UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

DEPARTAMENT DE PRODUCTES NATURALS, BIOLOGIA VEGETAL I EDAFOLOGIA

LABORATORI DE BOTÀNICA

Sistemàtica i filogènia d'*Artemisia* i gèneres relacionats: una aproximació citogenètica i molecular amb especial èmfasi en el subgènere *Dracunculus*

Jaume Pellicer Moscardó

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Sistemàtica i filogènia d'*Artemisia* i gèneres relacionats: una aproximació citogenètica i molecular amb especial èmfasi en el subgènere *Dracunculus*

Memòria presentada per Jaume Pellicer Moscardó per a optar al títol de Doctor per la Universitat de Barcelona

Dr. Joan Vallès Xirau

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Jaume Pellicer Moscardó
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Als meus pares i
a la meva germana, naturalment

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INTRODUCCIÓ

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1. MARC TAXONÒMIC: breu descripció dels grups estudiats

1.1 La família *Asteraceae* Martinov (*Compositae* Adans.)

La família de les *Asteraceae* o *Compositae* s'inclou dintre de l'ordre de les *Asterales,* a la subclasse de les *Asteridae* II (JUDD I OLMSTEAD 2004), essent una de les més diverses i nombroses de les angiospermes (FUNK *et al.* 2005). Aquesta diversitat és el resultat de la seva capacitat de colonització i d'adaptació a un gran ventall d'ecosistemes i ambients. Adaptades a viure arreu del globus, en moltes regions les *Asteraceae* arriben a integrar el 10% de la flora vernacular. El nivell de diversificació morfològica de la família és molt elevat (fig. 1), i el nombre d'espècies integrants és variable, depenent dels autors consultats, amb estimacions aproximades al voltant de 23.000 a 30.000 espècies, i de 1.500 a 1.700 gèneres segons les revisions més recents de la família (BREMER 1994; FUNK *et al.* 2005, 2009; KUBITZKI 2007).

La gran majoria de les *Asteraceae* són subarbusts, arbusts i herbes perennes, tot i que l'habilitat per a desenvolupar-se en ambients xèrics i en condicions extremes facilita l'aparició d'ecotips efímers de comportament anual o biennal. Un dels trets morfològics principals que caracteritza aquest grup és la inflorescència en capítol (HARRIS 1999), quasi específica d'aquesta família (fig. 2a), l'estructura bàsica del qual consta d'un receptacle comú on s'insereixen les diferents flors (flòsculs i/o lígules, fig. 2b), que queda protegit per un involucre de bràctees. Altres trets característics de les *Asteraceae* són la singenèsia (fusió de les anteres en forma d'anell al voltant de l'estil, fig. 2b), i el fruit en cípsela o aqueni (moltes vegades acompanyat d'un papus que n'afavoreix la dispersió, fig. 2c). L'aparició de tots aquests caràcters posa de manifest l'existència d'un equilibri entre les diferents variables biològiques implicades en la reproducció, la dispersió, la germinació, i la defensa front als predadors (STUESSY I GARVER 1996), per tal de garantir-ne la perpetuació. L'origen de les *Asteraceae* també ha estat llargament discutit. Els primers estudis basats en l'anàlisi de seqüències del DNA

de BREMER I GUSTAFSSON (1997) varen suggerir que els ancestres de l'ordre de les *Asterales* podrien haver estat originats a finals del cretaci a l'est de Gondwana (actualment la zona d'Australàsia) i que posteriorment s'haurien expandit cap al que ara coneixem com Amèrica del Sud (l'oest de Gondwana), on les *Asteraceae* haurien emergit abans de la separació final d'aquest territori i l'Antàrtida.

Figura 1. Diversitat morfològica en les *Asteraceae*: a) *Artemisia jacutica* Drobow, *Anthemideae* (J. Pellicer); b) *Aster alpinus* L., *Astereae* (aster.gforge.inria.fr); c) *Cheirolophus webbianus* (Sch. Bip.) Holub, *Cardueae* (J. Pellicer); d) *Cichorium intybus* L., *Cichorieae* (www.fickr.com); e) *Echinops sphaerocephalus* L., *Cardueae* (www.missouriplants.com); f) *Duseniella patagonica* Pilg. et Ulbr., *Barnadesieae* (www.plantsystematics.org); g) *Helenium autummale* L., *Helenieae* (www.missouriplants.com); h) *Helianthus angustifolius* L., *Heliantheae* (www.calfloranursery.com); i) *Helychrisum stoechas* DC., *Gnaphalieae* (www.cepcala.org); j) *Hesperomannia arbuscula* Hbd., *Vernonieae* (www.botany.hawaii.com); k) *Leontopodium alpinum* Cass., *Gnaphalieae* (www.fotonatura.com); l) *Mutisia decurrens* Cav., *Mutisieae* (www.slimiti.com); m) *Senecio aureus* L., *Senecioneae* (www.missouriplants.com); n) *Solidago canadensis* L., *Astereae* (J. Pellicer); o) *Tripleurospermum maritimum* (L.) V.D.J. Kock, *Anthemideae* (www.aphotoflora.com); p) *Wunderlichia mirabilis* Reidel ex Baker, *Mutisieae* (asteraceae.group.googlepages.com).

Aquesta hipòtesi va ser posteriorment confirmada també per BREMER *et al.* (2004), que postularen el final del cretaci, com el moment d'aparició de l'ordre de les *Asterales*. D'altra banda, estudis basats en troballes de pol·len fòssil de la família de les *Asteraceae* a Amèrica del Sud, bàsicament de tipus microequinat (subfamília *Mutisioideae*) i equinat (probablement de la subfamília *Asteroideae*), segons GRAHAM (1996), semblen apuntar cap a finals de l'eocè com a l'època més probable d'aparició de la família al continent sud-americà.

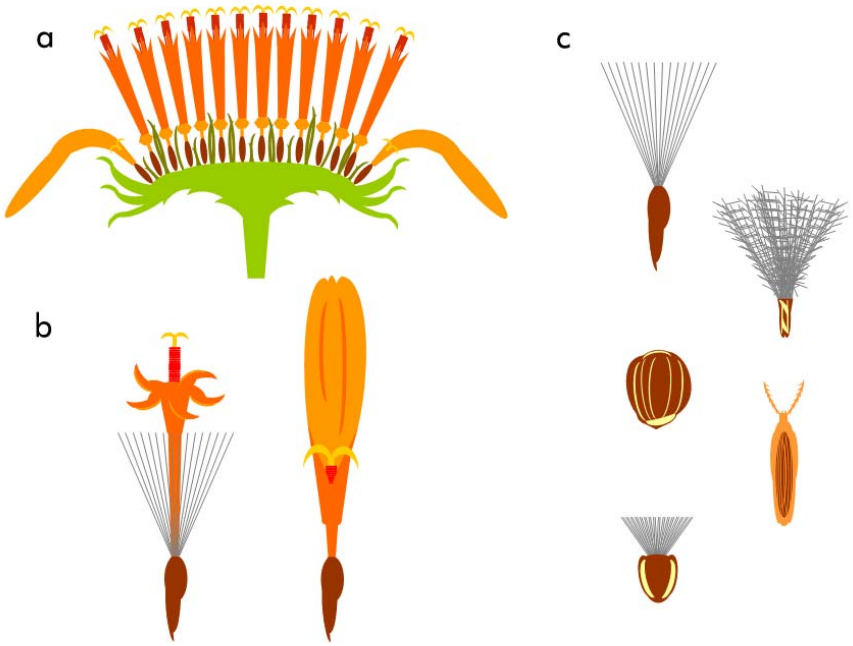

Figura 2. Esquema de les principals característiques de les *Asteraceae*. a) inflorescència en capítol, típica de les *Asteraceae*, b) flòsculs i lígules on es veuen els estams fusionats per les anteres, c) diferents tipus d'aquenis.

Les bases per a la classificació tribal de la família de les *Asteraceae* començaren a establir-se al segle XIX. Amb la incorporació de les eines d'estudi moleculars, s'han pogut realitzar reconstruccions filogenètiques de la família, on s'ha demostrat l'origen de la mateixa, donant suport també a la hipòtesi de la seva aparició al continent sud-americà. Els resultats de tots aquests treballs posen de manifest l'èxit colonitzador de la família, que posteriorment a la seva emergència, va expandir-se cap al continent africà, des d'on s'iniciaria la gran

explosió evolutiva, així com la posterior entrada a l'Àsia, Europa i Oceania (FUNK *et al.* 2005).

1.2 La tribu *Anthemideae* Cass.

La tribu de les *Anthemideae* pertany a la subfamília de les *Asteroideae* (Cass.) Lindl., i comprèn aproximadament unes 1.800 espècies, distribuïdes en 111 gèneres (BREMER i HUMPHRIES 1993; OBERPRIELER *et al.* 2007, 2009). És una tribu bastant cosmopolita i diversa (fig. 3), molts dels representants de la qual es troben àmpliament distribuïts als cinc continents. La hipòtesi d'un possible origen sud-africà és la més acceptada (NOYES i RIESBERG 1990; WATSON *et al.* 2000), des d'on s'hauria estès cap a l'hemisferi nord. Els grans centres de diversificació de la tribu es troben a la regió mediterrània, les zones meridional i central d'Àsia i a la zona del sud d'Àfrica, tot i que força gèneres han aconseguit arribar i ocupar amplis territoris al continent americà. La major part dels representants de la tribu apareixen repartits principalment a les zones temperades de l'hemisferi nord.

Els trets característics principals que permeten diferenciar els membres d'aquesta tribu són la fragància aromàtica que es desprèn de les fulles i la tija, deguda a la presència de compostos químics volàtils, que a més a més també poden tenir valor sistemàtic en alguns casos. Els principals compostos que podem trobar són acetilens, lactones sesquiterpèniques i flavonoides (GREGER 1977; SEAMAN 1982; MARCO i BARBERÀ 1990; BOHM i STUESSY 2001). Les fulles dissectes, el marge de les bràctees involucrals escariós i el papus no hirsut molt reduït (quan és present), són altres dels trets característics d'aquest grup.

La distribució dels tàxons també és variable, des dels gèneres amb una distribució més reduïda com *Argyranthemum* Webb. (endèmic de la regió macaronèsica), fins gèneres de distribució molt àmplia com *Artemisia*, que es pot trobar als cinc continents. Els treballs basats en l'anàlisi de diverses regions del DNA i les revisions més recents de la tribu (FUNK *et al.* 2005, OBERPRIELER *et al.* 2007, 2009) confirmen la seva monofília. Tot i això, encara cal aprofundir en el coneixement sistemàtic, la delimitació, i les relacions intergenèriques d'alguns

tàxons representatius com *Achillea* L., *Anthemis* L., *Artemisia* L. o *Tripleurospermum* Sch. Bip.

Moltes d'aquestes espècies també han estat usades des de temps immemorials pel seu valor medicinal, culinari o ornamental (com per exemple els gèneres *Artemisia*, *Chrysanthemum* L., *Matricaria* L. o *Santolina* Tourn.).

Figura 3. Representants de la tribu *Anthemideae*. A) *Anacyclus valentinus* L. (www.fotonatura.com); b) *Anthemis arvensis* L. (www.lacoctelera.com); c) *Artemisia maximovicziana* Krasch. ex Poljakov (J. Pellicer); d) *Chrysanthemum segetum* L. (www.floralimages.co.uk); e) *Eriocephalus africanus* L. (www.florasilvestres.es); f) *Leptinella dendyi* (Cockayne) D.G. Lloyd et C.J.Webb (www.flickr.com); g) *Leucanthemella serotina* (L.) Tzvelev (www.mobot.org); h) *Santolina chamaecyparissus* L. (www.habitas.org.uk); i) *Tanacetum vulgare* L. (www.plant-identification.co.uk); j) *Soliva sessilis* Ruiz et Pav. (www.stingerplace.com); k) *Tripleurospermum kotschyi* (Boiss.) E.Hossain (J. Pellicer); l) *Ursinia sp*. (www.pbase.com).

1.3 La subtribu *Artemisiinae* Less.

La subtribu de les *Artemisiinae* (fig. 4) tampoc no ha quedat exclosa de les disparitats taxonòmiques al llarg de la història. BREMER I HUMPRHIES (1993), en la seva revisió de les *Anthemideae*, delimitaren la subtribu *Artemisiinae* en base a l'anàlisi de caràcters morfològics (capítols homògams i discoides, o heteròg ams i disciformes, pol·len microequinat -espinulat- i aquenis sense costelles i amb la

testa prima). Aquesta classificació inclou el gènere *Artemisia* [i considera *Seriphidium* (Besser ex Less.) Fourr. com a gènere segregat, independent], a més de *Crossostephium* Less., *Elachantemum* Y. Ling et Y. R. Ling, *Filifolium* Kitam., *Kaschgaria* Poljakov, *Mausolea* Poljakov, *Neopallasia* Poljakov, *Stilpnolepis* Krasch., *Turaniphytum* Kitam. i dos gèneres endèmics d'Amèrica del Nord (*Picrothamnus* Nutt. i *Sphaeromeria* Nutt.) com a nucli central de la subtribu, i que segons SANZ *et al.* (2008) conformen una possible circumscripció monofilètica del grup *Artemisia*, des del punt de vista de la filogènia molecular. Altres gèneres relacionats que s'inclourien dintre de la subtribu són *Arctanthemum* (Tzvelev) Tzvelev, *Brachanthemum* DC., *Dendranthema* Des Moul. i *Tridactylina* Sch. Bip. [prèviament inclosos en *Chrysanthemum s. l.* per BREMER I HUMPHRIES (1993)], així com *Ajania* Poljakov, *Ajaniopsis* C. Sih. i *Phaeostigma* Muldashev. Les constants reordenacions taxonòmiques que hi ha hagut al llarg del temps en alguns grans gèneres com *Ajania*, *Artemisia* o *Chrysanthemum*, entre d'altres (BREMER I HUMPHRIES 1993; MASUDA *et al.* 2009) també ens ajuden a fer-nos la idea de la complexitat sistemàtica d'aquest grup, i de la necessitat d'aprofundir en el coneixement de les seves relacions filogenètiques.

Figura 4. Alguns representants de la subtribu *Artemisiinae* estretament relacionats amb *Artemisia*. a, b) *Crossostephium chinense* Makino (http://aoki2.si.gunma-u.ac.jp, J. Pellicer); c, d) *Filifolium sibiricum* Kitam. (J. Vallès); e) *Neopallasia pectinata* Pall. (J. Pellicer); f) *Sphaeromeria capitata* Nutt. (www.laporteavenuenursery.com); g) *Turaniphytum eranthemum* (Bunge) Poljakov (J. Vallès).

En aquest sentit, a les darreres revisions de les *Asteraceae* (KUBITZKI 2007; FUNK *et al*. 2009) es pot veure que el nucli de les *Artemisiinae* s'inclou dintre d'un gran grup asiaticosud-africà, que comprèn, entre d'altres, els grups d'*Ajania*, *Cancrinia* Kar. et Kir., *Handelia* Heimerl i altres gèneres afins (KUBITZKI 2007). Aquesta nova classificació i ordenació dels gèneres es basa principalment en aspectes biogeogràfics, a causa d'algunes de les incongruències que s'han trobat entre les classificacions morfològiques i aquelles basades en l'anàlisi de seqüències de DNA. Segons OBERPRIELER *et al*. (2007), la major part dels gèneres inclosos per BREMER I HUMPHRIES (1993) passen a formar part del grup *Ajania* L., i el grup *Artemisia* quedaria constituït pels gèneres *Artemisia* (incloent *Seriphidium*), *Artemisiella* Ghafoor, *Crossostephium*, *Filifolium*, *Mausolea*, *Neopallasia*, *Picrothamnus* i *Sphaeromeria*.

1.4 El gènere *Artemisia* L.

El gènere *Artemisia* és un dels més grans de la família de les *Asteraceae*, i el més gran de la tribu de les *Anthemideae*. Pel que fa al nombre de tàxons del gènere podem trobar certa variabilitat segons les referències, que van des de les 380 espècies (LING *et al*. 2006) fins les més de 500 (BREMER I HUMPHRIES 1993; KUBITZKI 2007). En algunes de les darreres revisions (KUBITZKI 2007), s'inclouen dintre del gènere *Artemisia* les espècies que en algun moment havien estat adscrites a gèneres independents [*Artanacetum* (Rzazade) Rzazade, *Oligosporus* Cass., i *Seriphidium*]. Aquesta heterogeneïtat i la falta de consens en la delimitació genèrica d'*Artemisia*, radica principalment tant en la disparitat de criteris com en el nivell d'exigència dels autors a l'hora de considerar els casos de sinonímia taxonòmica, així com de vicariança geogràfica.

La distribució del gènere *Artemisia* inclou principalment les zones temperades de tot l'hemisferi nord, tot i que també podem trobar algunes espècies a l'hemisferi sud (BREMER 1994). Segons WANG (2004), l'origen d'*Artemisia*, basant-se en dades palinològiques i filogeogràfiques, podria trobar-se en les estepes semiàrides de les muntanyes de l'Àsia temperada a mitjans del cenozoic, és a dir, fa aproximadament uns 20 milions d'anys. De fet, avui en dia

es considera l'Àsia Central com el principal centre d'especiació i diversificació del gènere, que s'estén fins altres regions com l'iranoturaniana, la mediterrània i la nord-americana.

Des del punt de vista morfològic les fulles, sovint dividides, es distribueixen de forma alternada o esparsa a la tija, i presenten un ventall molt ampli de formes, mides i textures. Els capítols són petits i de forma ovoide, semiesfèrica o esfèrica, compostos per flors tubuloses (flòsculs) i protegits per un involucre de bràctees. Es presenten solitaris o agrupats en inflorescències paniculades. Els flòsculs solen presentar la corol·la de color blanquinós, groc o porpra, molt poc vistosa, que s'inserta de forma vertical o obliqua a l'ovari, i es disposen organitzats en un receptacle pla o còmic (convex o còncau), que de vegades pot ser pilós (BREMER 1994). Els aquenis característics del gènere són petits, obovoides i lateralment comprimits. Presenten estries longitudinals i l'absència de papus és un tret comú a tot el gènere. D'altra banda, l'ornamentació del pol·len ha estat utilitzada com marcador sistemàtic per al gènere, ja que l'escassa, però variada, ornamentació verrucosa o espinulosa [segons JIANG *et al.* (2005)] que presenta l'exina és característica de tots els seus membres, fet que els diferencia de la majoria dels representants de la tribu que presenten una ornamentació de tipus equinat (MARTÍN *et al.* 2001, 2003).

Les artemísies són majoritàriament perennes (figs. 5b-d, f-i, m), encara que en podem trobar d'anuals (figs. 5e, j) o biennals (fig. 5a). Al voltant de 20 espècies del gènere presenten aquest comportament anual (POLJAKOV 1961a; BREMER I HUMPHRIES 1993; LING *et al.* 2006). Els tipus biològics també són bastant variables, éssent principalment classificades com a herbes (*Artemisia annua* L., *Artemisia vulgaris* L.), subarbusts o mates (*Artemisia changaica* Krasch., *Artemisia crithmifolia* L.) i arbusts, que arriben a presentar tiges fortament lignificades (*Artemisia tridentata* Nutt.). La plasticitat ecològica del gènere també queda patent quan s'observa l'extens rang d'ecosistemes que pot arribar a colonitzar, i que van des de les zones desèrtiques o semidesèrtiques (estepes, tundres, zones rocalloses), boscos oberts i zones antropitzades, fins a àrees humides, des del nivell del mar fins a l'alta muntanya. Podem trobar tàxons amb diferents nivells d'endemisme com *Artemisia klementzae* Krasch. (Mongòlia),

Artemisia edgeworthii Balakr. (nord de l'Índia) o *Artemisia barrelieri* Besser (península Ibèrica), fins a representants tan cosmopolites i ruderals com *Artemisia campestris* L. o *A. vulgaris*.

Figura 5. a) *Artemisia biennis* L.; b) *Artemisia desertorum* Spreng.; c) *Artemisia echegarayii* Hieron.; d) *Artemisia gmelinii* Stechm.; e) *Artemisia jacutica* Drobow; f) *Artemisia keiskeana* Miq.; g) *Artemisia mendozana* DC.; h) *Artemisia messerchmidtiana* Besser; i) *Artemisia nova* A. Nelson; j, k, l) *Artemisia palustris* L.; m) *Artemisia selengensis* Turcz. ex Besser. (Imatges: J. Pellicer).

Moltes espècies del gènere tenen cert interès econòmic en camps tan variats com la medicina (*A. annua*, de la qual s'extreu l'artemisinina per a combatre la malària), en alimentació (*Artemisia dracunculus* L., utilitzada com a condiment -l'estragó- o *Artemisia absinthium* L., per a la preparació del licor d'absenta), per a l'estabilització de sòls (*Artemisia sphaerocephala* Krasch.) o

com a plantes ornamentals (*Artemisia arborescens* L.). Característica de moltes espècies és la fragància, de gran intensitat en algunes d'elles (p. ex. *Artemisia mendozana* DC.), deguda a la presència de substàncies monoterpèniques i sesquiterpèniques localitzades en pèls i canals secretors esquizogènics (GREGER 1977).

La majoria de les espècies d'*Artemisia* floreixen cap a finals de l'estiu i durant la tardor, tret que les diferencia de la resta de les *Anthemideae*, que ho fan a la primavera i a l'estiu. A més, el fet de tenir una pol·linització quasi exclusivament anemòfila (encara que hi ha evidències d'entomofília en algunes espècies (GARNOCK-JONES 1986; VALLÈS *et al.* 1987), és una característica que sols comparteixen les artemísies amb alguns dels gèneres amb els quals estan estretament relacionades.

La classificació sistemàtica del gènere (taula 1), com la d'altres gèneres d'*Asteraceae*, és complexa i avui en dia encara no existeix un tractament taxonòmic del grup que siga globalment acceptat. Les espècies que actualment romanen sota el nom d'*Artemisia* foren prèviament agrupades en tres gèneres independents (*Abrotanum*, *Absinthium* i *Artemisia*) per TOURNEFORT (1700). Temps després, LINNÉ (1735) va agrupar-les subseqüentment sota el nom d'*Artemisia*. Al 1817 CASSINI i posteriorment LESSING (1832), transferiren part d'aquestes espècies a un nou gènere, *Oligosporus* Cass., que actualment coneixem com el subgènere *Dracunculus* (Besser) Rydb. BESSER (1829, 1832, 1834, 1835) va dividir el gènere en quatre grups (amb la nova creació de *Seriphidium* Besser) que rebrien el tractament de secció o subgènere (*Abrotanum* [actualment conegut com *Artemisia*], *Absinthium*, *Dracunculus* i *Seriphidium*). Aquesta divisió fou també acceptada per CANDOLLE (1837), i posteriorment reconsiderada per ROUY (1903). RYDBERG (1916) creà una nova secció que anomenà *Tridentatae*, que incloïa espècies prèviament integrades dintre de *Seriphidium*, i que més tard fou reconeguda com a subgènere per MCARTHUR *et al.* (1981). Temps després, LING (1982, 1991a, b, 1995a, b) proposà la segregació de *Seriphidium* (Besser ex Hook) Fourr. com a nou gènere independent. Aquesta segregació continuaria essent acceptada per BREMER I HUMPHRIES (1993) i BREMER (1994) en les seves revisions de les *Asteraceae*.

Actualment hi ha algunes revisions del gènere, com la de LING *et al.* (2006), per a la Flora de la Xina, que proposen una divisió infragenèrica basada en seccions, i en la qual es continua mantenint l'estatus genèric de *Seriphidium*, tot i que les filogènies moleculars (SANZ *et al.* 2008, i referències que conté), i els tractaments taxonòmics més recents (KUBITZKI 2007; FUNK *et al.* 2009) no hi donen suport.

Taula 1. Comparació entre les diferents classificacions infragenèriques d'*Artemisia*. Extret de VALLÈS I MCARTHUR (2001), amb lleugeres modificacions.

Categoria	Grups infragenèrics					Autor
Gèneres	*Absinthium*	*Abrotanum*	*Artemisia*			Tournefort (1700)
Gènere	*Artemisia*					Linné (1735)
Gèneres	*Artemisia*			*Oligosporus*		Cassini (1817); Lessing (1832)
Seccions	*Artemisia*	*Abrotanum*	*Seriphidium*	*Dracunculus*		Besser (1829, 1832, 1834, 1835); Candolle (1837)
Subgèneres	*Euartemisia*		*Seriphidium*	*Euartemisia*		Rouy (1903)
Subèneres	*Absinthium*	*Abrotanum*	*Seriphidium*	*Dracunculus*		Rydberg (1916)
Seccions			*Seriphidium*	*Tridentatae*		
Subgèneres	*Artemisia*		*Seriphidium*	*Dracunculus*		Poljakov (1961a)
Subgèneres	*Absinthium*	*Artemisia*	*Seriphidium*		*Dracunculus*	Persson (1974)
Seccions	*Artemisia*				*Dracunculus*	Tutin *et al.* (1976)
Subgèneres	*Artemisia*		*Seriphidium*	*Tridentatae*	*Dracunculus*	McArthur *et al.* (1981)
Subgèneres	*Artemisia*		*Seriphidium*		*Dracunculus*	Podlech (1986)
Gèneres	*Artemisia*		*Seriphidium*		*Artemisia*	
Subgèneres	*Artemisia*		*Seriphidium*		*Dracunculus*	Ling (1991a, b)

Tot i aquestes modificacions sobre les classificacions al llarg de la història, les agrupacions basades en la morfologia floral s'han mantingut més o menys constants al llarg del temps. Així doncs, les característiques florals de cada subgènere són (fig. 6):

- ***Absinthium***: Capítols heteròg ams amb flors radials femenines i flors centrals hermafrodites fèrtils. Receptacle pilós.
- ***Artemisia*** (= ***Abrotanum***): Capítols heteròg ams amb flors radials femenines i les centrals hermafrodites fèrtils. Receptacle glabre.

- ***Dracunculus***: Capítols heterògams amb flors radials femenines i les centrals hermafrodites però funcionalment masculines, és a dir, amb ovaris estèrils. Receptacle glabre.
- ***Seriphidium*** i ***Tridentatae***: Capítols homògams amb totes les flors hermafrodites i fèrtils. Receptacle glabre.

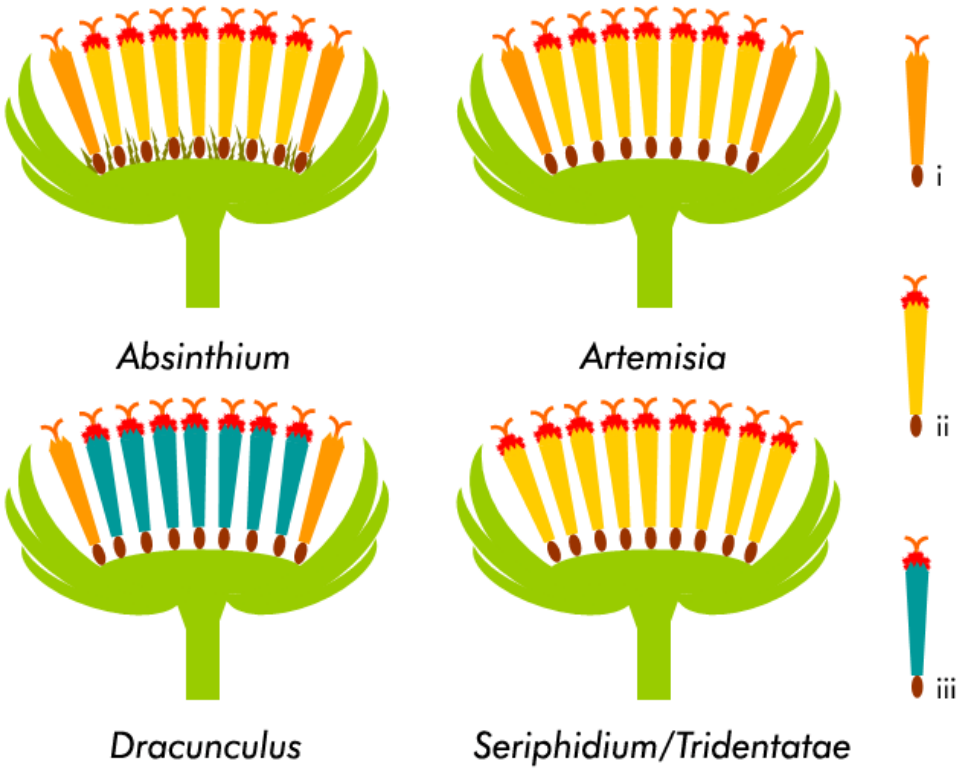

Figura 6. Esquema de la morfologia floral de cadascun dels subgèneres d'*Artemisia*. i) flors femenines; ii) flors hermafrodites fèrtils; iii) flors hermafrodites funcionalment masculines.

El gènere *Artemisia* ha estat estudiat intensament des del punt de vista de l'evolució cromosòmica. Des dels primers estudis fins a l'actualitat, s'han publicat nombrosos treballs sobre els nombres cromosòmics al gènere (aproximadament en unes 350 espècies), així com treballs adreçats a l'estudi de la distribució del DNA ribosòmic als cromosomes (VALLÈS I GARNATJE 2005, i referencies que conté; GARCIA *et al.* 2007, 2009). Al gènere hi trobem dos nombres cromosòmics bàsics: $x = 9$, present a tots els subgèneres, i $x = 8$, que solament s'ha trobat en algunes espècies dels subgèneres *Absinthium*, *Artemisia* i

Dracunculus (SOLBRIG 1977; SCHWEIZER I EHRENDORFER 1983; OLIVA I VALLÈS 1994; MCARTHUR I SANDERSON 1999). Sembla que una fusió cromosòmica robertsoniana podria ser la causa d'aquesta disploïdia descendent, que hauria reduït el nombre cromosòmic bàsic a $x = 8$ (VALLÈS I SILJAK-YAKOVLEV 1997). Ambdós nombres cromosòmics presenten sèries poliploides que arriben fins al dodecaploide ($12x$) per als $x = 9$, i fins als hexaploides ($6x$) per als $x = 8$ (EHRENDORFER 1964; ESTES 1969; PERSSON 1974; MCARTHUR I POPE 1979; MALAKHOVA 1990; OLIVA I VALLÈS 1994; MCARTHUR I SANDERSON 1999)

Mitjançant l'ús d'eines d'anàlisi molecular, que permeten la seqüenciació de diferents regions del DNA, la sistemàtica del gènere ha estat objecte d'estudi per a molts autors. S'han analitzat els espaiadors transcrits intern i extern (ITS, ETS), pel que fa al DNA ribosòmic (KORNKVEN I WATSON 1997; KORNKVEN *et al.* 1998; TORRELL *et al.* 1999; WATSON *et al.* 2002; D'ANDREA *et al.* 2003; VALLÈS *et al.* 2003; SANZ *et al.* 2008; TKACH *et al.* 2008a, b), el gen cloroplàstic *ndh*F (WATSON *et al.* 2000), llocs de restricció del DNA cloroplàstic (KORNKVEN *et al.* 1999), o polimorfismes del DNA amplificats a l'atzar (RAPD, MCARTHUR *et al.* 1998). Aquests treballs han permés obtenir una visió general de gènere, tot i que posen de manifest determinades incongruències amb la classificació tradicional. Fenòmens com la hibridació i la poliploïdia, freqüents a les plantes, així com la manca d'un mostreig representatiu, poden influir en aquests resultats, dificultant l'obtenció d'un marc filogenètic sòlid.

En el treball present, considerant els resultats obtinguts prèviament als estudis de SANZ *et al.* (2008 i referències incloses) i TKACH *et al.* (2008a, b), s'ha adoptat la classificació que divideix el gènere en cinc subgèneres: *Absinthium*, *Artemisia*, *Dracunculus*, *Seriphidium* i *Tridentatae*.

1.5 El subgènere *Dracunculus* (Besser) Rydb.

CASSINI (1817) va descriure el gènere *Oligosporus*, que incloïa el conjunt d'espècies que actualment conformen el subgènere *Dracunculus* (fig. 7). Aquest gènere acollia aquells tàxons que presentaven, com s'ha mencionat a l'apartat anterior, sexes funcionalment separats, és a dir, flòsculs radials femenins, i els

centrals masculins, com a conseqüència de l'avortament dels ovaris. El subgènere comprèn aproximadament 80 tàxons a nivell específic o subespecífic (POLJAKOV 1961a, b). Recentment LING *et al.* (2006) en la seva revisió de la tribu *Anthemideae* per a la Flora de la Xina, proposen un nou tractament sistemàtic per a la classificació de les espècies que conformen el gènere *Artemisia*, tradicionalment dividit en subgèneres. Així doncs, el subgènere *Dracunculus* es divideix en dues noves seccions, *Dracunculus* Besser i *Latilobus* Y. R. Ling, en base principalment a diferències de la morfologia foliar i de l'indument.

Figura 7. Representants del subgènere *Dracunculus*: a) *Artemisia crithmifolia* (J. Vallès); b) *Artemisia dracunculus* (J. Pellicer); c) *Artemisia eriopoda* Bunge (J. Pellicer); d) *Artemisia ledebouriana* Besser (J. Pellicer); e) *Artemisia macilenta* (Maxim.) Krasch. (J. Pellicer); f) *Artemisia monosperma* Delile (J. Pellicer); g) *Artemisia monostachya* Maxim. ex Bunge (J. Pellicer); h) *Artemisia pycnorhiza* Ledeb. (J. Pellicer); i) *Artemisia tomentella* Trautv. (J. Pellicer).

Cal posar atenció també a la inclusió en el subgènere *Dracunculus*, en base als resultats de la seqüenciació del DNA (VALLÈS *et al.* 2003; SANZ *et al.* 2008), d'alguns gèneres relacionats com *Mausolea*, *Neopallasia* i *Turaniphytum* Poljakov, i que prèviament ja havien estat inclosos dintre del gènere *Artemisia*

(amb els noms d'espècies *A. eranthema* Bunge, *A. eriocarpa* Bunge i *A. pectinata* Pall., respectivament). Tot i aquestes troballes, la manca d'un marc filogenètic representatiu del subgènere no ha permés resoldre completament les seves relacions interespecífiques. D'altra banda, estudis previs destinats a resoldre la sistemàtica del gènere, han confirmat la monofília del grup eurasiàtic (SANZ *et al.* 2008; TKACH *et al.* 2008a, b), així com la seva parafília interna, induïda per la discordança sistemàtica d'algunes de les espècies endèmiques d'Amèrica del Nord incloses per SHULTZ (2005) en el subgènere *Dracunculus* en base a caràcters morfològics i que apareixen agrupades en llinatges independents del nucli del subgènere (SANZ *et al.* 2008).

Pel que fa a la distribució, poden trobar-se representants del subgènere àmpliament distribuïts a l'hemisferi nord, principalment a les zones àrides i semiàrides des d'Europa a l'Àsia, on es troba el seu principal focus de diversificació, i arribant a colonitzar zones del continent nord-americà. Majoritàriament són subarbustives o herbàcies, principalment perennes, tot i que també inclou algunes espècies anuals com *Artemisia demissa* Krasch., *Artemisia edgeworthii* Balakr., *Artemisia pewzowii* C. Winkl. i *Artemisia scoparia* Waldst. et Kit.

D'entre les espècies del subgènere podem destacar l'espècie tipus, *A. dracunculus* (fig. 7b), que presenta un important valor econòmic degut al seu ús tradicional com a condiment culinari, així com altres espècies, com ja hem esmentat abans, que s'utilitzen per a fixar sòls (com *Artemisia sphaerocephala* Krasch. o *A. wudanica* Liou et W. Wang), o en medicina tradicional [*Artemisia capillaris* Thunb. (amb propietats detoxificants i diürètiques) i *Artemisia ordosica* Krasch. (amb propietats antiinflamatòries)].

Des del punt de vista cariològic, ambdós nombres cromosòmics bàsics en *Artemisia* ($x = 9$ i $x = 8$) són presents al subgènere, tot i que $x = 8$ té una incidència molt poc significativa, havent estat trobat solament en poblacions d'*A. scoparia* fins al moment (TORRELL *et al.* 2001b, i referències). Així mateix, la incidència de la poliploïdia en aquest grup té molta rellevància (GARCIA *et al.* 2006 i referències), fet que es tradueix en el gran nombre de poblacions poliploides que s'han descrit al subgènere. Una de les sèries poliploides més

completes és la descrita per a *A. dracunculus*, en la qual s'han trobat poblacions amb citotips que van des dels diploides fins els decaploides (WEINEDEL-LIEBAU 1928; ROUSI 1969; MURIN 1997; VALLÈS *et al.* 2001; KREITSCHITZ I VALLÈS 2003), i inclús, existeixen espècies de les quals només se'n coneixen poblacions poliploides, com per exemple *Artemisia klementzae* o *Artemisia xanthochroa* Krasch. (GARCIA *et al.* 2006).

1.6 El gènere *Ajania* Poljakov

Format per herbes perennes i subarbusts, el gènere *Ajania* Poljakov comprèn al voltant de 30-40 espècies (fig. 8; BREMER I HUMPHRIES 1993; BREMER 1994; KUBITZKI 2007; OBERPRIELER *et al.* 2009).

