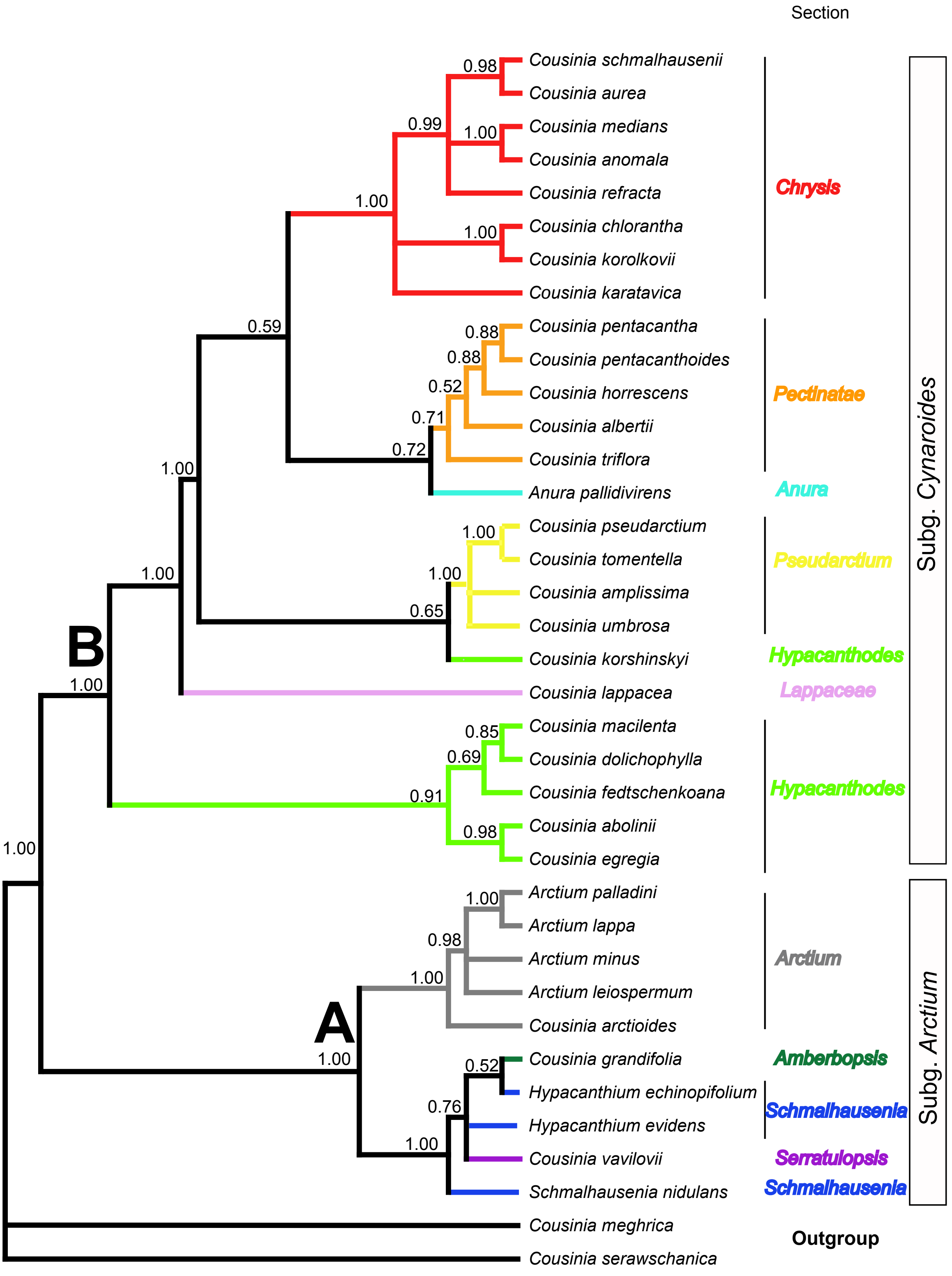

Fig. 3. 50% Majority rule consensus tree obtained from the Bayesian analysis of the ITS + *rpS4-trnT-trnL* + *rpl32-trnL* data set (namely combined tree). Numbers on branches are posterior probabilities. The sectional classification proposed is shown in different colors. Clade A, clade B.

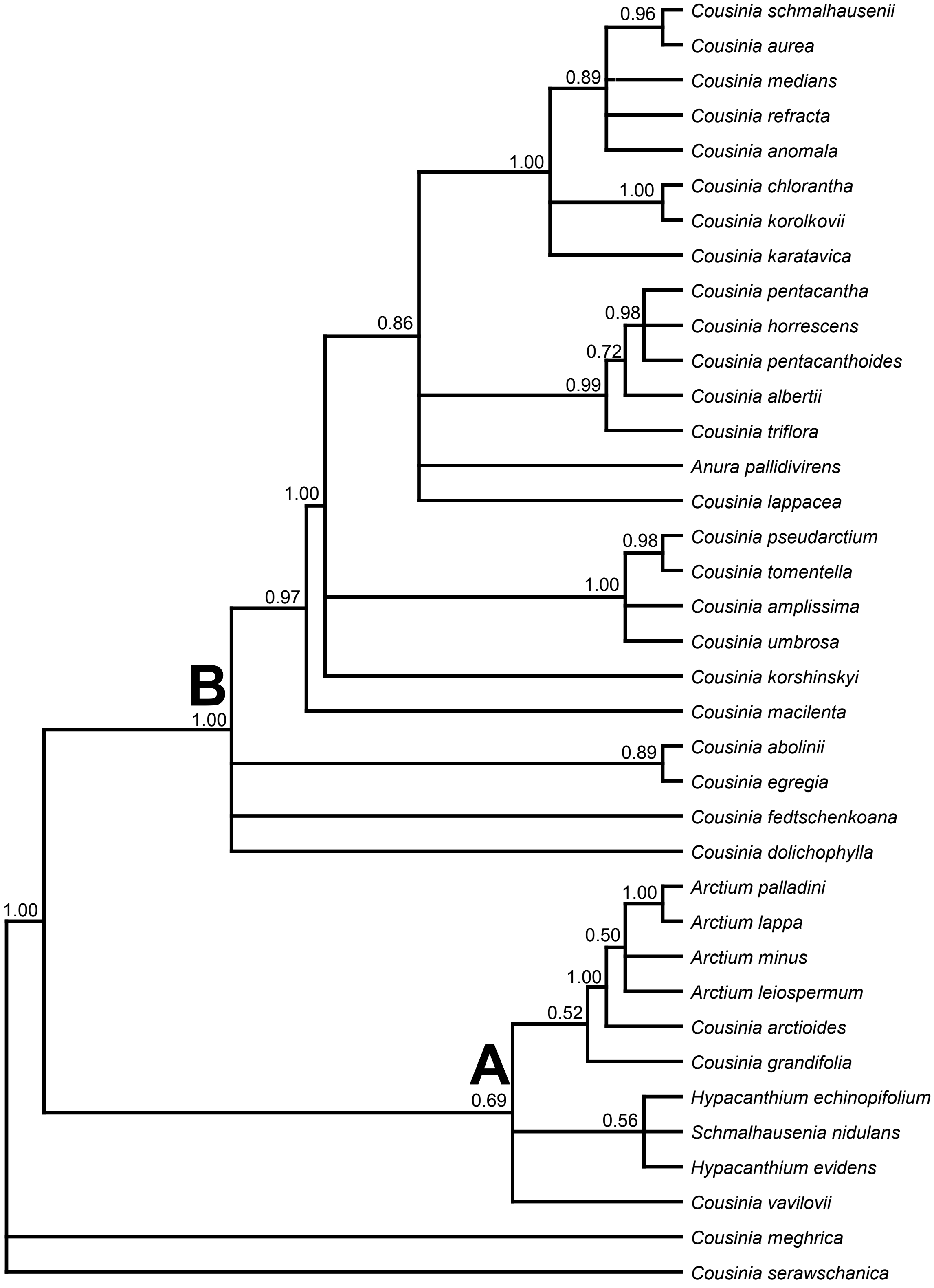

Fig. 1. 50% Majority rule consensus tree obtained from the Bayesian analysis of the ITS data set (namely nuclear tree). Numbers on branches are posterior probabilities. Clade A: subgenus *Arctium*, clade B: subgenus *Cynaroides*.

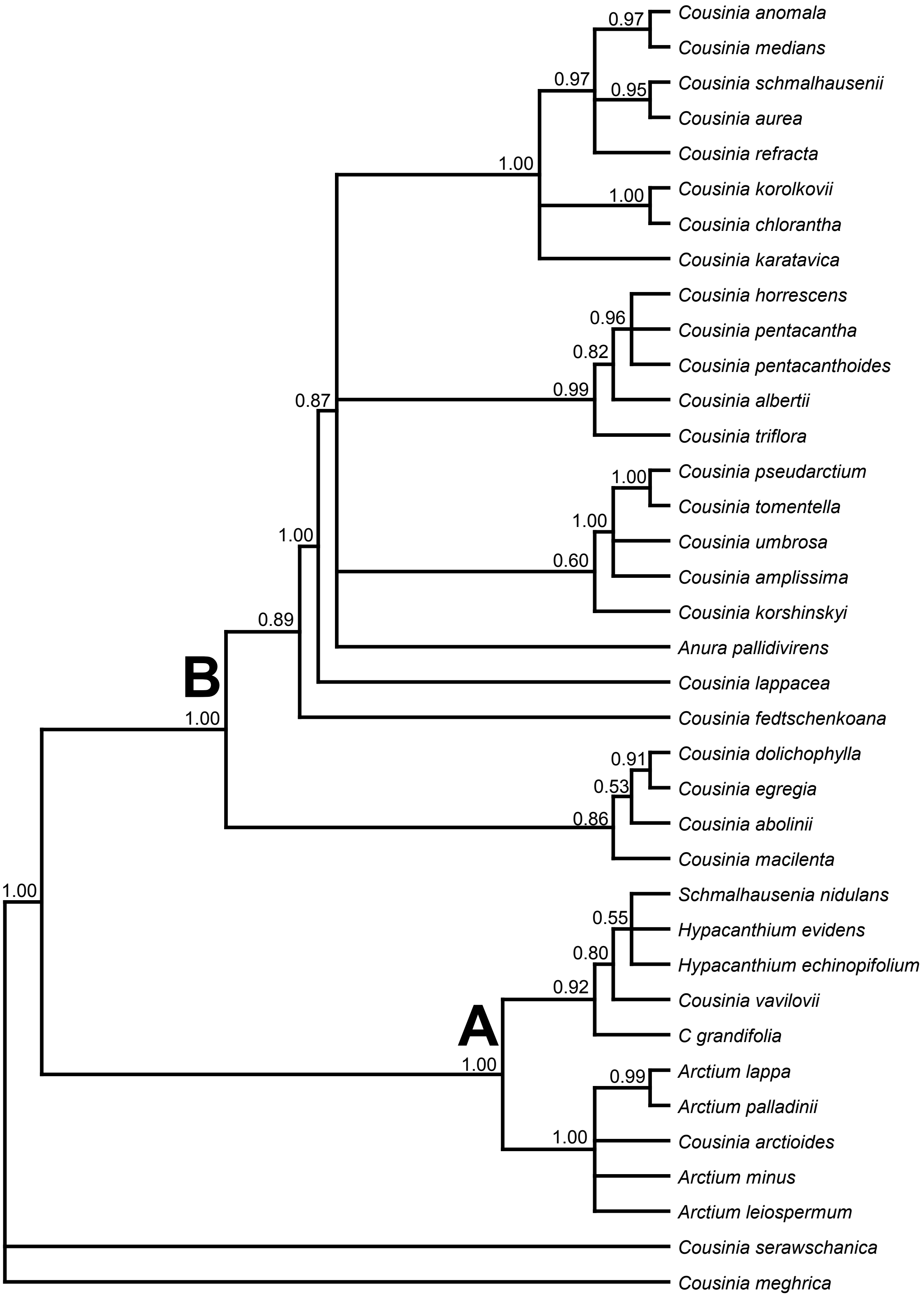

Fig. 2. 50% Majority rule consensus tree obtained from the Bayesian analysis of the ITS + rpS4-*trnT*-*trnL* data set (namely ITS-*trnT* tree). Numbers on branches are posterior probabilities. Clade A: subgenus *Arctium*, clade B: subgenus *Cynaroides*.

7. 3. Chromosome counts in the genera *Cousinia*, *Olgaea* and *Syreitschikovia* (Compositae)

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RESUM. Les escasses dades cariològiques disponibles tant per al complex *Arctium-Cousinia* com pel grup *Onopordum*, el qual mostra paral·lelismes amb el complex *Arctium-Cousinia*, han motivat aquest treball en que s'aporten recomptes cromosòmics amb l'objectiu de contribuir a entendre l'evolució cariològica d'aquests taxons. Es presenten 20 recomptes per al gènere *Cousinia* s. l. 13 dels quals són dades noves i els set restants complementen, confirmen o qüestionen recomptes anteriors. Tres de les 13 seccions presents a aquest estudi han estat comptades aquí per primera vegada. Els recomptes de les set espècies del llinatge Arctioide, $2n = 36$, mostren una gran uniformitat cariològica. En canvi, al grup Cousinioide els tretze recomptes que s'aporten coincideixen amb els tres principals nombres cromosòmics de la seva característica sèrie disploide $2n = 22$, 24 i 26. Tots aquests resultats confirmen la idea de que, malgrat que el grup Arctioide i Cousinioide conformen un grup natural, han seguit camins evolutius diferents. Finalment es presenten nous recomptes per als gèneres *Olgaea* i *Syreitschikovia*, $2n = 26$ i 24 respectivament, que mai havien estat comptats. Aquestes dades confirmen l'existència de dos llinatges també al grup *Onopordum*; els taxons colonitzadors ($x = 17$) i els perennes ($x = 12$, 13). No s'han trobat evidències de poliploïdia recent a cap dels grups estudiats.