Figura 8. Representants del gènere *Ajania*. a) *Ajania fastigiata* (C. Winkl.) Poljakov (flower.onego.ru); b) *Ajania fruticulosa* (Ledeb.) Poljakov (www.sbras.ru); c) *Ajania myriantha* (Franch.) Y.R. Ling ex C. Shih (www.chinese-plant.com); d) *Ajania pacifica* (Nakai) K. Bremer et Humphries (www.plantes-botanique.org); e) *Ajania purpurea* (www.tibetplant.com); f) *Ajania scharnhorstii* (Regel et Schmalh.) Tzvelev (flower.onego.ru); g) *Ajania tibetica* (Hook. f. et Thomson) Tzvelev (vandenbrink.demon.nl); h) *Ajania trilobata* Poljakov (flower.onego.ru).

Es caracteritza pel fet de presentar fulles dividides en major o menor grau, i per la presència d'inflorescències corimboses de capítols petits, amb flors de color groguenc principalment, tot i que hi podem trobar algunes espècies amb les flors de color porpra (p. ex. *Ajania purpurea* C. Shih, fig. 8e). Es tracta d'un gènere d'origen asiàtic; podem trobar gran nombre de representants

distribuïts àmpliament a la Xina i al Japó, així com a la zona del sud-oest asiàtic, Rússia i Mongòlia. Aquest gènere fou segregat d'*Artemisia* per POLJAKOV (1955). TZVELEV (1961), a la Flora de l'URSS, va acceptar aquesta segregació, tot mantenint l'estatus genèric d'*Ajania*, que agrupava al voltant de 25 espècies. Aquest reduït nombre d'espècies responia a la consideració que POLJAKOV (1955) havia classificat dintre d'*Ajania* diferents tàxons que amb el temps, i basant-se en el tipus pol·línic, Tzvelev va reincloure dins del gènere *Artemisia*. Aquest autor ja va apuntar la possibilitat que *Ajania* hagués evolucionat a partir d'un ancestre molt pròxim al gènere *Dendranthema* (actualment conegut com a *Chrysanthemum*), i que per adaptació a les àrides estepes i deserts d'Àsia s'hagués desenvolupat cert nivell de convergència morfològica amb alguns representants d'*Artemisia* que ocupaven aquestes terres.

Més tard, BREMER I HUMPHRIES (1993), en un intent de donar una explicació a les similituds existents entre aquests tres gèneres (*Ajania*, *Artemisia* i *Dendranthema*), postularen la possibilitat que a partir d'un mateix ancestre "dendrantemoide" s'hagueren originat línies independents d'evolució que haurien propiciat la segregació d'aquests gèneres. Aquestes reordenacions taxonòmiques del gènere es mantingueren fins que MULDASHEV (1982, 1983) segregà tres espècies d'*Ajania* per a constituir un nou gènere, *Phaeostigma* Muldashev. L'autor justificà aquest canvi en base a la morfologia del gra de pol·len, que en aquestes espècies presenta una ornamentació microequinada (espinulosa), més afí a la del pol·len d'*Artemisia*. En aquest sentit, proposà també la recombinació d'*Ajania junnanica* Poljakov com a *Artemisia junnanica* (Poljakov) Muldashev, entre d'altres motius, per la presència de pol·len amb espines molt petites, característic d'*Artemisia*.

2. ANTECEDENTS I OBJECTIUS

La tesi que aquí es presenta agrupa un compendi de treballs centrats en el gènere *Artemisia*, tot i que també ens hem interessat per l'estudi d'alguns aspectes de l'evolució d'altres gèneres afins com *Ajania, Crossostephium* o *Neopallasia*, entre d'altres. Aquests estudis s'inclouen dins de la línia de recerca

en organització i evolució del genoma i les seues implicacions sistemàtiques i evolutives en les *Asteraceae*, línia que es desenvolupa de forma conjunta al Laboratori de Botànica de la Facultat de Farmàcia (UB) i a l'Institut Botànic de Barcelona (CSIC-ICUB) (www.etnobiofic.cat). Amb una visió multidisciplinària, que inclou l'ús de tècniques tan variades com la palinologia, la citogenètica clàssica i molecular, la citometria de flux i la seqüenciació del DNA, es pretén donar resposta a tota una sèrie de qüestions que ens hem anat plantejant amb el temps sobre la sistemàtica i l'evolució d'aquests gèneres. Tot i que part d'aquests estudis aborden problemàtiques des d'un punt de vista generalista, hem considerat interessant l'opció d'estudiar alguns grups determinats, com ara el subgènere *Dracunculus*, les artemísies anuals o les espècies d'artemísies endèmiques d'Amèrica del Sud, per tal d'aprofundir en el coneixement dels processos evolutius i en la sistemàtica d'aquests tàxons. A continuació es descriuen les tècniques emprades durant la realització de la tesi, es recullen els antecedents sobre cada tema en els grups de plantes objecte del nostre estudi, es comenten els objectius concrets per a cada tècnica i es justifica la utilització de cadascuna de cara a una millor comprensió de l'evolució i sistemàtica del grup.

2.1 Cariologia i citogenètica

La citogenètica és una disciplina híbrida que combina la citologia (estudi dels cromosomes així com d'altres components cel·lulars) i la genètica (estudi de l'herència). Ens permet manipular i observar cromosomes (mitjançant tècniques de tinció cromosòmica), estudiar el comportament d'aquests a la cèl·lula (divisió cel·lular, mitosi, meiosi), fer recomptes i analitzar-ne l'estructura (anàlisi de cariotips), així com determinar nombroses modificacions de la seva estructura i modificacions relacionades amb fenòmens com la recombinació, la transmissió i l'expressió gènica. Aquestes tècniques permeten estudiar regions heterocromàtiques amb *loci* de DNA ribosòmic. Des dels primers estudis de WEINEDEL-LIEBAU (1928) fins a l'actualitat han estat molts treballs els que han vist la llum, pel que fa a la cariologia i la citogenètica, tant en el gènere *Artemisia* com en altres amb els quals està estretament relacionat (EHRENDORFER 1964;

KAWATANI I OHNO 1964; KOROBKOV 1972; MCARTHUR *et al.* 1981; OUYAHYA I VIANO 1981, 1988; VALLÈS I SILJAK-YAKOVLEV 1997; VALLÈS I GARNATJE 2005; GARCIA *et al.* 2006, 2007, 2009; MATOBA *et al.* 2007; TORRELL *et al.* 2001a, 2003). Des del punt de vista de la biosistemàtica, la citogenètica té aplicacions molt interessants, perquè al mateix temps ens aporta informació de tipus fenotípic (nombre, mida o simetria dels cromosomes) i genotípic (localització de bandes d'heterocromatina, nombre de *loci* ribosòmics i localització de gens, etc.). Tot i que les dades dels cariotips han estat utilitzades per a estudiar les relacions filogenètiques i evolutives en grups de tàxons pròxims, al gènere *Artemisia* cal tenir en compte que l'elevat grau de similitud cromosòmica interespecífica i intraespecífica, dificulta i de vegades no permet clarificar i justificar el grau de parentiu mitjançant l'anàlisi de cariotips (HOSHI *et al.* 2003; MATOBA *et al.* 2007).

2.1.1 Cariologia i citogenètica clàssica: Recomptes de cromosomes i bandatge amb fluorocroms

En aquesta disciplina s'inclouen les tècniques més bàsiques, i no per això menys interessants, de la cariologia i de la citogenètica, com els recomptes de cromosomes metafàsics, l'elaboració de cariotips, o el bandatge amb fluorocroms, entre d'altres. De fet, els primers treballs en el gènere, com ara els de WEINEDEL-LIEBAU (1928) o EHRENDORFER (1964), entre molts altres, aportaren dades cariològiques en un intent d'estudiar i posar de manifest diferents aspectes sistemàtics i evolutius en *Artemisia*. Des d'aquells temps fins a l'actualitat, les millores i els avenços qualitatius de les tècniques microscòpiques han permés la realització i la publicació de molts treballs. De fet, s'ha determinat el nombre de cromosomes aproximadament per al 50% de les espècies del gènere *Artemisia* (VALLÈS I GARNATJE 2005, i referències que conté).

Aquests treballs han constatat l'existència de dos nombres cromosòmics bàsics al gènere, $x = 9$, de gran incidència i present a tots els subgèneres, i $x = 8$, de menor incidència i restringit als subgèneres *Artemisia*, *Absinthium* i *Dracunculus*. VALLÈS I SILJAK-YAKOVLEV (1997) postularen que aquest segon

nombre cromosòmic podia tenir l'origen en una fusió cromosòmica de tipus robertsonià, mitjançant la qual, per disploïdia descendent, s'hauria generat aquest nou nombre $x = 8$. També, i gràcies al gran nombre de treballs publicats en aquesta disciplina, s'ha trobat que la incidència de la poliploïdia en el gènere que ens ocupa és bastant elevada (aproximadament en el 57% de les espècies del gènere s'han determinat poblacions poliploides). De fet, hi ha espècies de les quals sols es coneixen poblacions poliploides (p. ex. *Artemisia dracunculoides* Pursh i *A. klementzae*). Els nivells de ploïdia més elevats reportats per al gènere han estat el dodecaploide (12*x*), detectat per MALAKHOVA (1990) en una població russa *d'Artemisia macrantha* Ledeb., de nombre de base $x = 9$, i el nivell hexaploide (6*x*) en espècies que presentaven un nombre crosmosòmic bàsic $x = 8$, com per exemple *Artemisia austriaca* Jacq. També s'han trobat de forma més freqüent en el gènere aberracions cromosòmiques com les aneusomies (KREITSCHITZ I VALLÈS 2003; VALLÈS I GARNATJE 2005), a mesura que augmenta el nivell de ploïdia. Amb tots aquests precedents no és d'estranyar que fenòmens com la poliploïdia siguin considerats com un dels principals motors de l'evolució en plantes (SOLTIS I SOLTIS 2000; WENDEL 2000; SOLTIS *et al.* 2004; CUI *et al.* 2006; CHEN 2007).

La cromomicina A_3 és un fluorocrom àmpliament utilitzat en citogenètica degut a que produeix una bona diferenciació dels cromosomes, ja que permet detectar regions específiques del DNA riques en bases G-C. Com a resultat, ens facilita la detecció de l'existència d'un patró determinat de bandes, que pot ser més o menys variable, arribant inclús, a caracteritzar un grup de tàxons propers. En el gènere *Artemisia* s'han dut a terme estudis de bandatge en diferents subgèneres (VALLÈS I SILJAK-YAKOVLEV 1997; TORRELL *et al.* 2001a, 2003; GARCIA *et al.* 2007).

Així doncs, els objectius que ens hem plantejat a l'hora d'abordar els treballs que aquí es presenten són:

- Incrementar el nombre d'espècies i poblacions per a les quals es coneix el nombre de cromosomes, amb especial interès en els representants del subgènere *Dracunculus*. Principalment hem estudiat poblacions d'origen

asiàtic, regió que es considera el principal focus d'especiació i diversificació del gènere.

- Obtenir un nombre elevat de recomptes cromosòmics per tal que ens faciliti la comprensió dels resultats obtinguts en els estudis de la mida del genoma.
- Determinar i confirmar nous nivells de ploïdia per a tenir, junt amb les dades ja publicades, una idea global de la incidència d'aquest fenomen en el gènere *Artemisia*.
- Determinar el nombre i distribució de llocs de DNA rics en bases G-C mitjançant tincions de cromomicina A_3 i observar els canvis, pel que fa al nombre de bandes, en el complex poliploide d'artemísies d'Amèrica del Sud.

2.1.2 Citogenètica molecular: hibridació *in situ* fluorescent

Mitjançant les tècniques de citogenètica molecular es descobriren els cromosomes com els vehicles de la transferència gènica, fet que va contribuir a la millora del coneixement de la biologia dels organismes vius. Utilitzant com a base la metodologia emprada en la citogenètica clàssica, amb la citogenètica molecular s'ha pretès donar un pas endavant en l'estudi de la biosistemàtica i l'evolució. L'avanç en l'estudi dels cromosomes utilitzant eines moleculars, ha permés estudiar i entendre molts dels processos cel·lulars que hi tenen lloc.

Pel que fa als estudis que es presenten en aquesta tesi, ens hem centrat a realitzar diferents assaigs d'hibridació *in situ* fluorescent (FISH), tècnica que permet localitzar als cromosomes diferents famílies de gens del DNA ribosòmic, amb la finalitat de comparar el seu nombre, distribució i variació entre tàxons relacionats. El gènere *Artemisia* ha estat estudiat anteriorment des d'aquest punt de vista (TORRELL *et al.* 2001a, 2003; HOSHI *et al.* 2006; MATOBA *et al.* 2007; GARCIA *et al.* 2007, 2009). Alguns d'aquests treballs s'han centrat en grups específics com *Seriphidium* o *Tridentatae* (TORRELL *et al.* 2003; GARCIA *et al.* 2007, respectivament) mentre que n'hi ha hagut d'altres de més generalistes en què s'han estudiat espècies de diferents subgèneres (TORRELL *et al.* 2001a;

MATOBA *et al.* 2007). Amb tot i això, creiem que encara hi ha qüestions per resoldre al voltant de l'evolució del DNA ribosòmic en alguns grups, fet que ens ha fet plantejar-nos l'estudi més exhaustiu de grups concrets com el subgènere *Dracunculus*, les espècies anuals d'*Artemisia*, el grup d'artemísies endèmiques d'Amèrica del Sud o alguns dels poliploides més alts del gènere.

Els principals objectius que ens hem plantejat a l'hora de dur a terme aquests treballs han estat els següents:

- Determinar i avaluar mitjançant hibridació *in situ* fluorescent l'organització i disposició de les famílies de gens de l'RNA 18S-5.8S-26S i 5S per a estudiar la presència de possibles patrons de marcatge que caracteritzen els grups estudiats.
- Avaluar la utilitat de l'estudi de la localització del DNA ribosòmic com a possible marcador sistemàtic en el gènere *Artemisia*.
- Estudiar i analitzar els canvis en l'organització del DNA ribosòmic, així com qualsevol altre reordenament cromosòmic que puga estar relacionat amb un canvi en la ploïdia. Pel que fa al subgènere *Dracunculus* hem estudiat poblacions diploides, tetraploides, hexaploides i decaploides, tant d'espècies diferents com de la sèrie poliploide d'*A. dracunculus*. També hem estudiat aquests canvis en poliploides d'alt nivell de ploïdia com *Artemisia lagocephala* Fisch. ex Besser i *Artemisia medioxima* Krasch. ex Poljakov.
- Estudiar l'organització del DNA ribosòmic en artemísies anuals, i comparar els resultats amb les espècies perennes relacionades per tal de detectar possibles canvis en el nombre de *loci*.
- Caracteritzar des del punt de vista citogenètic, el complex poliploide d'espècies endèmiques d'Amèrica del Sud, *Artemisia copa* Phil., *Artemisia echegarayii*, *Artemisia magellanica* Sch. Bip., *Artemisia mendozana* DC. var. *mendozana* i *Artemisia mendozana* var. *paramilloensis* F. A. Roig et J. A. Ambrosetti.

2.2 La mida del genoma: evolució de la quantitat de DNA nuclear

La quantitat de DNA nuclear, entesa com la mida del genoma, és molt variable en les angiospermes. Aquest paràmetre sovint s'expressa com el valor C, terme utilitzat per primer cop per SWIFT (1950). Entenem per valor C d'un organisme la quantitat de DNA no replicat d'un gàmeta, independentment del nivell de ploïdia del tàxon, i és considerat constant per a una espècie -la C del terme prové de constància- (BENNETT I SMITH 1971). El podem trobar expressat en forma de valor 2C (quantitat de DNA nuclear per genoma 2*n*, independentment del nivell de ploïdia) o en forma de valor 1Cx (quantitat de DNA per complement bàsic de cromosomes). Amb el temps s'ha considerat una eina de gran importància en l'estudi de la biodiversitat. A la bibliografia han estat descrites diferències d'aproximadament 1.200 vegades entre espècies com *Gensilea margaretae* o *G. aurea* (2C = 0,128-0,130 pg; GREILHUBER *et al.* 2006), que presenten les estimacions de DNA nuclear més petites, fins als genomes més grans, trobats a *Fritillaria assyriaca* (2C = 254,80 pg; BENNETT I SMITH 1976).

El contingut de DNA nuclear és un caràcter amb un significat biològic fonamental, que es relaciona directament o indirectament amb diverses característiques fenotípiques i factors d'importància com la biologia reproductiva, l'ecologia, l'evolució o la distribució de les plantes, entre d'altres (BENNETT 1998; BENNETT I LEITCH 2005). S'han establert diferents relacions entre variables ecològiques i biològiques de les plantes, fet que ha posat de manifest la importància de l'estudi de la variació de la mida del genoma i la seva relació amb l'evolució de les plantes (p. ex. VINOGRADOV 2003; ALBACH I GREILHUBER 2004; GARCIA *et al.* 2004, 2008; GARNATJE *et al.* 2004; CHASE *et al.* 2005; PRICE *et al.* 2005; BEAULIEU *et al.* 2007). Malgrat la seva importància, el volum de dades de quantitat de DNA disponibles avui en dia, pel que fa a les angiospermes encara és escàs, al voltant d'un 4-5%. L'interès suscitat ha promogut la creació de bases de dades on s'han compilat tota una sèrie de valors, des del 1976, relacionats amb el valor C, com ara The Plant DNA C-values Database (http://www.rbgkew.org.uk/cval/homepage.html; BENNETT I LEITCH 2005), o FLOWer: A Plant DNA Flow Cytomety Database

(http://flower.web.ua.pt; LOUREIRO *et al.* 2008). Al llarg del temps, l'avanç de la genòmica ens ha permés conèixer de forma molt acurada l'estructura i el funcionament dels genomes, i ara podem entendre perquè moltes vegades aquest valor C no es correlaciona amb la complexitat d'un organisme [(paradoxa o enigma del valor C (THOMAS 1971; GREGORY 2001)], ja que en la majoria d'organismes més del 90% del DNA constitutiu d'un genoma és no codificant i, tot i que durant força temps es considerà DNA deixalla (DAWKINS 1976; PAGEL I JOHNSTONE 1992), actualment es pensa que és genèticament actiu i pot tenir funcions reguladores (MATTICK 2004). FLAVELL (1980) proposà que algunes de les angiospermes amb els valors C més elevats podien arribar a presentar el 90-99% del seu DNA nuclear no codificant. Aquest fet donaria suport a la idea que l'increment de seqüències altament repetitives i/o deixalla actua com a principal factor en la variació del valor C juntament amb els elements transposables (KELLOGG I BENNETZEN 2004).

Com ja s'ha avançat en paràgrafs anteriors, l'estudi de la mida del genoma té aplicacions en diferents camps de l'estudi de les plantes com l'ecologia, la biologia evolutiva, la sistemàtica, la taxonomia o la biogeografia (BENNETT I LEITCH 2005a, b, c). En aquest sentit, correlacions, tant positives com negatives, entre el DNA nuclear i paràmetres pertanyents a la citologia, la biologia reproductiva de les espècies, l'ecologia, la distribució, els factors ambientals o la producció de biomassa, entre d'altres, han estat àmpliament investigades en molts grups de plantes. És per aquest motiu que, amb el temps s'ha incrementat significativament el coneixement que es té d'aquest tema, i cada cop ha anat esdevenint més clar que la variació de la quantitat de DNA té conseqüències no solament a nivell cel·lular, sinó també ecològic i evolutiu (LEITCH I BENNETT 2007).

L'estimació de la quantitat de DNA nuclear pot realitzar-se utilitzant diferents tècniques com la microdensitometria amb tinció de Feulgen, la microscòpia confocal o la citometria de flux. Aquesta última ha estat la tècnica més utilitzada els últims anys per la seva facilitat, rapidesa i precisió, i és la que hem usat per a dur a terme les mesures de quantitat de DNA en les diferents

poblacions d'*Artemisia* estudiades. Els principals objectius dels treballs que s'han desenvolupat han estat:

- Aportar noves dades de quantitat de DNA per a espècies del gènere *Artemisia* i d'alguns altres gèneres afins (p. ex. *Crossostephium chinense* i *Neopallasia pectinata* Pall.), que no havien estat estudiades fins el moment, així com de noves poblacions d'espècies prèviament estudiades.
- Estudiar l'evolució de la mida del genoma en una mostra representativa del gènere *Artemisia*, amb el suport d'un marc filogenètic i amb l'anàlisi de dades inèdites així com de les publicades en treballs previs (TORRELL I VALLÈS 2001; GARCIA *et al.* 2004, 2006, 2008).
- Estudiar els canvis (increment o reducció) de la mida del genoma comparant els resultats en poblacions diploides i poliploides d'*Artemisia*.
- Predir la dinàmica del valor C amb la poliploïdia, ajustant diferents models matemàtics a les dades de què disposem, amb la finalitat d'avaluar l'existència d'un límit per a la quantitat de DNA.
- Estudiar la variabiliat intraespecífica (interpoblacional) en l'espècie *Artemisia crithmifolia*, amb la intenció d'avaluar la constància del valor C en una espècie de distribució fragmentada.
- Estudiar l'evolució de la mida del genoma en espècies del subgènere *Dracunculus*, amb el suport d'un marc filogenètic del grup, i amb especial atenció a la poliploïdia.
- Caracteritzar, des del punt de vista de la mida del genoma, el grup d'artemísies endèmiques d'Amèrica del Sud, que constitueixen un complex poliploide molt interessant.

2.3 Sistemàtica molecular: reconstrucció filogenètica

La necessitat d'estudiar i classificar les especies en funció dels seus caràcters i la seva història evolutiva ha generat la disciplina que coneixem per sistemàtica. Així doncs, la sistemàtica molecular tracta d'explicar l'origen i la diversificació dels organismes vius a través de l'estudi de la informació continguda en una

molècula de DNA, sota la premissa que aquests organismes presenten caràcters hereditaris que són potencialment informatius de la seva història evolutiva. Actualment és possible fer casar les idees de canvi evolutiu amb models estadístics, essent possible provar aspectes de l'evolució buscant l'explicació més plausible per al fet que s'estudia, la qual cosa queda plasmada en el que anomenem arbres filogenètics.

En el cas dels vegetals, podem trobar tres genomes susceptibles de ser utilitzats en estudis de filogènia molecular, el nuclear, el cloroplàstic i el mitocondrial. Tant el nuclear com el cloroplàstic són els que actualment més s'utilitzen amb aquesta finalitat. A la família de les *Asteraceae* una de les regions més utilitzades del DNA ribosòmic nuclear ha estat l'espaiador intern transcrit (ITS), així com també l'espaiador extern transcrit (ETS). Mentre la primera regió s'ha demostrat amb força eficiència a l'hora d'inferir les relacions filogenètiques a nivell intergenèric i intragenèric (BALDWIN *et al.* 1995), l'ETS afegeix nous caràcters per a incrementar la resolució filogenètica, tot i que en alguns casos l'elevat nivell de divergència observada en les seqüències de tàxons relativament propers fa difícil l'ús sencer de l'espaiador, i generalment sols s'utilitzen les zones més properes als extrems. Cada cop més s'estan utilitzant gens de còpia única o de baix nombre de còpies per a inferir relacions filogenètiques a nivell específic com el gen LEAFY (WEIGEL *et al.* 1992) o els gens de l'alcohol deshidrogenasa (*Adh*) (DENNIS *et al.* 1984; GAUT *et al.* 1999), ja que l'evolució concertada de l'ITS i l'ETS pot ser problemàtica a l'hora de realitzar reconstruccions filogenètiques (SOLTIS *et al.* 1998; ÁLVAREZ I WENDEL 2003) o a causa del baix poder de resolució d'aquestes regions nuclears. Pel que fa a les regions cloroplàstiques, també se n'han incorporat cada cop més en un intent d'incrementar la resolució filogenètica a nivells taxonòmics baixos. Algunes de les regions utilitzades són el *trn*L, *trn*L-F, *trn*T-*trn*L (TABERLET *et al.* 1991), i *psb*A-*trn*H (SANG *et al.* 1997) o el gen *mat*K (KHIDIR I HONGPING 1997) entre d'altres.

S'han publicat nombrosos treballs al voltant de les relacions filogenètiques en el gènere *Artemisia*, així com en altres gèneres afins de la subtribu *Artemisiinae* i de la tribu *Anthemideae* (KORNKVEN I WATSON 1997; KORNKVEN *et al.* 1998; TORRELL *et al.* 1999; WATSON *et al.* 2002; D'ANDREA *et al.* 2003; VALLÈS

et al. 2003; SANZ *et al.* 2008; TKACH *et al.* 2008a, b) basats en regions nuclears i WATSON *et al.* (2000) basat en el gen cloroplàstic *ndh*F. Amb tot això, s'ha vist que la sistemàtica del grup és bastant complexa i encara necessita d'una major prospecció i un major aprofundiment en el coneixement de les relacions filogenètiques de determinats grups. Hem mantingut l'anàlisi de les regions ITS i ETS (encara que coneixem les seves limitacions) i hem incorporat en alguns casos l'ús de noves regions cloroplàstiques amb la intenció d'incrementar la resolució de les filogènies. Els objectius generals que ens hem plantejat a l'hora d'abordar els treballs de reconstrucció filogenètica han estat:

- Obtenir un marc filogenètic representatiu del gènere *Artemisia*, complementari als treballs publicats amb anterioritat aportant noves dades, la qual cosa ens servirà com a base per als estudis de quantitat de DNA nuclear.
- Circumscriure el subgènere *Dracunculus* dintre del gènere *Artemisia* i estudiar les relacions filogenètiques d'una mostra representativa del subgènere, així com estudiar les espècies taxonòmicament conflictives per a intentar dilucidar-ne la posició sistemàtica.
- Intentar donar una resposta a les relacions sistemàtiques entre les espècies compreses al subgènere *Dracunculus* i aquells gèneres afins que han quedat embeguts dintre del grup, com apunten treballs previs (VALLÈS *et al.* 2003; SANZ *et al.* 2008)
- Determinar la posició taxonòmica de les artemísies endèmiques d'Amèrica del Sud i intentar correlacionar els resultats amb les dades citogenètiques de què disposem.

2.4 Palinologia

La palinologia és una disciplina botànica dedicada a l'estudi del pol·len i les espores (MOORE *et al.* 1991). Se centra principalment en l'anàlisi de la seva morfologia tant externa com interna, que presenta patrons estructurals diferents pel que fa a l'estructura de l'exina. L'estudi i les anàlisis als microscopis òptic i

electrònics de rastreig i transmissió, de la simetria, les obertures a les parets, la forma i la mida, tenen un valor taxonòmic rellevant i permeten diferenciar entre tàxons a diferents nivells (família, gènere, espècie).

Pel que fa al pol·len de les *Asteraceae* s'ha trobat gran variabilitat de formes i estructures, fet que queda reflectit en el gran nombre de tipus pol·línics descrits a la família (JEFFREY 2007). En les *Artemisiinae* se n'han descrit dos tipus en base a l'ornamentació de l'exina (STIX 1960), el tipus *Artemisia* (amb espínules) i el tipus *Anthemis* (amb espines). Aquesta reducció de les espines fou descrita per WODEHOUSE (1926) al grup d'*Artemisia* i gèneres afins, i ha estat posteriorment confirmada per tot un seguit de treballs on s'han citat espècimens del gènere provinents d'arreu del món (p. ex. MONOSZON 1948, 1950a, b; STRAKA 1952; STIX 1960; SINGH I JOSHI 1969; PRAGLOWSKI 1971; VALLÈS *et al.* 1987; MARTÍN *et al.* 2001, 2003; GRIGOREVA *et al.* 2009). També s'han realitzat estudis centrats en la distribució d'aquests pòl·lens en base a dades filogenètiques, on s'ha vist que cada tipus pol·línic caracteritza cadascun dels grans grups de les *Artemisiinae*: El grup de *Dendranthema* i gèneres relacionats presenta el pol·len tipus *Anthemis*, mentre que el grup d'*Artemisia* i gèneres afins es caracteritza pel tipus *Artemisia* (CHEN I ZHANG 1991; MARTÍN *et al.* 2001, 2003; JIANG *et al.* 2005 per als estudis de pol·len; VALLÈS *et al.* 2003; SANZ *et al.* 2008 per a les filogènies), amb algunes excepcions.

Els tipus pol·línics s'han utilitzat per a justificar la segregació de diferents gèneres d'*Artemisia*, per a incloure'ls en el grup de *Dendranthema*, així com al revés. Un d'aquests gèneres és *Ajania*, que centrarà un treball dedicat a la caracterització del seu pol·len en una mostra representativa del gènere, així com altres *Artemisiinae* relacionades. Els objectius d'aquest treball han estat:

- Aportar noves dades sobre el pol·len de la subtribu *Artemisiinae*, bàsicament del gènere *Ajania*, del qual s'estudiaren 25 espècies, així com dels gèneres *Brachanthemum*, *Cancrinia*, *Crossostephium*, *Dendranthema*, *Elachanthemum*, *Hippolytia*, *Kaschgaria*, *Poljakovia* i *Stilpnolepis*.

- Aprofundir en la caracterització dels dos tipus pol·línics de la subtribu mitjançant l'anàlisi conjunta de dades inèdites i de les publicades prèviament.
- Discutir els resultats en un marc filogenètic per tal de contribuir a l'esclariment de qüestions adreçades a la sistemàtica i a les relacions filogenètiques del grup
- Estudiar la possibilitat de l'existència d'una transició d'un tipus pol·línic cap a un altre.

3. Justificació del projecte de tesi

La tesi doctoral que aquí es presenta se centra bàsicament en el gènere *Artemisia* (*Asteraceae, Anthemideae*), tot abastant aspectes que ens han semblat interessants d'estudiar en altres gèneres pròxims com *Ajania*. Ens hem plantejat el treball adreçant-lo a resoldre diferents qüestions evolutives d'interès i que es poden agrupar en tres grans seccions: estudis relatius a *Artemisia* en sentit ampli, treballs centrats bàsicament en el subgènere *Dracunculus*, i estudis encaminats a resoldre problemàtiques diverses en gèneres afins.

3.1 Estudis centrats en el gènere *Artemisia s. l.*

Com ja s'ha comentat a l'apartat de la introducció, el gènere *Artemisia* és un dels més nombrosos de les *Asteraceae* (al voltant de 500 espècies) i distribuït arreu del globus arribant a ocupar grans extensions de terreny, en les quals domina el paisatge. Aquest fet de la gran extensió fa difícil plantejar-se estratègies de mostreig per a algunes espècies, particularment d'algunes que se situen en zones geogràfiques de difícil accés, bé siga per l'orografia o per la situació sociopolítica d'alguns dels indrets on habiten. Això fa que hi haja un cert coneixement bastant profund en determinats complexos del gènere, mentre que en moltes altres espècies hi ha diferents aspectes sobre els quals són pràcticament desconegudes.

Hem realitzat una aproximació filogenètica complementària a aquelles de les quals ja disposàvem per tal d'obtenir una visió global de la sistemàtica del gènere. Partint de la base de treballs previs, hem seqüenciat els espaiadors transcrits intern (ITS) i extern (ETS) per a obtenir un arbre filogenètic mitjançant inferència bayesiana. Paral·lelament hem estimat la grandària del genoma d'aquelles espècies incloses a la filogènia per tal d'analitzar la dinàmica d'aquest paràmetre amb el suport d'un marc filogenètic. Aquest estudi ha estat publicat a la revista científica inclosa a l'SCI *Plant Biology* amb el títol *Genome size dynamics in Artemisia L. (*Asteraceae, Anthemideae*): following the track of polyploidy*.

En les estades de tres mesos cadascuna, realitzades al *Laboratory of Plant Chromosome and Gene Stock,* a la Universitat d'Hiroshima i al *Département de Biodiversité, Systématique et Evolution* de la Universitat de Paris-Sud XI, hem intentat aprofundir en les tècniques de la citogenètica clàssica i molecular, per tal de complementar els estudis previs realitzats en les espècies del gènere. S'han dut a terme dos treballs centrats en espècies dels subgèneres *Artemisia* i *Absinthium*, un com a primera aproximació citogenètica als subgèneres estudiats i el segon basat en espècies poliploides dels subgèneres, amb la finalitat d'avaluar els canvis en la distribució de *loci* del DNA ribosòmic. Aquests treballs són *Molecular cytogenetic characterization of some representatives of the subgenera* Artemisia *and* Absinthium *(genus* Artemisia*, Asteraceae)*, publicat a la revista científica *Collectanea Botanica (Barcelona)*, i *Do polyploids require proportionally less rDNA loci than their corresponding diploids? Examples from* Artemisia *subgenera* Absinthium *and* Artemisia *(Asteraceae, Anthemideae)*, enviat a la revista inclosa a l'SCI *Plant Biosystems*, i que actualment es troba en fase de revisió.

Dos treballs que també hem inclòs dintre d'aquest apartat són *Life cycle versus systematic placement: cytogenetic implications in the genus* Artemisia *L. (Asteraceae, Anthemideae)* i *Origin and evolution of the South American endemic* Artemisia *species (Asteraceae): evidences from molecular phylogeny, ribosomal DNA and genome size data*, tots dos pendents d'enviar a revistes incloses a l'SCI. El primer treball (que enviarem a *Plant Systematics and Evolution*) omple

un buit que existia en el coneixement global del comportament sistemàtic i evolutiu de les artemísies anuals. Tot i que algun treball anterior ha incorporat de forma esporàdica algunes de les espècies anuals del gènere, hem vist la necessitat d'elaborar una anàlisi en profunditat de la seva sistemàtica en el conjunt global d'*Artemisia*, així com dels patrons de distribució de *loci* del DNA ribosòmic i de quantitat de DNA nuclear respecte a les perennes més pròximes. El segon treball (que enviarem a l'*Australian Journal of Botany*) se centra en l'estudi de l'origen i l'evolució del complex endèmic d'artemísies d'Amèrica del Sud, i aporta dades inèdites sobre la cariologia, la sistemàtica i l'evolució del genoma en aquestes grans desconegudes. A la majoria de revisions taxonòmiques del gènere es fa referència a l'existència d'aquestes espècies, però tret d'alguns treballs de taxonomia clàssica, de fitoquímica i etnobotànica, el coneixement a nivell evolutiu d'aquestes espècies és pràcticament nul.

3.2 Estudis centrats en el subgènere *Dracunculus*

Una de les primeres aproximacions que hem dut a terme, centrada majoritàriament en el subgènere *Dracunculus*, està constituïda per diversos estudis cariològics d'algunes de les espècies d'aquest grup, així com, secundàriament, d'altres subgèneres, i de les quals no hi havia dades d'aquest caire, o les existents eren molt escasses i necessitaven confirmació. Aquestes dades ens han resultat d'interès a l'hora de complementar estudis filogenètics i de mida del genoma, per als quals és molt interessant comptar amb aquest tipus d'informació per tal de deduir la història evolutiva del gènere. A més, tenint la informació prèvia de l'elevada incidència de la poliploïdia en el gènere *Artemisia*, ens ha semblat interessant realitzar aquests treballs en un intent d'aportar nous citotips als recomptes de què ja es disposa. Així doncs, els treballs que han vist la llum han estat *Chromosome counts in Asian* Artemisia *L. (Asteraceae, Anthemideae) species: from diploids to the first report of the highest polyploid in the genus*, publicat a la revista inclosa a l'SCI *Botanical Journal of the Linnean Society* i *Chromosome numbers in some* Artemisia *(Asteraceae, Anthemideae) species and genome size variation in its subgenus* Dracunculus*:*

karyological, systematic and phylogenetic implications, que ha estat publicat a *Chromosome Botany*.

Com que la sistemàtica i la filogènia d'aquest subgènere mai han estat estudiades en profunditat, tret de la inclusió dels representants més coneguts del grup, hi hem abordat una aproximació detallada. S'han analitzat, mitjançant inferència bayesiana i màxima parsimònia les regions del DNA ribosòmic nuclear ITS i ETS, així com del DNA cloroplàstic *trnS-trnC* i *trnS-trnfM* per tal d'esbrinar i aprofundir en les relacions filogenètiques, tant dintre del subgènere com en el conjunt del gènere *Artemisia*. També hem aportat noves dades de quantitat de DNA nuclear al subgènere, en un intent de poder estudiar les variacions que es produeixen en aquest paràmetre amb la poliploïdia, fenomen de gran incidència al grup. Aquest treball -en el qual fem propostes de reversió al gènere *Artemisia*, subgènere *Dracunculus*, d'alguns tàxons segregats en gèneres independents- serà enviat pròximament a la revista científica *Taxon*, inclosa a l'SCI, sota el títol el títol *Phylogenetic relationships and subgeneric delimitation of subgenus* Dracunculus *(Besser) Rydb. (genus* Artemisia*,* Asteraceae*) based on ribosomal and chloroplast DNA sequences*.