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Chromosome counts in the genera *Cousinia, Olgaea* and *Syreitschikovia* (Compositae)

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Abstract

The scarce karyological data available for both the *Arctium–Cousinia* complex and the *Onopordum* group has led us to provide more data essential to understand the karyological evolution of these taxa. Chromosome counts were made on somatic metaphases using the squash technique. The root tips were pretreated with 8–hydroxyquinoleine solution at 4ºC, fixed in fresh Carnoy I solution overnight at – 20ºC, hydrolysed with 5N HCl for 50 min at room temperature, stained with 1% acetic orcein and squashed in 45% acetic acid. We report twenty chromosome counts for the genus *Cousinia* s.l. Thirteen of them are new data and the remaining seven provide confirmation of scarce or uncertain previous data. Three of the thirteen sections present are counted for the first time. Our counts of the seven Arctioid species show karyological uniformity with $2n = 36$. In the Cousinioid group, our thirteen counts agree with the three major numbers of its characteristic dysploidy series $2n = 22$, 24 and 26. We also report two new chromosome counts for the genera *Olgaea* and *Syreitschikovia*, $2n = 26$ and 24 respectively, never counted before. Our results confirm the idea that, although the Arctioid and Cousinioid clades form a monophyletic group, they have followed different evolutionary paths. In the *Onopordum* group, our results confirm the existence of two lines; the colonizing taxa ($n = 17$) and the perennial genera ($n = 12$, 13). The evidence for recent polyploidization is absent in both *Arctium–Cousinia* complex and the *Onopordum* group.

Key words: dysploidy; karyology; polyploidy; Arctioid clade; Cousinioid clade.

Introduction

Our contribution deals with two of the informal (not taxonomic) groups defined in the latest survey of the large tribe Cardueae Cass. (Compositae): The *Arctium–Cousinia* complex and the *Onopordum* group. According to its most recent circumscription (Susanna and Garcia–Jacas 2007), the *Arctium–Cousinia* complex is a monophyletic group composed of four genera. In the last work focused on this complex (López–Vinyallonga et al. 2009), all the analyses performed, based on DNA sequences (nrDNA ITS and cpDNA *rps4–trnT–trnL*), consistently divided the complex in two major lineages: the Arctioid and the Cousinioid clades. This division agrees with pollen morphology and chromosome number.

The Arctioid group comprises the genera *Arctium* L. with 11 species, *Hypacanthium* Juz. with two species, the monotypic *Schmalhausenia* C. Winkl. and 24 species of *Cousinia* belonging to subgenera *Cynaroides* and *Hypacanthodes*. As stated by Knapp (1987), most species of *Cousinia* subg. *Cynaroides* and *Hypacanthodes* grow only in the mountainous terrain of the Pamir–Alai range and in the western Tien Shan in Central Asia. The two species of *Hypacanthium* are endemic to the western Tien Shan, and the monotypic *Schmalhausenia* is endemic to the subalpine and alpine zone in the northern Tien Shan. *Arctium* s.str. is Eurosiberian in distribution (Duistermaat 1996). The pollen type of the Arctioid species, named *Arctiastrum*, is orbicular and spiny. According to Tscherneva (1985) and Susanna et al. (2003a), the Arctioid group, is karyologically uniform since all the studied species have n = 18, despite being formed by four different genera and despite some morphological incongruences. This high number, the highest in all the Cardueae, suggests that the Arctioid group constitute an old polyploid complex (Tscherneva 1985) or paleopolyploid following the nomenclature in Wagner (1980) and Ramsey and Schemske (2002).

The Cousinioid group comprises only *Cousinia* subgenus *Cousinia* with ca. 500 species (Mehregan and Kadereit 2008; López–Vinyallonga et al. 2009). According to Rechinger (1986) and Knapp (1987), this genus is distributed in the Turkestan mountain region (Tien Shan and Pamir–Alai) and in the Irano–Turanian region. Its pollen, named *Cousinia* pollen type, is oblong and smooth. This group shows a dysploid series of x =

9, 10, 11, 12 and 13. Like other groups of tribe Cardueae dysploidy in *Cousinia* is probably descending as is generally accepted in the tribe. Frankton and Moore (1961), Fernández Casas and Fernández Morales (1979), Siljak–Yakolev (1986) and Garcia Jacas and Susanna (1992) pointed out that high basic numbers should be regarded as more primitive than the reduced ones. This was considered a general trend by Stebbins (1950, 1971) and Grant (1981).

In the literature there are only 149 species for which chromosome numbers are reported among the ca. 500 species belonging to the *Arctium–Cousinia* complex. This number represents only 55% of the species of the subg. *Cynaroides*, 30% of the subg. *Hypacanthodes* and 22.5% of the subg. *Cousinia*. Besides, some of these counts are unconfirmed. These percentages are very low, which demonstrates that this huge complex is not enough karyologically studied.

As regards to the second group of our present study, the *Onopordum* complex (Cardueae–Carduinae) is formed by two well–defined groups. The first group is formed by a single large genus of widespread biennials, *Onopordum* L. (60 species), native to the Irano–Turanian and Mediterranean regions and introduced as noxious weeds in Australia, California and South America (Susanna and Garcia–Jacas 2007). The second one is constituted by seven small genera of perennial herbs with a narrow Central and East Asian distribution: *Alfredia* Cass. (four species), *Ancathia* DC. (one species), *Lamyropappus* Knorring & Tamamsch. (one species), *Olgaea* Iljin (16 species), *Syreitschikovia* Pavlov (two species), *Synurus* Iljin (four species) and *Xanthopappus* C. Winkl. (one species). Previous counts on this group have reported $2n = 34$ in *Onopordum* and $2n = 26$ in *Ancathia* and *Synurus*. In *Alfredia*, two different numbers have been reported, $2n = 24$ and $2n = 26$. There are no counts in the literature for the rest of the genera of this complex.

The scarce karyological data available for both the *Arctium–Cousinia* complex and the *Onopordum* group has led us to provide more data, which are essential to understand the karyological evolution of these taxa.

Material and methods

Chromosome counts were made on somatic metaphases using the squash technique. Root tip meristems from germinating achenes, either collected in the field or from wild plants cultivated in pots in the Botanical Institute of Barcelona, were used. Voucher specimens are deposited in the herbarium of the Botanical Institute of Barcelona (BC).

The root tips were pretreated with 0.002M 8–hydroxyquinoline solution at 4ºC for 8 h. After a distilled water wash, the material was fixed in fresh Carnoy I solution (3 : 1 v/v absolute ethanol : glacial acetic acid) overnight at – 20ºC, and stored in 70% ethanol at – 20ºC. This material was hydrolysed with 5N HCl for 50 min at room temperature, washed with distilled water and stained with 1% acetic orcein and squashed in 45% acetic acid. For all the counts, at least five plates from five to ten different individuals were examined. Preparations were made permanent by freezing with CO_2, ethanol–dehydrating and mounting in Canada balsam. Metaphase plates were photographed using an Olympus 3030 digital camera mounted on an Olympus microscope U–TV1 X. The chromosome preparations are preserved in the Botanical Institute of Barcelona.

In this work, we follow the sectional classification proposed by Tscherneva (1962; 1988) for the species distributed in Central Asia and the one from Rechinger (1972) for the species distributed in the Irano–Turanian region.

Results and discussion

The Arctium–Cousinia complex

Cousinia subgenus Cousinia

Cousinia sect. Alpinae Bunge

Cousinia serawschanica C. Winkl.
Tadjikistan: Kishlak Magian settlement, 39º13’17’’ N, 67º39’24’’ E, 2200 m, 18 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2526* (BC). 2*n* = 24 (Fig. 1a).

According to our data, this is the first chromosome count for this species which agrees with the chromosome number established for *C.* sect. *Alpinae* in previous reports from Central Asia by Tscherneva (1985) and Susanna et al. (2003b). Our count confirms x = 12 as a basic chromosome number for this section. There is a single previous count by Podlech and Bader (1974) of x = 13 in a species of *C.* sect. *Alpinae* from Afghanistan, so this section has two different chromosome numbers.

Cousinia sect. Carduncellus (Juz.) Rech. f.

Cousinia ferruginea Kult.

Tadjikistan: mountains above Kara–Chuira, 39º05’55’’ N, 71º20’43’’ E, 3700 m, 25 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2560* (BC); Gergatal mountains, Surjov, 39º13’14’’ N, 71º10’11’’ E, 25 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2563* (BC). 2n = 26 (Fig. 1b).
According to the available data, this is the first chromosome count for this species which is consistent with the number given for *C.* sect. *Carduncellus* x = 13.

Cousinia princeps Franch.

Tadjikistan: Zimargh, 39º08’04’’ N, 68º41’36’’ E, 3400 m, 14 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2493* (BC). 2n = 26 (Fig. 1c).
According to our data, this is the first chromosome count for this species which agrees with the number established for *C.* sect. *Carduncellus* x = 13.

Our two counts confirm x = 13 as a basic chromosome number for *C.* sect. *Carduncellus* in accordance with five previous reports by Tscherneva (1985) and Susanna et al. (2003b). There are some different and conflicting previous counts for this section. The report of 2n = 18 by Chuksanova in Fedorov (1969) for *C. tianschanica* was in conflict with 2n = 26 by Tscherneva (1985) and Susanna et al. (2003b). Podlech and Bader (1974) reported 2n = 24 for *C. buphthalmoides* but according to Tscherneva (1985) this species has 2n = 26. There is one more count for this section by Chuksanova in Fedorov (1969): 2n = 36 for *C. glaucifolia*, a species considered a synonym of *C. outichaschensis* which disagrees with 2n = 26 reported by Tscherneva (1985). With all these previous reports, we think that x = 13 is confirmed as the basic chromosome number for *C.* sect. *Carduncellus* and $x = 9$, $x = 12$ and $x = 18$ must be discarded. The most likely explanation for the wrong previous counts are putative mistakes in the determination of the species counted.

Cousinia sect. Coronophora (Juz.) Rech. f.

Cousinia radians Bunge
Tadjikistan: Kondara river canyon, Vorzovski Rayon Nature Reserve, 38º48'34'' N, 68º48'45'' E, 11 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2452* (BC). $2n = 26$ (Fig. 1d).
Our report agrees with one by Tscherneva (1985) although disagrees with the report of $2n = 18$ by Chuksanova in Fedorov (1969).

Our report, together with the previous counts of *C. coronata* (Aryavand 1976, Tscherneva 1985, Susanna et al. 2003b) and *C. mulgediifolia* (Tscherneva 1985), confirm $x = 13$ as the basic chromosome number for *C.* sect. *Coronophora.*

Cousinia sect. Cousinia

Cousinia aleppica Boiss.
Turkey: Gaziantep, 4 Aug 2002, *K. Ertuğrul, N. Garcia–Jacas, A. Susanna 2317 & T. Uysal* (BC). $2n = 26$ (Fig. 1e).
According to our data, this is the first chromosome count for this species which agrees with one of the numbers established for *C.* sect. *Cousinia*, $x = 13$.

Cousinia congesta Bunge
Uzbekistan: between Samarkand and Kitov, Takhta–Karachi pass, 1600 m, 7 Nov 1999, *L. Kapustina, F. Khassanov, A. Susanna 2059 & J. Vallès* (BC). $2n = 24$ (Fig. 1f).
Our count agrees with previous reports from Iran (Aryavand 1975; Ghaffari et al. 2006) but not with the $2n = 26$ reported by Chuksanova in Fedorov (1969) and Susanna et al. (2003b) based on seed material of the same population. After a careful revision of the preparations used for this doubtful count, preserved in the Botanical Institute of

Barcelona, we think that some chromosomes might get broken and therefore the number of chromosomes for this species was overestimated.

There are three reported basic chromosome numbers for *C.* sect. *Cousinia*: one $x = 9$ by Chuksanova in Fedorov (1969), eight $x = 12$ by Aryavand (1975), Tscherneva (1985), Susanna et al. (2003b) and Ghaffari et al. (2000, 2006) altogether with the provided in the present work and finally two $x = 13$ by Poddubnaja–Arnoldi (1931) plus the reported here. In agreement with all these data, $x = 12$ and $x = 13$ are confirmed as basic chromosome numbers for *C.* sect. *Cousinia* and $x = 9$ needs confirmation.

Cousinia sect. Decumbentes Rech. f.

Cousinia decumbens Rech. f.
Iran: Kuh–e–Shavar, 3400 m, 24 Aug 2005, *K. Romashchenko & A. Susanna 2622* (BC). $2n = 26$ (Fig. 1g).
According to our data, this is the first chromosome count for this species and for *C.* sect. *Decumbentes* which basic chrosomome number is $x = 13$.

Cousinia sect. Eriocousinia Tscherneva

Cousinia franchetii C. Winkl.
Tadjikistan: Zimargh, 39º08’29’’ N, 68º42’09’’ E, 3400 m, 13 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2498* (BC). $2n = 26$ (Fig. 1h).
Our count confirms that by Tscherneva (1985) from Tadjikistan and agrees with one of the given basic chromosome numbers of *C.* sect. *Eriocousinia*, $x = 13$.

Cousinia libanotica DC
Lebanon: Jabal el Mekmel, 19 Sept 2005, *M. Bou Dagher Kharrat, O. Hidalgo & K. Romashchenko 408* (BC). $2n = 24$ (Fig. 1i).
According to our data, this is the first chromosome count for this species, which agrees with one of the established chromosome numbers of *C.* sect. *Eriocousinia*, $x = 12$.

In accordance with previous authors, *C.* sect. *Eriocousinia* has three basic chromosome numbers, x = 11, 12 and 13 reported by Susanna et al. (2003b), Ghaffari et al. (2006) and Tscherneva (1985) respectively, and the two latest are confirmed in this work.

Cousinia sect. Homalochaete C. Winkl.

Cousinia coerulea Kult.
Tadjikistan: Vorzov canyon, 38º57'52'' N, 68º46'12'' E, 12 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2459* (BC). 2n = 24 (Fig. 1j).
According to our data, this is the first chromosome count for this species and for *C.* sect. *Homalochaete*, the basic chrosomome number of which is x = 12.

Cousinia sect. Jurineopsis (Juz.) Tschern.

Cousinia submutica Franch.
Tadjikistan: Voru, 39º13'39'' N, 67º59'07'' E, 2000–2300 m, 16 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2515* (BC). 2n = 26 (Fig. 1k).
According to the available data, this is the first report for this species and for *C.* sect. *Jurineopsis*, the basic chrosomome number of which is x = 13.

Cousinia sect. Microcarpae Bunge

Cousinia pulchella Bunge
Tadjikistan: Guissar–Darvaz Mt., Takob area, Rog, 38°51'11'' N, 68°59'50'' E, 2442 m, 26 Aug 2007, *I. Kudratov, K. Romashchenko 614 & A. Susanna* (BC). 2n = 22 (Fig. 1l).
According to our data, this is the first chromosome count for this species.

Cousinia sewerzowii Regel
Kazakhstan: Aksu Dzabagly Nature Reserve, 1800 m, 29 Aug 2000, *A. Ivashchenko, A. Susanna 2178 & J. Vallès* (BC); Aksu Dzabagly Nature Reserve, Chimkentskaya, Tiulkubas, Mashat canyon, 31 Aug 2000, *A. Ivashchenko, A. Susanna 2207 & J. Vallès* (BC). 2n = 22 (Fig. 2a).

Our count confirms the previous one from Kirgizstan by Tscherneva (1985).

There are three chromosome numbers for this section; $x = 11$ by Aryavand (1976), Tscherneva (1985) and Susanna et al. (2003b) and the present work. The number $x = 12$ was reported for two species, *C. centauroides* Fisch. & Mey. ex Bunge and *C. integrifolia* Franch., by Tscherneva (1985) and for one, *C. arachnoidea* Fisch. & C. A. Mey., by Susanna et al. (2003b). Finally, $x = 13$ was reported by Koul (1964), Podlech and Dieterle (1969), Ghaffari (1984), Tscherneva (1985), Susanna et al. (2003b) and Ghaffari et al. (2006). In agreement with all these data, three basic chromosome numbers are confirmed for *C.* sect. *Microcarpae;* $x = 11$, 12 and 13.

Cousinia subgenus Cynaroides Tscherneva

Cousinia sect. Chrysis Juz.

Cousinia aurea C. Winkl.
Tadjikistan: Schtut, Penjikent road, 39º24’42’’ N, 68º02’34’’ E, 16 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2514* (BC). $2n = 36$ (Fig. 2b).
Our count confirms previous reports by Tscherneva (1985) and Chuksanova in Fedorov (1969) and agrees with the number given for C. subg. *Cynaroides* and all the Arctioid species of the *Arctium–Cousinia* complex.

Cousinia karatavica Regel and Schmalh.
Kazakhstan: Dzhambulskaya oblast, Karatau mountains, Kuyuk pass, 35 km from Dzhambul on the road to Tashkent, 1000 m, 28 Aug 2000, *A. Ivashchenko, A. Susanna 2162 & J. Vallès* (BC); Dzhambulsky reg., between Ajsha–Bibi and Shakbak–Ata, Kuik pass, 42°45’57’’ N, 70°59’29’’ E, 758 m, 22 Aug 2007, *K. Romashchenko 607* (BC). $2n = 36$ (Fig. 2c).
Our counts agree with the unique number established for C. subg. *Cynaroides* and confirms previous reports by Tscherneva (1985) and Susanna et al. (2003b) while is in conflict with the previous $2n = 26$ by Chuksanova in Fedorov (1969).

Cousinia refracta (Bornm.) Juz.

Tadjikistan: Dushanbe: Guissar–Darvaz region, Kondara river canyon, Vorzovski Rayon Nature Reserve, 38º48’43’’ N, 68º48’13’’ E, 11 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2456* (BC). 2*n* = 36 (Fig. 2d).

According to our data, this is the first chromosome count for this species which agrees with the number given for *C.* subg. *Cynaroides*.

Cousinia sect. Ctenarctium Juz.

Cousinia anomala Franch.

Tadjikistan: Khujand (Leninabad), Zeravshan reg., v. Rebat, Ispena, 39°22’18’’ N, 68°12’13’’ E, 1795 m, 31 Aug 2007, *I. Kudratov, K. Romashchenko 627 & A. Susanna* (BC). 2*n* = 36 (Fig. 2e).

According to the available data, this is the first chromosome count for this species which is consistent with the chromosome number established for C. subg. *Cynaroides*.

Cousinia sect. Pseudarctium Juz.

Cousinia tomentella C. Winkl.

Tadjikistan: Dushanbe: Guissar–Darvaz, Kondara river canyon, Vorzovski Rayon Nature Reserve, 38°48’35’’ N, 68°48’30’’ E, 1299 m, 28 Aug 2007, *I. Kudratov, K. Romashchenko 616 & A. Susanna* (BC). 2*n* = 36 (Fig. 2f)

This is the first report for this species, according to our data, which agrees with the number given for C. subg. *Cynaroides*.

Cousinia subgenus Hypacanthodes Tscherneva

Cousinia sect. Lacerae Tscherneva

Cousinia fedtschenkoana Bornm.

Tadjikistan: Guissar–Darvaz reg., Sioma river head, 38°56’18’’ N, 68°42’41’’ E, 2696 m, 02 Sept 2007, *I. Kudratov, K. Romashchenko 632 & A. Susanna* (BC). 2*n* = 36 (Fig. 2g)

According to the available data, this is the first chromosome count for this species which coincides with the number established for C. subg. *Hypacanthodes*.

Cousinia macilenta C. Winkl.
Tadjikistan: Kishlak Magian settlement,, 39º12’50’’ N, 67º39’18’’ E, 2200 m, 18 Aug 2004, *I. Kudratov, K. Romashchenko & A. Susanna 2530* (BC). 2*n* = 36 (Fig. 2h)
According to our data, this is the first chromosome count for this species which agrees with the chromosome number stated for C. subg. *Hypacanthodes*.

These two counts are the first reports for *C.* sect. *Lacerae*, whose basic chrosomome number is *x* = 18 as expected, as it belongs to *C.* subg. *Hypacanthodes*.

Our results regarding the *Arctium–Cousinia* complex confirm the idea that, even though the Arctioid and Cousinioid clades form a monophyletic group, they have followed strongly different chromosomal and pollen type evolutionary paths (Susanna et al. 2003a). As expected, all the counts for the Arctioid species are 2*n* = 36, previously stated as the somatic chromosome number for *Arctium* by Moore and Frankton (1974).

The Cousinioid group is an acute contrast to the sole 2*n* = 36 of the Arctioid group. On the basis of our results and the above cited previous works, somatic chromosome numbers in the Cousinioid lineage are 2*n* = 26, 24, 22, 20 and 18 in a dysploid series ranging from *x* = 13 to 9. Our molecular results do not provide data on whether dysploidy is ascendant or descendent in the Cousinioid group (López–Vinyallonga et al. 2009), but descending dysploidy predominates in other groups of the Cardueae (reviewed in the introduction). In subtribe Centaureinae, basal groups have chromosome numbers ranging from *x* = 16 to 13 whereas the complex of genera with derived features have *x* = 12 to 7 (Garcia–Jacas et al. 2001).

Ecological considerations support the descending trend. Selvi and Bigazzi (2002) suggested that in *Nonea* Med. (Boraginaceae) descending dysploidy was correlated to short life cycle as an adaptation to arid habitats. Watanabe et al. (1999) also found a relationship between low chromosome numbers, annual habit and dry habitats in *Pogonolepis* Steetz, *Sondottia* P. S. Short and *Trichantodium* Sond. & F. Muell.

(Asteraceae–Gnaphalieae). It seems possible that descending dysploidy is related to the adaptation to more extreme habitats in the Cousinioid clade. The species of the Arctioid group, which do not exhibit dysploidy, are found in mesophylous mountain areas. Instead, the Cousinioid species, where a dysploid series is found, grow mainly in arid zones (López–Vinyallonga et al. 2009).

The *Arctium–Cousinia* complex has six basic chromosome numbers, but not all of them are represented equally. On the basis of all the counts published by now, together with the reports presented here, we have verified that x = 12, 13 and 18 are the most common ones (with the relative abundances of 38.9%, 40.9% and 16.1% respectively) while x = 9, 10 and 11 are hardly found in the complex (with the relative abundances of 4.7%, 1.3% and 4.7% respectively).

We have found little correspondence between phylogeny and karyology by mapping the chromosome counts for the 63 species present in the Bayesian phylogenetic tree by López–Vinyallonga et al. (2009) for which chromosome number have been reported (Fig. 3). Only the species with $2n$ = 36, all belonging to the Arctioid group, merge in the same clade. The species from *Cousinia* s. str. appear scattered through the tree, and do not group according either to sectional classification or to chromosome numbers. Moreover, even some sections of the Cousinioid clade have more than one basic chromosome number; e.g., *C.* sect. *Alpinae*, *Cousinia*, *Eriocousinia* and *Microcarpae*, showing dysploidy at the sectional level too. The karyological data have failed in providing any help to resolve the problem of the evolution in this complex, where molecular reconstruction has also failed.

Regarding polyploidy, we have not found any confirmed case in the entire *Arctium–Cousinia* complex. This is unfrequent in the Cardueae, a group with many pioneer polyploid colonizers (e.g., *Carthamus*, Vilatersana et al. 2000). Hybrid polyploids should be evident in crosses involving species with different basic chromosome numbers, but they have not been detected. Either hybrids are scarce or they must occur only between species with the same chromosome number and they should be homoploid, as recently pointed out in the related genus *Centaurea* (Garcia–Jacas et al. 2009).

The Onopordum group

Olgaea Iljin

Olgaea pectinata Iljin
Kazakhstan: Chimkentskaya oblast, Boranchi–Asu mountain pass, near Il Tal village, 30 Aug 2000, *A. Ivashchenko, A. Susanna 2187 & J. Vallès* (BC). $2n = 26$ (Fig. 2i)
According to the available data, this is the first count for this species and for the genus *Olgaea*, the basic chrosomome number of which is $x = 13$.

Syreitschikovia Pavlov

Syreitschikovia spinulosa (Franch.) Pavlov
Kazakhstan: Chimkentskaya oblast, Lengerskii rayon, Aksu Dzabagly Nature Reserve, Darbassa canyon, 1840 m, 31 Aug 2000, *A. Ivashchenko, A. Susanna 2200 & J. Vallès* (BC). $2n = 24$ (Fig. 2j)
According to our data this is the first report for this species and for the genus *Syreitschikovia*, the basic chrosomome number of which is $x = 12$.

In the *Onopordum* group, our results confirm the existence of two well–separated lines. Colonizing taxa of the *Onopordum* group have $x = 17$ (Watanabe, http://www.lib.kobe-u.ac.jp/infolib/meta_pub/G0000003asteraceae_e). On the contrary, species from the perennial, Middle–Asian genera *Olgaea* and *Syreschikovia* have $x = 13$ or $x = 12$ (counted here for the first time). This is a fine parallelism with the *Arctium–Cousinia* complex: biennial, widespread *Onopordum* with $x = 17$ is comparable to *Arctium*, both in life cycle and chromosome number. The rest of the perennial genera of this group which have been counted (*Alfredia, Ancathia, Olgaea, Synurus* and *Syreitschikovia*) have $x = 13$ and $x = 12$, therefore is comparable with the *Cousinia* clade. Curiously, polyploidy is unknow in both cases.

Concluding remarks

It is possible that hybridization is much more frequent but undetected because of lack of sufficiently detailed taxonomic knowledge and the existence of several morphologically very similar species. However, the complete absence of polyploid chromosome numbers in *Arctium–Cousinia* complex among the 149 species analyzed (Watanabe, http://www.lib.kobe-u.ac.jp/infolib/meta_pub/G0000003asteraceae_e) implies that polyploid hybrid speciation played no role in the evolution of this complex (López–Vinyallonga et al. 2009).