Paral·lelament, s'ha realitzat un estudi de citogenètica molecular en algunes especies del subgènere *Dracunculus*. De fet, és el primer estudi centrat íntegrament en aquest grup, ja que les aportacions prèvies són bastant escasses i s'havien basat en altres subgèneres com *Tridentatae* i *Seriphidium* principalment. El nostre interès principal ha estat caracteritzar citogenèticament aquest grup, tot esbrinant la localització dels *loci* del DNA ribosòmic, i analitzar els canvis que es produeixen com a conseqüència de la poliploïdització. D'aquest estudi hem redactat un treball que enviarem pròximament a la revista *Annals of Botany*, inclosa a l'SCI, i que porta per títol *Relationships between* Artemisia *species (subgenus* Dracunculus*) assessed by FISH of 5S and 18S-5.8S-26S rRNA genes*.

Encara dins del subgènere *Dracunculus*, hem volgut estudiar els valors de quantitat de DNA nuclear en una espècie al llarg de tota la seva àrea de distribució. Es tracta, d'*Artemisia crithmifolia*, un tàxon hexaploide que viu a les dunes i altres zones arenoses de la costa atlàntica europea, d'Andalusia als

Països Baixos, i també en alguns punts d'Anglaterra i el País de Gal·les. Vam dur a terme una recol·lecció de 45 poblacions representatives de tota l'àrea esmentada de repartició de l'espècie i vam estimar la quantitat de DNA nuclear en totes elles. Els resultats han mostrat una variabilitat força petita, però s'han pogut establir diferències entre grups de poblacions, que han permés de discutir la influència de la fragmentació de l'àrea de distribució del tàxon en la variació de la mida del genoma. Aquests resultats s'han publicat a la revista inclosa a l'SCI *Caryologia*, amb el títol *Changes in genome size in a fragmented distribution area: the case of* Artemisia crithmifolia *L. (Asteraceae, Anthemideae)*.

3.3 Treballs relatius a gèneres afins: estudi palinològic en *Ajania*

A part del gènere *Artemisia* i els que li són més afins (alguns dels quals, com hem dit, anteriorment segregats, i que els nostres estudis mostren que s'hi han de tornar a incloure), el nostre grup de recerca s'ha interessat per altres gèneres de la tribu *Anthemideae*, especialment de la subtribu *Artemisiinae*. Així, s'han recollit força materials del gènere *Ajania*, que han estat estudiats des de diversos punts de vista (cariològic, seqüenciació del DNA) i dels quals nosaltres hem usat una espècie com a part del grup extern en el treball de filogènia molecular esmentat anteriorment. Ultra això, amb el nombrós material pol·línic d'espècies d'aquest gènere que havíem recol·lectat (al camp i en herbaris), es dugué a terme un estudi palinològic per tal de complementar els que el nostre equip de recerca ja havia dut a terme sobre *Artemisia* i gèneres propers. Així, vam estudiar al microscopi òptic i al microscopi electrònic de rastreig 31 poblacions de 25 espècies d'*Ajania* i 15 poblacions de nou gèneres més de les *Artemisiinae*. Aquesta recerca ha permés caracteritzar el gènere *Ajania* des del punt de vista palinològic, confirmar l'existència de dos models d'ornamentació de l'exina a la subtribu *Artemisiinae* i detectar-hi alguns tàxons de comportament aparentment anòmal, que seran objecte de futures recerques moleculars per a assegurar la seva posició filogenètica i taxonòmica. Els resultats d'aquest treball han sortit publicats a la revista inclosa a l'SCI *Botanical Journal of the Linnean Society* sota el títol *Palynological study of* Ajania *Poljakov and related genera (Asteraceae, Anthemideae)*.

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MATERIALS I MÈTODES

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1. MATERIALS

1.1 Tàxons estudiats

En el present treball hem estudiat 140 espècies d'*Artemisia*, així com altres gèneres estretament relacionats (*Crossostephium*, *Filifolium, Mausolea, Neopallasia i Turaniphytum*). S'han obtingut aquenis viables de la majoria d'espècies (principalment per als treballs de cariologia i citogenètica), a més de conservar exemplars vius als hivernacles de la Facultat de Farmàcia de la Universitat de Barcelona i de l'Institut Botànic de Barcelona (CSIC-ICUB). Tot i això, d'algunes espècies solament s'han pogut estudiar plecs d'herbari atesa la impossibilitat d'obtenir material fresc del camp. Les vies més comunes d'obtenció de material per al posterior estudi al laboratori han estat:

- Expedicions de camp realitzades pels investigadors i personal del Laboratori de Botànica de la Facultat de Farmàcia de la Universitat de Barcelona i de l'Institut Botànic juntament amb la col·laboració d'investigadors de diversos centres internacionals, com per exemple la Dra. Sh. Dariimaa (Department of Biology, Mongolian State University of Education, Ulaanbaatar, Mongòlia); els Drs. Sh. Tsooj, D. Samjid, E. Yatamsuren (Institute of Botany, Ulaanbataar, Mongòlia); el Dr. A. A. Korobkov (Komarov Botanical Institute, Sant Petersburg, Rússia); J. D. Twibell (N.C.C.P.G. *Artemisia* Collection, Devon, Regne Unit), els Drs. R. Cao, B. Liu, S. W. Zhao i L. Yan (Inner Mongolia University, Mongòlia Interior, República Popular de la Xina) o el Dr. M. Dematteis (Universidad Nacional del Nordeste, Corrientes, Argentina).
- Enviaments de material, principalment aquenis, per part d'investigadors amb els qui col·labora actualment el grup d'investigació, així com obtenció de llavors dels *Index Seminum* de jardins botànics internacionals.

- Visita a herbaris, així com la recepció de plecs d'herbari provinents d'institucions internacionals [herbaris (W) Viena; (E) Edinburg; (LE) Sant Petersburg), així com dels propis herbaris del Centre de Documentació de Biodiversitat Vegetal de la Universitat de Barcelona (BCN) i de l'Institut Botànic de Barcelona (BC)].

Les artemísies que s'han recol·lectat o de les quals s'ha rebut material són bàsicament d'origen asiàtic (fig. 9), de països com Iran, Israel, Japó, Kazakhstan, Mongòlia, Xina, Rússia (part asiàtica) i Uzbekistan, fet que no és estrany ja que allà trobem el seu principal centre de diversificació. Tot i això, també s'ha estudiat material provinent d'arreu del globus (fig 9.), com per exemple del continent americà (Argentina, Canadà i Estats Units), del Nord d'Àfrica (Egipte i Marroc) i del continent europeu [Regne Unit, Àustria, Bèlgica, França, Itàlia, Països Baixos, Península Ibèrica, Polònia i Rússia (part europea)].

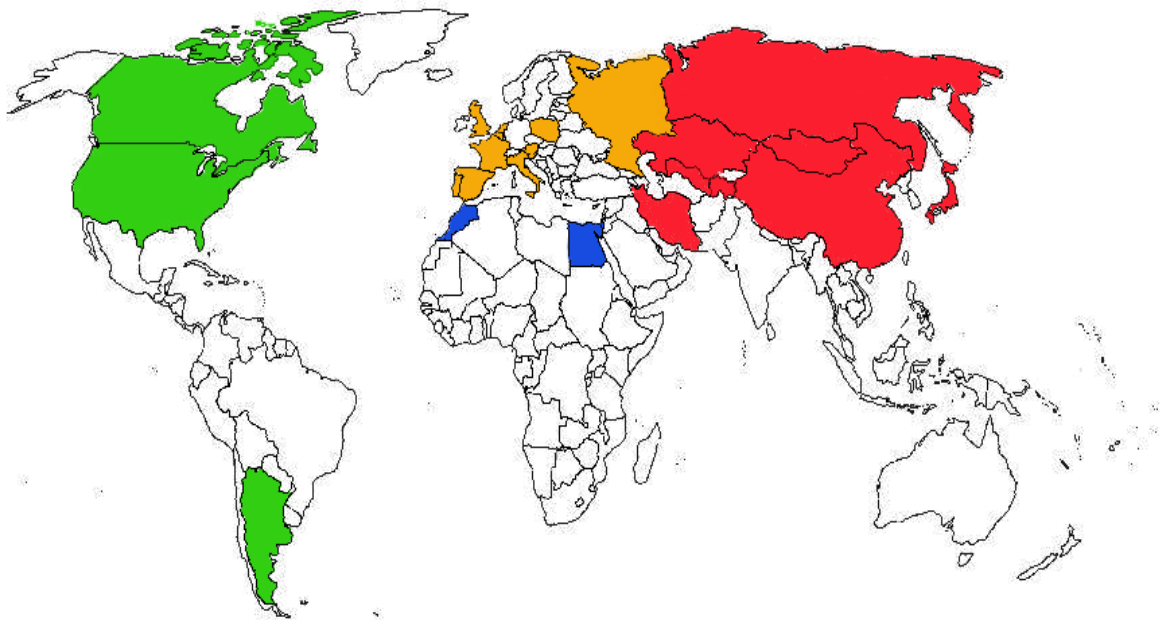

Figura 9. Principals regions de procedència de les espècies estudiades. Els diferents colors indiquen els països (agrupats per continents) d'on s'ha obtingut material.

2. MÈTODES

2.1 Cariologia i Citogenètica

2.1.1 Cariologia i citogenètica clàssica: recomptes de cromosomes i bandatge amb fluorocroms

Obtenció i preparació de plaques metafàsiques. Els aquenis madurs s'han sembrat en plaques de Petri sobre paper de filtre mullat i s'han conservat a temperatura ambient, en obscuritat. Aproximadament 24 h després de la geminació, els meristemes apicals de les plàntules germinades han estat pretractats amb una solució de colquicina aquosa al 0,05% a temperatura ambient o 8-hidroxiquinolina 0,002 M durant aproximadament 2,5-3 h. Posteriorment, el material s'ha fixat amb etanol absolut i àcid acètic glacial (3:1) durant 2-4 h a temperatura ambient, per a conservar-lo finalment a 4ºC. Un cop incubades en la solució fixadora durant 24 h aproximadament, les mostres s'han transferit a etanol 70º a 4ºC per a una millor conservació del material a llarg termini. Les mostres s'han hidrolitzat amb HCl 1N durant 3-4 min a 60ºC, s'han rentat amb aigua destil·lada i s'han tenyit amb orceïna acètica a l'1% durant 2-4 h. Per a observar el material al microscopi òptic, l'àpex de l'arrel s'ha separat amb l'ajuda d'un bisturí i s'ha muntat en un portaobjectes amb una gota d'àcid acètic al 45%/glicerol (9:1). Un cop s'ha col·locat el cobreobjectes s'ha aixafat durant uns segons la mostra per tal de facilitar la dispersió cel·lular. Les mostres han estat segellades amb DPX per a prolongar la conservació de les preparacions. Les millors plaques metafàsiques s'han fotografiat amb una càmera digital (AxioCam MRc5 Zeiss) acoblada a un microscopi Zeiss Axioplan. Les imatges han estat analitzades amb el programa Axio Vision Ac 4.2 i editades amb Adobe Photoshop CS2.

Obtenció i preparació de protoplasts.

PROTOCOL 1. Unes 5-7 puntes d'arrels fixades s'han rentat amb aigua desionitzada durant 10 min i s'han incubat en tubs de microcentrifugadora amb

200 µl d'una solució enzimàtica [cel·lulasa 3% Onozuka-RS (Yakult Honsha) i pectoliasa Y-23 0,5% (Kikkoman)] durant 30-50 min, depenent de les espècies, a 37ºC. Els meristemes s'han rentat amb aigua destil·lada durant 5 min, s'han col·locat en un portaobjectes per a homogeneïtzar-los amb una gota d'etanol absolut i àcid acètic glacial (3:1) amb l'ajuda d'unes pinces, i s'han assecat a l'aire. Per a comprovar l'existència de protoplasts amb cromosomes metafàsics, les preparacions s'han tenyit amb orceïna acètica al 0,01% i posteriorment s'han cobert amb un cobreobjectes. Les plaques s'han congelat durant 2 h a -80ºC per a facilitar la retirada dels cobreobjectes. Posteriorment s'han destenyit, primerament amb un rentat amb àcid acètic al 45% durant 1 h seguit de dos rentats amb aigua destil·lada durant 10 min. Les preparacions s'han conservat a -20ºC en una capsa amb un agent deshidratant (gel de sílice).

PROTOCOL 2. Entre 5 i 7 plàntules fixades s'han rentat amb tampó citrat 0,01 M (pH = 4,6) i posteriorment s'han incubat en vidres de rellotge que contenien 1ml d'una solució enzimàtica [cel·lulasa 3% Onozuka-RS (Yakult Honsha) i pectoliasa Y-23 0,5% (Kikkoman) preparada amb el mateix tampó citrat] durant 30-40 min, dintre d'una placa de Petri amb paper mullat, a 37ºC. Un cop digerides les plàntules s'han separat els àpexs de les arrels, s'han rentat amb tampó citrat durant 20 min i s'han col·locat en un portaobjectes per a homogeneïtzar-los amb una gota d'etanol absolut i àcid acètic glacial (3:1) amb l'ajuda d'unes pinces. Per a comprovar l'existència de protoplasts amb cromosomes metafàsics les preparacions s'han observat amb un microscopi òptic de contrast de fase. Les preparacions s'han congelat durant 20-24 h a -80ºC. Un cop retirats els cobreobjectes les mostres s'han deshidratat amb etanol absolut i s'han deixat assecar a l'aire. Les preparacions s'han conservat a -20ºC amb un agent deshidratant (gel de sílice).

Bandatge amb cromomicina A_3. S'han incubat les preparacions de protoplasts amb tampó McIlvaine (pH = 7) amb $MgCl_2 \cdot 6H_2O$ durant 15 min. Un cop retirat el tampó s'han tenyit amb 90-100 µl de cromomicina A_3 (0,02 g/100 ml) preparada amb el mateix tampó, i s'han deixat tenyir durant 90 min. Després

s'han rentat les mostres amb tampó McIlvaine (pH = 7) i s'ha efectuat una contracoloració amb una dilució de verd de metil (0,1%) en tampó McIlvaine (pH = 5,5) durant 7 min en obscuritat. Passat aquest temps, s'han retirat les restes de la tinció amb el mateix tampó McIlvaine (pH = 5,5) i s'ha deixat assecar a l'aire. Les preparacions han estat muntades amb una gota de Citifluor (Agar Scientific), i s'han observat al microscopi de fluorescència. Un cop fotografiades les plaques metafàsiques, per a eliminar la tinció de cromomicina (per tal d'efectuar posteriorment la hibridació *in situ* sobre les mateixes plaques), s'han incubat les preparacions durant 30-40 min en una solució d'etanol absolut i àcid acètic glacial (3:1) a temperatura ambient i amb agitació per a facilitar la separació dels cobreobjectes. Un cop destenyides les preparacions, s'han deshidratat durant 3 min amb sèries d'etanol de 70º, 90º i 100º i, finalment, s'han deixat assecar.

2.1.2 Citogenètica molecular

PROTOCOL 1

Preparació de les sondes del DNA ribosòmic

- **Sonda de DNA ribosòmic 5S.** El DNA genòmic de fulles joves d'*Artemisia princeps* Pamp. s'ha extret mitjançant el mètode de CTAB seguint les indicacions de DOYLE I DOYLE (1987) modificat per CULLINGS (1992). La regió 5S ha estat amplificada mitjançant PCR amb els encebadors 5'-CGGTGCATTAATGCTGGTAT-3' i 5'-CCATCAGAACTCCGCAGTTA-3' amb les següents condicions: cicle inicial de desnaturalització del DNA a 94,2ºC durant 5 min, 35 cicles de 30 s a 94, 2ºC, 30 s a 55,5ºC i 30 s a 72,2ºC, i un cicle final d'elongació del DNA durant 7 min a 72,2ºC. L'amplificació per PCR s'ha dut a terme en un volum de 50 µl de reacció amb 60 ng de DNA genòmic, 200 pmol de cadascun dels encebadors, 0,1 mM de cadascun dels dNTPs, 10 mM de tampó Tris-HCl pH = 8,3, 1,5 mM de $MgCl_2$, 50 mM de KCl i 1 unitat de *Taq* polimerasa (Tanaka, Scientific Ltd.). El producte de l'amplificació s'ha precipitat per centrifugació durant 30 min a 4ºC amb 5 µl d'acetat amònic 3M i 110 µl

d'etanol de 70º i ha estat tornat a suspendre en 50 µl de tampó TE a pH = 8. La sonda del DNA ribosòmic 5S fou marcada amb digoxigenina-dUTP (Roche Diagnostics) seguint les instruccions del fabricant.

- **Sonda de DNA ribosòmic 45S.** Per al gen del 18S-5.8S-26S de l'RNA ribosòmic s'ha utilitzat una sonda de pTa71 consistent en un fragment d'*Eco*RI de 9 kb de DNA ribosòmic obtinguda a partir de *Triticum aestivum* L. (GERLACH I BEDBROOK 1979), i reclonada en el plàsmid pUC 19. Aquesta sonda s'ha marcat amb Avidin-FITC mitjançant el sistema de *Nick translation* (BioNick Labelling System, Invitrogen).

Hibridació *in situ* fluorescent (FISH). Les preparacions amb protoplasts han estat incubades en 100 µg/ml de RNasa, lliure de DNasa, dissolta en tampó 2xSSC durant 1 h a 37ºC en una cambra humida, s'han rentat 1 cop amb 2xSSC (pH = 7) durant 10 min en agitació suau, i després durant 10 min en tampó 1xPBS (pH = 7). Seguidament, s'ha realitzat un tractament amb una solució de paraformaldehid 4% dissolt en 1xPBS durant 10 min a temperatura ambient. Les preparacions s'han desnaturalitzat a 72ºC durant 1,5 min amb una solució de formamida desionitzada al 70% diluïda amb 2xSSC, i posteriorment s'han deshidratat mitjançant rentats de 10 min en sèries d'etanol a 70º i 90º a -20ºC. De 15 a 20 µl d'una mescla d'hibridació* d'ambdues sondes 5S i 45S han estat dipositats a sobre de la mostra i coberts amb un cobreobjectes. Les preparacions s'han desnaturalitzat durant 5 min a 75ºC i posteriorment s'han hibridat a 37ºC durant 18-20 h en una cambra humida.

Transcorregut el període d'hibridació, s'han realitzat dos rentats post-hibridacionals astringents amb 4xSSC a 42ºC durant 10 min, seguits d'un rentat amb 2xSSCT (2xSSCT: 2xSSC amb 0,2% de tritó-100) a temperatura ambient i en agitació suau. Per a la detecció dels senyals de la sonda 5S, les mostres han estat tractades amb 1% (p/v) de sèrum d'albúmina bovina (BSA) diluït en 2xSSCT, durant 45 min a 37ºC, i posteriorment incubades durant 1,5 h a 37ºC amb fragments Fab d'anti-digoxigenina-rodamina 20 mg/ml (Roche Diagnostics) dissolts amb el mateix tampó (1:200). Les preparacions s'han rentat dos cops durant 10 min amb 2xSSCT a 42ºC, un cop amb 2xSSC a temperatura

ambient durant 5 min, un cop amb aigua desionitzada durant 5 min a temperatura ambient, i finalment s'han deshidratat amb sèries de tres min d'etanol de 70°, 90° i 100°. S'ha utilitzat Vectashield (Vector Laboratories), amb 500 ng/ml de DAPI (4',6-diamidino-2-fenilindol), com a medi per a muntar les preparacions, i s'han observat al microscopi de d'epifluorescència Nikon Eclipse E600, utilitzant diferents filtres (UV-1 (365/410), B-2A (450/490), G-2A (510/560). Les imatges s'han capturat amb una càmera CCD (Pixera, Pentium 600CL), i posteriorment manipulat amb un programa d'anàlisi d'imatges (Adobe Photosop CS2 v.9.0.2, Adobe Systems).

*MESCLA D'HIBRIDACIÓ: Un cop precipitades les sondes marcades mitjançant els diferents mètodes utilitzats, ambdues s'han tornat a suspendre per separat afegint-hi 50 µl d'una dissolució al 20% de sulfat de dextrà amb 4xSSC, i afegint-hi altres 50 µl de formamida desionitzada. Els còctels s'han desnaturalitzat durant 10 min a 95°C, i seguidament s'han conservat en gel. Una segona desnaturalització amb les mateixes condicions s'ha dut a terme un cop s'han mesclat a parts iguals ambdues sondes per a procedir a la hibridació.

PROTOCOL 2

Preparació de les sondes del DNA ribosòmic.

- **Sonda de DNA ribosòmic 5S.** La sonda utilitzada per a la detecció i localització de la regió 5S del DNA ribosòmic ha esta un clon del pTa794; un fragment de 410 kb del 5S del *Bam*HI aïllat del blat i clonat en pBR322 (GERLACH I DYER 1980). Aquesta sonda s'ha marcat amb digoxigenina-11-dUTP verda (Boehringer Mannheim) mitjançant PCR.
- **Sonda de DNA ribosòmic 45S.** S'ha utilitzat la mateixa sonda que la descrita a l'apartat anterior, seguint el protocol descrit a TORRELL *et al.* (2003). L'única modificació ha estat que en aquest cas la sonda ha estat marcada de forma directa amb roig Cy3 (Amersham) mitjançant PCR.

Hibridació *in situ* fluorescent (FISH). Les preparacions han estat incubades en 100 µg/ml de RNasa, lliure de DNasa, diluïda en 2xSSC durant 1h a 37°C en

una cambra humida. Posteriorment s'han rentat dos cops en 2xSSC durant 5 min amb agitació suau, un cop amb HCl 0,01N a temperatura ambient i s'han incubat afegint 80-100 µl/preparació de pepsina (0,1 mg/ml en HCl 0,01N) durant 10 min a 37ºC. Seguidament s'han rentat dos cops en 2xSSC durant 5 min, i s'han deshidratat amb sèries de 3 min amb etanol (70º, 90º i 100º). Un cop assecades, s'han dipositat 50 µl de còctel d'hibridació* a cada preparació, s'han cobert amb un cobreobjectes de plàstic i s'han desnaturalitzat les mostres durant 10 min a 75ºC, i després, a 55ºC durant 5 min. Tot seguit s'ha dut a terme la hibridació a 37ºC en cambra humida durant 15-18 h.

Transcorregut el temps d'hibridació, les preparacions s'han rentat amb agitació suau amb les següents solucions astringents: un rentat de 3 min amb 2xSSC a temperatura ambient, tres rentats de 5 min amb 2xSSC a 42ºC, dos rentats de 5 min amb formamida desionitzada 20% amb 0,1xSSC a 42ºC, un rentat de 5 min amb 0,1xSSC a 42ºC, dos rentats de 5 min amb 2xSSC a 42ºC, un rentat durant 5 min amb 4xSSCT (SSCT: 0,2% Tween 20) a 42ºC i tres rentats de 5 min amb 4xSSCT a temperatura ambient. Per a poder detectar els senyals de la sonda 5S les preparacions han estat pretractades durant 5 min a temperatura ambient amb 100 µl/mostra d'una dissolució BSA (concentració final 5%) amb 4xSSCT, i seguidament s'han dipositat 50 µl/mostra, de la mescla de detecció preparada amb 55 µl de la dissolució anterior (BSA i 4xSSCT) a la que s'han afegit 0,75 µl d'ADF (anti-digoxigenina-fluoresceïna; Boehringer Mannheim) a una concentració de 200 µg/ml, i s'han incubat durant 1 h a 37ºC. Tot seguit s'han rentat les preparacions tres cops durant 5 min amb 4xSCCT a temperatura ambient, i s'han muntat amb Vectashield (Vector Laboratories), que contenia 500 ng/ml de DAPI (4',6-diamidino-2-fenilindol). Les mostres s'han observat al microscopi de d'epifluorescència (Zeiss Axiophot), utilitzant diferents filtres d'excitació (01, 07, 15). La captura d'imatges s'ha fet amb una càmera CCD (Princeton Instruments), i posteriorment s'han manipulat amb un programa d'anàlisi d'imatges (Metavue, version 4.6, Molecular Devices Corporation).

*MESCLA D'HIBRIDACIÓ: La mescla d'hibridació ha estat preparada afegint aproximadament per a cada preparació d'1-1,5 µl de les sondes 46S i 5S (a una concentració aproximada de 25-100 ng/µl) 25 µl de formamida desionitzada (concentració final 50%, pH = 7), 10 µl de sulfat de dextrà al 50% (v/v), 3 µl de dodecilsulfat sòdic 10% (p/v), 1,5 µl d'esperma de salmó 250 µg/ml i 5 µl de 20xSSC. Un cop preparat el còctel s'ha desnaturalitzat durant 10 min a 75ºC i seguidament s'ha conservat amb en gel.

Tampons utilitzats:

- **20xSSC (pH = 7):** Clorur sòdic (NaCl) 3 M, citrat sòdic ($Na_3C_6H_5O_7 \cdot 2H_2O$) 0,3 M
- **10xPBS (pH = 7):** Clorur sòdic (NaCl) 1,3 M, hidrogenofosfat sòdic ($Na_2HPO_4 \cdot 12H_2O$) 0,07 M, dihidrogenofosfat sòdic ($NaH_2PO_4 \cdot 2H_2O$) 0,03 M.

2.2 Mesura de la quantitat de DNA: citometria de flux

S'han trossejat alhora porcions de fulles joves de la mostra a analitzar i del patró escollit per a fer les mesures (*Pisum sativum* 'Express Long' 2C = 8,37 pg, *Petunia hybrida* 'PxPc6' 2C = 2,85 pg, *Triticum aestivum* 'Chinese Spring' 2C = 30,90 pg; MARIE I BROWN 1993; MARUM *et al.* 2009). Generalment s'utilitza més o menys el doble de quantitat de mostra de l'espècie problema (uns 25 mm^2 aproximadament) que de patró. De cada població estudiada, hem analitzat cinc individus i s'han realitzat dues mesures independents per a cada individu. El trossejat de les mostres s'ha dut a terme en una placa de Petri amb 600-700 µl de tampó de lisi Galbraith (GALBRAITH *et al.* 1983), mitjançant una fulla d'afaitar per a provocar l'alliberament dels nuclis cel·lulars. El tampó (de pH = 7,0-7,2), que conté 10 µl de ribonucleasa A (RNasa A, Boehringer, Meylan, França) 100 µg/ml es guarda a 4ºC i s'afegeix a les mostres en fresc. La suspensió de nuclis en el tampó d'extracció s'ha filtrat amb una malla de niló d'un diàmetre de porus de 30 µm, amb la finalitat d'eliminar qualsevol traça de material vegetal

que pogués obturar el citòmetre. Un cop preparada i filtrada la suspensió de nuclis s'ha procedit a la tinció amb el fluorocrom d'elecció, iodur de propidi (1 mg/ml; Sigma-Aldrich Química), del qual se n'hi afegeixen 36-40 µl, amb una concentració final de 60 µg/ml, i s'han conservat en gel durant aproximadament 20 min abans de mesurar-les.

Els assaigs de citometria de flux es van dur a terme als Serveis Cientificotècnics de la Universitat de Barcelona, amb el citòmetre Epics XL (Coulter Corporation). L'instrument ha estat preparat en la configuració estàndard: l'excitació de la mostra s'ha fet utilitzant com a font de llum un làser d'argó a baixa intensitat (488 nm, 15 mW) i refrigerat per aire. S'han adquirit les dades proporcionades pel detector de FSC (*forward scatter*), que capta la llum dispersada en posició central, pel detector de SSC (*side scatter*), que capta la llum dispersada a 90º, i les de fluorescència vermella (620 nm) del iodur de propidi, el fluorocrom emprat. L'alineació es basa en un senyal optimitzat procedent de microesferes fluorescents de 10 nm (Immunocheck, Epics Division). El temps s'ha utilitzat com a control de l'estabilitat del instrument. La fluorescència vermella es projecta en un histograma monoparamètric de 1024 canals. S'exclouen els agregats seleccionant cèl·lules solitàries: àrea *vs.* senyal fluorescent. Es comparen el valor del pic (la moda) de l'estàndard i el de la mostra.

Tampó de lisi Galbraith (GALBRAITH *et al.* 1983, pH = 7): Clorur magnèsic ($MgCl_2$) 45 mM, citrat sòdic [$Na_3C_3H_5O(CO_2)_3$] 30 mM, MOPS (àcid 3-morfolinopropansulfònic) 20 mM, 0,1% (v/v) tritó X-100, mercaptoetanol 15 mM.

2.3 Filogènia molecular

2.3.1 Extracció del DNA genòmic

Mètode del CTAB [DOYLE I DOYLE 1987; amb modificacions de SOLTIS *et al.* (1991)]. Aquest mètode d'extracció de DNA s'ha dut a terme en material fresc i/o dessecat amb gel de sílice, l'estat de conservació del qual era òptim. S'han

pesat 0,01 g de material sec, o 0,05 g de material fresc i posteriorment s'han homogeneïtzat amb èmbols de vidre i/o mitjançant un aparell de trituració mecànica (Retsch GmbH). S'han afegit 500 µl de tampó d'extracció CTAB a la mostra que s'ha incubat d'una a quatre hores a 65°C, invertint els tubs cada 30-60 min. Posteriorment, s'han refredat les mostres en gel i s'han centrifugat durant 1 min a 13.300 rpm. S'han afegit 500 µl d'una mescla de triclorometà i alcohol isoamílic (24:1) i s'ha barrejat per inversió fins a obtenir una mescla homogènia. El contingut s'ha centrifugat durant 5 min a 13.300 rpm per recollir el sobrenedant i transferir-lo a un nou tub d'1,5 ml. Aquest pas s'ha tornat a repetir afegint 500 µl més de la mescla de triclorometà i alcohol isoamílic, i s'ha tornat a centrifugar 5 min a 13.300 rpm. Un cop més, el sobrenedant s'ha recuperat i transferit a un nou tub d'1,5 ml. Amb la finalitat de precipitar el DNA, s'han afegit acetat amònic 3 M i isopropanol fred en les proporcions adequades segons el volum que s'ha recuperat en el pas anterior (aproximadament 36 µl d'acetat i 262 µl d'isopropanol, respectivament, per a 400 µl de mostra). En aquest punt s'han seguit dues estratègies: i) deixar precipitar el DNA durant 20 h a 4°C (quan la qualitat de la mostra era dubtosa), ii) guardar les mostres a -80°C durant 15-20 min per a afavorir la precipitació del DNA (en mostres de qualitat òptima). El següent pas ha estat centrifugar les mostres durant 3 min a 13.300 rpm i retirar el sobrenedant amb una pipeta Pasteur amb cura de no perdre el sediment precipitat de DNA. Després s'ha afegit 1 ml d'etanol de 70° fred, s'ha mesclat per inversió amb la intenció de rentar el precipitat, i s'ha centrifugat durant 3 min a 13.300 rpm. L'etanol s'ha retirat dels tubs amb una pipeta Pasteur i les mostres s'han deshidratat durant 40-60 min al buit. El DNA s'ha tornat a suspendre afegint 50 µl de tampó TE i/o d'aigua desionitzada i incubant les mostres a 45 °C durant 20 min.

Mètode d'extracció de DNA genòmic amb *kit* d'extracció (Nucleospin Plant II; Macherey-Nagel, GmbH & Co.). S'han homogeneïtzat 0,01 g de teixit vegetal sec (principalment de plecs d'herbari antics) amb l'ajut d'èmbols de vidre i/o mitjançant un aparell de trituració mecànica (Retsch GmbH). S'han afegit 400 µl de tampó de lisi PL1, s'ha mesclat bé el contingut, i s'ha incubat al bany a 65°C

durant 30-60 min. La mostra incubada s'ha disposat en un tub especial per a filtrar el contingut i s'ha centrifugat durant 2 min a 11.000 rpm, tot conservant el volum resultat de l'elució. Aquest volum s'ha transferit a un tub d'1,5 ml nou, i s'han afegit 450 µl del tampó d'anellament (PC) al DNA, s'ha transferit aquest volum a un nou tub de filtració, i s'ha centrifugat durant 1 min a 10.900 rpm, tot descartant el volum eluït. El següent pas ha estat rentar mitjançant centrifugació durant 1 min a 10.900 rpm aquest DNA, que ha quedat adherit a la membrana del filtre, amb 400 µl d'un tampó de rentat PW1. S'ha repetit aquest pas amb l'addició de 700 µl d'un nou tampó de rentat PW2, i la posterior centrifugació, i finalment s'ha tornat a centrifugar sense afegir cap tampó per a eliminar-ne les restes i assecar la membrana del filtre. Per tal d'eluir el DNA retingut a la membrana, s'hi han afegit 50 µl de tampó d'elució (preescalfat a 70°C), i s'ha centrifugat durant 1 min a 10.900 rpm.

2.3.2 Amplificació, purificació i seqüenciació de les regions escollides de DNA nuclear i cloroplàstic

Les reaccions d'amplificació del DNA mitjançant una reacció en cadena per la polimerasa (PCR) s'han realitzat utilitzant diferents termocicladors [PTC-100, PTC-200 (MJ Research Inc., Massachussets, EUA), G-Storm (GRI Labcare, Essex, Regne Unit)], en un volum de reacció total de 25 µl [encebadors 5 µM, dNTPs 1 mM, tampó 10x, $MgCl_2$ 25 mM i 0,5 unitats de *Taq* polimerasa (Sigma), on s'han afegit aproximadament 50 ng de DNA genòmic]. Els productes de PCR han estat purificats mitjançant QIAquick PCR Purification Kit (Qiagen), i en aquells casos en què les bandes de DNA observades eren dèbils s'ha utilitzat el kit DNA Clean & Concentrator-5 D4003 (Zymo Research), seguint les instruccions dels fabricants. Un cop purificat el DNA, la reacció de seqüenciació s'ha realitzat amb els mateixos termocicladors que les amplificacions, en un volum de reacció de 20 µl (tampó de seqüenciació 5x, tampó de seqüenciació RR-100, encebador 5 µM, 1-1,5 µl de DNA purificat). La seqüenciació directa dels fragments amplificats s'ha realitzat utilitzant Big Dye Terminator Cicle sequencing v 3.1 (PE Biosystems), en un ABI Prism 3700 DNA analyzer (PE Biosystems) a les instal·lacions de la Unitat de Genòmica dels

Serveis Científicotècnics de la Universitat de Barcelona. Les seqüències de DNA s'han editat mitjançant el programa Chromas Lite (Technelysium Pty Ltd.) i s'han alineat utilitzant ClustalW (THOMPSON *et al.* 1994) amb les opcions per defecte del programa Bioedit 7.0 (HALL 1999).

REGIONS DEL DNA ANALITZADES

Espaiador intern transcrit (ITS). L'ITS1 i ITS2, incloent el gen 5.8S, han estat amplificats conjuntament mitjançant PCR amb els encebadors ITS1 (WHITE *et al.* 1990) i 1406f (NICKRENT *et al.* 1994) com a encebadors directes, i ITS4 (WHITE *et al.* 1990) com a encebador revers. Les condicions de PCR utilitzades han estat, un primer pas de desnaturalització de 2 min a 94ºC, trenta cicles d'1 min a 94ºC, 30 s a 55ºC i 3 min a 72ºC, i un pas d'elongació del DNA de 15 min a 72ºC. Un cop acabada la reacció les mostres s'han conservat a 4ºC. Per a la reacció de seqüenciació solament s'ha utilitzat només l'encebador revers (ITS4) a causa de la mida reduïda de la regió (al voltant de 620 pb). Tot i això, l'encebador ITS1 també s'ha utilitzat en alguns casos específics.

Espaiador extern transcrit (ETS). La regió ETS ha estat amplificada utilitzant els encebadors ETS1f i 18SETS com a directe i revers respectivament (BALDWIN I MARKOS 1998). Les condicions d'amplificació utilitzades han estat, 5 min de desnaturalització a 95ºC; trenta cicles de 45 s a 94ºC, 45 s a 50ºC, 40 s a 72ºC i 7 min d'elongació a 72ºC. Per a alguns tàxons s'han utilitzat encebadors interns a l'hora d'amplificar la regió, amb la intenció d'amplificar més fàcilment fragments més petits. Aquests han estat l'AST1f i l'AST1R (MARKOS I BALDWIN 2001), i les condicions d'amplificació han estat les mateixes. Per a la seqüenciació dels fragments amplificats s'han utilitzat l'ETS1f i 18SETS per a les mostres amplificades amb aquests dos encebadors, i els AST1R i 18SETS per a les mostres en les quals s'ha amplificat la regió amb dos fragments de mida més reduïda.

***trnS*^{UGA}*-trnfM*^{CAU}. Aquesta regió cloroplàstica ha estat seqüenciada seguint el treball de SHAW *et al.* (2005) i utilitzant els encebadors descrits en aquest estudi.