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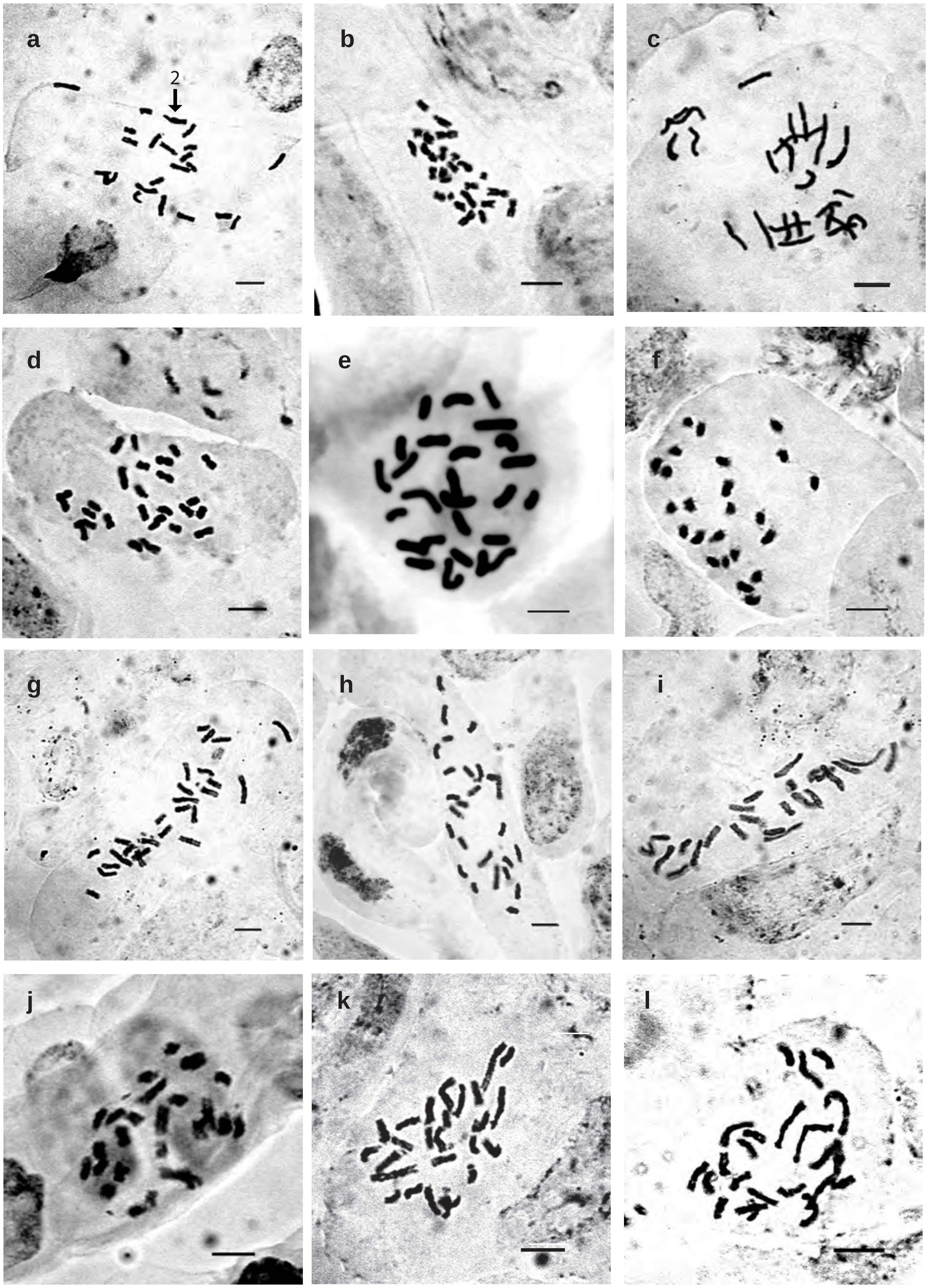
Figure captions

Fig. 1 Somatic metaphases of *Cousinia* spp. Scale bars = 10 µm. Fig. a *Cousinia serawschanica* (2*n* = 24). Fig. b *C. ferruginea* (2*n* = 26). Fig. c *C. princeps* (2*n* = 26). Fig. d *C. radians* (2*n* = 26). Fig. e *C. aleppica* (2*n* = 26). Fig. f *C. congesta* (2*n* = 24). Fig. g *C. decumbens* (2*n* = 26). Fig. h *C. franchetii* (2*n* = 26). Fig. i *C. libanotica* (2*n* = 24). Fig. j *C. coerulea* (2*n* = 24). Fig. k *C. submutica* (2*n* = 26). Fig. l *C. pulchella* (2*n* = 22). Numbers in arrows indicate overlapping chromosomes

Fig. 2 Somatic metaphases of *Cousinia*, *Olgaea* and *Syreitschikovia* spp. Scale bars = 10 µm. Fig. a *Cousinia sewerzowii* (2*n* = 22). Fig. b *C. aurea* (2*n* = 36). Fig. c *C. karatavica* (2*n* = 36). Fig. d *C. refracta* (2*n* = 36). Fig. e *C. anomala* (2*n* = 36). Fig. f *C. tomentella* (2*n* = 36). Fig. g *C. fedtschenkoana* (2*n* = 36). Fig. h *C. macilenta* (2*n* = 36). Fig. i *Olgaea pectinata* (2*n* = 26). Fig. j *Syreitschikovia spinulosa* (2*n* = 24). Numbers in arrows indicate overlapping chromosomes

Fig. 3 50% majority–rule consensus tree obtained from the Bayesian analysis of the regions ITS and *rps4–trnT–trnL* after López–Vinyallonga et al. (2009). Numbers above branches are posterior probabilities. The chromosome numbers are mapped in the branches of the tree with the following patterns: thin black line with grey squares, *x* = 18; thick solid black, *x*= 13; dashed black, *x* = 12; thick solid grey, *x* = 11; thin black line with thick dashed black, *x* = 10; thin dashed black, *x* = 9; thin solid black, no data available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c
d
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h
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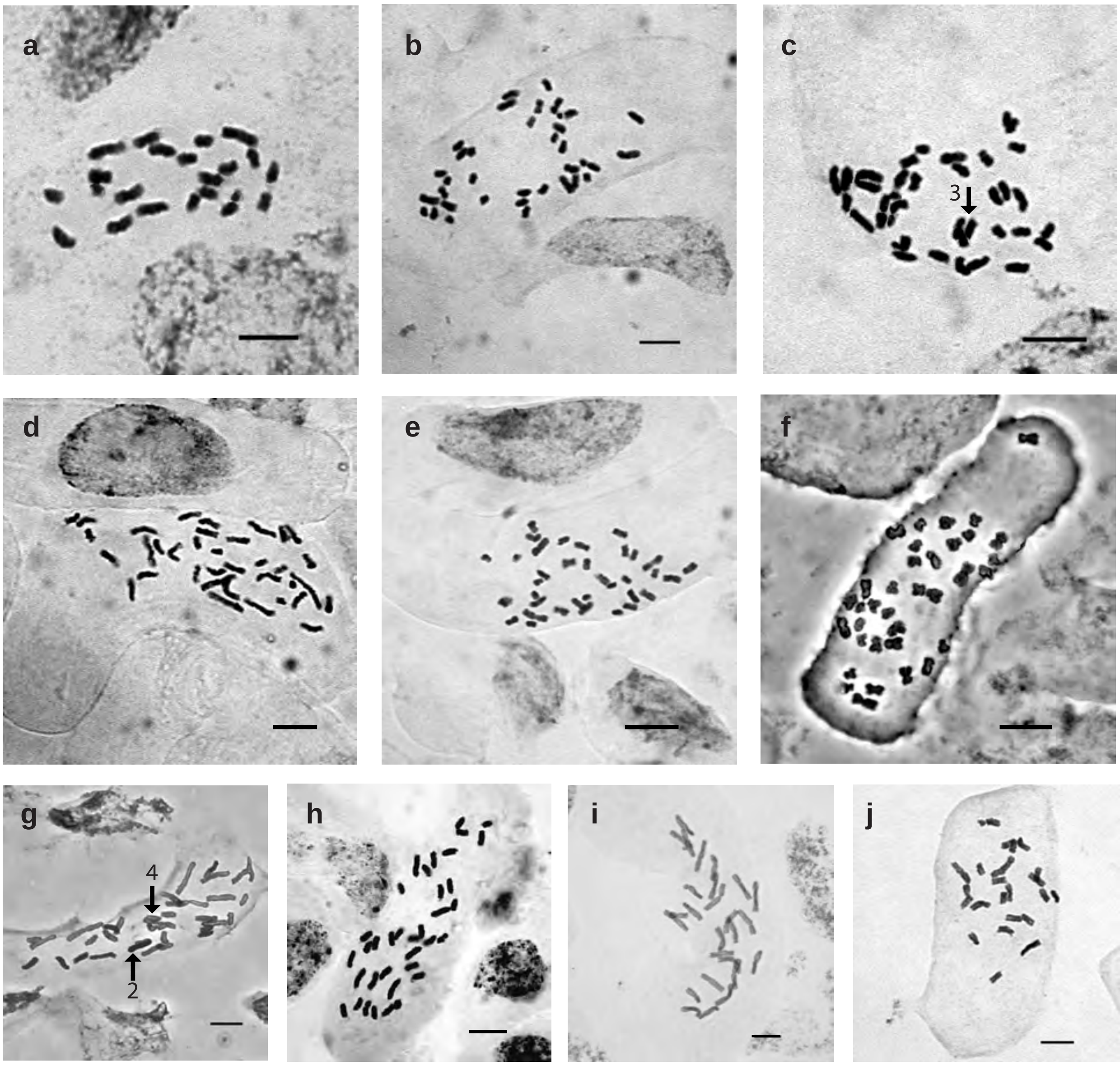
j
k
l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3
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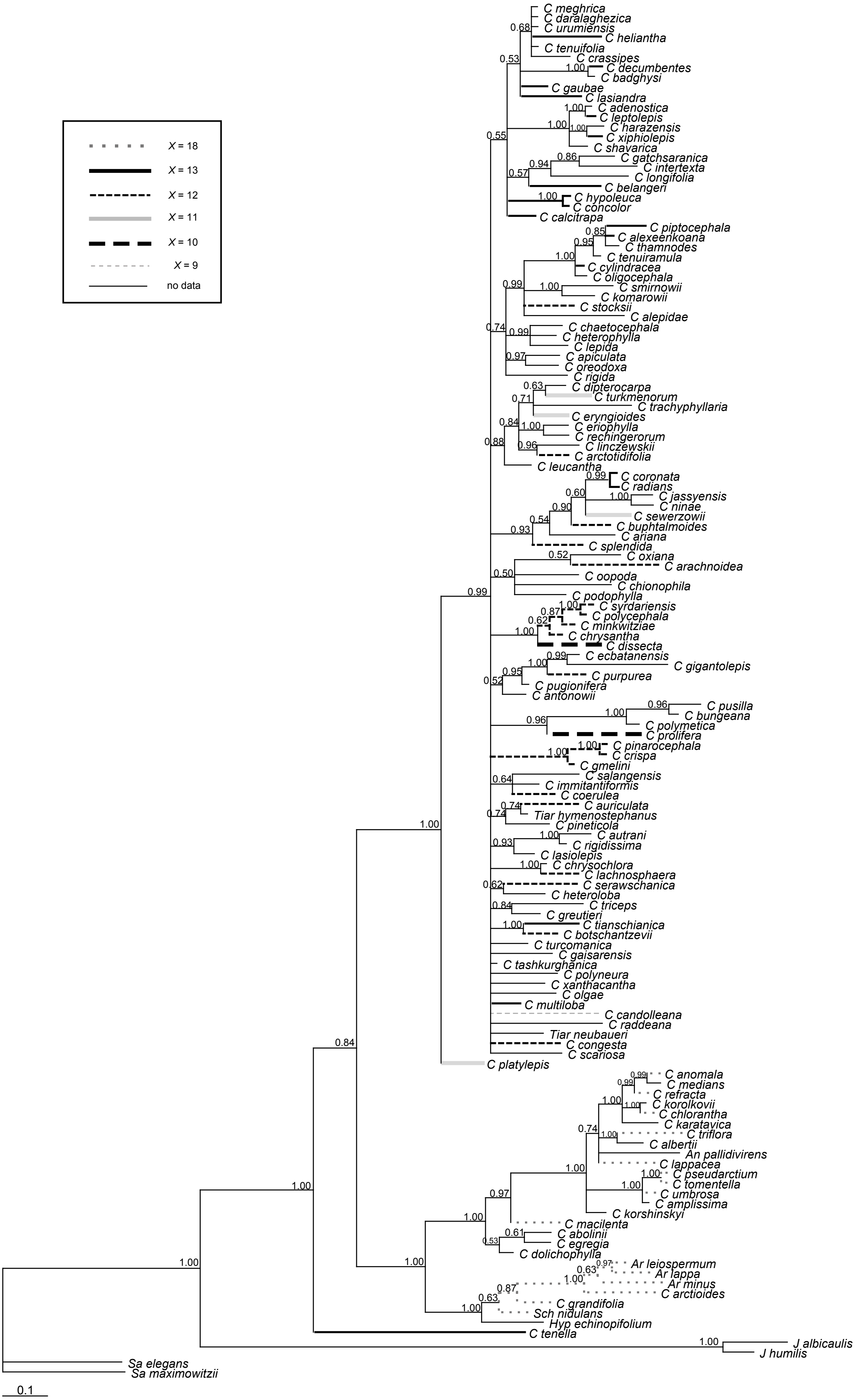
2
h
i
j