Les condicions de PCR programades han estat, 5 min de desnaturalització a 80ºC; trenta cicles de 30 s a 94ºC, 1 min 30 s a 62ºC, 2 min a 72ºC, i 5 min d'elongació a 72ºC. Ambdós encebadors s'han fet servir per a la seqüenciació d'un fragment d'aproximadament 1.200 pb.

trnS^{GCU}-trnC^{GCA}. Els encebadors trnS^{GCU} (directe) i trnC^{GCA} (revers), extrets del treball de KIM *et al.* (2005), han estat utilitzats per a amplificar aquesta regió cloroplàstica, tot utilitzant les mateixes condicions de PCR que per a la regió precedent. Sols s'ha utilitzat l'encebador trnC^{GCA} per a seqüenciar un fragment de 850 pb aproximadament. Hem de fer notar que aquest fragment cloroplàstic és exclusiu de les *Asteraceae* a causa de les modificacions estructurals sofertes pel seu genoma cloroplàstic, com a resultat de dues inversions, una de 22,8 kb, i la segona de 3,3 kb, que han tingut lloc a la família (KIM *et al.* 2005).

2.4 Anàlisis estadístiques i filogenètiques

2.4.1 Anàlisis estadístiques

Les comparacions entre les variables estudiades s'han dut a terme mitjançant tests d'anàlisi de variància (ANOVA simple) sempre que les dades complien els següents requisits; seguir una distribució normal, homogeneïtat de les variàncies, i independència de les observacions. En cas contrari, hem dut a terme tests no paramètrics. El test escollit en aquests casos ha estat el de Kruskal-Wallis que es basa en un contrast de la igualtat de les medianes. Aquests tests s'han dut a terme amb el programa estadístic Statgraphics Plus v.5.1 (Statistical Graphic Corp.). En altres casos, s'ha utilitzat el paquet informàtic PHYLOGR R per a dur a terme els test *t* i els basats en dades filogenètiques (PGLS; *Phylogenetic Generalized Least Squares*). Per a estudiar l'anàlisi de l'evolució del la quantitat de DNA amb l'increment de ploïdia s'han realitzat aproximacions matemàtiques amb la intenció de trobar un model matemàtic que pogués explicar-ne la dinàmica de les dades. S'han provat el coeficient quadràtic d'una funció polinòmica, $y = \beta_0 + \beta_1 x + \beta_2 x^2$, així com el model Michaelis-Menten, $y = \beta_0 x / (\beta_1 + x)$. Els càlculs han estat desenvolupats amb el programa R (versió 2.5.0), mitjançant l'ús del llenguatge S (VENABLES I RIPLEY 2003).

2.4.2 Anàlisis filogenètiques

S'han analitzat les matrius amb els diferents criteris seleccionats, que s'especifiquen a cada treball, i que millor s'han adaptat al tipus d'anàlisi escollida. Cadascuna de les regions amplificades, tant nuclears com cloroplàstiques, s'han analitzat de forma independent i posteriorment combinada, sempre que la congruència de les dades ho hagi permés. Les matrius han estat analitzades utilitzant PAUP v.4.0b10 (SWOFFORD 2003) per als estudis de màxima parsimònia. El suport de les branques s'ha avaluat mitjançant *bootstrap*.

Per a determinar els models d'evolució més afins a les nostres dades s'utilitzaren els criteris d'informació AIC (*Akaike Information Criterion*) i hLRT (*hierarchical Likelihood Ratio Test*) (POSADA I CRANDALL 1998; POSADA I BUCKLEY 2004), implementats al programa MrModeltest 2.2 (NYLANDER 2004). Un cop seleccionats el models d'evolució, s'han analitzat les matrius de dades amb el programa Mr Bayes v.3.1.2 (HUELSENBECK I RONQUIST 2006) per a inferència bayesiana. En aquest cas, el suport de les branques s'ha avaluat mitjançant probabilitat posterior.

Les condicions específiques utilitzades per a les anàlisis filogenètiques de les dades, així com els coeficients obtinguts per a cadascuna d'elles apareixen especificats a cadascun dels estudis inclosos en el compendi de treballs.

2.5 Palinologia

El material per a l'estudi pol·línic es va obtenir de plantes seques recol·lectades en el camp i de plecs d'herbari dipositats en els herbaris de la Universitat de Barcelona (BCN), de la Universitat de Mongòlia interior (HIMC) i de l'herbari Komarov de Sant Petersburg (LE).

El pol·len s'obtingué disseccionant les anteres prèviament deshidratades amb alcohol de 96°. Les observacions es dugueren a terme en els Serveis Cientificotècnics de la Universitat de Barcelona amb un microscopi de rastreig

Hitachi 52300 a 15 kV després d'haver estat acetolitzades seguint el micromètode d'AVETISSIAN (1950).

Per tal de dur a terme les mesures biomètriques, les mostres de pol·len s'acetolitzaren seguint el mètode abans esmentat, es muntaren en glicerogelatina i se segellaren les preparacions. Les mesures es dugueren a terme usant un aparell Visopan (Reichert). Per a cada espècimen es mesuraren 15 grans de pol·len completament desenvolupats (amb algunes excepcions que es fan constar a l'apartat corresponent). Els paràmetres considerats, d'acord amb ERDTMAN (1969), FAEGRI I IVERSEN (1964) i REITSMA (1970) foren: diàmetre polar (P), diàmetre equatorial (E) i esfericitat (P/E). Es calularen la mitjana i la desviació estàndard per a cadascun dels paràmetres. En els casos dels grans de pol·len amb ornamentació amb espines, es calculà l'altura de l'espina des de la punta fins al començament de la base multiperforada. La densitat d'espines supratectals es calculà en la zona del mesocolpi en una àrea de 25 μm^2. El volum aproximat dels grans de pol·len es calculà utilitzant la fórmula de l'el·lipsoide: $V = (4\Pi/3)(P/2)(E/2)^2$. S'utilitzà la terminologia proposada per REITSMA (1970).

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COMPENDI DE PUBLICACIONS

ARTEMISIÁ EVOLUCIÓ

Dinàmica de la quantitat de DNA en el gènere *Artemisia* L. (*Asteraceae*): seguint el rastre de la poliploïdia

Plant Biology (en premsa)

Jaume Pellicer, Sònia Garcia, Miguel Á. Canela, Teresa Garnatje, Aleksandr A. Korobkov, John D. Twibell i Joan Vallès

La poliploïdia és un factor clau en l'evolució de les plantes superiors i juga un paper important en la variació dels seus genomes, afavorint, en alguns casos, els processos d'especiació. Durant la poliploïdització, tenen lloc, a nivell genòmic, diferents processos equilibradors que poden promoure la variació de la quantitat de DNA nuclear. Hem estimat la quantitat de DNA nuclear mitjançant citometria de flux en 84 poblacions corresponents a 67 espècies d'*Artemisia*, i en una població de *Crossostephium chinense*. Un total de 73 seqüències de les regions ITS i 3'-ETS de l'rnDNA foren generades i analitzades junt amb les seqüències ja publicades prèviament, per tal de poder estudiar l'evolució de la quantitat de DNA nuclear del gènere en un marc filogenètic representatiu. Es varen trobar diferències significatives del valor 2C entre llinatges, així com un increment de l'heterogeneïtat de les dades de quantitat de DNA en aquells subgèneres on les relacions filogenètiques encara no són clares. Vàrem confirmar que l'increment dels valors 2C en espècies poliploides d'*Artemisia* no és proporcional al nivell de ploïdia i, a més, que el valor 1Cx tendeix a decréixer significativament en assolir alts nivells de ploïdia. Aquests resultats ens permeteren postular la hipòtesi que la quantitat de DNA en poliploides tendeix a un màxim, com si se seguís una corba de saturació, en concordança amb el model de Michaelis-Menten. Ajutàrem diferents funcions aritmètiques amb les nostres dades, que corroboraren una relació no linear entre l'increment de la quantitat de DNA i el nivell de ploïdia, fet que ens ha permès suggerir un límit superior teòric per al contingut de DNA en el gènere.

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RESEARCH PAPER

Genome size dynamics in *Artemisia* L. (Asteraceae): following the track of polyploidy

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ABSTRACT

Polyploidy is a key factor in the evolution of higher plants and plays an important role in the variation of plant genomes, leading to speciation in some cases. During polyploidisation, different balancing processes take place at the genomic level that can promote variation in nuclear DNA content. We estimated genome size using flow cytometry in 84 populations of 67 *Artemisia* species and one population of *Crossostephium chinense*. A total of 73 sequences of nrDNA ITS and 3′-ETS were newly generated and analysed, together with previously published sequences, to address the evolution of genome size in a phylogenetic framework. Differences in 2C values were detected among some lineages, as well as an increase of genome size heterogeneity in subgenera whose phylogenetic relationships are still unclear. We confirmed that the increase in 2C values in *Artemisia* polyploids was not proportional to ploidy level, but 1Cx genome size tended to decrease significantly when high ploidy levels were reached. The results lead us to hypothesise that genome size in polyploids tends to a maximum as it follows saturation behaviour, in agreement with the Michaelis–Menten model. We tested different arithmetic functions with our dataset that corroborated a non-linear relationship of genome size increase in polyploids, allowing us to suggest a theoretical upper limit for the DNA content of this genus.

INTRODUCTION

The nuclear DNA content, understood as genome size, is very variable across flowering plants, and has been revealed as an important character in biodiversity. Differences of more than 1200-fold have been reported between species such as *Genlisea margaretae* or *G. aurea* (2C = 0.128–0.130 pg; Greilhuber *et al.* 2006), which has the lowest genome size estimate, to the largest genome in *Fritillaria assyriaca* (2C = 254.80 pg; Bennett & Smith 1976). Swift (1950) coined the term 'C value' to refer to the DNA content of the unreplicated haploid complement. We use the term 'monoploid genome size' (1Cx) for the DNA content in a basic chromosome set (x) of a somatic cell (Greilhuber *et al.* 2005), and 2C for the whole genome size of a somatic cell. The study of genome size has applications in many fields, *i.e.* ecology, evolution and systematics (*e.g.* Bennett & Leitch 2005a,b). Relationships found between genome size and ecological and biological features have shed light on important evolutionary effects of genome size variations (*e.g.* Vinogradov 2003; Albach & Greilhuber 2004; Garcia *et al.* 2004b, 2008; Garnatje *et al.* 2004; Chase *et al.* 2005; Price *et al.* 2005; Beaulieu *et al.* 2007).

Polyploidy is one of the main evolutionary forces that influence the large variation in nuclear DNA content. In fact, continuous polyploidisation events in plants are common (*e.g.* Soltis & Soltis 2000; Wendel 2000; Cui *et al.* 2006; Chen 2007). It is known that different reorganisations at the genomic level can occur during and after polyploid formation, many of which involve genome size changes (Leitch & Bennett 2004), although the

mechanisms are still under investigation. Hence, polyploidy implies much more than a simple multiplication of the chromosome complement; it is one of the determining factors in the direction of genome changes (Leitch & Bennett 2004), and an important mechanism that can influence evolution and speciation of plants (Otto & Whitton 2000; Abbott & Lowe 2004; Soltis *et al.* 2004). Indeed, allopolyploid formation may be the most common mechanism of sympatric speciation in plants. It is thought that up to 70% of angiosperm species have experienced one or more episodes of polyploidy (Masterson 1994; Soltis & Soltis 2000); even some species with low chromosome numbers and classically considered as diploids are currently believed to be ancient polyploids (*e.g. Arabidopsis thaliana*, Vision *et al.* 2000).

Artemisia L. is the largest genus of the tribe *Anthemideae* and one of the broadest genera of the Asteraceae, with more than 500 species (Oberprieler *et al.* 2007). The genus has been subject to different taxonomic rearrangements since the first systematic treatments, and at present five large groups are considered at the subgeneric level, mostly based on floral characters: *Absinthium* DC., *Artemisia*, *Dracunculus* Besser, *Seriphidium* Besser and *Tridentatae* (Rydb.) McArthur. Molecular studies based on analysis of chloroplast DNA and nuclear ribosomal DNA sequences (Torrell *et al.* 1999; Watson *et al.* 2002; Vallès *et al.* 2003; Sanz *et al.* 2008; Tkach *et al.* 2008) have shown that these subgenera (and some of them in particular) do not represent natural groups, but an alternative classification has not yet been achieved. The genus is widely dispersed across the Northern Hemisphere (mainly in Eastern Europe and Asia, reaching North America), and there are few species in the Southern Hemisphere (Ling 1982; Vallès & Garnatje 2005). Two basic chromosome numbers have been detected in the genus, x = 9 (present in all subgenera), and the less frequent x = 8. Genome size variation in *Artemisia* and related genera has been studied (Garcia *et al.* 2008 and references therein), with estimates of more than 100 species. In most cases, differences in 2C values of about sevenfold have been detected between species, which are directly related to polyploidisation events.

Polyploidy is very common in *Artemisia* and reflects the great genetic plasticity of this genus, where ploidy levels up to 16x have been reported (Pellicer *et al.* 2007b). From the available published data, it is estimated that *ca.* 43.5% of species are exclusively diploid, *ca.* 29.7% are exclusively polyploid, and around 26.8% include species known at both diploid and polyploid levels. The high percentage of polyploids, ability of the genus to colonise different ecosystems from sea level to high mountains, as well as adaptation to wide and variable habitat conditions may be the main traits that favoured its successful expansion across the landscape.

In the present study, we provide estimates of C values for 67 *Artemisia* species (with new data on 52) and a first estimate for the related species *Crossostephium chinense* Makino. The distribution of C values is discussed in the light of phylogenetic and taxonomic relationships, as well as to emphasise the implications and evolution of genome size in polyploid representatives. The principal aims of the present investigation are to: (i) analyse the evolution of genome size, for the first time within a phylogenetic framework, in a representative sample of the genus, composed of about 220 populations extracted from the present and previous works (Torrell & Vallès 2001; Garcia *et al.* 2004b, 2006, 2008; Pellicer *et al.* 2007a); (ii) study changes (increase or decrease) in genome size from diploid to polyploid *Artemisia* species; (iii) predict dynamics of C values across ploidy levels by adjusting different mathematical functions to the dataset in order to evaluate the possible existence of an upper limit to genome size and ploidy level in *Artemisia*; and (iv) assess intraspecific (inter-population) genome size variation.

MATERIAL AND METHODS

Plant material

Table S1 shows the 85 populations belonging to 68 species (including *C. chinense*) studied and grouped at subgeneric level under traditional classification criteria, with their location, collector and herbarium voucher (refer to the Supporting Information section). Leaf material was obtained from culture of ripe achenes in greenhouses of the Facultat de Farmàcia (Universitat de Barcelona) and the Institut Botànic de Barcelona (CSIC-ICUB). *Petunia hybrida* Vilm. 'PxPc6' (2C = 2.85 pg), *Pisum sativum* L. 'Express long' (2C = 8.37 pg) and *Triticum aestivum* L. 'Chinese spring' (2C = 30.90 pg) were used as internal standards to cover the range of 2C values found (Marie & Brown 1993). Seeds of the standards were provided by the Institut des Sciences du Végétal (CNRS), Gif-sur-Yvette (France).

Karyological information

Most chromosome counts were obtained from our previous works (Garcia *et al.* 2004a; Pellicer *et al.* 2007a,b and references therein). New counts (22) were made following methods described in these papers. For species in which only leaf material was available, to find whether chromosome counts had been reported previously, we consulted the most common chromosome number indices (cited in Vallès *et al.* 2001) and the following chromosome number databases: Index to Plant Chromosome Numbers (Missouri Botanical Garden, http://mobot.mobot.org/W3T/Search/ipcn.html) and Index to Chromosome Numbers in the Asteraceae (Watanabe 2002; http://www-asteraceae.cla.kobe-u.ac.jp/index.html).

Flow cytometry assessment

Leaf tissue of five individuals for each studied population was chopped with a razor blade in 600 µl of Galbraith's isolation buffer (Galbraith *et al.* 1983), together with

the chosen internal standard, and supplemented with 100 $\mu g \cdot ml^{-1}$ ribonuclease A (RNase A; Boehringer, Meylan, France). For each individual, two independent samples were extracted for cytometric assessment. Samples were subsequently stained with 36 µl propidium iodide (1 $mg \cdot ml^{-1}$) to a final concentration of 60 $\mu g \cdot ml^{-1}$ (Sigma-Aldrich Química, Madrid, Spain), kept on ice for 20 min and measured in an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL, USA). Measurements were made at the 'Serveis Cientificotècnics' of the Universitat de Barcelona. To ascertain that the instrument gave a linear response across the range of genome sizes studied, we performed several assays that included both internal standards and one of the populations with the highest genome size at the same time. The difference between the obtained results with respect to each standard was negligible (less than 2% deviation), thus verifying the linearity of the flow cytometer for this interval and use of the chosen internal standards. We also calculated the mean half peak coefficient of variation (HPCV) corresponding to ten samples. The HPCVs obtained were 2.54% in *Petunia hybrida*, 1.87% for *Pisum sativum* and 1.63% for *Triticum aestivum*. Further details are described in Garcia *et al.* (2004b).

DNA amplification and sequencing

In order to analyse genome size variation in a phylogenetic framework, we constructed a molecular phylogenetic tree that included representatives of all subgenera for which the nuclear DNA content had been estimated. Phylogenetic analysis was based on sequences of the ITS1, ITS2 and the 3′-ETS ribosomal nuclear DNA regions. Most sequences were available from GenBank, and to complete the sampling, 73 sequences were newly generated. The double-stranded DNA ITS region was amplified with primers ITS1 and ITS4 (White *et al.* 1990), and the 3′-ETS was amplified using ETS1f and 18SETS (Baldwin & Markos 1998), although some smaller fragments were amplified using AST1f as the forward primer. The profile used for amplification was the same as that used in Vallès *et al.* (2003). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). ITS4 and 18SETS were used as sequencing primers, and direct sequencing of the amplified DNA fragments was performed using the Big Dye Terminator Cycle sequencing v3.1 (PE Biosystems, Foster City, CA, USA). Nucleotide sequencing was carried out at the Serveis Cientificotècnics at the Universitat de Barcelona, on an ABI PRISM 3700 DNA analyser (PE Biosystems). DNA sequences were edited using Chromas 1.56 (Technelysium PTy) and aligned visually. We were not able to amplify the above-cited DNA regions in *A. anomala, A. californica* and *A. pauciflora*, and these species are therefore not present in the phylogenetic analysis. The sequence alignment matrix is available from the corresponding author, and Genbank accession numbers and specimen provenances are given in Appendix S1.

Phylogenetic and statistical analyses

Phylogenetic analyses were carried out using Bayesian inference (BI). ITS and ETS data were analysed separated and then combined. To determine models under the Akaike and hLRT information criteria (Posada & Crandall 1998; Posada & Buckley 2004), the combined dataset was analysed using MrModeltest 2.2 (Nylander 2004). The model GTR+G+I best fitted our data, and was used to perform the Bayesian analysis with MrBayes 3.1.1 (Huelsenbeck & Ronquist 2001). Four Markov chains were run simultaneously for 1,000,000 generations, and these were sampled every 100 generations. Data from the first 1000 generations were discarded as the *burn-in* period, after confirming that likelihood values had stabilised prior to the 1000th generation. Posterior probabilities were estimated through the construction of a 50% majority rule consensus tree. The outgroup species, *Ajania fastigiata*, *Dendranthema zawadskii*, *D. maximoviczii*, *Kaschgaria brachanthemoides* and *K. komarovii* were chosen on the basis of previous works (Vallès *et al.* 2003; Sanz *et al.* 2008).

Analyses between mean DNA content of the different selected groups were performed using the phylogenetically-based generalised least squares (PGLS) algorithm, as implemented in the PHYLOGR R package, as well as Kruskal–Wallis and *t*-tests for comparative purposes. Note that statistics do not include annual species; given that these 11 species present heterogeneous behaviour at phylogenetic, genome size and cytogenetic levels (Pellicer J., Garnatje T., Hidalgo O., Kondo K., Vallès J., unpublished results), and these results will be fully discussed in a future study.

Various approaches were chosen for modelling the relationship between ploidy level and DNA content. First, to discard a possible linear relationship, we tested the quadratic coefficient of a polynomial function $y = \beta_0 + \beta_1 x + \beta_2 x^2$, to search for significance. However, the quadratic function provides a parabola, meaning that genome size increases with ploidy level until a maximum and then starts to decrease, which is difficult to explain in biological terms. Therefore, alternative curvilinear models were investigated and, in particular the Michaelis–Menten function $y = \beta_0 x/(\beta_1 + x)$, which is well known in enzyme kinetics, provides a model for the asymptotic pattern discussed in this paper, since y has an upper limit β_0/β_1 when x tends to infinity. Provided that these equations were fitted by a least squares algorithm, the fit could be assessed by the respective percentages of variance explained. The calculations were carried out with R (version 2.5.0), a public domain implementation of the S language (Venables & Ripley 2003).

RESULTS AND DISCUSSION

To provide a global overview of C value dynamics in *Artemisia*, genome size data obtained in the present study were combined with those previously reported (see Appendix S2). Only entities at specific and subspecific levels

and in which genome size had been calculated by flow cytometry were considered. In species where more than one population were assessed, we analysed differences between 2C values in order to detect any significant intraspecific variation. These differences did not exceed 4%, being predominantly below 3%, and thus their mean nuclear DNA content was used for calculations. Doležel & Bartoš (2005) stated that differences in genome size around 5% could be considered acceptable in some groups, assuming that a fraction of genuine variability in a species can exist.

2C values in the genus *Artemisia*

Table 1 gives the 2C and 1Cx values and ploidy levels estimated for the species and populations studied in picograms and megabase pairs, and a histogram illustrating the results obtained by flow cytometry is presented in Fig. 1. The 2C DNA content measured varied about 7.4-fold, from 4.20 pg in *Artemisia dolosa* (2n = 2x = 18) to 31.14 pg in *A. medioxima* (2n = 16x = 144); monoploid genome size varied 2.3-fold, from 1Cx = 1.93 pg in *A. medioxima* to 4.44 pg in *A. xerophytica*.

Genome size variation within and between groups in the phylogeny

Three major lineages in *Artemisia* appeared in the phylogenetic tree (Fig. 2; clades A, B and C). Clade A basically comprised species from the subgenus *Dracunculus* in its current circumscription plus the closely related *Mausolea eriocarpa* (originally described as *Artemisia eriocarpa*). The 2C values of the diploids in the group ranged from 4.2 pg in *A. dolosa* to 6.54 pg in *A. arenaria*, while tetraploid ranged from 8.46 pg in *A. desertorum* to 11.97 pg in *A. glauca*. In clade B representatives of subgenus *Artemisia* are embedded, with the exception of *Filifolium sibiricum*, which appears to be closely related to this small complex although its genome size (2n = 2x; 2C = 9.44 pg; Garcia *et al.* 2004b) is larger than its diploid related species [*A. atrata* (2C = 7.50 pg); *A. laciniata* (2C = 7.93 pg)]. The tetraploid species also included in the clade had 2C values of 14.81 and 14.6 pg, suggesting that their diploid ancestors had a similar nuclear DNA content (around 7–8 pg) to those found in their current diploid relatives.

Clade C comprised the remaining subgenera, agreeing with the phylogenetic groups previously noted by Watson *et al.* (2002), Vallès *et al.* (2003) and Sanz *et al.* (2008). This notwithstanding, the distinct lineages are only coincident with the traditional systematics at subgeneric level at some points. Clade C1, which mainly included species originally placed in the subgenus *Absinthium*, is divided into two monophyletic groups. In addition, species considered to belong to this group (*A. lagocephala*, *A. kruhsiana* and *A. lithophila*) appear segregated as sister group to the main clade of subgenera *Artemisia* (Fig. 2, C5). In this sense, misplacements of species of subgenus *Artemisia* in subgenus *Absinthium* have also previously been reported in species such as *A. judaica* and *A. lucentica* (Torrell *et al.* 1999; Vallès *et al.* 2003). Genome size of diploid species phylogenetically comprising this group (C1) ranged from 5.25 pg in *A. frigida* to 11.52 pg in *A. judaica*, and tetraploids ranged from 9.41 pg (*A. frigida*) to 13.59 pg (*A. splendens*). The reduced number of species with incorrect placement (three) did not allow statistical testing, but these species tend to have larger 2C values than their related species. In contrast, genome sizes of the three *Absinthium* species located as sister group to *Artemisia* in clade C5 (2C 5.85–6.75 pg) fall within the range of C values of both *Artemisia* (C5) and *Absinthium* (C1) clades. This makes it impossible to discriminate misplacements on the basis of divergent genome sizes.

The subgenus *Artemisia* is paraphyletic and, as mentioned above, appears segregated in the phylogenetic tree into three main groups (Fig. 2; clades B, C2, C5). Genome size of diploid species placed in the largest clade (C5) ranged from 5.39 pg in *A. feddei* to 8.75 pg in *A. argyi*, and from 9.11 pg (*A. momiyamae*) to 12.99 pg (*A. mongolica*) in the tetraploid species. Between these three lineages, only C5 includes x = 8 specimens. This clade is the most heterogeneous in terms of 2C values, compared with the other two *Artemisia* groups (B and C2), and the presence of both x = 8 and x = 9 species intermixed may contribute to this larger degree of C value variability. Not significantly, but meaningfully different (P = 0.051), genome sizes have been found among the diploids included in those three lineages (B, C2, C5), mainly between clade B and the two remaining clades.

Two of the most homogeneous clades are C4 (Fig. 2), which holds the North American endemic species (subgenus *Tridentatae*), and C3 (Fig. 2), comprising representatives of subgenus *Seriphidium*. The 2C values for diploids in these *Seriphidium* ranged from 5.10 pg in *A. taurica* to 6.66 pg in *A. caerulescens*, and represented one of the most homogeneous groups in terms of genome size data, as well as cytological (Torrell *et al.* 2003) and DNA sequence similarity (Sanz *et al.* 2008). The subgenus *Tridentatae* was thoroughly studied by Garcia *et al.* (2008), where the narrow range of genome size variation between its representatives was pointed out, revealing the North American group as one of the most uniform at this level.

The distribution of genome size data for diploid representatives is consistent with the different lineages that were phylogenetically defined, but cannot be used to discriminate between clades because of a great overlap in C values between some of these groups. Bearing in mind that the range of 2C values obtained was influenced by sample size, possible hybridization events or simply random chance, the subgenera *Dracunculus*, *Seriphidium* and *Tridentatae*, in which molecular and traditional systematic delimitations are basically congruent, showed a narrow range of 2C values (Fig. 3), *i.e.* their genome sizes are quite homogenous.

Related genera

The generic delimitation of *Artemisia* has long been controversial, and exclusion of several small genera such as *Filifolium*, *Crossostephium* and *Mausolea* from the genus is questionable, at least when it is known that some of them

Table 1. Nuclear DNA content and karyological information of the populations studied, grouped by subgenera on the basis of their traditional classification.

taxa	number of individuals	2n[1]	ploidy level	2C (pg)[2]	2C (Mbp)[3]	1Cx (pg)[4]	HPCV (%)[5]	standard
subgenus *Absinthium*								
A. absinthium	5	18[a]	2x	8.67 (0.11)	8.479	4.34	2.37	*Petunia*
A. argentea	4	36	4x	9.91 (0.14)	9.691	2.48	2.42	*Pisum*
A. austriaca	3	16	2x	5.73 (0.09)	5.603	2.87	2.21	*Petunia*
A. austriaca	3	48	6x	16.72 (0.22)	16.352	2.79	1.43	*Pisum*
A. austriaca	4	16	2x	5.68 (0.06)	5.555	2.84	1.53	*Petunia*
*A. caespitosa**	5	18	2x	6.69 (0.23)	6.542	3.35	2.00	*Pisum*
*A. czekanowskiana**	5	89	10x	23.45 (0.28)	22.934	2.35	0.24	*Triticum*
A. frigida	5	36	4x	9.41 (0.14)	9.202	2.35	3.42	*Pisum*
*A. hololeuca**	5	18[a]	2x	7.81 (0.12)	7.638	3.91	2.65	*Petunia*
*A. kruhsiana**	5	18	2x	5.85 (0.25)	5.721	2.93	6.04	*Petunia*
A. lagocephala	5	54	6x	17.24 (0.25)	16.860	2.87	1.74	*Pisum*
*A. lithophila**	5	18	2x	6.38 (0.11)	6.239	3.19	5.95	*Pisum*
*A. niitakayamense**	4	18	2x	4.39 (0.08)	4.293	2.20	2.08	*Petunia*
*A. schmidtiana**	3	18[a]	2x	5.74 (0.11)	5.613	2.87	3.01	*Petunia*
*A. sericea**	5	90	10x	24.71 (0.32)	24.166	2.47	0.86	*Triticum*
A. sericea	5	90	10x	23.95 (0.24)	23.423	2.40	0.95	*Triticum*
A. sericea	5	90	10x	23.33 (0.25)	22.816	2.33	0.97	*Triticum*
*A. xerophytica**	5	18	2x	8.88 (0.12)	8.684	4.44	1.87	*Petunia*
subgenus *Artemisia*								
A. abrotanum	5	18	2x	5.76 (0.03)	5.633	2.88	2.59	*Petunia*
A. abrotanum	5	36	4x	11.03 (0.11)	10.787	2.76	1.58	*Pisum*
*A. anomala**	3	18	2x	8.20 (0.06)	8.019	4.10	2.64	*Petunia*
*A. argyi**	2	18	2x	8.85 (0.20)	8.655	4.43	2.31	*Petunia*
A. argyi	5	18[a]	2x	8.65 (0.13)	8.459	4.32	1.05	*Petunia*
*A. atrata**	5	18	2x	7.50 (0.10)	7.335	3.75	4.50	*Petunia*
*A. californica**	4	18	2x	8.77 (0.07)	8.577	4.39	2.95	*Petunia*
A. californica	3	18	2x	8.42 (0.08)	8.234	4.21	2.54	*Petunia*
*A. freyniana**	5	18	2x	5.51 (0.09)	5.467	2.80	1.72	*Petunia*
*A. feddei**	5	16[a]	2x	5.39 (0.16)	5.271	2.70	1.54	*Petunia*
*A. gmelinii**	5	36[a]	4x	14.53 (0.23)	14.210	2.42	2.23	*Pisum*
*A. kawakamii**	4	18	2x	5.48 (0.15)	5.359	2.74	1.64	*Petunia*
*A. keiskeana**	5	18[a]	2x	7.29 (0.07)	7.129	3.65	1.73	*Pisum*
*A. koidzumii**	5	36[a]	4x	10.56 (0.09)	10.327	2.64	1.93	*Pisum*
*A. laciniata**	5	18[a]	2x	8.04 (0.14)	7.960	4.07	3.96	*Petunia*
A. laciniata	5	18	2x	7.82 (0.13)	7.647	3.91	4.31	*Pisum*
*A. lactiflora**	3	36	4x	11.51 (0.29)	11.256	2.88	3.18	*Pisum*
A. lactiflora	3	54	6x	14.96 (0.12)	14.630	2.49	3.00	*Pisum*
*A. leucophylla**	5	16[a]	2x	5.50 (0.08)	5.379	2.75	2.78	*Petunia*
*A. macrantha**	5	108	12x	26.63 (0.29)	26.044	2.22	2.38	*Triticum*
*A. medioxima**	5	36	4x	14.60 (0.21)	14.278	3.65	1.79	*Pisum*
A. medioxima	5	144	16x	31.14 (0.39)	30.454	1.95	0.95	*Triticum*
A. medioxima	5	144[a]	16x	30.88 (0.38)	30.200	1.93	1.27	*Triticum*
*A. messerschmidtiana**	5	54[a]	6x	14.71 (0.26)	14.386	2.45	2.38	*Pisum*
A. messerschmidtiana	5	36[a]	4x	10.53 (0.13)	10.298	2.63	2.63	*Pisum*
*A. michauxiana**	4	18	2x	7.50 (0.03)	7.335	3.75	2.55	*Pisum*
*A. momiyamae**	3	18	2x	9.11 (0.09)	8.909	4.56	2.01	*Petunia*
*A. mongolica**	5	34[a]	4x	12.99 (0.16)	12.704	3.25	2.74	*Pisum*
*A. montana**	5	54[a]	6x	14.93 (0.20	14.601	2.48	2.09	*Pisum*
*A. pontica**	5	18[a]	2x	4.59 (0.16)	4.489	2.30	4.86	*Petunia*
A. princeps	5	34	4x	9.06 (0.26)	8.860	2.02	1.95	*Petunia*
A. princeps	5	34	4x	9.48 (0.26)	9.271	2.37	1.75	*Petunia*
*A. rubripes**	5	18[a]	2x	5.57 (0.12)	5.447	2.79	2.94	*Petunia*
*A. selengensis**	5	36	4x	13.36 (0.40)	13.066	3.34	2.06	*Pisum*
A. selengensis	5	36[a]	4x	13.16 (0.16)	12.870	3.29	1.62	*Pisum*
*A. stolonifera**	5	36[a]	4x	10.53 (0.08)	10.298	2.63	1.72	*Pisum*

Table 1. Continued.

taxa	number of individuals	2n[1]	ploidy level	2C (pg)[2]	2C (Mbp)[3]	1Cx (pg)[4]	HPCV (%)[5]	standard
*A. subulata**	5	54	6x	12.45 (0.19)	12.176	2.08	2.12	*Pisum*
*A. sylvatica**	5	16	2x	5.58 (0.13)	5.457	2.79	2.21	*Petunia*
*A. tanacetifolia**	5	36	4x	14.81 (0,25)	14.484	3.70	1.19	*Pisum*
*A. umbrosa**	5	50	6x	12.39 (0.20)	12.117	2.07	0.70	*Pisum*
A. umbrosa	5	54	6x	12.73 (0.31)	12.449	2.12	1.05	*Pisum*
subgenus *Dracunculus*								
A. arenaria	5	36	4x	10.53 (0.11)	10.298	2.63	2.25	*Pisum*
A. arenaria	5	18	2x	6.54 (0.16)	6.396	3.27	2.68	*Pisum*
A. campestris	5	54[a]	6x	15.04 (0.08)	14.709	2.51	1.96	*Pisum*
*A. commutata**	5	18	2x	6.05 (0.22)	5.916	3.03	0.99	*Pisum*
A. commutata	5	36	4x	9.00 (0.11)	8.802	2.25	1.32	*Petunia*
A. depauperata	5	36	4x	8.75 (0.26)	8.557	2.19	2.02	*Petunia*
A. desertorum	5	36	4x	8.66 (0.31)	8.469	2.17	2.12	*Petunia*
*A. dolosa**	5	18	2x	4.20 (0.08)	4.107	2.10	1.76	*Petunia*
A. dracunculus	5	36	4x	11.90 (0.21)	11.638	2.98	2.42	*Pisum*
A. glauca	5	18	2x	5.66 (0.06)	5.535	2.83	2.59	*Petunia*
A. marschalliana	5	36	4x	10.57 (0.09)	10.337	2.64	3.00	*Pisum*
A. monostachya	5	36	4x	8.91 (0.12)	8.713	2.23	1.25	*Petunia*
*A. pamirica**	4	18	2x	6.03 (0.24)	5.897	3.02	3.25	*Pisum*
*A. sphaerocephala**	5	18	2x	5.52 (0.16)	5.398	2.76	1.79	*Petunia*
subgenus *Seriphidium*								
*A. cretacea**	3	18	2x	6.30 (0.06)	6.161	3.15	1.85	*Pisum*
*A. ferganensis**	5	36	4x	10.47 (0.35)	10.239	2.62	0.85	*Pisum*
*A. gobica**	5	18[a]	2x	5.49 (0.17)	5.369	2.75	5.67	*Petunia*
*A. inculta**	5	18	2x	5.71 (0.15)	5.584	2.86	2.68	*Petunia*
*A. maritima**	5	54	6x	15.54 (0.21)	15.198	2.59	3.00	*Pisum*
*A. nitrosa**	5	36	4x	10.54 (0.25)	10.308	2.64	2.47	*Pisum*
*A. pauciflora**	5	18	2x	5.00 (0.05)	4.890	2.50	2.56	*Petunia*
*A. ramosa**	5	54	6x	14.53 (0.29)	14.210	2.42	0.97	*Pisum*
*A. schrenkiana**	5	18	2x	5.19 (0.10)	5.075	2.60	1.13	*Petunia*
*A. taurica**	4	18	2x	5.10 (0.08)	4.987	2.55	3.42	*Petunia*
*A. vallesiaca**	4	36	4x	9.13 (0.21)	8.929	2.28	2.12	*Petunia*
genus Crossostephium								
*C. chinense**	5	18[a]	2x	2.54 (0.09)	2.484	1.24	1.99	*Pisum*

The order of species is the same as in Table S1.

*Indicates species in which nuclear DNA content was assessed for the first time. [1]Somatic chromosome number ([a] indicates new count). [2]Nuclear DNA content [2C value (standard deviation)]. [3]Nuclear DNA content; 1 pg = 978 Mbp (Doležel *et al.* 2003). [4]Monoploid genome size. [5]Mean half-peak coefficient of variation.

were previously recognised as *Artemisia* species. In the light of molecular studies (Watson *et al.* 2002; Sanz *et al.* 2008), the re-inclusion of these genera in *Artemisia* appears plausible and is required under cladistic principles. Thus, the C value obtained in *Crossostephium* is the smallest found in a diploid population of the *Artemisiinae*, and a future re-inclusion of all the segregated genera in *Artemisia* would highlight the genus as one of the most diverse in terms of genome size, with differences of about 4.5-fold among diploid populations.

Genome size and ploidy level

As two basic chromosome numbers have been reported in *Artemisia*, a comparison of nuclear DNA content between diploid x = 8 and x = 9 species was carried out (Table 2), but differences were not found to be significant ($P > 0.05$) in either ordinary or phylogenetically-based generalised least squares algorithm tests. In order to elucidate whether the nuclear DNA content of a given diploid species limits its 'ability' to evolve into a polyploid series, we searched for differences among strict and non-strict diploids, but no significance was found (Table 2).

The 2C values of species known exclusively at the tetraploid level were significantly different from tetraploid taxa also reported at different ploidy levels (Table 2). In the case of hexaploids, while the ordinary test showed significant differences between taxa currently only known as hexaploids and those with different reported ploidy levels, the PGLS algorithm was not significant (Table 2). For both of these ploidy levels, exclusively polyploid species had smaller mean genome sizes.

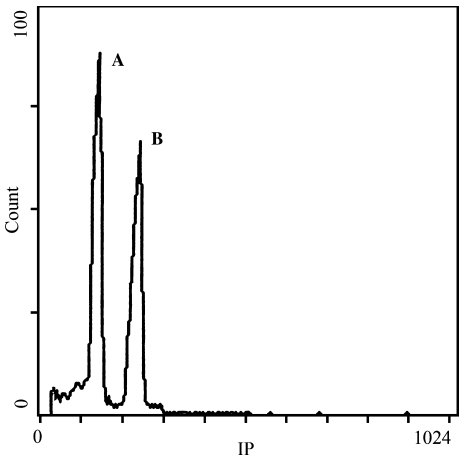

Fig. 1. Fluorescence histogram of the genome size assessment for *Artemisia dolosa*. A. Peak of the standard *Petunia hybrida* (2C = 2.85 pg; HPCV = 2.90). B. Peak of *A. dolosa* (2C = 4.20 pg; HPCV = 1.86).

Non-proportional increase of genome size in polyploids

When all *Artemisia* accessions available were considered, mean nuclear DNA content increased with ascending ploidy level, but not proportionally, as enlargement of the difference between observed and expected 2C values indicates (Fig. 4). Hence, monoploid genome size (1Cx value) is also a parameter that is quite sensitive to polyploidisation in this genus. Changes at this level are smaller, with mean 1Cx ranging from 3.54 pg in diploids (2x) to 1.93 in the hexaidecaploid (16x) population of *A. medioxima*. This genome downsizing after polyploidisation, which appears to be a general trend in angiosperms (Kellogg & Bennetzen 2004; Leitch & Bennett 2004), is particularly apparent in genera such as *Artemisia*, in which the highest ploidy levels within the Asteraceae have been described (Malakhova 1990; Pellicer *et al.* 2007b). Several studies have highlighted the genomic changes that can occur during and after polyploid formation, pointing towards a possible need for harmonisation of genome constituents after joining and removal of some unnecessary genomic redundancies (Petrov 2001; Bennetzen *et al.* 2005). Mechanisms leading to a decrease in genome size in polyploids may include non-random elimination of specific low-copy sequences (Feldman *et al.* 1997) and chromosome- and genome-specific sequences (Ozkan *et al.* 2001; Shaked *et al.* 2001), illegitimate crossing over (Devos *et al.* 2002) or unbalanced deletion–insertion rates (Petrov 2001, 2002). It has been suggested that the extra DNA in the genome arising from polyploidy may be eliminated when it imposes excessive metabolic cost (Gregory & Hebert 1999). Counterbalancing mechanisms are probably also involved to reduce the genetic and structural instabilities that accompany DNA loss.

As stated above, mean 2C values of the different species studied grouped by ploidy level are, in all but one case, lower than expected if this gain is proportional to the ploidy level (Fig. 4). The exception is *A. cana* (2n = 8x = 72, 2C = 27.04 pg; Garcia *et al.* 2008), the single representative at the octoploid level (from subgenus *Tridentatae*). This non-concordant behaviour can be explained if we remember that this subgenus has the highest mean nuclear DNA amount in the genus (Garcia *et al.* 2008), so the available sample at the octoploid level does not represent variability in the whole genus.

When changes in genome size of polyploids were evaluated at the species level, a generalised trend towards genome downsizing was also detected between diploid and tetraploid cytotypes (Fig. 5). Exceptions to this are found in the tetraploid species *A. abrotanum*, *A. dracunculus*, *A. glauca*, *A. marschalliana* and *A. frigida*, where only slight differences between theoretic expected genome size (considering a DNA gain proportional to ploidy level increase from 2x to 4x) and observed values in the tetraploids after a polyploidisation have been detected. As there is no available information about the age and origin of these polyploid species, it is difficult to propound any conclusive hypothesis on a possible relationship between genome size changes and time of polyploid formation. In the genus *Nicotiana*, polyploid species showing genome downsizing (*N. tabacum*, *N. rustica*), together with others showing additivity (*N. clevelandii*, *N. quadrivalvis*) have also been reported. In this genus, genome size increase has been related to species age, ancient species showing DNA addition while younger species show genome downsizing (Leitch *et al.* 2008).

Although a degree of heterogeneous behaviour was observed in this first stage of polyploidisation (4x), when higher ploidy levels are attained the trend towards genome downsizing becomes more obvious. One species that clearly illustrates these changes is *A. medioxima*. Estimates from different populations of this taxon gave two different cytotpes (2n = 4x and 2n = 16x); the nuclear DNA content assessed for the tetraploid population was 2C = 14.60 pg, whereas the hexaidecaploid has a mean 2C = 31.01 pg. The 2C value obtained for the second population is about 47% less than expected if the DNA gain was proportional to ploidy level increase. A possible explanation for the substantial fraction of DNA elimination at high ploidy levels could be that mechanisms inducing a genomic constraint are more efficient when polyploidisation is attained at these upper stages, mainly in allopolyploid species.

An upper limit for genome size in Artemisia

The quantitative changes observed in genome size lead us to hypothesise the existence of an upper limit for the DNA content. In order to dismiss a linear relationship between number of chromosomes and DNA content within polyploids, a quadratic function was tested ($y = 1.512 + 0.314x - 0.0007x^2$). As expected, the quadratic coefficient was significant ($P = 0.002$), confirming non-linearity of the dataset. The Michaelis–Menten model, another curvilinear function [$y = 64.106x/(151.494 + x)$], yielded a satisfactory coefficient ($P < 0.001$), also accounting for a likely asymptotic pattern.

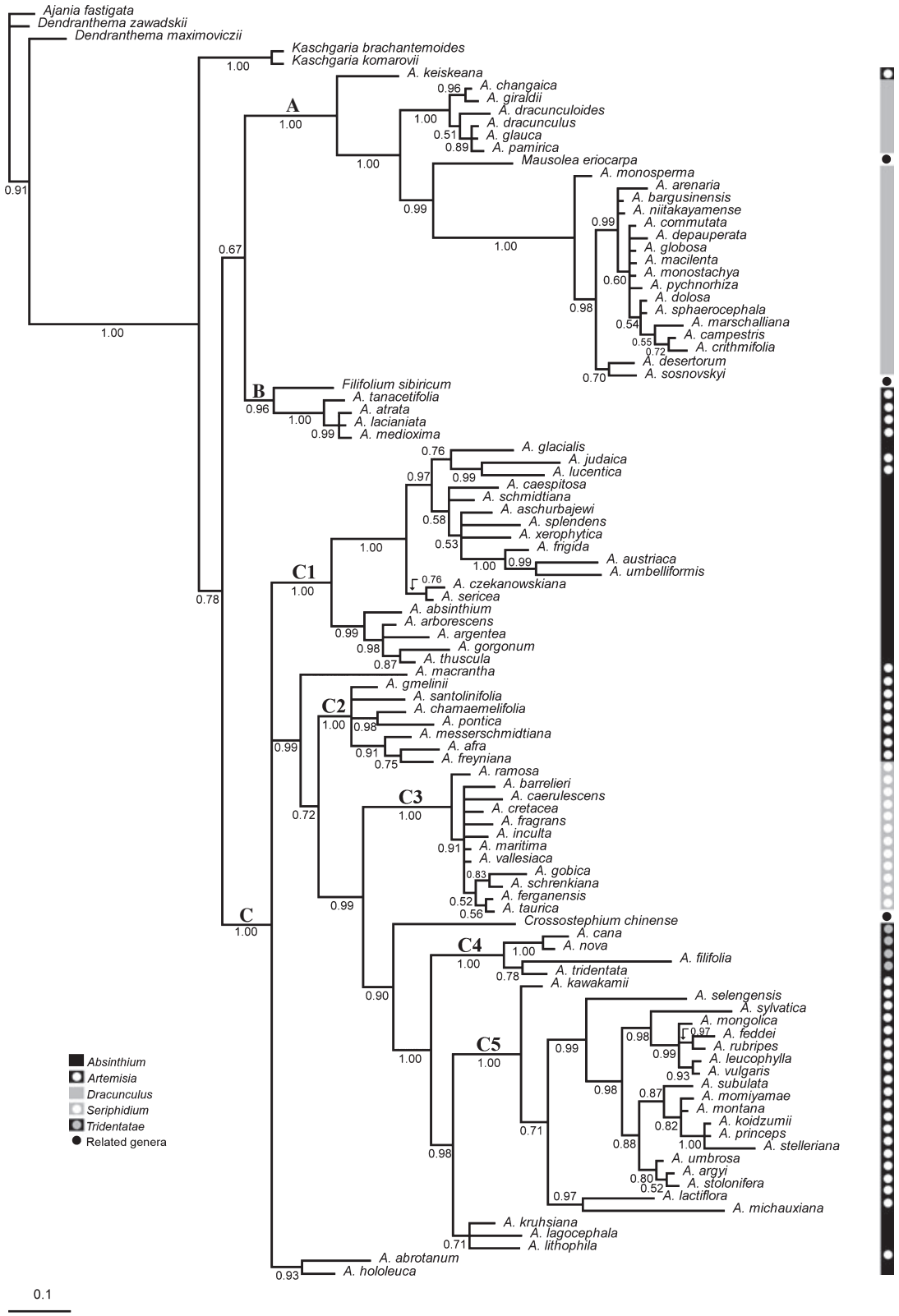

Fig. 2. Phylogenetic tree resulting from the Bayesian analysis (PP values are indicated above and below branches), with indication of traditional subgeneric classification.

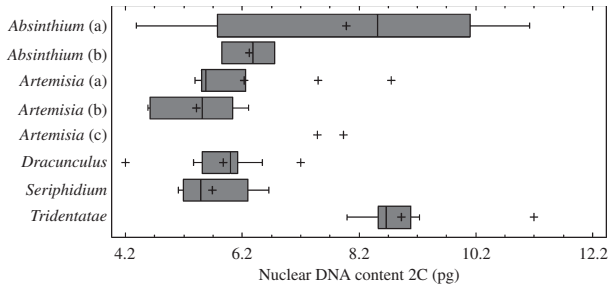

Fig. 3. Distribution of genome size (2C values of diploid taxa) in *Artemisia* according to phylogenetic lineages and with traditional classification. Note: *Absinthium*: **a**, core of the subgenus (clade C1); **b**, *A. lagocephala* complex (sister group of clade C5). *Artemisia*: **a**, *A. vulgaris* and relatives (clade C5); **b**, *A. atrata* complex (clade B); **c**, *A. afra* group (clade C2). *Dracunculus* (clade A). *Seriphidium* (clade C3). *Tridentatae* (clade C4).

Table 2. Mean 2C values and results of comparisons, using *t*-tests and phylogenetically-based generalised least squares (PGLS) algorithm.

	mean 2C	*t*-test	PGLS test
basic chromosome number			
x = 8	7.02	P = 0.607	P = 0.483
x = 9	6.66		
diploid species			
1	6.99	P = 0.282	P = 0.370
2	6.39		
tetraploid species			
1	10.26	P = 0.025*	P = 0.033*
2	11.85		
hexaploid species			
1	13.88	P = 0.041*	P = 0.518
2	15.51		

Note: 1; species known exclusively at a specific ploidy level, 2; species that evolved into polyploid series; *, indicates statistically significant values.

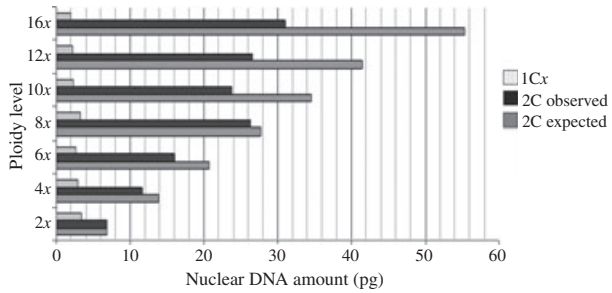

Fig. 4. Evolution of DNA content at different ploidy levels in the genus *Artemisia*.

The trend towards a reduction in genome size at 1Cx level in polyploid *Artemisia* observed in this study suggests the existence of an upper limit for nuclear DNA content (Fig. 6). Previous studies established correlations between cell size, nucleus size and C value (Mirsky & Ris 1951; Price & Bachmann 1976; Bharathan 1996; Vinogradov 2003). It would be interesting to examine the existence of limiting factors to genome expansion. In this study, our approach to elucidating the possible existence of an upper limit to genome size tied to ploidy level was based on testing the coefficients of different regression models.

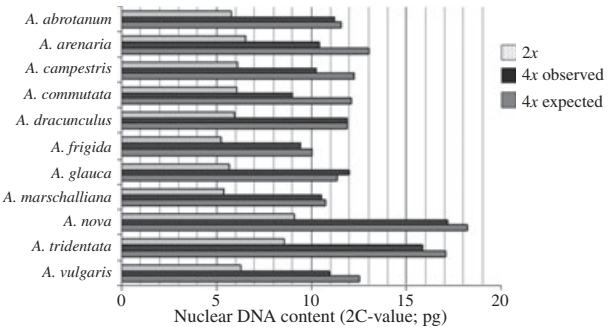

Fig. 5. Evolution of genome size between diploid and tetraploid levels in representatives of genus *Artemisia* with both ploidy levels available.

Based on the Michaelis–Menten model, a species with 2n = 18x = 162 chromosomes would have a 2C value of about 32.35 pg, which would be half the upper limit theoretically attainable, and similar to the maximum value known for the genus (*A. medioxima* with 2n = 16x = 144, mean 2C = 31.01 pg). Thus, we can expect that with increasing ploidy level, genome size would approach asymptotically to a theoretical maximum, as if it followed a saturation pattern (Fig. 6). Accordingly, it could be possible to establish a particular ploidy level for this maximum genome size by fitting mathematical models, but we think that it is practically impossible to determine exactly either this upper limit or the maximum ploidy level that *Artemisia* could achieve because we cannot predict which will be the maximum ploidy level viable in nature. Moreover, until now no ploidy level higher than 16x has been reported in the literature for *Artemisia*. It would be interesting to examine whether other plant groups follow the same behaviour in terms of DNA loss in their own polyploid series.

CONCLUSION

Although several molecular studies in the genus *Artemisia* have been undertaken (Torrell *et al.* 1999; Watson *et al.* 2002; Vallès *et al.* 2003; Sanz *et al.* 2008; Tkach *et al.* 2008) to elucidate its systematic relationships, the phylogenetic position of some species is still not clear. It seems that, in general, the well-defined clades in the phylogenies do present homogeneous, similar genome size data, whereas conflicting, non-resolved subgenera with species dispersed in different clades along the phylogenetic tree have more fluctuating genome size values among their species.

Hence, the degree of heterogeneity observed at C value level in some of the currently established subgenera (*e.g.*

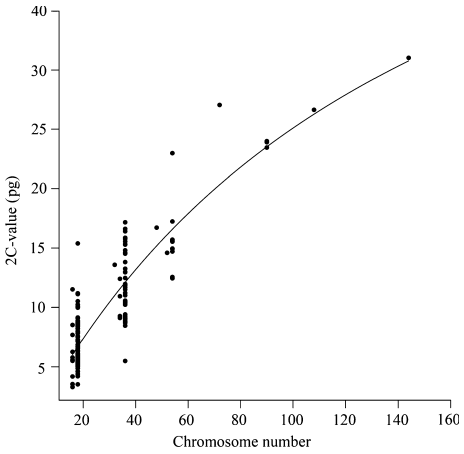

Fig. 6. Nuclear DNA content *versus* chromosome number.

Absinthium, *Artemisia*) indicates that in spite of all previous research, a complete phylogenetic framework that reflects natural relationships is not yet fully achieved. Furthermore, evolutionary forces such as genetic drift, hybridisation events, genome introgression and environmental changes may influence placement of the species and also genome size variation. The genome size dataset should be taken into account in establishment of a definite structuring of the genus.

Different patterns of genome size variation linked to different kinds of evolutionary mechanisms have been reported (Bennetzen & Kellogg 1997; Soltis *et al.* 2003), sometimes pointing towards genomic reduction, or to an increase during speciation and diversification processes. The current study of natural and synthetic polyploids has led to a better understanding of changes that take place at chromosome and genome level during polyploidy (Levy & Feldman 2004; Kovařík *et al.* 2005; Dadejová *et al.* 2007), although many of the features involved in molecular aspects are still unresolved. Our results also indicate a clear reduction in monoploid genome size in the formation of polyploid species in *Artemisia*, which is most apparent when the highest ploidy levels are reached. We have established the possibility of approaching a theoretical upper limit for nuclear DNA content in this genus, and we proposed a mathematical model that satisfactorily describes the observed decrease at 1Cx level with increasing polyploidy. In summary, the correlations observed between C value data and polyploidy reveal genome size as a valuable tool for better understanding of the evolutionary processes in plants.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Genbank numbers of species included in the phylogenetic analysis. For species sequenced for the first time, voucher references and/or collection numbers are indicated.

Appendix S2. Published chromosome numbers and C values of different representatives of the genus *Artemisia* used for calculations.

Table S1. Provenance of the populations studied.

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Origen i evolució de les espècies endèmiques sud-americanes d'*Artemisia* (*Asteraceae*): evidències provinents de dades de filogènia molecular, del DNA ribosòmic i de la mida del genoma

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Hom creu que el gènere *Artemisia* va arribar a Amèrica del Nord des d'Àsia a través de l'estret de Bering a les darreries del terciari, però la posició sistemàtica de les espècies endèmiques sud-americanes del gènere, així com les vies de migració cap al sud no han estat estudiades. Hem usat seqüències del DNA nuclear per a aclarir les relacions específiques entre les *Artemisia* sud-americanes i també amb les restants espècies del gènere, així com hibridació *in situ* fluorescent i estimacions de la mida del genoma per a caracteritzar aquest complex poliploide. La major part de les espècies del gènere endèmiques d'Amèrica del Sud s'agrupa en una clada monofilètica que s'insereix en la clada d'espècies endèmiques americanes, amb l'excepció d'*A. magellanica*, que apareix segregada de la resta, tot constituint una clada amb *A. biennis*. La hibridació *in situ* fluorescent i les dades de mida del genoma revelen que, contràriament a allò esperat i comú en el gènere estudiat i en molts altres grups de plantes, la quantitat de DNA nuclear monoploide roman força constant al llarg dels diferents nivells de ploïdia i hi ha un increment proporcional dels *loci* del DNA ribosòmic, una dinàmica poc corrent en el gènere. Els resultats han sigut discutits a la llum dels processos evolutius que s'esdevenen en les plantes, i hem proposat una hipòtesi per a la diferenciació i la migració de les espècies sud-americanes d'*Artemisia*.

Origin and evolution of the South American endemic *Artemisia* species (Asteraceae): evidence from molecular phylogeny, ribosomal DNA and genome size data.

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Abstract

Artemisia is thought to have reached the Americas across the Bering Strait from Asia during the late Tertiary, but the systematic position of the South American endemic species and the migration ways towards the South have not yet been studied. We used nuclear DNA sequences to unravel specific relationships between the South American *Artemisia* and also with the remaining species of the genus, as well as fluorescent *in situ* hybridization and genome size assessments to characterize this polyploid complex. Most species of this complex group in a monophyletic clade, merged in the American endemics clade, with the exception of *Artemisia magellanica*, which appeared segregated, constituting a clade together with *Artemisia biennis*. Fluorescent *in situ* hybridization and genome size data revealed that, contrary to what might expect, monoploid genome size remained quite constant along the ploidy levels and a proportional increase of ribosomal loci was detected, a dynamics not usually found in the genus. The results have been discussed in the light of evolutionary processes which occur in plants, and plausible origins for the South American endemic species have been hypothesized.

Key words: American endemic species, Compositae, DNA amount, FISH, sequencing, 2C values

Introduction

The genus *Artemisia* L. (Asteraceae, Anthemideae) is a worldwide distributed genus, growing in the temperate zones of the Northern Hemisphere, with few species spread across the Southern Hemisphere (Bremer and Humphries 1993; Kubitzki 2007, Oberprieler *et al*. 2009). The mechanisms and migration pathways followed by *Artemisia* to colonize new habitats have been formerly hypothesized. On the one hand, the Asian species belonging to the subgenus *Seriphidium* have been suggested as possible ancestors of the North American endemic *Artemisia* and a likely migration pathway across the Bering strait has been pointed out (Ling 1991, 1995), and on the other hand McArthur and Plummer (1978) and McArthur *et al*. (1981), agreeing with this migration route, proposed that the herbaceous members of the subgenus *Artemisia* could have been differentiated in North America during the Pleistocene in response to climatic changes, originating the species of the subgenus *Tridentatae* and other endemics. The origin of the *Tridentatae* from subgenus *Artemisia* is also supported by Jeffrey (1995) on a phytochemical basis.

In the Southern Hemisphere a complex of South American endemic species of *Artemisia* exists, occurring basically in Argentina (Ariza 1997), but a few of them reaching Chile (Zuloaga *et al*. 2008). These taxa are *A. copa* Phil. var. *copa*, *A. copa*. var. *trifida* Acevedo, *A. echegarayi* Hieron., *A. magellanica* Sch. Bip., *A. mendozana* DC. var. *mendozana* and *A. mendozana* DC. var *paramilloensis* F.A. Roig et J. A. Ambrosetti. The knowledge of these species is scarce, except in the case of *A. mendozana*, which was assigned to the genus *Seriphidium*, by Candolle (1837) and later renamed as *Seriphidium mendozanum* by Bremer and Humphries (1993) in their revision of the Anthemideae without taking into account the opinions of Roig (1982) and Roig and Ambrosetti (1989) who reported the presence of heterogamous capitula. These plants are perennial shrubs or subshrubs. In the case of *A. magellanica*, although being described as perennial (Cabrera 1971; Ariza 1997), has been also reported to behave as a biennial/perennial herb (J. Molero, pers. obs.). This species is restricted to a few provinces of Argentina and has clear ecological and environmental requirements (e.g. altitude ranges or soil structure).

Although the knowledge of these species at a molecular systematic level is poor, studies concerning their traditional uses are common in the literature (Manfred 1959; Ratera and Ratera 1980; Roig 2002).

The South American endemic *Artemisia* species are of particular interest for the evolutionary history of the genus because of their geographical distribution and the scarcity of *Artemisia* taxa in the Southern Hemisphere. A phylogenetic circumscription of these species within the genus might shed light on their origin and the interspecific relationships within this polyploid complex. The ITS and ETS nuclear regions have been extensively used for phylogenetic reconstructions in the genus (Kornkven *et al.* 1999; Torrell *et al.* 1999; Vallès *et al.* 2003; Sanz *et al.* 2008; Tkach *et al.* 2008; Pellicer *et al.* 2009), as for many other plant groups (Baldwin *et al.* 1995; Baldwin and Markos 1998, Garnatje *et al.* 2005, 2007; Hidalgo *et al.* 2006), though not being exempt from disagreement because of problems derived from concerted evolution (see review on problems using ITS by Álvarez and Wendel 2003). Nevertheless, the combination of both regions represents a source of useful data for phylogenetic reconstructions, and leads us to suggest hypotheses worthy of further analysis. Other techniques which can be addressed to resolve evolutionary questions on plant genome organization are fluorescent *in situ* hybridization (FISH) with ribosomal DNA probes and genome size data (e.g. Vinogradov 2003; Pires *et al.* 2004; Bennett and Leitch 2005a, b; Price *et al.* 2005; Lim *et al.* 2007). In the genus *Artemisia*, these techniques have provided valuable tools for a better understanding of its systematics, as well as for the changes taking place at the genome size level (Torrell *et al.* 2003; Garcia *et al.* 2004, 2007, 2008, 2009; Hoshi *et al.* 2006; Pellicer *et al.* 2009).

The aims of the present study are addressed to shedding light on the origin and evolution of this group of endemic taxa, through the following specific points: i) to circumscribe and determine the phylogenetic relationships among the South American endemic *Artemisia* species; ii) to characterize the patterns of distribution and evolution of GC-rich bands and the ribosomal gene families; iii) to elucidate the evolutionary changes of rDNA and C values with the polyploidy

and, iv) to suggest a hypothesis about the evolution of this complex from the combination of all the obtained results.

Table 1. Provenance of the populations studied.

Taxa	Localities, collectors and herbarium vouchers
Artemisia copa Phyl.	Argentina. Salta. Los Andes Dept. San Antonio de los Cobres, on the way to the "tren de las nubes", Concordia mine. 4117 m. 19-III-2009. J. Molero & A. Rovira (BCN3324)
Artemisia copa Phyl.	Argentina. Salta. Los Andes Dept. Quiron slope, 25 km W from Santa Rosa de los Pastos Grandes. 3600 m. 15-II-2007. M. Dematteis 2549 (CTES)
A. echegarayi Hieron.	Argentina. Mendoza. Las Heras Dept. 5.3 Km NE from Uspallata, on the way to Villavicencio. 1800 m. 12-II-2007. M. Dematteis 2481 (CTES)
A. echegarayi Hieron.	Argentina. Mendoza. Las Heras Dept. Uspallata valley, on the way to Calingasta. 1900 m. 30-II-2007. M. Dematteis 2663 (CTES)
A. magellanica Sch. Bip.	Argentina. Santa Cruz. Lago Buenos Aires Dept. 79 km NW from the Perito Moreno. 5-II-1975. Boelcke *et al*. 16125 (LP)
A. magellanica Sch. Bip.	Argentina. N. Patagonia, Chubut Dept. Route 40 from Esquel to El Bolsón, km 1807, Madera stream (sandy bank with round slopes). 925 m. 15-III-2009. J. Molero & A. Rovira (BCN3326)
A. mendozana DC. var. *mendozana*	Argentina. Mendoza. Las Heras Dept. Natural Reserve of Villavicencio. Quebrada de Villavicencio. 1600 m. 30-IX-2006. J. Molero & A. Rovira (BCN3325)
A. mendozana DC. var. *mendozana*	Argentina. Mendoza. Las Heras Dept. Natural Reserve of Villavicencio. Quebrada del Toro. 2000 m. 30-IX-2006. J. Molero & A. Rovira (BCN3327)
A. mendozana DC. var. *paramilloensis* F.A. Roig & J. A. Ambrosetti	Argentina. Mendoza. Las Heras Dept. Moorlands of Uspallata hills. 3100 m. 12-II-2007. M. Dematteis 2459 (CTES)
A. mendozana DC. var. *paramilloensis* F.A. Roig & J. A. Ambrosetti	Argentina. Mendoza. Las Heras Dept. Moorlands of Uspallata, close to the Darwin's *Araucaria*. 2980m. 30-IX-2006. J. Molero & A. Rovira (BCN3328)

Materials and methods

Plant material

Table 1 shows the populations of *Artemisia* studied, with their location, collectors and herbarium vouchers. All the South American endemic *Artemisia* taxa have been studied, except the Chilean *A. copa* var. *trifida* of which we could not obtain material. Leaf material for cytometric assessments came from fresh leaves collected in the field, and chromosome counts were carried out from either achenes grown in plastic Petri dishes or plants cultivated in the greenhouse of the Institut Botànic de Barcelona (CSIC-ICUB). *Petunia hybrida* Vilm. 'PxPc6' (2C =

2.85 pg), *Pisum sativum* L. 'Express long' (2C = 8.37 pg) and *Triticum aestivum* L. 'Chinese spring' (2C = 30.90 pg) were used as internal standards (Marie and Brown 1993) to cover the range of 2C-values found. Seeds of the standards were provided by the Institut des Sciences du Végétal (CNRS), Gif-sur-Yvette (France).

Chromosome counts and protoplast preparation

Root tips from both germinated seedling and adult plants used for chromosome counts were treated as described in our previous work (Pellicer *et al.* 2007). For protoplast preparations, pre-treated seedlings stored in ethanol 70º were washed in agitation in 0.01 M citrate buffer (0.01 M citric acid - sodium citrate, pH = 4.6) for 15 min and subsequently digested during 40 min in 1 ml of digestion buffer [cellulase 3 % Onozuka-RS (Yakult Honsha) and pectolyase Y-23 0.5 % (Kikkoman) diluted in 0.01 M citrate buffer]. Slides were prepared following the instructions described in Pellicer *et al.* (2008).

Genome size assessments

Leaf tissue of five individuals for each studied population was chopped in 600 µl of Galbraith's isolation buffer (Galbraith *et al.* 1983) with a razor blade, together with the chosen internal standard, and supplemented with 100 µg/ml ribonuclease A (RNase A, Boehringer, Meylan, France). For each individual, two independent samples were extracted to be processed under the cytometric assessment. Samples were subsequently stained with 36 µl of propidium iodide (1mg/ml) to a final concentration of 60 µg/ml (Sigma-Aldrich Química), kept on ice for 20 min and measured in an Epics XL flow cytometer (Coulter Corporation). Measurements were made at the 'Serveis Cientificotècnics' of the Universitat de Barcelona. To ascertain that the instrument showed a linear response across the range of genome sizes studied, we performed several assays which included both internal standards and one of the populations with the highest genome size at the same time. The difference between the obtained results with respect to each standard was negligible (less than 2% of deviation) hence we can certify the linearity of the flow cytometer in this interval and the convenience of the use of the chosen internal standards. We also calculated the mean half peak coefficient of variation (HPCV) corresponding to ten samples.

The HPCV obtained was 2.54% in the case of *Petunia hybrida*, 1.87% for *Pisum sativum*, and 1.63% for *Triticum aestivum*.

Fluorochrome banding and fluorescent in situ *hybridization (FISH)*

When possible, we used the same slides for fluorochrome banding with chromomycin and for FISH after destaining with fixative followed by dehydratation in ethanol series (70º, 90º and 100º) and dried overnight. For revealing GC-rich DNA bands, the procedures from Schweizer (1976) and Cerbah *et al*. (1995) with minor modifications were followed. The slides were incubated in McIlvaine buffer with $MgCl_2 \cdot 6H_2O$ 20 mM during 15 min; incubated with 100 µl of chromomycin A_3 (0.02g/100ml in McIlvaine buffer pH = 7) for 90 min; rinsed in the same buffer; counterstained with 0.1 % methyl green in McIlvaine (pH = 5.5) for 7 min; rinsed in the same buffer; and mounted with a drop of Citifluor (Agar Scientific).

The probe used for 18S-5.8S-26S rDNA localization was a clone of a 9 kb *Eco*RI fragment of 18S-5.8S-26S rDNA and the intergenic sequence cloned from *Triticum aestivum* (Gerlach and Bedbrook 1979). This probe was labelled with direct Cy3 red (Amersham) by the polymerase chain reaction (PCR). The probe used for 5S rDNA localization was a clone of a pTa794, a 410 kb *Bam*HI fragment of 5S rDNA isolated from wheat and cloned in pBR322 (Gerlach and Dyer 1980). It contains the 5S rRNA gene (120 bp) and the noncoding intergenic spacer (290 bp). This probe was labelled with digoxigenin-11-dUTP-green (Boehringer Mannheim) by PCR.

Preparations were incubated in 100 µg/ml DNase-free RNase in 2xSSC (salt sodium citrate: 0.03 M sodium citrate and 0.3 M sodium chloride) for 1 h at 37ºC in a wet chamber, washed twice in 2xSSC for 5 min with slow shaking, once with HCl 0.01 N for 2 min, incubated in pepsin (0.1 mg/ml in 0.01N HCl) for 10 min at 37ºC, washed 3 times in 2xSSC for 5 min, dehydrated through an ethanol series (70º, 90º and 100º) and air dried. The rDNA probes were mixed each at a concentration of 25-100 ng/µl for simultaneous hybridization with 50% (v/v) formamide, 10% (w/v) dextran sulfate, 0.1% (w/v) sodium dodecylsulfate, 250 µg/ml salmon sperm, and 20xSSC. The probe mixture was denatured at 75ºC during 10 min and immediately chilled on ice for 5 min to inhibit

renaturation. Approximately 50 µl of probe mixture were loaded on the slide and covered with coverslides. The preparations were then denatured during 10 min at 75°C and transferred for 5 min to 55°C. Hybridization was carried out overnight at 37°C in a wet chamber. Posthybridization stringency washes were carried out with agitation as follows: after a first wash in 2xSSC at room temperature for 3 min, several washes for 5 min were done at 42°C (three in 2xSSC, two in 20% formamide, one in 0.1xSSC, three in 2xSSC and one in 4xSSC with 0.2% Tween 20), and a final wash was performed for 5 min in 4xSSC with 0.2% Tween 20 at room temperature. For 5S signal detection, the slides were treated with 5% (w/v) bovine serum albumine (BSA) in 4xSSC with 0.2% Tween 20 for 5 min, incubated for 1 h at 37°C in 20 µg/ml antidigoxigenin-fluorescein (Boehringer Mannheim) in the same buffer, and washed three times for 5 min also with the same buffer at room temperature. Counterstaining was done with Vectashield, a mounting medium containing DAPI. FISH preparations were observed with an epifluorescent Zeiss Axiophot microscope with different combinations of Zeiss excitation and emission filter sets (01, 07 and 15). Hybridization signals were analysed and photographed using a highly sensitive CCD camera (Princeton Instruments), and an image analyser software (Metavue, version 4.6, Molecular Devices Corporation). As shown, in some cases, chromomycin staining and FISH have been photographed on the same metaphases (Figures 1: e, f and n, o) whereas in other cases we show different ones. This is mainly because some chromomycin metaphases were overdenatured after hybridization and chromosome morphology was partially lost, so we preferred showing more intact chromosomes from other metaphase plates. Correlation between chromomycin and FISH signals was made based on at least ten different metaphases from different individuals.

Molecular phylogenetics

Procedure for PCR amplification, sequencing and for editing the matrix were as used in Pellicer *et al.* (2009). Genbank accessions and provenance for both formerly published and newly generated sequences are listed in Appendix 1. Bayesian inference (BI) was carried out with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). The most appropriate nucleotide substitution models were

chosen with MrModeltest version 2.3 (Nylander 2004). Separated analyses were carried out on both markers resulting of GTR + I + G model (hLRT and AIC criteria) for the ITS region, HKY + G model (hLRT and AIC) for the ETS, and GTR + I + G (hLRT and AIC) for the combined ITS + ETS. Four Markov chains were run simultaneously for $6x10^6$ generations, and these were sampled every 100 generations. Data from the first 6000 generations were discarded as the *burn-in* period, after confirming that likelihood values were stabilized prior to the 6000th generation. The 50% majority rule consensus trees and posterior probability (PP) of nodes were calculated from the pooled samples. Most parsimonious trees were found using heuristic searches with PAUP* (Swofford 2003) version 4.0b10, after excluding uninformative characters 1000 random sequence addition replicates, holding one tree at each step during the stepwise addition and saving no more than 500 trees greater or equal than length 1 in each sequence addition replicate (following a similar approach to Schneeweiss *et al.* 2004; Nelsen and Gargas 2008). The strict consensus tree was calculated from the resulting trees and used as a constraint in a second search of 10,000 random addition replicates with one tree saved at each replicate, saving only trees that were not compatible with the consensus. This strategy should ensure that no shorter trees exist, even if not all the shortest trees from the initial search were covered (Catalán *et al.* 1997; Berry *et al.* 2005).

Congruence of the regions ETS and ITS

The congruence of the ETS and ITS datasets was addressed by conducting a partition homogeneity test (incongruence length difference "ILD", Farris *et al.* 1994) as implemented by PAUP*, using 1000 homogeneity replicates and an heuristic search of 100 random sequence addition replicates, saving one tree at each step during the stepwise addition and no more than 1 tree greater or equal than length 1 in each sequence addition replicate. The ILD test depends on the length of the most parsimonious trees and not on recovering all of them (Berry *et al.* 2005), this being the reason why we adopted here a strategy that favours the increased number of ILD replicates and thus a lower variance in ILD P value. Uninformative sites were excluded from the ILD test (Lee 2001).

Table 2. Karyological information, nuclear DNA content and Genbank accession numbers of the populations studied.