X = 18
X = 13
X = 12
X = 11
X = 10
X = 9
no data
0.68
C meghrica
C daralaghezica
C urumiensis
C heliantha
C tenuifolia
0.53
C crassipes
1.00
C decumbentes
C badghysi
C gaubae
C lasiandra
1.00
C adenostica
C leptolepis
1.00
1.00
C harazensis
C xiphiolepis
C shavarica
0.55
0.94
0.86
C gatchsaranica
C intertexta
0.57
C longifolia
C belangeri
1.00
C hypoleuca
C concolor
C calcitrapa
0.85
C piptocephala
C alexeenkoana
0.95
C thamnodes
1.00
C tenuiramula
C cylindracea
C oligocephala
0.99
1.00
C smirnowii
C komarowii
C stocksii
C alepidae
0.74
C chaetocephala
0.99
C heterophylla
C lepida
0.97
C apiculata
C oreodoxa
C rigida
0.63
C dipterocarpa
C turkmenorum
0.71
C trachyphyllaria
C eryngioides
0.84
1.00
C eriophylla
C rechingerorum
0.88
0.96
C linczewskii
C arctotidifolia
C leucantha
0.99
C coronata
C radians
0.60
1.00
C jassyensis
C ninae
0.90
C sewerzowii
0.54
C buphtalmoides
0.93
C ariana
C splendida
0.52
C oxiana
C arachnoidea
0.50
C oopoda
C chionophila
C podophylla
0.99
1.00
C syrdariensis
0.87
C polycephala
0.62
C minkwitziae
1.00
C chrysantha
C dissecta
0.99
C ecbatanensis
1.00
C gigantolepis
0.95
C purpurea
0.52
C pugionifera
C antonowii
0.96
C pusilla
1.00
C bungeana
0.96
C polymetica
C prolifera
1.00
C pinarocephala
1.00
C crispa
C gmelini
0.64
C salangensis
C immitantiformis
C coerulea
0.74
C auriculata
0.74
Tiar hymenostephanus
C pineticola
1.00
C autrani
0.93
C rigidissima
C lasiolepis
1.00
C chrysochlora
C lachnosphaera
0.62
C serawschanica
C heteroloba
0.84
C triceps
C greutieri
1.00
C tianschianica
C botschantzevii
C turcomanica
C gaisarensis
C tashkurghanica
C polyneura
C xanthacantha
C olgae
C multiloba
C candolleana
C raddeana
Tiar neubaueri
C congesta
C scariosa
1.00
C platylepis
0.84
0.99
C anomala
0.99
C medians
C refracta
1.00
1.00
C korolkovii
C chlorantha
C karatavica
0.74
1.00
C triflora
C albertii
An pallidivirens
C lappacea
1.00
1.00
C pseudarctium
C tomentella
1.00
C umbrosa
C amplissima
0.97
C korshinskyi
1.00
C macilenta
0.61
C abolinii
0.53
C egregia
C dolichophylla
1.00
0.97
Ar leiospermum
0.63
Ar lappa
1.00
Ar minus
0.87
C arctioides
0.63
C grandifolia
1.00
Sch nidulans
Hyp echinopifolium
1.00
C tenella
1.00
1.00
J albicaulis
J humilis
Sa elegans
Sa maximowitzii
0.1

7. 4. Isolation and characterization of novel microsatellite markers for *Arctium minus* (Hill) Bernh. (Compositae)

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RESUM. En aquest treball es presenten nou microsatèl·lits polimòrfics dissenyats per *Arctium minus* a partir d'una llibreria genòmica enriquida. La caracterització d'aquests loci es basa en sis poblacions, de més de 20 individus cadascuna, procedents d'Eslovàquia, Espanya (2 poblacions), França, Polònia i Turquia. Pel que fa a la caracterització dels loci, el nombre d'al·lels per locus va de dos a 10, els valors d'heterozigositat esperada i observada varien de 0.015 a 0.487 i de 0.016 a 0.694, respectivament i el valor mitjà de F_{IS} (indicador de consanguinitat) és de 0.316. Sis loci mostren una desviació estadísticament significativa de l'equilibri Hardy-Weinberg (HW a partir d'ara) degut a una deficiència d'heterozigots, i nou de 36 comparacions entre parelles de locus mostren un significatiu desequilibri en el lligament. S'ha detectat la presència d'al·lels nuls, amb freqüències baixes o moderades, als loci Am31 i Am34 per cinc i tres poblacions respectivament. Pel que fa a la caracterització de les poblacions considerades, només la de Turquia està en equilibri HW i les de Polònia, Eslovàquia i nord-oest d'Espanya mostren un significatiu desequilibri en el lligament. Complementàriament, s'aporten les seqüències de cinc parells de primers que amplifiquen loci monomòrfics, potencialment útils per a altres espècies o per poblacions amb més variabilitat i de quatre parells de primers assajats en menys de 20 individus per població però que amplifiquen loci polimòrfics. Els microsatèl·lits presentats a aquest treball poden ser d'utilitat per a examinar l'estructura genètica de les poblacions d'*A. minus* i per a investigar aspectes relacionats amb la colonització del continent Americà per part d'aquesta espècie.

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Isolation and characterization of novel microsatellite markers for *Arctium minus* (Hill) Bernh. (Compositae)

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Keywords: *Arctium minus*, enriched library, lesser burdock, microsatellites, population genetics, SSR

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Running title: New microsatellite markers for *Arctium minus*

Abstract

Nine polymorphic microsatellite markers were developed in lesser burdock (*Arctium minus* L.) from an enriched genomic library. The number of alleles per locus ranged from two to 10, expected and observed heterozygosities ranged from 0.015 to 0.487 and from 0.016 to 0.694, respectively, and the mean value of F_{IS} was 0.316. Six loci showed a significant departure from Hardy-Weinberg equilibrium, and nine out of 36 pairwise locus comparisons showed significant linkage disequilibrium. Presence of null alleles was found in some populations at two loci with low or moderate frequencies. In addition, sequences for four primer pairs that were tested in fewer than 20 individuals per population but yielded polymorphic loci and for five primer pairs that amplified monomorphic loci for the samples examined are also provided. Microsatellite markers reported here will be useful for examining population genetic structure and for addressing questions regarding the colonization of the Americas by this species.

Arctium minus (Hill) Bernh.is a diploid biennial plant, native to Eurasia. It also grows in Africa, where it is rare, and it is widespread and often naturalized as a weed in many parts of North and South America. This species is monocarpic, growing vegetatively as broad-leaved rosettes and then dying after producing a tall (0.5-2 m) flowering stalk (Gross & Werner 1983). *Arctium minus* is allogamous, although it can be self-pollinated if insect pollination fails (Fenner *et al.* 2002). In previous phylogenetic studies (López-Vinyallonga *et al.* 2009), little sequence divergence among species of the *Arctium-Cousinia* complex was found, most likely due to rapid and recent speciation in the group. The high polymorphism found in microsatellite markers can be a helpful tool for the study of the population genetics of this complex, as it has been for the study of other recently speciated groups (e. g. Chirhart *et al.* 2005, Gugerli *et al.* 2001, Edwards *et al.* in press). Hence, we report here a set of novel polymorphic microsatellites that will be useful for assessing genetic variability and divergence as well as gene flow within and among populations of *A. minus*.

Genomic DNA was extracted from dried leaf tissue of specimens collected from wild populations, representing the majority of the distribution of *A. minus*, using the NucleoSpin® Plant Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Two genomic libraries enriched for 1) (AAG/TTC) and 2) (CA/GT) were constructed from *A. minus* following Symonds (pers. comm.). The genomic DNA was digested with *Sau*3AI and enriched for repeats by hybridization to 3'-biotinylated $(AAG)_{14}$ and $(CA)_{15}$ TATAAGATA oligonucleotides, respectively, followed by magnetic capture with streptavidin-coated magnetic beads (Promega Corp, Madison, WI, USA). Enriched fragments were made double-stranded by polymerase chain reaction (PCR) and were ligated into a TOPO TA pPCR 4.0 vector, transformed into One Shot *Escherichia coli* competent cells (Invitrogen, Carlsbad, California, USA) and grown on Luria Broth (LB) agar plates with Kanamycin. Replicas were transferred to LB/Kanamycin 96-well plates and grown overnight. Screening for positives was PCR-based, involving two PCRs per sample, with a repeat primer (either AAG or CA) and a primer for the vector (M13F or M13R).

A total of 264 positive clones for AAG and 235 for CA were detected; 192 and 160 of these, respectively, were sequenced in an ABI 3730xl DNA Analyzer (Applied

Biosystems, Foster City, California, USA). Using the criterion of at least six repeat units in the target sequence, we were able to design primers from 28 (for AAG) and 14 (for CA) of these sequences using PRIMER3 version 0.4.0 (Rozen & Skaletsky 2000) and Operon (Operon Biotechnologies, Inc., Huntsville**,** Alabama, USA). Forward primers had universal M13 tails added to their 5'ends following Boutin-Ganache *et al*. (2000). Amplifications were performed in 10 µl reactions containing 0.5 U of Go*Taq* Flexi DNA polymerase (Promega), 1x Promega colorless Go*Taq* Flexi Buffer, 2.0 mM $MgCl_2$, 50 µM of each dNTP, 0.45 µM of the reverse primer, 0.012 µM of the extended forward primer, 0.45 µM of the labelled M13 primer (6-FAM, VIC, NED or PET, Applied Biosystems) and 25 ng/µl of template DNA. The profile used for amplifications consisted of a denaturation step of 94 ºC for 3 min, followed by 34 cycles of 94 ºC for 30 s, 52 ºC for 30 s, and 72 ºC for 45 s and a final elongation time of 20 min at 72 ºC. Labeled PCR products were diluted 1:20 and pool-plexed by using 1-4 µl of each PCR (up to four loci). One µl of the diluted PCR mixture, 9.9 µl of formamide, and 0.1 µl LIZ 600 size standard (Applied Biosystems) was loaded and run on an ABI 3730xl DNA Analyzer (Applied Biosystems) at the Interdisciplinary Center for Biotechnology Research (ICBR) facility at the University of Florida. Four loci were monomorphic and 12 loci required further optimization; therefore, we did not pursue them further. Specific amplification and polymorphism were achieved for nine primer sets. We genotyped at least 20 individuals per population, belonging to six different populations. Fragment analysis was performed using GENEMARKER 1.5 (Soft Genetics, LLC) and Peak Scanner Software 1.0 (Applied Biosystems) and scored manually in both cases. The data were analyzed with GenAlEx6 (Peakall & Smouse 2006), and the results are shown in Table 1.

The number of alleles (*N*a) observed at each locus ranged from two to 10, with observed heterozygosities (H_o) ranging from 0.016 to 0.694, and expected heterozygosities (H_e) from 0.015 to 0.487 with a mean value of 0.173, showing low allelic diversity for *A. minus*. Estimates of the inbreeding coefficient F_{IS} (Weir & Cockerham 1984) ranged from -0.752 to 0.890, with a mean value of 0.316 for the studied populations.

GENEPOP version 3.4 (Raymond & Rousset 1995) was used to test for departure from Hardy-Weinberg equilibrium and linkage disequilibrium. Six loci showed a significant departure from Hardy-Weinberg equilibrium ($P < 0.005$) due to heterozygote deficiency, and nine out of 36 pairwise locus comparisons showed significant linkage disequilibrium ($P < 0.005$).

Given the high levels of F_{IS} and linkage disequilibrium detected in our data, we performed additional analyses at the population level in order to investigate the reason for these high values. All populations but the one from Turkey showed a significant departure from Hardy-Weinberg equilibrium ($P < 0.005$). In addition, we observed significant linkage disequilibrium ($P < 0.005$) in the populations from Poland (seven out of 36 pairwise locus comparisons), Slovakia and Spain (one out of 36 pairwise locus comparisons each). There was no significant linkage disequilibrium in any of the other populations.

Furthermore, tests for the presence of null alleles were performed using MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004). There was no evidence for scoring errors due to stuttering and no evidence for large allele dropout at any of the nine loci tested. In addition, seven out of these nine loci did not show any presence of null alleles; null alleles were detected at two loci, but in low or moderate frequency and only in some of the populations tested. Locus Am31 showed evidence of null alleles in the five populations that are not under HWE, with estimated frequencies lower than 0.45. Locus Am34 showed evidence of null alleles in three populations, one of them under HWE, with estimated frequencies lower than 0.34.

The results of all these HWE and LD tests are consistent with the presence of null alleles and the facultative ability of self-pollination in *A. minus* as well.

Given the low diversity detected in the six populations studied, we also give details for four additional primer pairs that yielded polymorphic loci when tested in populations with fewer than 20 individuals (Appendix 1). For those loci that we genotyped and scored, no significant departure from HWE ($P < 0.005$) nor significant LD was found ($P < 0.005$).

We also provide details of five additional primer pairs that appeared to generate monomorphic loci (Appendix 2), although they might be polymorphic when tested in more populations of *A. minus*, or more individuals, or in closely related species.

Microsatellite markers reported here for *A. minus* are suitable for population genetic studies, such as understanding past evolutionary and demographic events. Extensive sampling of more populations has been carried out, and genetic analyses of these populations are in progress in order to answer questions regarding the colonization of the Americas and other population processes in this species.

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Table1 Primer sequence and configuration of 9 microsatellite loci in *Arctium minus*.