Taxa	Life cycle	2n[1]	2C (pg)[2]	2C (Mbp)[3]	1Cx (pg)[4]	45S sites	5S sites	CMA + signals
A. copa (BCN3324)	P	108(12*x*)	31.51(0.54)	30,817	2.63	10	10	ca. 46
A. echegarayi (2481)	P	72(8*x*)	21.13 (0.37)	20,665	2.64			
A. echegarayi (2663)	P	72(8*x*)	20.69 (0.30)	20,235	2.59	8	8	8
A. magellanica (BCN3326)	B/P	18(2*x*)	6.18 (0.26)	6,044	3.09	2	2	2
A. mendozana var. *mendozana* (BC3327)	P	72(8*x*)	20.56 (0.24)	20,107	2.57	8	8	8
A. mendozana var. *mendozana* (BCN3325)	P	72(8*x*)	19.87 (0.42)	19,432	2.48			
A. mendozana var. *paramilloensis* (BCN3328)	P	54(8*x*)	16.50 (0.32)	16,137	2.77			
A. mendozana var. *paramilloensis* (2459)	P	54(8*x*)	15.53 (0.15)	15,188	2.59	6	6	6

Note: [1]Somatic chromosome number (ploidy level). [2]Nuclear DNA content [2C value (standard deviation)]. [3]Nuclear DNA content; 1pg = 978 Mbp (Doležel *et al.,* 2003). [4]Monoploid genome size.

Table 3. Numerical results for the phylogenetic trees.

Dataset		ITS	ETS	ITS - ETS
Total characters		484	370	854
Informative characters		103	72	175
Number of MPTs		493150	292204	324114
Number of steps		262	132	412
Consistency index (CI)		0.4885	0.6453	0.5146
Retention index (RI)		0.8842	0.9342	0.8991
Homoplasy index (HI)		0.5115	0.5435	0.4854
Rescaled consistencyindex (RC)		0.4320	0.6022	0.4626
Selected models for Bayesian Inference	AIC	GTR + I + G	HKY + G	GTR + I + G
	hLRT	GTR + I + G	HKY + G	GTR + I + G

Note: The consisteny index, retention index and homoplasy index were calculated excluding the uninformative characters.

Results

Chromosome numbers

The chromosome numbers found in the species studied are listed in Table 2, and metaphase plates are presented for all of these taxa (Fig. 1 a, d, g, j, m). Ploidy levels among the species range from diploid (2*n* = 18*; A. magellanica*) to dodecaploid (2*n* = 108; *A. copa*). The presence of B-chromosomes, in a

variable number, is related to the polyploid species being from one to three in *A. copa* and from two to three in *A. echegarayi* and *A. mendozana* including its subspecific entities.

Flow cytometry measurements

Genome size data varies about 5-fold between the species studied, ranging from 6.18 pg in *A. magellanica* to 31.51 pg in *A. copa*. Monoploid genome size variation is drastically reduced to 1.23-fold, ranging from 2.52 pg (mean value) in *A. mendozana* var. *mendozana* to 3.09 pg in *A. magellanica*. Values obtained are included in Table 2, expressed in picograms and megabase pairs. Note that for the species where more than one population's genome size has been assessed, the mean C value has been used for calculations.

GC-banding and fluorescent in situ *hybridization*

The cytogenetic results are described in Table 2; images of GC-banding and FISH assays are presented in Fig. 1. All the studied species show a colocalized pattern of the 5S and 45S regions. In addition, FISH signals have always been colocalized with CMA+ bands with the exception of the species *A. copa*, where a larger number of CMA+ signals (ca. 46) than FISH sites (10) was found (Fig. 1b, c). Those GC-rich bands are distributed in chromosome ends (sometimes in both), and absent at the centromeric position. Fluorescent *in situ* hybridization has revealed a similar pattern in the physical mapping of rDNA in the octoploid species (Fig. 1e-f, k-l). Both *A. echegarayi* and *A. mendozana* var. *mendozana* present four 5S and 45S loci, which are located at the terminal position of chromosomes, sometimes at sub-terminal position. *Artemisia magellanica* has two 5S and 45S sites, and also two CMA+ signals (Fig. 1h, i). In the hexaploid species *A. mendozana* var. *paramilloensis* six CMA+ signals have been detected, as well as three ribosomal DNA loci, either at terminal or sub-terminal position. In all cases, B-chromosomes, when detected, bore neither CMA+ signals nor 5S-45S ribosomal loci. DAPI-positive bands were detected in the species studied, but as these signals are the result of the counterstaining after FISH with Vectashield, and the intensity depends strongly on the quality of materials (after denaturation) and on the methodology, the presence of these bands (constitutive heterochromatin) has not been taken into account for systematic purposes.

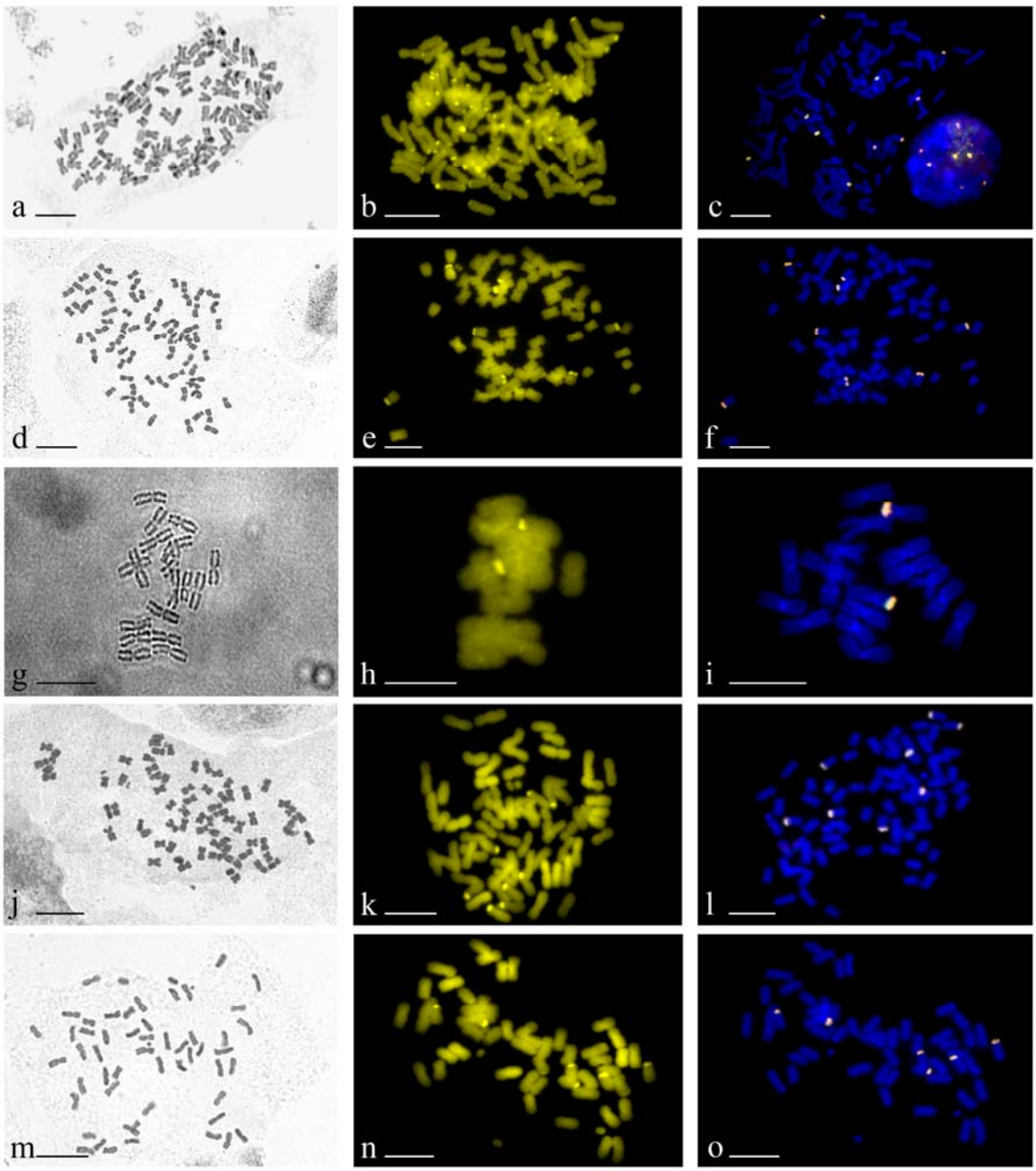

Figure 1. a-c, *A. copa*, metaphase plate (a), CMA banding (b), FISH (c); **d-f**, *A. echegarayi*, metaphase plate (d), CMA banding (e), FISH (f); **g-i**, *A. magellanica*, metaphase plate (g), CMA banding (h), FISH (i); **j-l**, *A. mendozana* var. *mendozana*, metaphase plate (j), CMA banding (k), FISH (l); **m-o**, *A. mendozana* var. *paramilloensis*, metaphase plate (m), CMA banding (n), FISH (o). Scale bars 10 μm.

Molecular phylogenetic approach

Data on numerical results from the phylogenetic analyses and selected models for Bayesian inferences are presented in Table 3. Trees obtained from Bayesian inference were of better resolution than those carried out by parsimony, but their topologies were congruent without discordances between significantly supported

branches. That is the reason why we show only the Bayesian tree of the combined data set even though the ILD P-value of the combined data set is 0.008, indicating that a certain degree of incongruence between data exists. The subsequent P-value calculations carried out removing different species lead us to think about the species which can directly contribute to this incongruence. Test results after removing the species *A. biennis*, *A. magellanica* and *A. tournefortiana* was P = 0.026, increasing to P = 0.087 when *Filifolium sibiricum* was also removed. When *F. sibiricum* alone was removed from the data set, the test result was P = 0.046; and if *F. sibiricum* and *A. tournefortiana* were removed, the result was P = 0.056. These results indicate that the species phylogenetic position, even when not being statistically incongruent between regions, does not reflect a clear consistency between markers, and is directly related to the significance of the ILD tests. Furthermore, the level of homoplasy of the data might also influence results (see Table 3). The phylogenetic results have confirmed the close relationships between the South American and the North American *Artemisia* endemic species (PP = 100%; BS = 95%; Fig. 2), which appear merged in the same clade, providing the phylogenetic position of these species. *Artemisia copa* is placed at sister position to the remaining species (*A. mendozana* and *A. echegarayi*; PP = 100%; BS = 100%) performing a monophyletic group, with the exception of *A. magellanica*, which is clearly separated and grouped with *A. biennis* (PP = 100%; BS = 100%). Both accessions of *A. mendozana* var. *paramilloensis* perform a monophyletic clade (PP = 98%; BS = 65%) while for those of *A. mendozana* var. *mendozana* and *A. echegarayi* we cannot confirm the monophyly (Fig. 2). The phylogenetic position of *A. magellanica* (Fig. 2) close to the subgenus *Dracunculus* is only supported by the ETS and the combined analyses and the ITS region has not defined the position of this species (clade in dotted line).

Discussion

Chromosome counts and polyploidy incidence

The counts presented are new for the species, with the exception of *A. magellanica* and *A. mendozana*. A previous count in the former species is

available in Moore (1981), reporting a diploid cytotype. Our count, even indicating a diploid population, does not agree with Moore's results, as he found 22 chromosomes in a population from Isla Grande (Tierra del Fuego, Argentina), and the present count reveals 18 chromosomes, which is more common in the diploid *Artemisia* species (Vallès and Garnatje 2005, and references therein). In the case of *A. mendozana*, our result in *A. mendozana* var. *mendozana* ($2n = 72$) is consistent with a previous report of Kawatani and Ohno (1964), without any indication as to the variety they counted. As the populations counted of *A. mendozana* var. *paramilloensis* resulted in hexaploid cytotypes ($2n = 54$) and its distribution range is restricted to the Uspallata moorlands, it makes sense to think that the taxon studied by Kawatani and Ohno was *A. mendozana* var. *mendozana*. The counts in *A. echegarayi* and *A. copa* which have reported octoploid ($2n = 8x = 72$) and dodecaploid ($2n = 12x = 108$) cytotypes respectively, support the consideration of the South American endemic representatives of *Artemisia* as performing a polyploid complex, with the premise that events such as autopolyploidy and allopolyploidy, possibly as well as hybridization and genome introgression, are presumably processes that took place during the evolution of these species.

Phylogenetic circumscription and conflictive placement of the South American endemics within the genus Artemisia*: evidence for a likely North to South migration route*

The different ILD tests that have been carried out to detect incongruence between both ribosomal markers have revealed that some of the species studied clearly influenced the significance of these test (Fig. 2). Nevertheless, we have combined (after individual analyses) both data sets, as many authors have previously considered (Englund *et al.* 2009; Hidalgo *et al.* 2008; López-Vinyallonga *et al.* 2009) but keeping in mind how those species behave. In any case, we must take into account that ILD shows only the different evolutionary stories between both regions, but can be influenced by several factors and is not forcibly a good representation of the phylogeny's accuracy (Darlu and Lecointre 2002; Dowton and Austin 2002). That procedure helps to improve our knowledge of the

relationships within the species, and allows us to investigate into which of the molecular markers accounts for a higher phylogenetic score, that is, a better understanding of the evolutionary history of each species. One of the candidates which showed a certain degree of incongruence between both ITS and ETS regions has been *A. magellanica*. This species splits from the rest of the South American endemic group, together with the three accessions of *A. biennis* from Europe and North America, constituting a well supported clades in the combined analysis (Fig. 2; PP = 100 %; BS = 100 %), but while the ETS region embedded the species in a phylogenetic position close to the subgenus *Dracunculus* Besser (data not shown), an unresolved position within *Artemisia* can be extracted for these species from the ITS analysis (Fig. 2; dotted clades). The phylogenetic inconsistencies and unresolved placements between trees of different DNA regions might be induced by phenomena such as hybridization, long branch attraction, lineage sorting or a high rate of mutations. As mentioned in the introduction, such a relationship between *A. biennis* and *A. magellanica* might be reasonably expected if we take into account the morphological similarities between both species, which also lead Ling (1995) to consider them as synonyms. So it would not be strange to hypothesize about two possible ways of colonization. On the one hand, such a relationship might be the result of a previous colonization event of the species *A. biennis* from Eurasia to North America (via Bering strait), reaching South America, and subsequent isolation in the Patagonia (South Argentina and Chile) zone, where possibly adaptive morphological changes to the environment induced Schultz-Bipontinus (1855) to consider it as an independent entity. On the other hand, a migration to the Southern lands might be originated from North America, taking into account the considerations of Thomas (1961) and Munz and Keck (1968), later compiled by Kegode and Christoffers (2003), who found *A. biennis* as a native to North America (originated from Asian primitive representatives migrated through the Bering Strait). Thus, it is feasible to confirm the colonization from North to South America but we are not able to confirm the direction of migrations between the Old and the New World.

As stated in the results section, the core of the South American endemic *Artemisia* appeared embedded into a robust clade (Fig. 2; PP/BS = 100%) with the North American endemics, thus performing a monophyletic group (Fig. 2; PP = 100%; BS = 95%). Although some authors have hinted at some difficulties for morphological differentiation between *A. mendozana* varieties and also with *A. echegarayi*, all of them are placed in well defined clades, but with a certain degree of heterogeneity among accessions (Fig. 2), maybe induced by an allopolyploid origin.

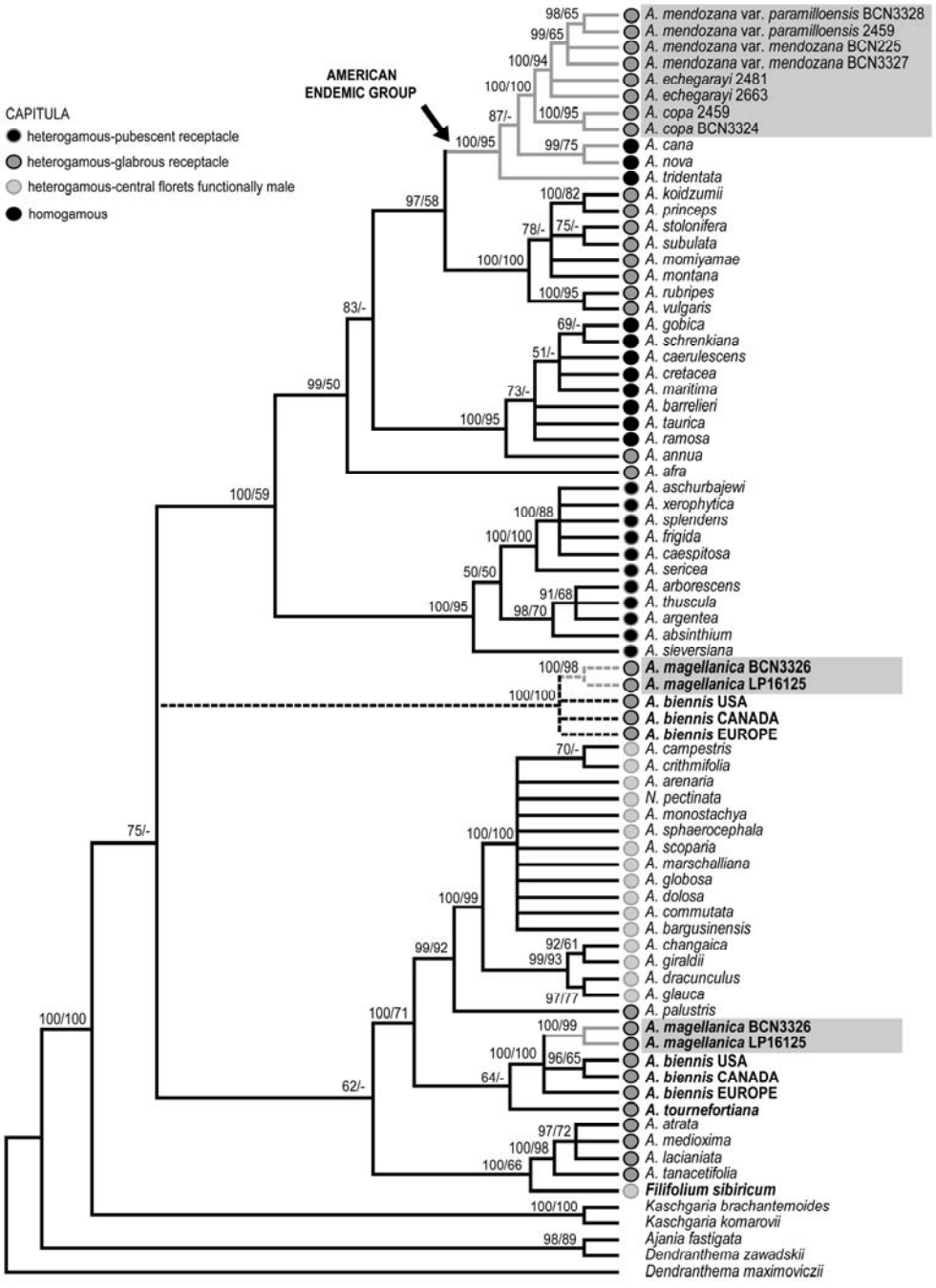

Figure 2. Majority rule consensus tree resulting from Bayesian analysis of the combined ITS + ETS data set; numbers in branches are Bayesian posterior probabilities (PP, front) and branches Bootstrap (BS, rear). **Note:** Taxa squared in grey correspond to the South American endemic *Artemisia*. Species in bold highlight those taxa with conflictive phylogenetic positions between molecular markers, and dotted line clade refers to the phylogenetic position resulted from the ITS analysis.

This close relationship between the species of the complex with the North American ones can be understood when considering biogeographic evidence. While traditionally, the capitula structure has been used to characterize taxonomical groups (Besser 1829, 1832, 1834, 1835; Candolle 1837), and this fitted quite well at a molecular phylogenetic level (with some exceptions in the subgenera *Artemisia* and *Absinthium*; see Fig. 2 and Sanz *et al*. 2008), within the North American endemics the classification under these taxonomical characters does not reveal phylogenetic relationships between these species and the Eurasian *Artemisia*. Thus, although the South American ones presented the characteristic capitula traits related to the subgenus *Artemisia*, a direct relationship with the Eurasian relatives cannot be confirmed because still needs to investigate how they behave the North America endemic (non *Tridentatae*) species with the South American ones. Furthermore, the phylogenetic tree revealed a close relationship between the American species and the *Artemisia vulgaris* L. group, which is placed at sister position of the American clade (Fig. 2; PP = 100 %; BS = 95 %), giving support to the hypothesis of McArthur and Plummer (1978). Even though, the phylogentic position of the subgenus *Seriphidium* (with homogamous capitula) does not allow to discard the Ling's (1991, 1995) proposals of a *Seriphidum* ancestor to the North American endemics.

The South American endemic core: cytogenetic traits in a polyploid complex

The results of 5S and 45S ribosomal DNA mapping in *Artemisia* showed both ribosomal RNA genes embedded, that is, colocalized, as a common feature in the genus (Torrell *et al*. 2003; Garcia *et al*. 2007, 2009). Such an unusual organization has been confirmed by different techniques in previous works [PCR assays or Southern blot hybridization, Garcia *et al*. (2009)]. During

polyploidization, changes at genome level involving ribosomal DNA and genome downsizing in the newly generated polyploidy have been reported in plants (Leitch and Leitch 2008). Hence, one of the aims of the present study is to characterize cytogenetically this polyploid complex, but comparisons between different ploidy levels are difficult, as each species has been reported at a single ploidy level and also because the number of South American endemic representatives is scarce. Nevertheless, we have studied two varieties of the species *A. mendozana*, which have been found to be related to different ploidy levels (Fig. 1j, m), and results from both nrDNA mapping and genome size assessments do not indicate either a clear genome downsizing or a loss of ribosomal loci (Table 2; Fig. 1l, o). Thereby, on the one hand, mean monoploid genome size of the hexaploid *A. mendozana* var. *paramilloensis* and *A. mendozana* var. *mendozana* (2n = 72) are quite similar, being respectively of 2.68 pg and 2.52 pg, with only slight differences. Genome downsizing, even to a large extent, has frequently been reported in polyploid series (Leitch and Bennett 2004), and particularly in *Artemisia* (Pellicer *et al.* 2009), but this is not the case in the currently studied complex. On the other hand, the increase of the ribosomal loci number from hexaploid level to octoploid has occurred as expected for a proportional gain. Hence, in some cases, weak signals have been detected (Fig. 1l, o) which can be related to loss of gene copies at the loci, as reported in other polyploid plants despite genome additivity having been found (Leitch and Bennett 2004; Leitch *et al.* 2008). Minor genome downsizing and proportional gain of ribosomal loci between ploidy levels have also been found in a few representatives of the genus *Artemisia*, but always related to low ploidy levels (e.g. *A. dracunculus*; Pellicer *et al.* unpublished). When reaching higher ploidy levels, DNA loss becomes greater, even when the number of ribosomal loci resulted in a proportional increase. In the North American subgenus *Tridentatae*, both tendencies (increase/loss related to genome size and ribosomal loci) have been reported between diploid and tetraploids, but pointing to loci elimination in higher ploidy levels (Garcia *et al.* 2009).

As mentioned before, genome size and nrDNA loci changes at an interspecific level have to be cautiously considered. Even so, the findings in *A.*

copa where about 10 nrDNA signals have been found (though the fluorescent signal might not reveal weaker sites that could exist), along with and its great genome size (2C = 31.51 pg; 1Cx = 2.62 pg), supported the results in lower ploidy levels of the South American complex where a trend to a proportional genome expansion in lower ploidy levels seems to be a common feature in this group. In other genera such as *Carthamus* (Garnatje *et al.* 2006) an additive pattern of genome size increase has been reported in polyploidy. Furthermore, proportional increases of nrDNA loci number have also been confirmed in *Tragopogon* (Pires *et al.* 2004) and *Nicotiana* (Matyásek *et al.* 2003).

Fluorochrome banding with chromomycin revealed heterochromatin composed of GC-rich DNA. In all the studied species, we have found it located at the distal ends of the chromosomes, in some plates clearly located in satellites (data not shown). Both octoploid taxa (*A. mendozana* var. *mendozana* and *A. echegarayi*) presented the same number of CMA+ bands (eight ; Fig. 1e, k), and a substantially increased number of bands have been detected in *A. copa* (ca. 46) with some chromosomes marked at both ends (Fig. 1b). Different patterns in heterochromatin staining exist. We have found either polyploid species showing only GC-bands in the same position as nrDNA sites (*A. echegarayi* and *A. mendozana s. l.*) or as in the case of *A. copa*, where significantly higher number of CMA+ sites as opposed to nrDNA have been revealed. The same increased number of GC-rich bands has been found in other polyploid North American *Artemisia* (Garcia *et al.* 2009), as well as in other Eurasian representatives (Vallès and Siljak-Yakovlev 1997; Torrell *et al.* 2003) and other polyploid angiosperms (e.g. *Urvillea;* Urdampilleta *et al.* 2006), indicating the presence of new clusters of heterochromatin in those polyploid populations, and also pointing to a certain independence between GC-rich bands and nrDNA sequences (Hamon *et al.* 2009). This gain of novel heterochromatic DNA can be associated with new satellite repeats (Lim *et al.* 2007) or to massive processes of amplification of transposable elements (McClintock 1984; Cheng and Murata 2003) among others.

Artemisia magellanica *and* A. biennis. *Are they true independent species?*

As commented previously, the phylogenetic position of *A. magellanica*, which is placed at a segregated position from the South American endemics (Fig. 2), leads us to hypothesise its origin, seeing such a close parallel phylogenetic behaviour to *A. biennis*. In this sense, the number and distribution of telomeric CMA+ bands and the results of FISH for both 5S and 18S-5.8S-26S nrDNA probes in *A. magellanica* (Fig. 1h, i) are also coincidental with those previously mapped for *A. biennis* (Pellicer *et al.* unpublished): a single locus of ribosomal DNA colocated with two CMA+ bands, and therefore, an uncommon number of ribosomal genes for diploid *Artemisia* only detected previously in other annual species of *Artemisia* and in the diploid, also annual, *Neopallasia pectinata* (Pall.) Poljakov (Pellicer *et al.* unpublished). *Artemisia biennis* belongs to the subgenus *Artemisia*, a group cytogenetically characterized by the presence of two ribosomal loci (four sites) in diploid populations, as previously found in *Artemisia annua* L. (an annual as well, thus the life cycle is apparently not related with the loci number), *Artemisia chamaemelifolia* Vill. or *Artemisia vulgaris* L., among others (Torrell *et al.* 2003; Hoshi *et al.* 2006; Pellicer *et al.* 2007), in spite of its complex systematics (Tkach *et al.* 2008; Pellicer *et al.* unpublished). Also *Artemisia tournefortiana* Reich., an annual/biennial species belonging to the same subgenus and morphologically close to *A. biennis* -but placed separated from *A. biennis* and *A. magellanica* in the molecular phylogeny, Fig. 2-, shows the typical subgenus *Artemisia* pattern (two nrDNA loci; Pellicer *et al.* unpublished), differing also by this cytogenetic character from the cluster formed by the latter two species. Besides this, their similar genome sizes also support a really close relationship between those species; being 6.18 pg in *A. magellanica* and 6.37 pg (mean 2C-value; Pellicer *et al.* unpublished.) in *A. biennis*. In summary, the coincident results concerning the systematics, cytogenetics and the previous taxonomical suggestions through a possible synonymy between both taxa (Ling 1995) have to be taken into account, since it seems we might be referring either to the same species, or to the result of speciation processes after geographic isolation, as scarce morphological divergences have been reported in the literature (Cabrera 1971; Ariza 1997).

Concluding remarks

According to the present results, two groups can be established in the South American endemic *Artemisia* species. On the one hand, *A. magellanica*; this species is grouped, according to DNA sequence analyses, with *A. biennis*, belonging to subgenus *Artemisia*, with which it also shares the cytogenetic characteristics (not in agreement with the subgenus *Artemisia* typical pattern). On the other hand, the remaining South American *Artemisia* taxa, which are grouped in the molecular phylogeny with the North American endemic taxa of subgenus *Tridentatae*. This suggests at least two different introduction and differentiation processes of the genus from North to South America: i) the one that originated *A. magellanica*, with scarce differentiation from some subgenus *Artemisia* representatives, and ii) the one that gave rise to *A. copa*, *A. echegarayi*, *A. mendozana* and their infraspecific taxa, related to the already differentiated North American endemics. From the current characterization, further studies including an extensive representation of the North American endemic *Artemisia* species, from the *Tridentatae* and other subgenera (Garcia *et al*. unpublished.), will shed definite light on this matter. Furthermore, a morphometric study of *A. magellanica*, *A biennis* and the morphologically similar *A. tournefortiana*, as well as the different subspecies and varieties of *A. copa* and *A. mendozana*, is envisaged (Molero *et al.*, unpublished) to round up on the taxonomic and nomenclatural aspects.

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Appendix 1. Genbank numbers of the species included in the phylogenetic analysis. For the species sequenced for the first time, voucher references and/or collection numbers are indicated.

TAXON	Herbarium collection n°, Bibl. Ref.	ITS1	ITS2	ITS1-5.8S-ITS2	Herbarium collection n°, Bibl. Ref.	3'-ETS
A. absinthium	Torrell *et al*. 1999	AF045394	AF079946		Sanz *et al.* 2008	DQ028850
A. afra	Torrell *et al*. 1999	AF045392	AF140484		Sanz *et al.* 2008	DQ028881
A. annua	Tkach *et al*. 2008			AM398847	Tkach *et al.* 2008	AM397956
A. arborescens	Torrell *et* al. 1999 (unpub.)	AF045393	AF079945		Pellicer *et al.* 2009	FJ642934
A. arenaria	Sanz *et al*. 2008	EF063639	EF063640		Sanz *et al.* 2008	DQ028897
A. argentea	Pellicer *et al*. 2009	FJ642971	FJ643007		Pellicer *et al.* 2009	FJ642935
A. aschurbajewi	Torrell *et al.* 1999	AF504170	AF504143		Sanz *et al.* 2008	DQ028838
A. atrata	Pellicer *et al.* 2009	FJ642973	FJ643009		Pellicer *et al.* 2009	FJ642937
A. barrelieri	Torrell *et al.* 1999	AF045410	AF79961		Sanz *et al.* 2008	DQ028875
A. biennis	Tkach *et al.* 2008			AM398851	Tkach *et al.* 2008	AM398032
A. biennis	BCN-USA				BCN-USA	
A. biennis	BCN-CANADA				BCN-CANADA	
A. caerulescens	Torrell *et al.* 1999	AF045409	AF079960		Sanz *et al*. 2008	DQ28872
A. caespitosa	Tkach *et al.* 2008			AM398855	Tkach *et al.* 2008	AM397957
A. campestris	Torrell *et al.* 1999	AF045398	AF079950		Sanz *et al.* 2008	DQ028854
A. cana	Torrell *et al.* 1999	AF045413	AF079965		Sanz *et al.* 2008	DQ028882
A. changaica	Tkach *et al.* 2008			AM398858	Tkach *et al*. 2008	AM397965
A. commutata	Tkach *et al.* 2008			AM398860	Tkach *et al.* 2008	AM397967
A. copa	BCN3324				BCN3324	
A. copa	Dematteis 2549 (CTES)				Dematteis 2549 (CTES)	
A. cretacea	Pellicer *et al.* 2009	FJ642975	FJ643011		Pellicer *et al*. 2009	FJ642939
A. crithmifolia	Torrell *et al.* 1999	AF045399	AF079962		Sanz *et al.* 2008	DQ028856
A. dolosa	Tkach *et al.* 2008			AM398864	Tkach *et al*. 2008	AM397971
A. dracunculus	Torrell *et al.* 1999	AF504172	AF504145		Sanz *et* al. 2008	DQ028859
A. echegarayi	Dematteis 2481 (CTES)				Dematteis 2481 (CTES)	
A. echegarayi	Dematteis 2663 (CTES)				Dematteis 2663 (CTES)	

A. giraldii	Pellicer *et al.* 2009	FJ642979	FJ643015		Pellicer *et al.* 2009	FJ642943
A. glauca	Tkach *et al.* 2008			AM398871	Tkach *et al.* 2008	AM397978
A. globosa	Pellicer *et al.* 2009	FJ642980	FJ643016		Pellicer *et al.* 2009	FJ642944
A. gobica	Tkach *et al.* 2008			AM398876	Tkach *et al.* 2008	AM397983
A. koidzumii	Tkach *et al.* 2008			AM398884	Tkach *et al.* 2008	AM308035
A. laciniata	Tkach *et al.* 2008			AM398886	Tkach *et al.* 2008	AM397991
A.magellanica	(BCN3326)				(BCN3326)	
A. magellanica	16125 (LP)				16125 (LP)	
A. maritima	Pellicer *et al.* 2009	FJ642987	FJ643023		Pellicer *et al.* 2009	FJ642951
A. marschalliana	Torrell *et al.* 1999	AF505177	AF504150		Sanz *et al.* 2008	DQ028858
A. medioxima	Pellicer *et al.* 2009	FJ642988	FJ643024		Pellicer *et al.* 2009	FJ642952
A. mendozana var. *mendozana*	(BCN3325)				(BCN3325)	
A. mendozana var. *mendozana*	(BCN3327)				(BCN3327)	
A. mendozana var. *paramilloensis*	(BCN3328)				(BCN3328)	
A. mendozana var. *paramilloensis*	Dematteis 2459 (CTES)				Dematteis 2459 (CTES)	
A. momiyamae	Pellicer *et al.* 2009	FJ642989	FJ643025		Pellicer *et al.* 2009	FJ642953
A. monostachya	Tkach *et al.* 2008			AM398896	Tkach *et al.* 2008	AM398000
A. montana	Pellicer *et al.* 2009	FJ642991	FJ643027		Pellicer *et al.* 2009	FJ642955
A. nova	Torrell *et al.* 1999	AF045412	AF060462		Sanz *et al.* 2008	DQ028883
A. palustris	Tkach *et al.* 2008			AM398902	Tkach *et al.* 2008	AM398006
A. princeps	Tkach *et al.* 2008			AM398905	Tkach *et al.* 2008	AM398009
A. ramosa	Pellicer *et al.* 2009	FJ642994	FJ643030		Pellicer *et al.* 2009	FJ642958
A. rubripes	Pellicer *et al.* 2009	FJ642995	FJ643031		Pellicer *et al.* 2009	FJ642959
A. schrenkiana	Pellicer *et al.* 2009	FJ642997	FJ643033		Pellicer *et al.* 2009	FJ642961
A. sericea	Tkach *et al.* 2008			AM398916	Tkach *et al.* 2008	AM398019
A. sieversiana	Tkach *et al.* 2008			AM398917	Tkach *et al.* 2008	AM398020
A. sphaerocephala	Tkach *et al.* 2008			AM398918	Tkach *et al.* 2008	AM398021
A. stolonifera	Pellicer *et al.* 2009	FJ643000	FJ643036		Pellicer *et al.* 2009	FJ642964

A. subulata	Pellicer *et al.* 2009	FJ643001	FJ643037		Pellicer *et al.* 2009	FJ642965
A. tanacetifolia	Tkach *et al.* 2008			AM398923	Tkach *et al.* 2008	AM398026
A. taurica	Pellicer *et al.* 2009	FJ643003	FJ643039		Pellicer *et al.* 2009	FJ642967
A. thuscula	Tkach *et al.* 2008			AM398924	Tkach *et al.* 2008	AM39027
A. tournefortiana	Pellicer *et al.* in prep				Pellicer *et al.* in prep	
A. tridentata	Torrell *et al.* 1999	AF045411	AF079963		Sanz *et al.* 2008	DQ028884
A. vulgaris	Tkach *et al.* 2008			AM398927	Tkach *et al.* 2008	AM398029
A. xerophytica	Tkach *et al.* 2008			AM398929	Tkach *et al.* 2008	AM398031
Ajania fastigata	Torrell *et al.* 1999	AF504169	AF504142		Sanz *et al.* 2008	DQ028868
Dendranthema maximoviczii	Sanz *et al.* 2008	DQ028923	DQ028910		Sanz *et al.* 2008	DQ028899
Dendranthema zawadskii	Sanz *et al.* 2008	DQ028924	DQ028911		Sanz *et al.* 2008	DQ028901
Filifolium sibiricum	Pellicer *et al.* 2009	FJ643006	FJ643042		Pellicer *et al.* 2009	FJ642970
Kaschgarria brachantemoides	Torrell *et al.* 1999	AF504189	AF504162		Sanz *et al.* 2008	DQ028865
Kaschgaria komarovii	Sanz *et al.* 2008	DQ028925	DQ028912		Sanz *et al.* 2008	DQ028902
Neopallasia pectinata	Sanz *et al.* 2008	DQ028927	DQ028927		Sanz *et al.* 2008	DQ028914

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Caracterització citogenètica molecular d'alguns representants dels subgèneres *Artemisia* i *Absinthium* (gènere *Artemisia*, *Asteraceae*)

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S'ha desenvolupat un estudi basat en tècniques de citogenètica molecular en tres espècies del gènere *Artemisia*, complementant treballs previs en dos subgèneres que no han estat estudiats en profunditat des d'aquest punt de vista, *Artemisia* (*A. chamaemelifolia*, *A. vulgaris*) i *Absinthium* (*A. absinthium*). S'han dut a terme assaigs de bandeig amb cromomicina A_3 i 4',6-diamidino-2-fenilindole (DAPI), així com hibridació *in situ* fluorescent (FISH) del DNA ribosòmic 5S i 18S-5.8S-26S. S'han obtingut paràmetres morfomètrics dels cariotips i s'han construït els idiogrames, amb la posició de les zones riques en bases A-T i G-C, així com dels *loci* del DNA ribosòmic. S'ha observat que la majoria d'aquests marcatges són colocalitzats, fet que confirma troballes anteriors en aquest gènere. Ambdues regions del DNA ribosòmic també són colocalitzades, un tret distintiu respecte a la majoria de les angiospermes, on se situen en posicions diferenciades als cromosomes. Les espècies estudiades presenten un cariotip bastant simètric. *Artemisia absinthium* té cromosomes bastant grans i sense bandes centromèriques, que, per contra, si que es troben a les espècies *A. vulgaris* i *A. chamaemelifolia*. En aquesta última també s'han trobat cromosomes B, en algun dels quals s'ha detectat DNA ribosòmic i heterocromatina. A pesar d'aquestes diferències, la morfologia del cariotip i el patró de senyals d'aquestes tres espècies és bastant semblant. Això pot reflectir una estreta relació filogenètica entre ambdós subgèneres, fet que coincideix amb les dades moleculars de què disposem, on representants de tots dos apareixen entremesclats.

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Molecular cytogenetic characterization of some representatives of the subgenera *Artemisia* and *Absinthium* (genus *Artemisia*, Asteraceae)

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Abstract
A molecular cytogenetic study has been performed in three species of the genus *Artemisia*, complementing previous works on two subgenera that had been scarcely studied from this standpoint, *Artemisia* (*A. chamaemelifolia*, *A. vulgaris*) and *Absinthium* (*A. absinthium*). Chromomycin A_3 and 4',6-diamidino-2-phenylindole (DAPI) banding have been carried out, as well as fluorescent *in situ* hybridization (FISH) of 5S and 18S-5.8S-26S ribosomal DNA. Morphometrical data of karyotype characters were calculated and idiograms with the position of the AT- and GC-rich regions as well as rDNA loci were constructed. Colocalization of most of these regions has been observed, confirming previous findings in this genus. Both ribosomal DNA appear always colocalized, which is a distinct feature with respect to most angiosperms surveyed. Regarding the differential characteristics of each species, a symmetrical karyotype has been found in the species studied. *Artemisia absinthium* shows long chromosomes and absence of centromeric banding signals that, conversely, are absent in *A. vulgaris* and *A. chamaemelifolia*. The last species also presents B-chromosomes in which ribosomal DNA and heterochromatin have been detected. Despite these differences, karyotype morphology and signal pattern of the three species are quite coincidental. This might reflect a close phylogenetic relationship between both subgenera, which is consistent with the available molecular phylogenies presenting species of the subgenera *Artemisia* and *Absinthium* intermixed.

Keywords: *Artemisia*, Asteraceae, colocalization, fluorescent *in situ* hybridization, fluorochrome banding, karyotype analysis.

Introduction

The genus *Artemisia* L. (Asteraceae, Anthemideae) comprises more than 500 species, basically perennial and worldwide distributed (a variable number depending on the authors consulted: McArthur, 1979; Mabberley, 1990; Ling, 1991a, b, 1995a, b; Bremer & Humphries, 1993; Kubitzki, 2007). Many species of this genus are useful in different fields: food (*A. absinthium* L., *A. dracunculus* L., *A. genipi* G. Weber in Stechm.), medicine (*A. annua* L., *A. santonica* L.), forage (*A. herba-alba* Asso, *A. tridentata* Nutt.), ornamentals (*A. arborescens* L., *A. vulgaris* L.). After different rearrangements and systematic treatments, *Artemisia* has been divided into five large groups treated at sectional or subgeneric levels: *Absinthium*, *Artemisia* (= *Abrotanum*), *Dracunculus*, *Seriphidium* and *Tridentatae* (Torrell *et al*., 1999 and references therein). Nevertheless, this classification does not accurately represent natural groups. Molecular phylogenetic studies, based on the analysis of chloroplast (cpDNA) and nuclear ribosomal (nrDNA) sequences, have helped elucidating the

systematic relationships within *Artemisia* (KORNKVEN *et al*., 1998; TORRELL *et al*., 1999; WATSON *et al*., 2002; VALLÈS *et al*., 2003; SANZ *et al*., 2008), although important questions still remain unresolved.

The study of chromosomes has provided essential information for the systematics and the evolution of the genus, since the first studies (WEINEDEL-LIEBAU, 1928; EHRENDORFER, 1964; KOROBKOV, 1972) until present (VALLÈS & SILJAK-YAKOVLEV, 1997; TORRELL *et al*., 2003; VALLÈS & GARNATJE, 2005; GARCIA *et al*., 2007). Two different basic chromosome numbers have been described for the genus: $x = 9$, which is detected in all the subgenera, and the less frequent $x = 8$, only reported in the subgenera *Absinthium*, *Artemisia* and *Dracunculus* (SOLBRIG, 1977; OLIVA & VALLÈS, 1994; MCARTHUR & SANDERSON, 1999). Both basic chromosome numbers show polyploid series with known levels up to hexadecaploid for $x = 9$-based species and hexaploid for $x = 8$ (EHRENDORFER, 1964; PERSSON, 1974; VALLÈS *et al*., 2001; GARCIA *et al*., 2006; PELLICER *et al*., 2007).

Fluorochrome banding and fluorescent *in situ* hybridization (FISH) with rDNA are useful markers for chromosome identification (LEITCH & HESLOP-HARRISON, 1993) and have provided a valuable tool in order to determine genome organization in plants (RAINA *et al.*, 2001; SINGH *et al*., 2001) and the phylogenetic relationships between close taxa (ANSARI *et al*., 1999). The detection of 18S-5.8S-26S and 5S ribosomal DNA by fluorescent *in situ* hybridization and fluorochrome banding have become frequently used techniques for establishing the physical mapping of a certain type of repeated DNA, and in *Artemisia* previous researches have been carried out in this sense (TORRELL *et al*., 2001, 2003; HOSHI *et al*., 2006; GARCIA *et al*., 2007).

Within the framework of systematic and evolutionary studies in *Artemisia*, in particular those providing molecular cytogenetic data (TORRELL *et al*., 2007; GARCIA *et al*., 2007), the present work uses fluorochrome banding to characterize AT- and GC-rich chromosome regions, and fluorescent *in situ* hybridization (FISH) to obtain the distribution patterns of 18S-5.8S-26S and 5S rDNA loci in two subgenera scarcely studied from this point of view: two species from subgenus *Artemisia* (*A. chamaemelifolia* Vill., *A. vulgaris*) and one from subgenus *Absinthium* (*A. absinthium*). In previous works (TORRELL *et al*., 2001, 2003; GARCIA *et al*., 2007) we have studied from molecular cytogenetic viewpoint some representatives of subgenera *Seriphidium* and *Tridentatae* (plus one annual member of the subgenus *Artemisia*, related to *Seriphidium* in the molecular phylogeny (TORRELL *et al*., 1999; VALLÈS *et al*., 2001). Now we are presenting the results of the first investigation of two more subgenera, *Artemisia* and *Absinthium*.

MATERIALS AND METHODS

Plant material

Table 1 shows the species studied, grouped by subgenera, with their origins and vouchers, deposited at the herbarium of the Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona (BCN).

Root-tip meristems were obtained from wild-collected achenes germinated on wet filter paper in Petri dishes at room temperature in the dark.

Chromosome preparations

For all the techniques used, root tips were pretreated with 0.05% aqueous colchicine at room temperature for 2 hours 30 minutes to 3 hours. The material was fixed in absolute ethanol and glacial acetic acid (3:1) and then stored at 4ºC for 48 hours. Subsequently, the materials were transferred to 70% ethanol and stored at 4ºC.

Chromosome preparation and staining for counts and karyotype elaboration

Root tips were hydrolysed in 1N HCl at 60ºC during 2 minutes. Subsequently they were washed in distilled water, stained in 2% aceto-orcein during 2-8 hours at room temperature, and squashed in a drop of 45% acetic acid and glycerol (9:1).

Fluorochrome banding

Chromosome preparations for fluorochrome banding and fluorescent *in situ* hybridization (FISH) were done using the air-drying technique of GEBER & SCHWEIZER (1987), with some modifications: root tips were washed with agitation in citrate buffer (0.01 M citric acid – sodium citrate, pH = 4.6) for 15 minutes, excised, and incubated in an enzyme

Table 1. Origin and herbarium vouchers of the populations studied.

Taxon	Location and herbarium voucher
Subgenus *Absinthium*	
Artemisia absinthium L.	Armenia, Ekhegnadzor, Vernashen: between the village of Vernashen and the church of Gladzor, 1,300 m, *G. Faivush, E. Gabrielian, N. Garcia-Jacas, M. Guara, M. Oganesian, A. Susanna S-1507, K. Tamanian, J. Vallès,* 20.viii.1995 (BCN 11601)
Subgenus *Artemisia*	
Artemisia chamaemelifolia Vill.	Andorra, Canillo: margins of the path from the church of Sant Joan de Caselles towards Vall de Riu, 1,600 m, *M. Torrell, J. Vallès*, 17.xi.1996 (BCN 13222)
Artemisia vulgaris L.	France, Paris: Bois de Boulogne, margins of a forest, 100 m, *M. Torrell, J. Vallès*, 8.ii.1997 (BCN 15297)

solution [4% cellulase Onozuka R10 (Yakult Honsha), 1% pectolyase Y23 (Sigma) and 4% hemicellulase (Sigma)] at 37ºC for 20 to 25 minutes, depending on the species and meristematic thickness. The lysate of 8-10 root-tips was centrifuged twice in 100 µl buffer and once in 100 µl fixative, at 4,000 rpm for 5 minutes for each centrifugation, and removing the supernatant each time. The final pellet was resuspended in 50 µl of fixative, about 10 µl were dropped onto a clean slide, and air-dried. In order to reveal GC-rich bands, chromomycin A_3 was used, following the protocols of SCHWEIZER (1976) and CERBAH *et al.* (1995).

Fluorescent in situ *hybridization (FISH)*

DNA hybridization was carried out according to TORRELL *et al.* (2003), with minor changes: the 18S-5.8S-26S rDNA probe was labelled with direct Cy3 -red- (Amersham) and the 5S rDNA probe with digoxigenin-11-dUTP -green- (Boehringer Mannheim). Preparations were mounted in a medium containing DAPI, which revealed AT-rich DNA bands.

FISH preparations were observed with an epifluorescent Zeiss Axiophot microscope with different combinations of Zeiss excitation and emission filter sets (01, 07 and 15). The best metaphase plates were photographed with a digital camera (AxioCam MRc5 Zeiss) mounted on a Zeiss Axioplan microscope, and images were analysed with Axio Vision Ac software version 4.2. Hybridization signals were analysed and photographed using the highly sensitive CCD camera (Princeton Instruments), and an image analyser software (Metavue, version 4.6, Molecular Devices Corporation).

Karyological analyses

Chromosome counts and measurements were done and subsequent analyses were performed (see Table 2 for details). The total karyotype length was also calculated, and compared with nuclear DNA content provided in previous works (TORRELL *et al*., 2001; GARCIA *et al*., 2004) is included for comparative purposes. These data were used to construct idiograms. Mean values were obtained from at least five metaphase plates corresponding to five different individuals for each taxon (Fig. 1).

RESULTS

Results of the karyological and molecular cytogenetic assays are presented in Fig. 1 and Table 2. The chromosome counts carried out in the species studied revealed the presence of both basic chromosome numbers commonly reported in the genus, $x = 9$ (*A. absinthium*, Fig. 1A; *A. chamaemelifolia*, Fig. 1E) and $x = 8$ (*A. vulgaris*, Fig. 1I). All the species studied are diploid, *A. absinthium* and *A. chamaemelifolia* having $2n = 18$ chromosomes

Table 2. Karyological data. The superscripts indicate: [1]Nuclear DNA content (from TORRELL *et al.*, 2001; GARCIA *et al.,* 2004); [2]chromosomal formula according to LEVAN *et al.* (1964); [3]mean chromosome length; [4]chromosome length range; [5]symmetry class according to STEBBINS (1971); [5]ROMERO (1986) indexes; A1: intrachromosomal asymmetry index, A2: interchromosomal asymmetry index.

Taxon	2*n*	Ploidy level	Mean 2C (pg)[1]	Chromosomal formula[2]
A. absinthium	18	2*x*	8.75	12m + 2m^{sat} + 4sm
A. chamaemelifolia	18 + (1-5)B	2*x*	6.04	10m + 2m^{sat} + 4sm + 2sm^{sat}
A. vulgaris	16	2*x*	6.26	14m + 2sm^{sat}

				Romero indexes[6]	
Taxon	MCL[3]±(SD) (µm)	CLR[4] (µm)	Stebbins class[5]	A1	A2
A. absinthium	4.27 ± 0.23	38.46	2A	0.30	0.15
A. chamaemelifolia	3.08 ± 0.10	27.76	2A	0.09	0.09
A. vulgaris	3.69 ± 0.25	29.51	2A	0.24	0.18

(with the presence of one to three B-chromosomes in the case of *A. chamaemelifolia*) and *A. vulgaris* 2*n* = 16.

Artemisia absinthium presents long chromosomes (MCL: 4.27 ± 0.23 µm) and the values of the asymmetry indexes (STEBBINS, 1971) reveal a very symmetric karyotype (Table 2). We have detected a secondary constriction in pair VII, constituting a satellite in its short arm, which is positively stained both with chromomycin and the two FISH probes (Fig. 1A-D). The same pattern is found in the short arm of pair III, although no satellite is visible in this chromosome. Two loci with both rDNA (18S-5.8S-26S and 5S) colocalized were detected for this specimen. Several AT-rich bands, stained with DAPI, have also been detected at chromosome ends, as shown in Fig. 1D.

Artemisia chamaemelifolia displays again a very symmetric karyotype. The presence of CMA+ and DAPI+ heterochromatin signals has been detected at centromeric and telomeric position (Figs. 1F; 1G). As found in *A. absinthium*, four GC-rich DNA bands located at satellites coincide with the two rDNA loci, with both 5S and 18S-5.8S-26S rDNA colocalized. The presence of up to three B-chromosomes has been also observed in the studied population but these kind of chromosomes are normally variable in number depending on the specimen studied. Some of these B's show heterochromatic bands and, occasionally, ribosomal DNA loci have been detected in some of these supernumerary chromosomes (data not shown).

Artemisia vulgaris also presents a symmetrical karyotype, with abundant GC-rich bands, mainly at telomeric position, but also near the centromeres (Figs. 1J; 1K). As observed in the other taxa considered, both kinds of rDNA are colocalized (also in this case, two rDNA loci are found), and coincidental with some CMA-rich regions. Additionally, as in the other species studied, some AT-rich bands are detected.

DISCUSSION

Chromosome number

For both *A. absinthium* and *A. chamaemelifolia* previous studies have detected diploid and tetraploid populations respectively. For *A. vulgaris*, either *x* = 8- or *x* = 9-based cytotypes have previously been reported, with 2*n* = 16 as a predominating chromosome number and, in addition, 2*n* = 18 and several unusual chromosome

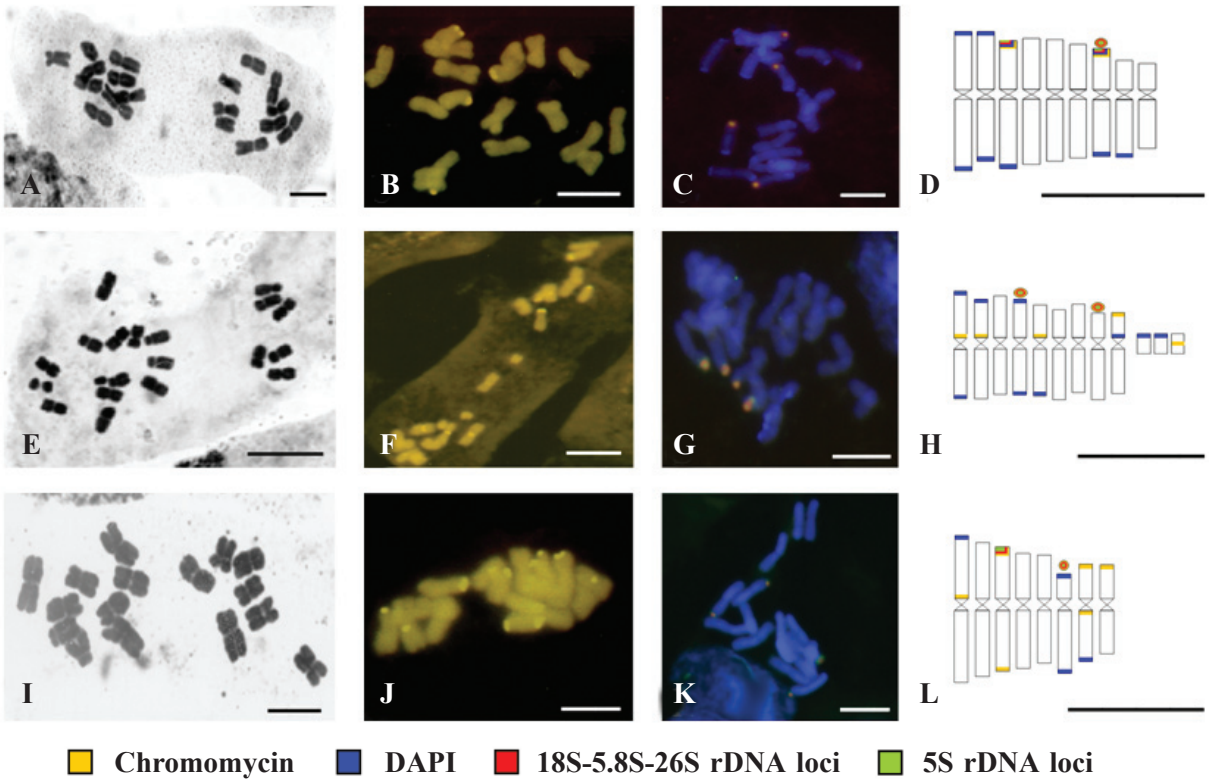

Figure 1. Metaphase plates stained with aceto-orcein (A, E, I), fluorochrome banding with chromomycin (B, F, J), fluorescent *in situ* hybridization (C, G, K) and haploid idiograms of the species studied (D, H, L). *Artemisia absinthium* (A, B, C, D), *A. chamaemelifolia* (E, F, G, H), *A. vulgaris* (I, J, K, L). Scale bars = 5 µm.

numbers ($2n$ = 24, 34, 40, 45), including the occasional presence of B-chromosomes (http://www-asteraceae.cla.kobe-u.ac.jp/search.html; 14/07/2007). In the latter species, Vallès & Siljak-Yakovlev (1997) reported the evidence of a chromosomal fusion and a certain centromeric fragility of the longest chromosome pair, which could explain the supposed $2n$ = 18 counts. The other numbers may belong to other species of the *A. vulgaris* complex (such as *A. princeps* Pamp. or *A.* x *wurzellii* C.M. James & Stace in C.M. James, Wurzell & Stace, James *et al.*, 2000, Vallès *et al.*, 2005), since many counts performed in genuine *A. vulgaris* populations have consistenly yielded $2n$ = 16 (Vallès *et al.*, unpubl. data).

Kayotype features

Indexes of asymmetry

All three studied species present a very symmetrical karyotype, which is a common trait in the genus (Vallès & Garnatje, 2005, and references therein), and is consistent with the assumption of a symmetrical karyotype characterizing the species included in tribe Anthemideae (Schweizer & Ehrendorfer, 1983). Furthermore, *A. chamaemelifolia* shows a particulary low index of interchromosomal asymmetry, which is smaller than the average for the genus (Vallès & Garnatje, 2005, and references therein).

Karyotype morphology

Among the taxa studied, karyotype morphology is rather homogeneous. As a general pattern in these diploid species, we have detected the presence of six to eight metacentric (m) and one to three submetacentric (sm) chromosome pairs. Furthermore, the presence of secondary constrictions (SC) and satellites (SAT) has been observed. It is possible that more SC and SAT exist but they are not always detectable, depending on the degree of chromatin condensation, so they have not been used for chromosome identification (Vischi *et al.*, 2003).

These results are rather similar to those recorded in *Artemisia* in previous works (MCARTHUR & SANDERSON, 1999; TORRELL *et al*., 2003; GARCIA *et al.*, 2007). Karyotype homogeneity is another feature of the species included in the present study, as PERSSON (1974) stated for the whole genus. Only slight differences in size between chromosomes have been detected.

Karyotype size

Although MCL of the species studied are quite similar, some differences between each other are detected, which are mirrored in genome size differences. This illustrates once more the remarkable correlation between genome size and karyological data (BENNETT & LEITCH, 2005, and references therein). *Artemisia absinthium* is the species with the largest MCL (4.27 ± 0.23 μm) of the three, and also presents a higher nuclear DNA content (2C = 8.75 pg, Table 2). Contrarily, *A. chamaemelifolia* accounts for the smallest MCL (3.08 ± 0.10 μm), and its genome size (2C = 6.04 pg, Table 2) correlates negatively with the karyotype size. *Artemisia vulgaris*, with an intermediate karyotype length (3.69 ± 0.25 μm), presents a nuclear DNA amount (2C = 6.26 pg, Table 2) which ranges between those of the two other species.

Presence of B-chromosomes

B- or supernumerary chromosomes have been detected in one of the species studied, *A. chamaemelifolia* (Figs. 1E-H), as already pointed in TORRELL *et al*. (1999). The presence of B-chromosomes has been frequently observed in other subgenera of *Artemisia*, e. g. *Absinthium* (PELLICER *et al*., 2007) or *Tridentatae* (GARCIA *et al*., 2007). B's are extra chromosomes found in some, but no all individuals within a species, and have been described in many plants and animals (JONES & REES, 1982). Their function, composition and origins are not still well understood (TRIVERS *et al*., 2004). The number of B-chromosomes found is also variable, from 1 to 3, when present. The frequency of occurrence of this kind of chromosomes in *Artemisia* is also a subject of discussion (MCARTHUR & SANDERSON, 1999; GARCIA *et al*., 2007). Although different kind of B's might be found in plants linked to their origin (VALLÈS & SILJAK-YAKOVLEV, 1997), in *A. chamaemelifolia* we have only detected some compact, small chromatin bodies, in which the centromere is hardly seen. Most B-chromosomes are heterochromatic, composed of repetitive DNA sequences (CUADRADO & JOUVE, 1994), suggesting the idea that they are genetically inert. Nonetheless, some of them show the presence of ribosomal genes, as in *A. chamaemelifolia*, which might imply transcriptional activity (GREEN, 1998), although this point is still to be confirmed in this genus.

The three studied species present strongly homogenous karyotype traits. Although rather consistent at the genus level, karyotype resemblance generally does not allow identification and discrimination of *Artemisia* species (HOSHI *et al*., 2003). In this sense, fluorochrome banding and FISH of rDNA can provide useful data for karyotype characterization and distinction in closely related species.

Fluorochrome banding and fluorescent *in situ* hybridization (FISH)

Banding pattern

The banding and FISH patterns here shown are coincidental to a high degree with those established hitherto in different molecular cytogenetic researches centred in *Artemisia* (TORRELL *et al*., 2001, 2003; GARCIA *et al*., 2007). Fluorochrome banding with chromomycin revealed heterochromatin regions composed of GC-rich DNA (Figs. 1B; 1F; 1J), and in all cases these regions were located at distal ends, in some cases in satellites. Only in the species belonging to subgenus *Artemisia* (*A. chamaemelifolia*, *A. vulgaris*), centromeric GC-regions were detected, and sometimes the B-chromosomes of *A. chamaemelifolia* also revealed positive regions when treated with chromomycin. In a previous work (TORRELL *et al*., 2003), only four positive chromomycin bands were detected in diploid individuals of a species belonging to subgenus *Artemisia*, *A. annua*. In the present study we detected six or seven CMA+ signals. *Artemisia annua* being annual, the divergence observed with regard to CMA+ signal number of this taxa with respect to its perennial relative could be related to their difference in life cycle. Such a difference has been also found in other Asteraceae (e. g. HIDALGO *et al*., submitted).

Artemisia chamaemelifolia and *A. vulgaris* belong to the same subgenus, *Artemisia*, and both present positive CMA signals at centromeric position, which might reflect a closer phylogenetic relationship between these two taxa than with *A. absinthium*, as indicated by molecular phylogenetic studies (VALLÈS *et al*., 2003; SANZ *et al*., 2008). However,

phylogenetic inferences from cytogenetical data should be cautiously done because subgenus *Artemisia* may not to have a unique CMA signal pattern, and AT-rich (DAPI-positive) bands are presented much more variable among these three taxa. DAPI-positive signals are always telomeric except in the case of *A. chamaemelifolia*, in which a centromeric band has been detected in a single chromosome pair. In most cases, this kind of banding coincides or is located very close to GC-rich signals.

FISH pattern: colocalization of the 18S and 5S rDNA

Our FISH results show that all three *Artemisia* species present four signals (two loci) for 18S-5.8S-26S and 5S regions (Figs. 1C; 1G; 1K). In all cases these signals are colocalized, so 18S-5.8S-26S and 5S rDNA signals are always in the same number and in the same position. This peculiar distribution of the 45S (18S, 5.8S and 26S rRNA genes) and 5S rDNA, which in animals and plants are usually placed separately and transcribed by different RNA polymerases (SRIVASTAVA & SCHLESSINGER, 1991), has been previously reported in former investigations in *Artemisia* (TORRELL *et al*., 2003; HOSHI *et al*., 2006; GARCIA *et al*., 2007) and in other Anthemideae genera, e. g. *Chrysanthemum* L. (ABD EL-TWAB & KONDO, 2006), which is quite close to *Artemisia*. The overlapped position of these rDNA sites might be a frequent feature of chromosome evolution in *Artemisia*, and probably in some other Anthemideae (GARCIA *et al*., unpubl. res.). Hence, the results obtained in former studies and in the present one suggest that the existence of 18S-5.8S-26S rDNA linked to 5S rDNA is not an experimental artifact; previous works hypothesize a casual insertion of the 5S rDNA into the 45S repeat unit, (DROUIN & MONIZ DE SA, 1995), although a clear interpretation of this phenomenon is not still achieved.

CONCLUDING REMARKS

The present study reports a typical *Artemisia* and *Absinthium* signal pattern at the diploid level, with four rDNA loci located at telomeric positions, and sometimes in satellites of metacentric and submetacentric chromosomes. The different pattern of the distribution of GC-rich regions at telomeric and centromeric position between these two subgenera may reflect the different evolutionary histories of these species. All these results contribute to the cytogenetical knowledge of *Artemisia*, and, according to former investigations, we demonstrate that the patterns of distribution of the rDNA loci might be subject to the different evolutionary episodes that have taken place in the genus. The extensive colocalization of the 18S-5.8S-5S rDNA and 5S rDNA found in *Artemisia* should be considered as a particular trait of the evolution of ribosomal DNA in the genus, as well as in other Anthemideae genera. Further investigations would be needed to elucidate when and how these changes at genome level took place and what information they convey on the relationships at the species level within the *Artemisia* as well as at the tribal level.

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Requereixen els poliploides proporcionalment menys *loci* d'rDNA que els seus corresponents diploides? Exemples en el gènere *Artemisia*, subgèneres *Absinthium* i *Artemisia* (*Asteraceae, Anthemideae*)

Plant Biosystems (enviat)

Jaume Pellicer, Teresa Garnatje, Oriane Hidalgo, Norikazu Tagashira, Joan Vallès i Katsuhiko Kondo

S'ha dut a terme hibridació *in situ* fluorescent (FISH) de les regions del DNA ribosòmic 5S i 18S-5.8S-26S en dues espècies del gènere *Artemisia* pertanyents als subgèneres *Artemisia* (*A. medioxima*) i *Absinthium* (*A. lagocephala*), cadascuna amb poblacions de baixos i alts nivells de ploïdia (2*x*, 4*x* i 16*x*, i 2*x*, 4*x* i 6*x*, respectivament). Ambdues espècies presenten un nombre cromosòmic bàsic $x = 9$. Els gens de l'rRNA són colocalitzats, com ja s'ha observat en recerques prèvies. La població diploide ($2n = 18$) d'*A. lagocephala* en presenta tres *loci* i l'hexaploide, sis. Així mateix, en *A. medioxima* el nombre de *loci* de l'rDNA no s'incrementa proporcionalment al nivell de ploïdia. En aquesta espècie, la població diploide presenta dos *loci* d'rDNA, la tetraploide en mostra quatre i l'hexaidecaploide en té al voltant de 20. Aquests resultats evidencien una pèrdua relativa de *loci* d'rDNA i d'heterocromatina, i aquest fenomen es dóna de manera més pronunciada en poliploides alts. No obstant, el patró de bandes de DAPI no segueix aquesta tendència en *A. lagocephala*, on trobem un increment espectacular de bandes d'heterocromatina al nivell hexaploide. Aquests resultats han estat discutits a la llum de l'existència de possibles fenòmens com reestructuracions i mecanismes de silenciament gènic que poden tenir lloc durant la poliploïdització i, més freqüentment, durant la formació d'al·lopoliploides.

Do polyploids require proportionally less rDNA loci than their corresponding diploids? Examples from *Artemisia* subgenera *Absinthium* and *Artemisia* (Asteraceae, Anthemideae)

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Abstract

Fluorescent *in situ* hybridization (FISH) of 5S and 18S-5.8S-26S ribosomal DNA has been carried out in two species of the genus *Artemisia*, belonging to the subgenera *Artemisia* (*A. medioxima*) and *Absinthium* (*A. lagocephala*) each one showing both low and high ploidy levels (2*x* and 16*x*, and 2*x*, 4*x* and 6*x*, respectively). Both species have a base chromosome number of $x = 9$. Linkage of both rDNA genes has been observed confirming previous researches. Diploid *A. lagocephala* ($2n = 18$) shows three rDNA loci and the hexaploid six. Also in *A. medioxima*, the number of rDNA loci does not increase in the proportion given by the ploidy level, and a relative loss is found. In this species, the diploid population shows two rDNA loci, the tetraploid four, and the hexadecaploid has around 20. The results evidence a relative loss of rDNA loci and heterochromatin, a phenomenon more pronounced at higher ploidy levels. Nevertheless, the DAPI banding pattern of *A. lagocephala* does not follow this trend, as it shows a spectacular increase of heterochromatic bands at the hexaploid level. These results are discussed in the light of possible chromosome restructurations and gene silencing mechanisms that take place during polyploidy, and more especially the allopolyploids formation.

Keywords: *Absinthium*, *Artemisia*, Asteraceae, constitutive heterochromatin, fluorescent *in situ* hybridization, rDNA.

Introduction

The genus *Artemisia* L. (Anthemideae, Asteraceae) comprises ca. 500 species and constitutes one of the largest genera in this family (Vallès & Garnatje 2005). This genus is very abundant in the Northern hemisphere and dominates many landscapes, indicating the ability of these plants to colonize different habitats. The plasticity of *Artemisia* genome is revealed especially from the genome size point of view, as evidenced in previous studies carried out by several authors (Vallès & Torrell 2001; Garcia et al. 2004, 2006b, 2008; Pellicer et al. 2008). Polyploidy, a phenomenon with high incidence in plant evolution (Otto & Whitton 2000), has been particularly relevant in *Artemisia* (Pellicer et al. 2007b and references therein). Recently, Soltis (2005) pointed out that all angiosperms are ancient or recent polyploids.

Artemisia is currently divided into five subgenera (Vallès & Garnatje 2005). Two of them, *Absinthium* and *Artemisia*, appear mixed in all molecular phylogenetic reconstructions obtained (Torrell et al. 1999; Vallès et al. 2003; Watson et al. 2002; Sanz et al. 2008; Tkach et al. 2008). This work focuses on two representatives of those subgenera with different ploidy levels, *A. lagocephala* (Bess.) DC. (subgenus *Absinthium*, 2*x* and 6*x* ploidy levels) and *A. medioxima* Krasch. ex Poljakov (subgenus *Artemisia*, 2*x*, 4*x* and 16*x* ploidy levels), with the purpose to compare the rDNA loci number and localization throughout the polyploid series. *Artemisia lagocephala* is a perennial undershrub growing in stony and rocky places, endemic to the Russian Arctic, Siberia and Far East regions (Poljakov 1961). *Artemisia medioxima* is also a perennial species with long rhizomes occupying the forestry zones, leaf-bearing forests and meadows in the Russian Far East and Northern China (Poljakov 1961). The phylogenetic position of these species is not yet solved. In a more complete phylogenetic framework than the above cited (Pellicer et al. 2009), *A. lagocephala* is completely merged into a clade constituted by species belonging to subgenus *Absinthium*, along with *A. krushiana* Besser and *A. litophila* Turcz. ex DC., whereas *A. medioxima* appears closely related to *A. atrata* Lam., *A. laciniata* Willd. and *A. tanacetifolia* L., both taxa belonging to the subgenus *Artemisia*

Chromosome banding and fluorescent *in situ* hybridization (FISH) have demonstrated to be a valuable tool for studies on genome organization and evolution in plants (Sumner 1990; Jiang & Gill 1994). In particular, the detection of sites 18S-5.8S-26S and 5S ribosomal DNA is useful for chromosome identification and for evolutionary studies of *Artemisia* species (Vallès & Siljak-Yakovlev 1997; Torrell et al. 2001, 2003; Garcia et al. 2008, 2009; Pellicer et al. 2008). In these works, the FISH pattern was not always clearly related with the phylogenetic position of the species but in some cases, it was more likely associated to specific features as the life cycle.

Until now, the works dealing with rDNA loci mapping in *Artemisia* have been carried out mostly in low ploidy levels (mainly 2*x* and 4*x*, and only in one case in 8*x*; Garcia et al. 2008 and references therein). The objective of the present paper is to compare the rDNA loci distribution within a species showing different ploidy levels, including the one in which the highest ploidy level has been detected in the genus (16*x*, *A. medioxima*, Pellicer et al. 2007b).

Material and methods

Plant material

The populations of the species studied are listed in Table 1, classified by subgenera, with the indication of their origins, vouchers (deposited in the herbarium BCN, Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona) and collectors. Root tips were obtained from achenes germinating in wet paper in Petri dishes and subsequently pretreated in 0.05% aqueous colchicine solution at room temperature during 2 h 15 min. After this first step, the root tips were fixed in 3:1 absolute ethanol and glacial acetic acid, stored at 4°C and transferred in a solution of ethanol 70% after a few days in the fixative.

Chromosome (protoplast) preparation

Root tips were washed in distilled water during 10 min and prepared following the method described in Leitch & Heslop-Harrison (1993), with minor modifications. The tips were incubated in a microcentrifuge tube containing 200 µl of an enzymatic solution [3% Cellulase Onozuka-RS (Yakult Honsha) and 0.5% Pectolyase Y-23 (Kikkoman)] during 40-50 min, depending on species, at 37°C.

They were washed in distilled water for a 5 min, placed in a clean slide, crushed into a drop of fixative and air-dried. Staining of the slides with 1% acetic orcein was carried out to find the metaphase plates. The slides were frozen overnight at – 80ºC to make easier the cover slides removal, and for acetic orcein distaining, they were washed with 45% acetic acid for 1 h and subsequently two times with distilled water during 10 min and air-dried.

Table 1. Origin, collectors and vouchers of the *Artemisia* species and populations studied.

Species	Origin, collectors and voucher
Subg. *Absinthium* (Mill.) Less.	
Artemisia lagocephala (Fischer ex Besser) DC.	Russia, Snezhnaya mountain. Index Seminum Vladivostok nº 53 (BCN S-805). 2*n* = 18
Artemisia lagocephala (Fischer ex Besser) DC.	Russia, Sokha Republic (Yakutya), Aldan raion: Ugoyan, near the mouth of Tommozh river, forest, V.N. Zakharova, 26.viii.2005 (LE-Korobkov 06-29). 2*n* = 54
Subg. *Artemisia*	
Artemisia medioxima Krasch. ex Poljakov	Mongolia, Tuv (Central) aimag: Mungunmort sum, 10 km north-west of the sum, path margins, steppe, Sh. Dariimaa, Sh. Tsooj, J. Vallès & E. Yatamsuren, 7.ix.2004 (BCN 23792). 2*n* =18, 36
Artemisia medioxima Krasch. ex Poljakov	Russia, Khabarovskii krai: Baninskii raion, Tumnin river basin, upper Akur river valley, base of rocky slopes, Larix and Betula forest, A.A. Korobkov, 21.x.2004 (LE-Korobkov). 2*n* =144

Probe preparation and fluorescent in situ *hybridization*

The 18S-5.8S-26S rRNA gene of wheat, pTa71, which contained the intergenic spacer region, was used as 45S rDNA probe (Gerlarch and Bedbrook, 1979). Total genomic DNA of *Artemisia princeps* Pamp. was extracted from young leaves following the method of Doyle & Doyle (1987) modified by Cullings (1992). The 5S rDNA probe was amplified by PCR using the primers described in Hoshi et al. (2006) and with the following PCR conditions: one cycle of 5 min at 94.2ºC, 35 cycles of (30 sec at 94.2ºC, 30 sec at 55.5ºC and 30 sec at 72.2ºC) and one cycle for final extension at 72.2ºC during 7 min. The PCR reaction mixture (final volume of 50 µl) contained 60 ng of DNA template, 200 pmol of each primer, 0.1 mM of each dNTP, 10 mM of Tris-HCl pH 8.3 buffer, 50 mM of KCl, 1.5 mM of $MgCl_2$ and 1 unit of Taq polymerase (Tanaka). The 5S and 18S probes were labelled with random primed DNA labelling with digoxigenin-dUTP (Roche Diagnostics, Mannheim, Germany) and with Avidin-FITC BioNick

labelling system (Invitrogen, USA), respectively, following the manufacturer's instructions. Hybridization mixtures contained 50% formamide, 10% dextran sulfate, and each probe (concentration of 4 ng/µl in 2xSSC) were subsequently denatured during 10 min at 95°C.