Locus	Primer Sequence (5’→3’)	T_a (ºC)	Repeat Motif	Size Range (bp)	N	N_a	H_o	H_e	F_{IS}
Am8	F: *ATCGCCATCGAAGACAAGAC R: AATAAAATCCTGAGCCGGAAA	54	$(AAG)_9$	108-152	162	4	0.074	0.134 †	0.448
Am26	F: *TTGGGGTGAGGTAGAAGCAG R: TTCCACCAAGTTGGGTTAGC	52	$(AAG)_6$	260-265	131	2	0.016	0.015	-0.050
Am30	F: *GCAAAGGGGTCCTAGAGCAT R: TCGAAGTGTTATCGGTTGCT	52	$(CA)_8$	180-189	186	3	0.033	0.077 †	0.572
Am31	F: *TGTGCAACTGCTCCTTCAGT R: CTCCAACAATGCAGAAACCA	52	$(CA)_9$	182-272	142	8	0.053	0.487 †	0.890
Am32	F: *GCTGTTGCCATGACTCTAAGG R: CGGAAAAGGACGACAAAGAA	52	$(CA)_8$	212-226	157	4	0.334	0.304	-0.099
Am33	F: *TCCCTTGTGAAACGCAATTT R: CGGTCAGATCCATTTATCACG	52	$(CA)_9$	197-203	154	3	0.038	0.210 †	0.820
Am34	F: *CCATGCTCACCTCCATTTCT R: CAGCATAATGATACGGCAACA	52	$(GA)_9$	158-185	133	4	0.117	0.262 †	0.555
Am35	F: *AGTTAGTGTCATTGTTGAGAGAACTTA R: TGTGATAGCATCCAAACTCCA	50	$(TATG)_6(TG)_4[(TATG)_2(TG)_4]_4$	174-239	158	10	0.201	0.374 †	0.463
Am39	F: *TGCAGACACGCATTACAACA R: TGGCCCTAGAATGATGGAAA	52	$(CA)_3CGC(CA)_5$	248-258	170	4	0.694	0.396	-0.752

* M13 tail (CACGACGTTGTAAAACGAC), optimized annealing temperature (T_a), repeat motifs, size ranges of PCR products, number of individuals genotyped (N), number of alleles observed (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F_{IS}). Significant departures from HWE: † $P < 0.005$.

Appendix 1 Primer pairs polymorphic for *Arctium minus* tested in fewer than 20 individuals per population.

Locus	Primer Sequence (5'→3')	T_a (ºC)	Repeat Motif	Size Range (bp)	N_a	H_o	H_e	F_{IS}
Am2	F:* AGAAAGGAAAGGGGGAGCTT R: TCTTCTGGATCTGCCTCGAT	50	$(AAG)_6$	212-247	2	0.020	0.018	-0.111
Am17	F:* TCGTGGGACTCTACCACCTC R: TCCTGGACCCAGATCGTACT	52	$(AAG)_6$	254-288	3	0.076	0.054	-0.421
Am37	F:* TCTCACCGGCGATAGAAACT R: ATACCGGAAGACGGAATGTG	50	$(TC)_9$	175-204	3	0.107	0.078	-0.362
Am40	F:* CACTGTTGTGGTGGTGGTTT R: GTGGGTGGAGCTAATGTGGA	52	$(CA)_3G(CA)_2N(CA)_5$	182-266	8	0.402	0.409	0.016

[*] M13 tail (CACGACGTTGTAAAACGAC), optimized annealing temperature (T_a), repeat motifs, size ranges of PCR products, number of alleles observed (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F_{IS}).

Appendix 2 Primer pairs that successfully amplified microsatellite-containing loci in *Arctium minus*. Given the low genetic diversity in the populations used in this study, these monomorphic loci could be assessed for polymorphism in genetically more diverse populations of *A. minus* or in closely related species.

Locus	Primer Sequence (5’→3’)	T_a (ºC)	Repeat Motif	Expected size (bp)
Am16	F:[*] TGTTTCTGCATAGTTCCAAGGTT R: AGGCATTCAAATCAACAATCC	45	$(AAG)_6$	216
Am23	F:[*] ATCGCCATCGAAGACAAGAC R: AATAAAATCCTGAGCCGGAAA	54	$(AAG)_9$	153
Am25	F:[*] GGTTTGGTTCTCCCTCAGGT R: ATCAAGCCGGTGACCATATC	45	$(TC)_8$	278
Am27	F:[*] CGGGTTCTGACTTAGCTTGC R: GGTAACATCCGTTTCGTTCG	50	$(AAG)_6$	361
Am36	F:[*] TGTTATTTCAGCCCTGGATTG R: CAACTTACAATTTCAATGGTATCTCTC	54	$(CA)_{11}$-$(TA)_6$-$(GA)_5$	204

[*] M13 tail (CACGACGTTGTAAAACGAC), optimized annealing temperature (T_a), repeat motifs, expected size ranges of PCR products.

7. 5. Genetic structure and diversity in *Arctium minus* (Compositae): effects of historical climate change and life history

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RESUM. La història biològica de les plantes s'ha postulat com un dels principals factors que afecten la seva estructura genètica. Al present treball, s'ha estudiat la variabilitat genètica d'*Arctium minus* per tal d'investigar com hi han pogut afectar les següents característiques biològiques: autogàmia facultativa (efecte del sistema d'aparellament), epizoocòria (efecte de l'estratègia de dispersió) i biennualitat (efecte de la durada del cicle vital). Per a dur a terme aquesta recerca, s'han analitzat vuit loci microsatèl·lits i 14 poblacions representatives de la major part de l'àmplia àrea de distribució de l'espècie. Els patrons de variació genètica detectats són coherents amb l'estil de vida d'*A. minus*. La baixa diversitat genètica, els alts valors d'endogàmia i l'excés d'homozigots estadísticament significatiu són resultats que s'expliquen per la capacitat d'autofecundació d'*A. minus* i la curta durada del seu cicle de vida. També s'ha observat que la major part de la variabilitat genètica de l'espècie es troba entre poblacions degut a un baix flux gènic entre elles juntament amb la capacitat d'autofecundació de l'espècie i fenòmens de dispersió de llavors a llarga distància. Els esdeveniments de dispersió de llavors a llarga distància també es postulen com l'explicació més plausible per a dos patrons detectats: i) absència d'aïllament per distància, tant dins del rang natural d'*A. minus* com a les poblacions del límit de la seva àrea de distribució, i finalment ii) l'aparent manca d'influència dels canvis climàtics esdevinguts durant el Pleistocè a les poblacions del seu rang natural. No s'han trobat diferències entre les poblacions europees i les americanes. Se suggereix que els patrons biogeogràfics d'*A. minus* han quedat esmorteïts i emmascarats per influència de l'activitat humana.

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Genetic structure and diversity in *Arctium minus* (Compositae): effects of historical climate change and life history

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Abstract

Plant life history is one of the main factors affecting the genetic structure of organisms. In the present work the genetic variation in *Arctium minus* is investigated in order to test the effect of mating system (facultative self-pollination), dispersal strategy (efficient seed dispersal by epizoochory) and life cycle (biannuality) on its genetic structure. The analysis of eight microsatellite loci and 14 populations from most of its worldwide distribution has been used for this purpose. The observed patterns of genetic variation are consistent with *A. minus* life style. Low gene diversity, high inbreeding values and significant homozygote excess were recovered, factors linked with the self-pollination ability of *A. minus* and its short-life cycle. Long distance seed dispersal is suggested as the main cause for three patterns observed: i) most of the genetic variability is found among instead of within populations, ii) absence of isolation by distance across Europe and iii) the lack of influence of Pleistocene climate changes in the European populations. No differences are found between European and American populations. It is suggested that original biogeographic patterns in *A. minus* may have been blurred by human activity.

INTRODUCTION

Nonrandom spatial distribution of genotypes, i.e. spatial genetic structure, is more the rule than an occasional phenomenon in the natural populations of plant species (i.e. McCauley 1997; Balloux & Lugon-Moulin 2002; Palmé & Vendramin 2002; Rendell & Ennos 2002; Leblois *et al.* 2006). A variety of agents or processes such as environmental and historical factors and life histories can affect the partitioning of genetic variability among plant populations. Regarding life history, plant breeding system, modes of seed dispersal, and length of life cycle are important nonexclusive factors affecting the spatial genetic structure in wild populations (Austerlitz *et al.* 2000;

Nordborg 2000; Charlesworth 2003; Clauss & Mitchell-Olds 2006). Differences in self-fertilization rates are among the main factors affecting the genetic diversity. As a trend, lower genetic variability is expected for inbreeding than outcrossing plants, and in inbreeding species the variability is found between populations rather than within them (*cf.* Charlesworth 2003).

Different modes of seed dispersal provide diverse capacities for the species to spread their propagules to long distances and therefore determine both the distribution of plant populations and the species range. Likewise, dispersal distances affect the spatial genetic structure of the species, since longer dispersal distances yield more separate populations and stronger founder effects (long dispersions are rare events in which few colonizers are involved, and the gene flow with other populations is low or it does not exist). However, long dispersions can also increase the gene flow between previously isolated populations (Nichols & Hewitt 1994; Petit *et al.* 2003).

The effect of colonization processes on genetic diversity and population structure also depends on the life cycle of the species, having a lesser impact in long life-cycle species than in short-lived plants. Long life-cycle species have a longer juvenile phase and therefore a delayed first reproduction which allows a large increase in the number of initial founders (by immigration) of a given population before reproduction begins (Austerlitz *et al.* 2000).

In this context, *Arctium minus* (Hill) Bernh. provides a useful system to analyze the effect of the different life history factors on the spatial structure of the genetic diversity, since this species combines a mixed mating system with short-life cycle and the ability of long distance dispersal as we shortly comment.

Arctium minus (Lesser Burdock) is a diploid herbaceous plant, native to Eurasia. It also grows in the northernmost Africa, where it is rare, and it is widespread and often naturalized as a weed in many parts of South and North America, where is considered as an invasive species in many areas of US and Canada. In spite of its wide distribution, *A. minus* populations are patchily distributed due to its ecological requirements. This species has a broad ecological range with regard to climate, altitude and soil type although it prefers moderate to high levels of moisture as well as high soil nitrogen content. As a consequence, populations of *A. minus* are restricted to nitrified-mesic places like open and disturbed woods, disturbed areas and pastures, abandoned fields and stream banks, and even roadsides.

Lesser burdock has a mixed mating system with predominant allogamy, although it can be self-pollinated if insect pollination fails (Gross *et al.* 1980; Fenner *et al.* 2002). According to Gross *et al.* (1980) high selfing levels lead to an increase of achene abortion. In addition, hybridization between sympatric *Arctium* species has been largely described (*cf.* Repplinger *et al.* 2007). However, Repplinger *et al.* (2007) have suggested the existence of pre- and postzygotic isolation mechanisms to explain the few hybrids detected in mixed populations of *A. minus*, *A. lappa* L. and *A. tomentosum* Mill.

Seed dispersal is epizoochorous by the attachment of burrs to the coats of animals and therefore achenes are dispersed altogether as a whole. Between 29 and 47 seeds are produced per head, but more than 60% of the seeds are predated before dispersion (Hawthorn & Hayne 1978). In contrast, the germination success of this species is of 90% (Gross *et al.* 1980).

Long-distance dispersion of lesser burdock burrs of up to 700 m (166 m in average) has been reported (Couvreur *et al.* 2008). The main vectors of dispersion of this species are medium-sized or big mammals, both wild and domestic, although some birds and small mammals have been reported as well. In addition, it is very likely that the dispersion of *A. minus* has been favoured by humans. Actually, lesser burdock is hypothesized to be introduced to North America by early English and French settlers in the mid 17th century (Gross *et al.* 1980).

Regarding life cycle, *A. minus* is a biennial monocarpic species growing vegetatively and then dying after flowering during the second year. However, several authors have reported that under unfavourable conditions many plants may take four or more years to flower or even not flower (Gross *et al.* 1980; Gross & Werner 1983). Moreover, Rollo *et al.* (1984) observed that this species colonizes habitats in early stages of succession but over the following 3 years it declines rapidly and at the 4th year it becomes rare, suggesting an ephemeral character for lesser burdock populations.

Nothing is known about the genetic diversity of *A. minus* and how this genetic diversity is structured among its populations.

The current distribution of *A. minus* throughout Eurasia, including the glacial refugia of South Europe, North Africa and Middle East (i.e. Turkey), indicate that this species likely endured during the glacial and interglacial periods occurred in the Pleistocene. As many other species now widespread in Europe, lesser burdock could recolonize the continent from southern glacial refugia during the present interglacial period, and thus its present range may be consequence of glacial advance and retreat. This migration pattern across Europe during Pleistocene has been detected in many plants using molecular markers, including microsatellites (e.g. Rendell & Ennos 2002; Grivet & Petit 2003; Sharbel *et al*. 2006), and a common pattern for genetic diversity partition has been established (Petit *et al*. 2003). Thus populations in different glacial refugia, as a consequence of prolonged isolation, should be highly divergent. Moreover, the intrapopulation diversity should decline away from refugia as a consequence of successive founder events during European recolonization. However, this cline can become blurred if populations contact during the recolonization period.

In the present work we analyze the spatial model of genetic variation within and among 11 populations from the native range of *A. minus*, namely Europe, as well as from the limits of its distributional area (Turkey, Morocco and Norway) using eight microsatellite loci (López-Vinyallonga *et al*. in prep). Three populations from Americas are also analyzed in order to compare them with those of the native range of this species. In this study we try to evaluate the effect on the genetic structure of the mating system, seed dispersal strategy and life cycle of *A. minus*. We test if the current spatial genetic structure of lesser burdock across its natural range fits to the expected pattern for one species which has underwent the past Pleistocene climatic changes or, in contrast, that pattern has been masked by the effect of the life history factors. If the latter occurs, the spatial genetic structure should fit that expected for a species with long-distance seed dispersion, short life cycle and mixed mating system.

MATERIAL AND METHODS

Sampling

We sampled 14 populations of *Arctium minus* representing its distribution area (Table 2). Eleven populations represented the natural range of the species (Table 2; Fig. 1): nine from Europe, one from Morocco and one from Turkey. Populations from Morocco, Turkey, South of Italy and the Iberian Peninsula were selected in order to include populations from places which were important refugia during Pleistocene climatic changes. Five populations were included from Centre and North Europe, where the diversification centre of *Arctium* is thought (Tscherneva 1962; Duistermaat 1996), and one population from Norway was used to represent the northernmost edge of *A. minus* (where is a rare species). Finally, three populations from America (two from North America and one from South America; Table 2) were included in the study to compare them with the populations from the natural range of the species.

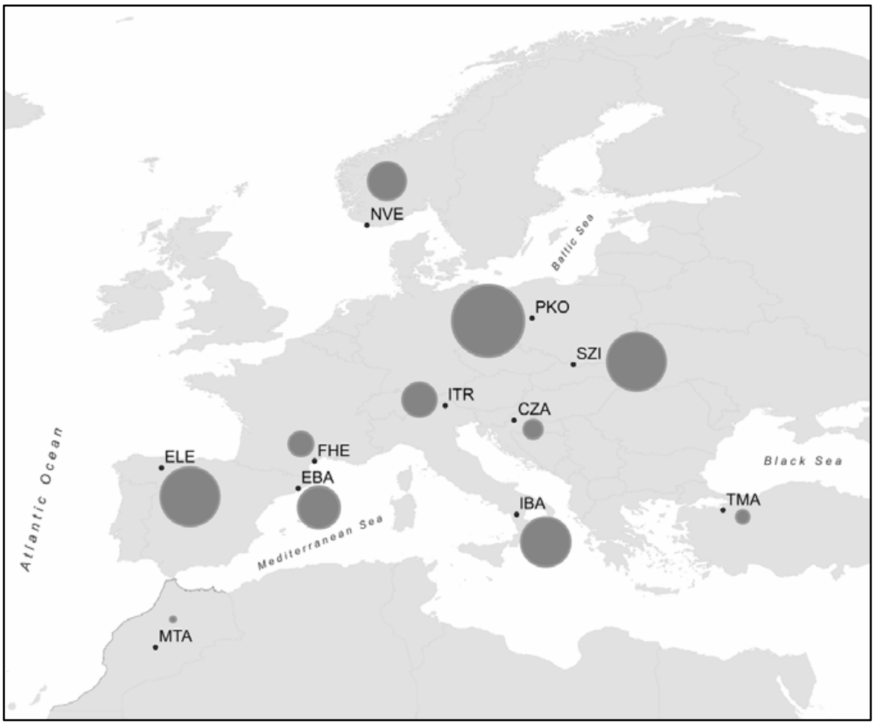

Fig. 1. Native area of distribution of *A. minus*. Circles represent H_E (expected heterozygosity under Hardy-Weinberg equilibrium) values observed for each population. CZA: Croatia, EBA and ELE: Spain, FHE: France, IBA and ITR: Italy, MTA: Morocco, NVE: Norway, PKO: Poland, SZI: Slovakia, TMA: Turkey.

Thirty individuals per population (368 individuals in total) were sampled, except for those populations with lower number of plants (CAN, MTA, NVE, and USA), for which all individuals in the population were sampled (Table 2).

DNA isolation and Microsatellite loci

Genomic DNA was extracted from dried leaf tissue of up to 30 specimens, collected from each wild population, using the NucleoSpin® Plant Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

Eight microsatellite loci, showing different polymorphism levels, were used in this work (Table 1). Microsatellite loci were amplified using FAM, NED, PET and VIC fluorescently labelled forward primers as explained in López-Vinyallonga *et al*. (in pres.). Genotyping was performed on an ABI 3730xl DNA Analyzer (Applied Biosystems) using LIZ600 size standard at the Interdisciplinary Center for Biotechnology Research (ICBR) facility at the University of Florida. Fragment analysis was performed with GENEMARKER 1.5 (SoftGenetics, LLC) software.

Table 1. Configuration of 8 micosatellite loci used in this work. Optimized annealing temperature (T_a), repeat motifs, size ranges of PCR products, number of alleles observed (*NA*).

Locus	T_a (ºC)	Repeat Motif	Size Range (bp)	*NA*
Am30	52	$(CA)_8$	180-189	3
Am31	52	$(CA)_9$	182-272	9
Am32	52	$(CA)_8$	198-226	8
Am33	52	$(CA)_9$	197-223	10
Am34	52	$(GA)_9$	158-185	7
Am35	50	$(TATG)_6(TG)_4[(TATG)_2(TG)_4]_4$	152-238	16
Am37	50	$(TC)_9$	171-179	4
Am39	52	$(CA)_3CGC(CA)_5$	252-256	2

Data analysis

For each population the mean number of alleles per locus (*NA*), the observed heterozygosity (H_O), the unbiased expected heterozygosity (H_E; Nei 1978), and the fixation index (F_{IS}; Weir & Cockerham 1984) were calculated for each locus using GENETIX software (Belkhir *et al.* 1996-2004). Significant deviations from Hardy-Weinberg equilibrium expectations were assessed in each population (GENETIX; 10,000 permutations of F_{IS}). In order to test for significant differences in H_E and F_{IS} between populations a Mann-Whitney *U*-test was used for each pairwise comparison. This test was also implemented between American and Eurasian-North African populations.

Nei's (1973) population structure statistics (unbiased for sample size; Nei & Chesser 1983) were calculated for all 14 *A. minus* populations as a whole and for the 11 populations of the natural range of the species. The differentiation between pairs of populations was quantified with θ (F_{ST}; Weir & Cockerham 1984) using GENETIX, and a permutation test of the pairwaise θ differentiation was performed (GENETIX; 10,000 permutations). A chord distance matrix (Dc; Cavalli-Sforza & Edwards 1967) among populations was constructed from allele frequency data using MICROSAT ver. 1.5b (Minch *et al* 1995), from which 1,000 bootstrapped replicate matrices were computed. Bootstrap analysis was carried out with the software package PHYLIP ver. 3.66 (Felsenstein 2006) using the module NEIGHBOR to compute Neighbor-Joining dendrograms for all bootstrapped matrices, and the module CONSENSE to produce an extended majority-rule consensus tree.

Isolation-by-distance among populations was investigated by computing the correlation between the matrix of pairwise genetic differentiation [$F_{ST}/(1-F_{ST})$] and the matrix of the logarithm of geographical distances (Rousset 1997) by applying the Mantel test (1,000 permutations) using GENETIX. The geographical distances between pairs of populations were calculated from the longitudes and latitudes given in Table 2. An analysis of molecular variance (AMOVA), using the program ARLEQUIN version 3.11 (Excoffier *et al.* 2005), was performed to estimate the geographical structure of genetic variation. AMOVA was carried out at different hierarchical levels between the Eurasian and North African populations: 1) among and within populations, without regional grouping, 2) among geographical groups (regions), among populations within regions and among individuals within populations. Regions were defined: a) according to the sampling scheme and b) according to the phylogenetical result. Moreover, an AMOVA analysis was performed with all 14 populations, nested in two regions (America vs. Eurasia-North Africa).

RESULTS

In total, 59 alleles were observed from the eight loci surveyed. The number of alleles per locus ranged from two (Am39) to 16 (Am35), being the mean number of alleles per locus 7.13. All loci were monomorphic in at least one of the studied populations. A high significant homozygote excess overall was detected ($F_{IS} = 0.347 \pm 0.185$; $P < 0.001$). All loci except two (Am32 and Am37) showed significantly positive inbreeding coefficients (data not shown).

At the population level, values of the inbreeding coefficient were significantly greater than zero for seven of the 14 populations, while in four populations (CZA, ITR, MTA and TMA) we recovered $F_{IS} < 0$ suggesting heterozygote excess (Table 2). The Mann-Whitney *U*-test indicated significant differences in F_{IS} for five pairwise comparisons (EBA-IBA, EBA-MTA, MTA-IBA, MTA-PKO and MTA-SZI; $P < 0.05$).

Different levels of polymorphism (at 95%) were found between the populations, being TMA, MTA and ITR the less polymorphic, while the most polymorphic were SZI and ELE. The number of alleles per population ranged from nine (ITR; 1.12 in average) to 26 (PKO; 3.25 in average), and 16 (18 when U.S.A. population was considered) privative alleles were detected in seven populations (Table 2). All eleven Eurasian-North African populations showed low gene diversity (from 0.037 for MTA to 0.444 for PKO; Table 2, Fig. 1), but significant differences ($P < 0.05$; Mann-Whitney *U*-test) were found for 10 pairwise comparisons (PKO-CZA, PKO-MTA, PKO-TMA, ELE-CZA, ELE-MTA, ELE-TMA, SZI-CZA, SZI-FHE, SZI-MTA, SZI-TMA). Non-significant difference in H_E was found when the *U*-test was performed between American *versus* Eurasian-North African populations.

Table 2 Sampling localities for 14 populations from across the range of *Arctium minus* and main parameters of genetic diversity within populations. Lat., latitude; Long., longitude; N, number of individuals; *K*, number of alleles (na; mean number of alleles); Priv., number of privative alleles; P_{95}, proportion of polymorphic loci at 95%; H_O, observed heterozygosity; H_E, unbiased expected heterozygosity (Nei, 1978); F_{IS}, inbreeding coefficient. *$P < 0.05$.

Code	Population	Country	Lat.	Long.	N	*K*(na)	Priv	P_{95}	H_O	H_E	F_{IS}
ABA	La Plata	Argentina	S34°55'	W57°57'	30	16 (2)	0	0.500	0.049	0.108	0.553*
CAN	-	Canada	-	-	16	12 (1,5)	0	0.375	0.102	0.115	0.118
CZA	Medveščak	Croatia	N45º49'	E15º59'	30	14 (1,75)	0	0.375	0.150	0.113	-0.333
EBA	Canyamars	Spain	N41°35'	E2°24'	30	16 (2)	0	0.625	0.162	0.258	0.380*
ELE	Murias de Paredes	Spain	N42º52'	W6º11'	30	20 (2,5)	1	0.875	0.185	0.363	0.496*
FHE	Plateau de l'Escandorque	France	N43°16'	E3°26'	30	15 (1,87)	0	0.375	0.089	0.154	0.424*
IBA	Prastio	Italy	N39º56’	E16º7’	30	17 (2,12)	7	0.500	0.107	0.306	0.655*
ITR	Kloster Neustift	Italy	N46º44’	E11º38’	30	9 (1,12)	0	0.286	0.243	0.209	-0.167
MTA	Demnate	Morocco	N31º37’	W6º33’	17	10 (1,25)	4	0.286	0.038	0.037	-0.028
NVE	Farsund	Norway	N58°4'	E6°45'	8	15 (1,87)	1	0.500	0.205	0.241	0.156
PKO	Kórnik	Poland	N52º14'	E17º5'	30	26 (3,25)	1	0.750	0.222	0.444	0.505*
SZI	Liesek	Slovakia	N49º21'	E19º40'	30	21 (2,62)	2	0.857	0.287	0.367	0.223
TMA	Bursa	Turkey	N40º11'	E29º3'	30	13 (1,62)	0	0.125	0.112	0.085	-0.329
UWA	Washington DC	USA	N38°58'	W77° 1'	28	11 (1,37)	2	0.375	0.102	0.183	0.446*

Total gene diversity (H_T) through all loci and populations was 0.58, ranging from 0.167 (Am30) to 0.837 (Am35) (Table 3). The population structure statistics (Table 3) showed that the interpopulation component explains most of the genetic variation detected in *A. minus* ($G_{ST} = 0.599$).

Table 3 Main parameters of gene diversity across loci among populations for *A. minus*. H_T, total gene diversity; H_S, within-population gene diversity; D_{ST}, mean gene diversity among populations; G_{ST}, Nei's coefficient of gene diversity among populations. *$P < 0.001$

	H_T	H_S	D_{ST}	G_{ST}
Am30	0.167	0.048	0.118	0.709
Am31	0.772	0.354	0.417	0.541
Am32	0.736	0.314	0.422	0.573
Am33	0.831	0.332	0.499	0.601
Am34	0.642	0.243	0.399	0.621
Am35	0.837	0.350	0.488	0.583
Am37	0.170	0.132	0.038	0.225
Am39	0.484	0.087	0.397	0.820
Multilocus	0.580	0.233	0.347	0.599

In the pairwise θ analysis all populations showed high significant differentiation from each other ($P < 0.001$), except the NVE and PKO comparison which was non-significant (Table 4). PKO and SZI were the most related populations (excluding the comparison NVE-PKO), while MTA and ITR were the populations most genetically differentiated from the rest of populations.

Table 4. Pairwise comparisons showing differentiation between populations based on θ values (Weir & Cockerham 1984). *P< 0.001. ABA: Argentina, CAN: Canada, CZA: Croatia, EBA and ELE: Spain, FHE: France, IBA and ITR: Italy, MTA: Morocco, NVE: Norway, PKO: Poland, SZI: Slovakia, TMA: Turkey, UWA: USA.

	ABA	CAN	CZA	EBA	ELE	FHE	IBA	ITR	MTA	NVE	PKO	SZI	TMA	UWA
ABA	0.000													
CAN	0.678*	0.000												
CZA	0.772*	0.564*	0.000											
EBA	0.507*	0.598*	0.717*	0.000										
ELE	0.445*	0.436*	0.482*	0.428*	0.000									
FHE	0.479*	0.509*	0.680*	0.484*	0.411*	0.000								
IBA	0.699*	0.689*	0.640*	0.629*	0.425*	0.638*	0.000							
ITR	0.849*	0.860*	0.866*	0.808*	0.723*	0.819*	0.748*	0.000						
MTA	0.889*	0.850*	0.862*	0.775*	0.657*	0.887*	0.778*	0.853*	0.000					
NVE	0.629*	0.347*	0.532*	0.538*	0.381*	0.431*	0.552*	0.807*	0.756*	0.000				
PKO	0.504*	0.386*	0.544*	0.452*	0.348*	0.371*	0.490*	0.518*	0.583*	0.251	0.000			
SZI	0.642*	0.582*	0.657*	0.548*	0.462*	0.593*	0.546*	0.755*	0.707*	0.545*	0.297*	0.000		
TMA	0.833*	0.811*	0.719*	0.768*	0.581*	0.797*	0.632*	0.868*	0.897*	0.782*	0.649*	0.633*	0.000	
UWA	0.729*	0.651*	0.763*	0.653*	0.469*	0.699*	0.669*	0.825*	0.727*	0.563*	0.461*	0.629*	0.823*	0.000

The bootstrap consensus neighbor-joining tree of populations showed four population groups (I-IV; Fig. 2). Only two of them were congruent with the geographical distribution of the populations gathered. Thus, group I included the Iberian and French populations which are 233 km aside, and group III included the Central European populations from Poland and Slovakia which are separated by 355 km with no evident barrier between them. The Mantel test detected no correlation between increase in genetic differentiation with the log of geographic distance, and therefore an absence of isolation by distance was corroborated across all 11 populations (r = 0.190, P = 0.182).

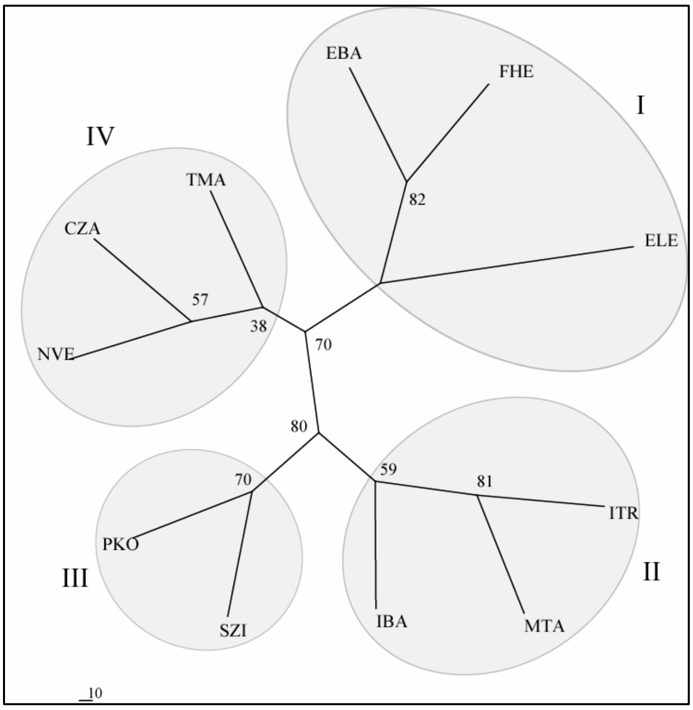

Fig. 2 Extended majority-rule consensus neighbor-joining tree based on bootstrap analysis of the 11 populations from the natural range of *A. minus*. I to IV highlight the groups recovered. CZA: Croatia, EBA and ELE: Spain, FHE: France, IBA and ITR: Italy, MTA: Morocco, NVE: Norway, PKO: Poland, SZI: Slovakia, TMA: Turkey.

Table 5 shows the results for the AMOVA test. When AMOVA was performed among populations without considering higher levels of groupings, the majority of variation was partitioned among populations (60.49%), with a highly significant F_{ST} value (0.605, $P < 0.001$), and with only 39.51% variation within populations. The three-level hierarchical AMOVA considering regional groupings according to the sampling scheme (putative glacial refugia *vs*. Central European populations) yielded in a partition where most of the variation was among populations within regions (62.16%), 39.87% of the variation was within populations, and no variation was found between regions. Only when we considered the four groups obtained in the neighbor-joining tree as regions, the test detected a significant partition of the variation between regions (17.19%; F_{CT} = 0.172, $P < 0.01$). Finally, when the AMOVA analysis was performed with all 14 populations, nested in two regions (America *vs*. Eurasia-North Africa), no variation was detected between both regions.

Table 5. Analisis of Molecular Variance (AMOVA) in *Arctium minus*. Models and populations included: Natural area of distribution (CZA, EBA, ELE, FHE, IBA, ITR, MTA, NVE, PKO, SZI, TMA); Putative refugia *vs*. Central Europe: (EBA, ELE, IBA, MTA, TMA) - (CZA, FHE, ITR, NVE, PKO, SZI); Natural area of distribution partitioned in 4 groups according to Fig. 2 (IBA, ITR, MTA) – (PKO, SZI) – (CZA, NVE, TMA) – (EBA, ELE, FHE); Americas *vs*. Europe (CZA, EBA, ELE, FHE, IBA, ITR, MTA, NVE, PKO, SZI, TMA) – (ABA, CAN, UWA). n.s. no significative.

Model	Partitioning	Variance (%)	*F*-statistic	*P*
Natural area of distribution	Among populations	60.49	$F_{\mathrm{ST}} = 0.605$	< 0.001
	Within populations	39.51		
Putative refugia vs. Central Europe	Among groups	-2.04	$F_{\mathrm{CT}} = -0.020$	n.s.
	Among populations within groups	62.16	$F_{\mathrm{SC}} = 0.609$	< 0.001
	Within populations	39.87	$F_{\mathrm{ST}} = 0.601$	< 0.001
Natural area of distribution partitioned in 4 groups	Among groups	17.19	$F_{\mathrm{CT}} = 0.172$	< 0.01
	Among populations within groups	44.49	$F_{\mathrm{SC}} = 0.537$	< 0.001
	Within populations	38.32	$F_{\mathrm{ST}} = 0.617$	< 0.001
Americas vs. Europe	Among groups	2.83	$F_{\mathrm{CT}} = 0.028$	n.s.
	Among populations within groups	60.80	$F_{\mathrm{SC}} = 0.626$	< 0.001
	Within populations	36.37	$F_{\mathrm{ST}} = 0.636$	< 0.001

DISCUSSION

Our results show that the spatial genetic structure found for *Arctium minus* in Europe does not conform the expected pattern for Europe recolonization from southern glacial refugia, i.e. (i) decline in genetic diversity northwards from refugied populations, and (ii) strong genetic differentiation between populations in different refugia in contrast with a lesser population differentiation between the recolonized areas (*c.f.* Petit *et al.* 2003). (i) The former pattern is not found since populations of *A. minus* throughout Europe do not show a clear pattern with regard to the genetic diversity; much in contrast, the populations show different levels of genetic diversity independently of the European region considered (Table 2; Fig. 1). Thus, for example, the most diverse populations are PKO and SZI, while other populations from centre Europe show little diversity (i.e. ITR and CZA). This is also the case for the populations located in the commonly accepted glacial refugia where both moderately diverse (EBA, ELE, IBA) and poor populations (MTA, TMA) are found. (ii) The last pattern is neither found although significant differences in H_E are obtained for comparisons between the Iberian Peninsula (ELE) respect the populations from Morocco (MTA) and Turkey (TMA). This result may suggest the existence of glacial refugia in South Europe, North Africa and Middle East although this pattern has not been detected for any other pairwise comparison between the remaining putative refugia (i.e. IBA, EBA and ELE).

Petit *et al.* (2003) shown that the most simple expected predictions for genetic diversity distribution in recolonizing species can be altered, being the most diverse populations those at intermediate latitudes, instead of the southernmost ones, due to the admixture of divergent lineages from separate refugia. We have found significant differences in H_E for comparisons between Poland (PKO) and Slovakia (SZI) respect the populations from Morocco (MTA) and Turkey (TMA), which agrees with Petit *et al.* (2003), but not respect the populations from the remaining putative refugia, namely Italy and the Iberian Peninsula.

It is worth to mention that many authors have proposed the existence of cryptic northern refugia in Europe, i.e. Hungary (Willis *et al.* 2000), Slovakia (Litynska-Zajac 1995), Belgium (Leroi-Gourhan 1992) and a more controversial one in Norway (Stewart & Lister 2001, Kullman 2001, 2002, Birks *et al.* 2005, Tollefsrud *et al.* 2008). These refugia would have been in areas of sheltered topography that provided suitable stable microclimates during the Last Cold Stage (Stewart & Lister 2001). This would have been the case for *A. minus* since this cold-tolerant species shows its maximum diversity in the populations from Poland and Slovakia, in Central Europe, which likewise have privative alleles.

Regarding population divergence, a strong differentiation was detected between *A. minus* populations, as the population structure statistics and pairwise θ analysis showed. However, as found with H_E, the detected population genetic structure did not agree with the expected pattern under the glacial refugia model. AMOVA analysis indicated that most of the genetic variation was partitioned among populations, independently if refugee or not refugee areas were considered. Moreover, the spatial genetic structure detected does not fit with an isolation by distance model (Mantel's test not significant), which should be the expected during the Europe recolonization process, as has been shown in other European plants (e.g. Sharbel *et al*. 2000, Schönswetter *et al*. 2003). In spite all these considerations, there are few signs which may suggest that actually *A. minus* adapted to the Pleistocene climatic changes like many other European species. Thus, the presence of seven privative alleles in the population from South Italy and four in the population from Morocco points out to putative refugia in these areas from where the species might radiate northwards.

To sum up, according to our results the current population genetic structure of *A. minus* does not reflect the demographic processes associated with the Pleistocene climatic changes although some signs suggest the likely existence of refugia in North and South Europe. Instead, the detected structure seems to fit well with the life history of this species which combines long-distance seed dispersal with mixed breeding system and short generation time. All three factors have been shown to affect the spatial genetic structure of plant species (Austerlitz *et al*. 2000; Nordborg 2000; Charlesworth 2003; Clauss & Mitchell-Olds 2006), and as a consequence they can blur the genetic signature of colonization and range expansion during Pleistocene. Another factor that may confound genetic patterns is hybridization. This is a frequent phenomenon in *A. minus* and although the pollen produced by hybrid specimens is usually viable, hybrid achenes are often abortive (Duistermaat 1996). Thus hybrid populations are no permanent but there is interespecific genetic flow through pollen grains. And of course we can not ignore human influence since the dispersion of this species is highly correlated with movements of livestock. In addition to this, the lack of information from the fossil record hinders a good reconstruction of the past distribution of *A. minus*.

Range expansion via multiple long-distance dispersal events can result in population genetic structure with little or no isolation by distance. McCauley *et al*. (2003) showed that the best scenario to explain the genetic structure of *Silene vulgaris* in North America (high population differentiation and no isolation by distance) was that in which the range expansion was by long-distance dispersal events. Distances of 166 m in average for seed dispersal in *Arctium minus* have been reported being frequent dispersal distance of 700 m (Couvreur *et al*. 2008), and distances for adhesive-dispersed seeds up to 2.9 km (including *A. minus*; Mouissie *et al*. 2005) and 4.4 km (Cain *et al*. 1998) have also been reported. Therefore, the mode of seed dispersion fits well with the population genetic structure observed in *A. minus*, especially if we consider that this species behaves as a weed in nature, being adapted to disturbed habitats and having ephemeral populations with frequent events of local extinctions and recolonizations.

Isolated populations, as a consequence of long-distance seed dispersion events, frequently will undergo bottlenecks and founder effects which will cause low within-population diversity (*c.f.* Charlesworth 2003) which is congruent with the low intrapopulation genetic diversity found in *A. minus*. Moreover, this species only produces 29-47 seeds per head, from which more than 60% are predated before dispersion (Hawthorn & Hayne 1978). Therefore, the founder effect in the new colonizing populations must be higher due to few numbers of initial plants. This situation agrees with the high inbreeding coefficient detected in all populations of *A. minus*, since the isolation and the few breeders increase the probability of self-pollination in a species with mixed breeding system. In addition, inbreeding interacts with population isolation, diversity and structure, decreasing within-population diversity while increasing among population differentiation (*c.f.* Charlesworth 2003).

The short-life cycle of *A. minus* may contribute to the high inbreeding values recovered in the present work. Austerlitz *et al.* (2000) stated that short-lived plants arriving to a new site can reproduce the next year and therefore the offspring of these first occupants, which are genetically similar to parental founder individuals, have the opportunity to colonize the whole space. This behaviour leads to a genetically homogeneous population and thus results in more variability among populations than within them.

This pattern where most of the genetic variability accounts between populations, as found in *A. minus*, has been reported for worldwide distributed organisms (Sivasundar & Hey 2003) and autogamous species (Hamrick & Godt 1996), characteristics found in lesser burdock. Low gene flow is been stated as another cause of differentiation among populations and in spite of the highly efficient seed dispersal in *A. minus*, its present patchy distribution may actually restrict gene flow among populations.

Although most of the genetic differentiation in *A. minus* is found among populations, differences are independent from the regions where the populations are located, even if they are separated by important geographic barriers. This is the case for the American populations which are not significantly differentiated from those in Europe, Morocco and Turkey. Furthermore, the populations from Americas are supposed to date from the mid 17th century (Gross *et al.* 1980) while a fossil *Arctium sp.* achene from Germany was dated to 7-9 mya (Wähnert, pers. comm.). The most likely explanation for these evidences is a combination of recent long distance dispersal events and human influence, both shaping the genetic structure of *A. minus* populations.

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