Slide preparations were incubated in 100 µg/ml DNase-free RNase in 2xSSC for 1 h at 37°C in a wet chamber, washed once in 2xSSC (pH 7) for 10 min with slow shaking and then 10 min in 1xPBS (pH 7), treated with 4% paraformaldehyde in 1xPBS during 10 min, denatured at 72°C with 70% deionized formamide in 2xSSC 1.5 min, and dehydrated through an ethanol series (70°, 90° and 100°) and air dried. After denaturation of probe mixtures, approximately 15-20 µl of probe were loaded on the slide and covered with coverslips. The preparations were then denatured during 5 min at 75°C, and transferred down to 37°C overnight for hybridization in a wet chamber. Posthybridization stringency washes were done with agitation as follows: two washes in 4xSSC at 42°C for 10 min followed by a wash in 2xSSC (with 0.2% Triton-100) at room temperature. For 5S signal detection, the slides were treated with 1% (w/v) bovine serum albumine (BSA) in 2xSSC with 0.2% Triton-100 for 45 min at 37°C, and then incubated for 1.5 h at 37°C in 20 µg/ml Anti-digoxigenin-rhodamine Fab fragments (Roche Diagnostics, Mannheim, Germany) in the same buffer. Slides were washed two times for 10 min in 2xSSC with 0.2% Triton-100 at 42°C, once in 2xSSC at room temperature 5 min, once in distilled water at room temperature for 5 min, and finally dehydrated (ethanol 70°, 90° and 100°). Counterstaining was done with Vectashield (Vector Laboratories, Burlingame CA, USA), a mounting medium containing 500 ng/ml DAPI (4',6-diamidino-2-phenylindole). FISH preparations were observed with an epifluorescence Nikon Eclipse E600 microscope using the following filters: UV-1A (365/410), B-2A (450/490), G-2A (510/560), Dia-ill. Hybridization signals were analysed and photographed using a CCD camera (Pixera, pengium 600CL), and an image analyser software (Metavue, version 4.6, Molecular Devices Corporation).

Results

The chromosome number, DNA amount and number of bands visualised with DAPI (after FISH denaturation), as well as rDNA FISH signals for each studied population, are summarized in Table 2. Somatic metaphases with FISH signals and DAPI marks of both taxa at the different ploidy levels are presented in Figs. 1 and 2. All populations show a very symmetric karyotype, typical in *Artemisia* (Torrell & Vallès, 2001 and references therein) and, in general, in the tribe Anthemideae (Schweizer & Ehrendorfer 1983). Data on constitutive heterochromatin and rDNA loci of previously studied *Artemisia* species belonging to subgenera *Absinthium* and *Artemisia* has been used for comparative purposes.

Table 2. Karyological and cytogenetic features of the studied species and populations.

Species	Chromosome number (2*n*)	DAPI (constitutive heterochromatin)	18S-5.8S-26S	5S
Subg. *Absinthium*				
A. lagocephala	18	2	6	6
A. lagocephala	54	78	12	12
Subg. *Artemisia*				
A. medioxima	18	12	4	4
A. medioxima	36	ca. 18	8	8
A. medioxima	144	≥ 34	ca. 20	ca. 20

Discussion

FISH pattern

The 18S-5.8S-26S and 5S rRNA genes are co-localized in both studied taxa, confirming the reports of all previous works on this subject in the genus *Artemisia* (Torrell et al. 2003; Garcia et al. 2007, 2009; Pellicer et al. 2008), in which both ribosomal RNA genes are linked in a single unit, a structure recently described for the first time in angiosperms (Garcia et al. 2009).

There is no correlation between the number of rDNA loci and ploidy level. It seems that the number of FISH marks does not increase proportionally to the ploidy level and moreover in the highest polyploids the number of FISH signals is much lower than expected (Fig. 3b,d). In *A. lagocephala*, six signals are detected in the diploid populations, whereas the hexaploid one presents only 12, some of them weak, instead of the expected 18 rDNA sites (Fig. 3b). The situation is

similar in *A. medioxima*, where the proportionality, although maintained between the diploid and the tetraploid levels (with four and eight signals, respectively), is broken in the hexadecaploid population, which only shows ca. 20 marks (instead of the 32 expected, Fig. 3d).

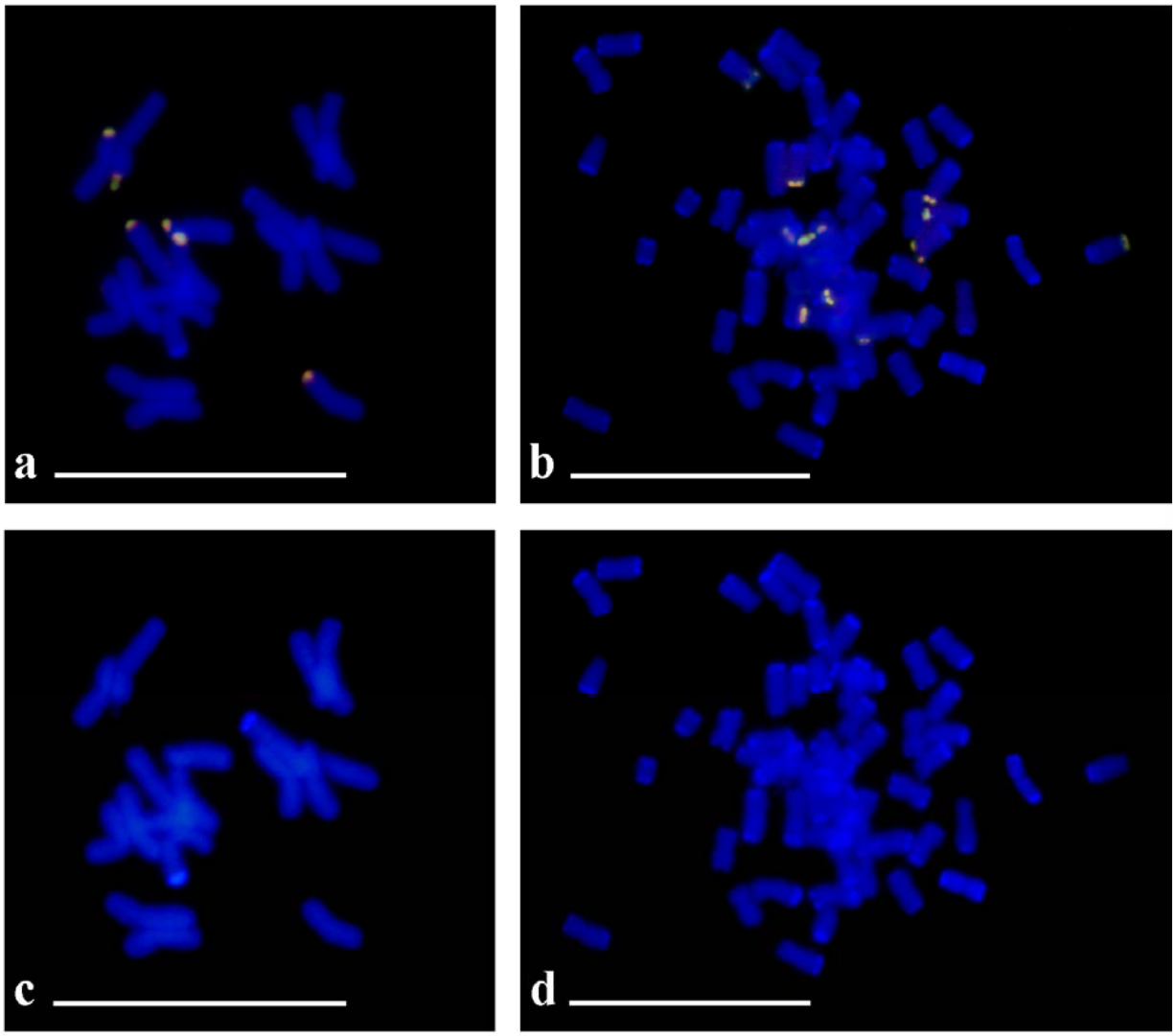

Figure 1. Physical mapping of 5S and 18S-5.8S-26S rDNA loci revealed by FISH and DAPI signals in diploid (a, c) and tetraploid (b, d) populations of *Artemisia lagocephala* (subgenus *Absinthium*).

A variation of the number of rDNA loci or copy number variation have been related to the polyploid formation, as well as genome downsizing, in which nuclear DNA amount of the polyploid is less than the sum of genome sizes of the parental diploids (Lim et al. 2000; Leitch et al., 2008; Leitch and Leitch 2008). On the one hand, the presence of some weaker ribosomal signals in the case of

A. lagocephala could indicate that loss of gene copies might be taking place at these loci, as was documented in polyploid *Nicotiana* by (Leitch et al. 2008), even with an additive loci pattern. On the other hand, the hypothesis of gene loss may be also contemplated at the same level as other different consequences of polyploidy, such as evolution in function in duplicate genes or gene silencing (Adams & Wendel 2004). When considering that, the exact cause and the mechanisms of the rDNA loci loss in polyploids remain unclear, the simple excess of dispensable genetic material being one of the most plausible explanations for sequence elimination. In this respect, according to Reeder (1999), the total number of rDNA copies present in a genome is much beyond the necessary to supply the requirements of ribosomes, as many copies remain transcriptionally inactive, so the loss of rDNA loci (or copy number) in polyploids might not represent a problem of transcriptional requirements to these species. The present results corroborate this fact, suggesting that some rRNA gene copies may not be strictly necessary in polyploids, so that they can be eliminated in the frame of DNA loss associated with polyploid formation and stabilization.

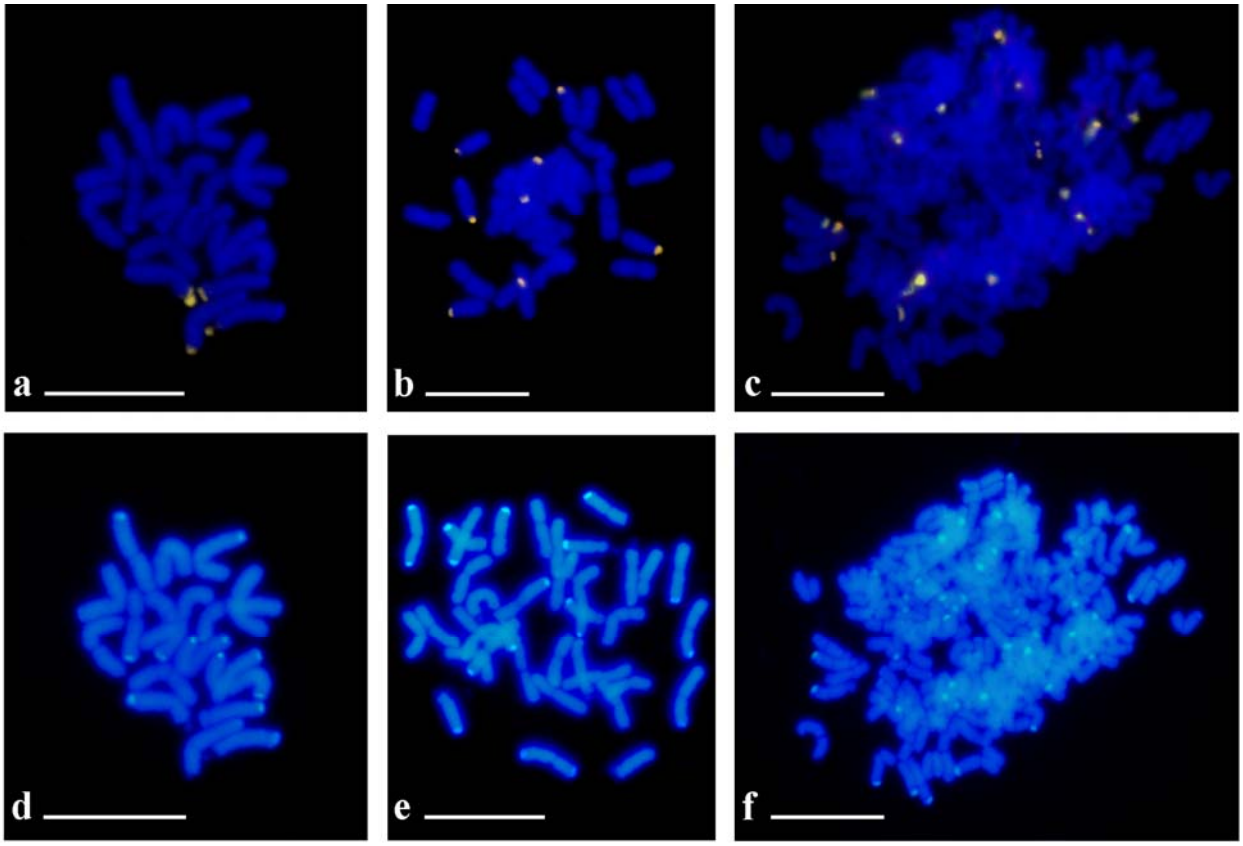

Figure 2. Physical mapping of 5S and 18S-5.8S-26S rDNA loci revealed by FISH in diploid (a, d), tetraploid (b, e), and hexaidecaploid (c, f) populations of *Artemisia medioxima* (subgenus *Artemisia*).

The polyploid populations of both studied species exhibit a relevant ratio of vegetative multiplication and an aptitude to occupy new habitats; particularly, the 16*x* *A. medioxima* population colonized a large area in a territory after being affected by a fire (A. A. Korobkov, pers. comm.). Vegetative reproduction, which gives the plants the ability to colonize new environments, is often linked to polyploidy (Urbanska 1986; Lumaret et al. 1997). Additionally, this would be consistent with findings in *Nicotiana* polyploids, in which, although the number of rDNA loci was additive, considerable sequence elimination of individual copies had taken place (Leitch et al. 2008), cytogenetic instability has been described in plants with vegetative reproduction (Duncan 1945; Lewis 1970; Persson 1974; Couderc et al. 1985). In particular, aneusomaty has been reported in the hexaploid *Artemisia verlotiorum* Lamotte (Martinoli & Ogliotti 1970; Vallès 1987), a plant that usually reproduces vegetatively. The vegetative-spreading plants can better stand cytogenetic abnormalities, because meiosis is not so significant for them. Although a high amount of rDNA may be necessary for a higher protein synthesis in these plants that quickly develop in new habitats, a certain rate of rDNA loci or gene copy loss (in the context of the chromosomal irregularities proper to asexually-reproducing plants) should not be problematic.

This rDNA loci decreasing could be, in part, considered in the framework of the above-mentioned frequent occurrence of genome downsizing in polyploids (Leitch & Bennett 2004). This phenomenon has been repeatedly reported in *Artemisia* (Torrell & Vallès 2001; Garcia et al. 2004, 2008; Pellicer et al. 2007b). It is also confirmed for the species studied in the present paper, with a 1C*x*-value downsizing around 15% in the hexaploid *A. lagocephala* and around 47% in the hexaidecaploid *A. medioxima* with respect to their diploid relatives (Pellicer et al. submitted). Restructuration during homoeologous chromosome pairing in allopolyploids, leading sometimes to deletions and the elimination of specific DNA sequences after polyploid formation are two of the possible explanations for this non-proportional DNA content increase in polyploids (Leitch

& Bennett 2004, and references therein). Furthermore, evidences have been reported in several genera of gene loss in polyploids, including rDNA loci (Wendel 2000; Ozkan et al. 2001; Leitch & Bennett 2004). Reduction of rDNA loci in polyploids has also been documented in *Artemisia*. Diploid *A. herba alba* Asso subsp. *valentina* (Lam.) Mascl. and tetraploid subsp. *herba-alba* present the same number (four) of rDNA loci (Torrell et al. 2003), and tetraploid *A. umbelliformis* exhibits eight rDNA loci instead of the 12 expected considering the six present in the related diploid *A. eriantha* (Garcia et al. unpubl. res.).

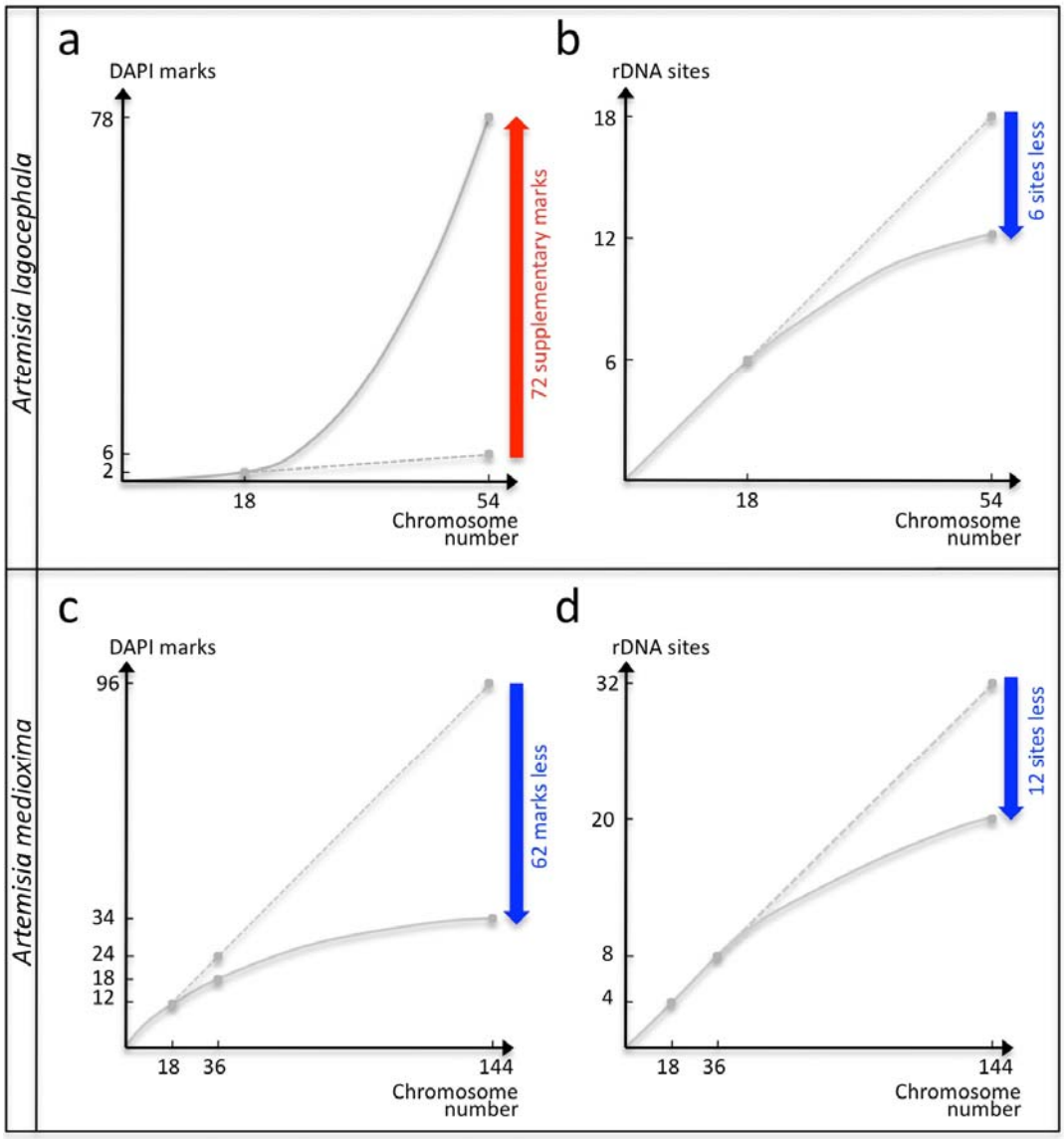

Figure 3: Graphical representation of the variation of DAPI marks and rDNA sites with increasing ploidy levels. **a-b** *Artemisia lagocephala*, DAPI marks ft. chromosome number (a), rDNA sites ft. chromosome number (b). **c-d** *Artemisia medioxima*, DAPI marks ft. chromosome number (c), rDNA sites ft. chromosome number (d). Full lines represent de observed values and dotted lines the expected ones

In the studied *Artemisia* species, the basic pattern of the variation of rDNA loci number with polyploidy is consistent with the findings in other genera, as it corresponds either to the exact proportionality (e.g. tetraploid *A. medioxima*, Fig. 3d; in *Tragopogon*, Pires et al. 2004; in *Nicotiana*, Leitch et al. 2008) or to a relative loss of sites (e.g. highest polyploids of *A. lagocephala* and *A. medioxima*; loss of a 5S sites in *Sanguisorba*, Mishima et al. 2002; loss of 18S-5.8S-26S loci in *Thinopyrum*, Li et al. 2004).

DAPI banding pattern

Since DAPI was used after chromosome denaturation, the regions detected with this fluorochrome correspond to constitutive heterochromatin and are equivalent to those obtained with Giemsa C-banding (Bogunic et al. 2006). The pattern of DAPI banding observed in the studied species is less homogeneous than for rDNA loci, as the number of heterochromatin marks in polyploids can be either more (*A. lagocephala*; Fig. 3a) or less (*A. medioxima*; Fig. 3c) than expected with respect to the diploids .

A dramatic increase of DAPI bands from the diploid (two telomeric marks) to the hexaploid (76 telomeric plus two subtelomeric marks) levels occurs in *A. lagocephala* (Fig. 1c,d; Fig. 3a). The appearance of new heterochromatic blocks with polyploidy is rather unfrequent in the genus, and apart from *A. lagocephala*, it has been documented only in some representative of the subgenus *Tridentatae* (Garcia et al. 2007). Chromatin remodelling mechanisms, and especially the heterochromatinization of euchromatin, are known to play an important role in plant epigenetics (Meyer 2000). When euchromatin becomes condensed in heterochromatin, the genes are not accessible to the transcription machinery, providing a gene silencing mechanism fast, effective, and potentially reversible (Chen 2007). Epigenetic regulation of gene expression is particularly active in the early stages of polyploid formation (because of its fastness), and in the case of allopolyploids it enables the match of different genomes by solving the regulatory incompatibilities and gene dosage problems (Chen 2007). Other hypotheses to explain the emerging heterochromatin blocks might be that these correspond to

some novel satellite, as Lim et al. (2007) found in *Nicotiana*, maybe also to species-specific repeats, that have appeared during the polyploidization.

Artemisia medioxima shows less DAPI marks (than expected from the diploid population) at tetraploid and hexadecaploid levels (Fig. 3c). The diploid population ($2n = 18$) presents 12 bands, about 18 are observed in the tetraploid ($2n = 36$), and around 34 in the hexaidecaploid ($2n = 144$; Fig. 2d, e, f; Table 2). The same trend is found in the subgenus *Absinthium*, between the diploid *A. eriantha* Ten., which shows 36 C-bands, and its related tetraploid *A. umbelliformis* Lam., which has 56 (Garcia et al. unpubl. res.). In another subgenus, *Dracunculus*, a tetraploid population of *A. campestris* L. has 34 C-bands, the same number of its related hexaploid *A. crithmifolia* L. (Torrell et al. 2001). This relative loss of heterochromatic bands follows the same tendency than rDNA loci or 1Cx genome size, and may respond to the same pressures acting toward a genome diploidization

Conclusions

The study of two diploid-polyploid pairs in two closely related *Artemisia* subgenera (*Absinthium* and *Artemisia*) has basically shown that polyploidy is associated with a proportional decrease of rDNA loci, and either a decrease or increase of constitutive heterochromatin regions. These results agree with previous reports on other subgenera of *Artemisia* (Torrell et al. 2001, 2003; Garcia et al. 2008, unpubl. res.), particularly *Seriphidium* and *Tridentatae* (and *Dracunculus* only for the heterochromatin).

Ongoing research aiming to complement the knowledge of the subgenera *Absinthium* and *Tridentatae* and especially an extensive review of subgenus *Dracunculus* (Pellicer et al. unpubl. res.) will allow us to depict precisely the evolution of heterochromatin and rRNA genes in the genus.

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Cicle vital *versus* posició sistemàtica: implicacions filogenètiques en el gènere *Artemisia* L. (*Asteraceae, Anthemideae*).

Plant Systematics and Evolution (en preparació)

Jaume Pellicer, Oriane Hidalgo, Teresa Garnatje, Katsuhiko Kondo i Joan Vallès

Onze de les vora 20 espècies anuals del gènere *Artemisia*, a més de l'espècie anual relacionada *Neopallasia pectinata*, han estat estudiades des del punt de vista citogenètic. El mostreig inclou representants de tots els subgèneres llevat *Tridentatae*, el qual és compost únicament de tàxons perennes. La mida del genoma ha estat estimada i s'ha dut a terme hibridació *in situ* fluorescent (FISH) per a obtenir el mapa físic del DNA ribosòmic 18S-5.8S-26S i 5S. A més, s'ha elaborat un marc filogenètic basat en l'anàlisi de seqüències de les regions ITS i ETS del DNA ribosòmic nuclear per a poder discutir els atributs de les plantes vinculats al cicle vital en un context evolutiu. Els *loci* de l'rDNA són localitzats en la part terminal dels cromosomes -a vegades en satèl·lits- i les dues regions són sempre coincidents entre elles. Tenint en compte el marc filogenètic del gènere, observem que, depenent del subgènere i, a vegades, del nombre bàsic de cromosomes, el comportament de les plantes anuals difereix del de les perennes. En alguns casos, s'ha detectat un guany de *loci* de l'rDNA en les espècies anuals, la qual cosa suggereix que s'han esdevingut importants reorganitzacions del cariotip durant l'evolució de les formes anuals, les quals poden haver aparegut independentment en cadascun dels subgèneres que en contenen. Aquestes dades reflecteixen l'adaptabilitat d'aquestes espècies a condicions com ara l'aridesa, la salinitat dels sòls o les inundacions.

Life cycle versus systematic placement: cytogenetic implications in the genus *Artemisia* L. (Asteraceae, Anthemideae)

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ABSTRACT. Eleven of the ca. 20 annual species of *Artemisia*, plus the annual related species *Neopallasia pectinata* have been studied from cytogenetic point of view, the sampling including representatives of all subgenera but *Tridentatae* (which is only composed by perennial species). Genome size has been assessed and fluorescent *in situ* hybridization (FISH) has been performed to obtain the physical mapping of ribosomal DNA (18S-5.8S-26S and 5S). A nrDNA ITS and ETS molecular phylogenetic framework of the genus was elaborated in order to discuss in an evolutionary context the plant attributes with regards to life cycle. The rDNA loci are located in the terminal position of the chromosomes, sometimes in the satellites and always colocalized among them. Depending on the phylogenetic position of the species, the behaviour of most of annual plants differs from the perennial ones, either as the result of a phylogenetic linkage of annual species with perennial ones included in different subgenus species, or by accounting for different chromosome numbers. In some cases gain of rDNA loci number in the annual species has been detected, suggesting that important karyotypic reorganizations have taken place during the evolution of the annual forms, which may have appeared independently en each subgenus. These data can reflect the adaptability of these species to arid conditions, soil salinity and floodings.

Keywords: Compositae, fluorescent *in situ* hybridization, genome size, life cycle, rDNA

INTRODUCTION

The genus *Artemisia* L. (Asteraceae, Anthemideae) comprises around 500 basically allogamous species, distributed mainly in the Northern Hemisphere. Nowadays, five groups -usually considered as subgenera- are accepted within this genus: *Artemisia*, *Absinthium* (Mill.) Less., *Dracunculus* (Besser) Rydb., *Seriphidium* Besser ex Less. and *Tridentatae* (Rydb.) McArthur. Representatives of the genus are mostly perennial species, however, ca. 20 of them are annual (Poljakov 1961; Ling et al. 2006). The annual species are distributed in all subgenera but *Tridentatae*, accounting for multiple independent acquisitions of annual habit throughout the evolutionary history of the genus. This made *Artemisia* an interesting subject to study the evolution of plant life-cycle in a phylogenetic context.

Artemisia and related genera have been profusely studied from several points of view. Many studies have been carried out in order to establish an appropriate phylogenetic framework to improve the understanding of the karyological and cytogenetical results (Kornkven et al. 1999; Torrell et al. 1999; Watson et al. 2002; Vallès et al. 2003; Sanz et al. 2008 Tkach et al. 2008; Pellicer et al. unpublished). The karyology of this genus has been largely studied (Weinedel-Liebau 1938; Kawatani and Ohno 1964; Korobkov 1972; McArthur and Sanderson 1999; Torrell et al. 2001a; Vallès et al. 2005; Garcia et al. 2006; Pellicer et al. 2007a, b; and references therein) and also the molecular cytogenetics (Vallès and Siljak-Yakovlev 1997; Torrell et al. 2001b, 2003; Garcia et al. 2004, 2007, 2009a; Hoshi et al. 2006; Pellicer et al. 2008). A notable finding in the cytogenetic studies is that 18S-5.8S-26S (45S) and 5S sites of ribosomal DNA are all colocalized (Torrell et al. 2003; Garcia et al. 2007, 2009b; Pellicer et al. 2008).

Two basic chomosome numbers have been found in the genus *Artemisa* linked to polyploid series. Ploidy levels up to hexaidecaploid for $x = 9$ and hexaploid for $x = 8$ are known (Ehrendorfer 1964; Persson 1974; Vallès et al. 2001; Garcia et al. 2006; Pellicer et al. 2007b), which reveals the important role played by polyploidy in all the subgenera of this genus. The subgenera *Absinthium*, *Artemisia* and *Dracunculus* exhibit both $x = 9$ and $x = 8$ base

numbers, whereas *Seriphidium* and *Tridentatae* are only *x* = 9-based. The sense of dysploidy in the genus has been postulated to be descending (Vallès and Siljak-Yakovlev 1997), as in other Asteraceae such as the Centaureinae (Fernández Casas and Susanna 1986; Garcia-Jacas et al. 1996). These phenomena suggest that the evolutionary history of this genus is linked to a complex genome organization and that several events have taken place as an outcome or to facilitate the successful habitat colonization of these species. Life cycle, biogeographical distribution and habitat are factors which could induce changes in the genome of the species.

Annual species present different adaptive mechanisms than perennial ones and due to its brief life cycle a different genome organization can be expected (Bennett and Leitch 2005). Conditions that are predicted to favour annual versus perennial life-histories are environments which have a low survival of parents and high survival of seedlings (Silvertown and Charlesworth 2001). The distribution of annual and perennial habit within the phylogenies suggests that a switch in habit may have occurred several times during the evolution of the genus *Nemesia* Vent. (Datson et al. 2008). Fiz et al. (2002) proposed that the shift to annual life-history in *Bellis* L. was an adaptation to dry conditions following the Messinian crisis (5 mya) and related to establishment of summer drought in the Mediterranean (2.8 mya). In this sense, annual species occur in regions with lower and seasonal rainfall, suggesting that the development of annual forms has allowed the spread into drier environments (Datson et al. 2008).

Fluorescent *in situ* hybridization (FISH) of ribosomal DNA is an appropriate marker, as deals with conserved DNA sequences that are present in high copy numbers (Datson and Murray 2006) and has been used to identify the chromosomes and their changes taking place during plant evolution (Torrell et al. 2003). FISH signal pattern can be variable even in closely related species meaning that the position of these genes is not fixed. Some authors hypothesize that this phenomenon takes place as a transposition and by means of a transposase (Schubert and Wobus 1985) contrarily to the spread hypothesis

supporting that the genome changes are produced by translocation or inversion (Datson and Murray 2006).

The aims of the present study were: i) to provide a cytogenetic characterization of twelve diploid annual species using genome size assessments and FISH of 45S and 5S rRNA genes, ii) to evaluate the possible systematic and phylogenetic implications of genome organization in these taxa, iii) to compare the results obtained with those already published in other annual *Artemisia* species, iv) to discuss the cytogenetic changes in the light of life cycle, habitat and adaptability of these species, and v) to make some conclusions about possible prerequisites, causes and consequences for the shifts in life cycle.

MATERIAL AND METHODS

Plant material

The studied species are listed in Table 1, classified by subgenera under the traditional systematics, with their origins, collectors and vouchers. Vouchers are deposited in the herbaria of either the Centre de Documentació de Biodiversitat Vegetal de la Universitat de Barcelona (BCN) or the Botanical Institute "V. L. Komarov" of the Russian Academy of Sciences (LE).

Genome size assessments

Leaf tissue of five individuals for each studied population was chopped in 600 µl of Galbraith's isolation buffer (Galbraith et al. 1983) with a razor blade, together with the chosen internal standard, and supplemented with 100 µg/ml of ribonuclease A (RNase A, Boehringer). For each individual, two independent samples were extracted. Samples were subsequently stained with 36 µl of propidium iodide (1mg/ml) to a final concentration of 60 µg/ml (Sigma-Aldrich Química), kept on ice for 20 min and measured in an Epics XL flow cytometer (Coulter Corporation). Measurements were made at the Serveis cientificotècnics of the Universitat de Barcelona. To ascertain that the instrument showed a linear response across the range of genome sizes studied, we performed several assays which included both internal standards and one of the populations with the highest genome size at the same time. The difference between the obtained

results with respect to each standard was negligible (less than 2% of deviation) hence we can certify the linearity of the flow cytometer in this interval and the convenience of the use of the chosen internal standards. We also calculated the mean half peak coefficient of variation (HPCV) corresponding to ten samples which resulted of 2.54% in the case of *Petunia hybrida* and 1.87% for *Pisum sativum*.

Table 1. Origin, collectors and herbarium vouchers of the *Artemisia* species studied.

Taxa	Origin, collectors and voucher
Subg. *Absinthium* (Mill.) Less.	
A. anethifolia Weber ex Stechm.	Russia, Republic of Buryatia, Selenge raion: Path from Selenduma to Shanan, bottom of a dried lake. 18.ix.2005. Leg. A.A. Korobkov (LE-Korobkov 06-22)
A. anethoides Mattf.	Mongolia, Selenge aimag: Shaamar sum, 3 km west of the sum, Buureg Tolgoi hills, near river Okhon. 700 m. 9.ix.2004. Leg. Sh. Dariimaa, Sh. Tsooj, J. Vallès & E. Yatamsuren (BCN 23790)
A. jacutica Drob.	Russia, Sakha Republic (Yakutya). Ust-Aldans camp, near the village of Oner, ruderal. 10.ix.2005. Leg. V. N. Zakharova (LE 06-31).
A. jacutica Drob.	Russia, Republic of Buriatya, Eravnin district, between lakes Bolshoe Eravnoe and Maloe Eravnoe. 4.x.2007. Leg. A.A. Korobkov (LE)
A. macrocephala Jacq. ex Bess	Mongolia, Uvur Khangi aimag: Arvaykheer city, ruderal in streets. 30.viii.2004. Leg. Sh. Dariimaa, Sh. Tsooj & J.Vallès (BCN 23801)
A. sieversiana Nakai	Russia, Chitin oblast, Kyr raion: northern part of Onon-Baldzhin mountain system, southern slope, valley of a small river. 24-viii.2005. Leg. A.A. Korobkov (LE-Korobkov 06-24)
A. sieversiana Nakai	Mongolia, Ulaan Baatar, within the city. 7.ix.2004. Leg. Sh. Dariimaa, Sh. Tsooj & J. Vallès (BCN-Mong.80)
Subg. *Artemisia*	
A. biennis Willd.	United States of America, Utah: Uinta National Forest, Santaquim canyon, on ruderal soils. 31.viii.2008. Leg. S. Garcia, ED McArthur, SC Sanderson & J. Vallès (BCN-SC28)
A. biennis Willd.	Canada, Mississauga: University campus, on a road margin. 22.x.2008. Leg. J. Pellicer (BCN)
A. blepharolepis Bunge	Mongolia, Umnu (South) Gobi aimag: Bulgan sum,1 km north of the sum, desert steppe. 26.viii.2004. Leg. Sh. Dariimaa, D. Samjid, Sh. Tsooj & J. Vallès (BCN 34490)
A. palustris L.	Mongolia, Uvur-Khangai aimag: Khotont sum, 10 km east, margins of cultivated fields in steppe area. 26.viii.2004. Leg. Sh. Dariimaa, Sh. Tsooj & J. Vallès (BCN 34847)
A. palustris L.	Russia, Republic of Buriatya, distrit of Selengi. Near Bilyutai. 18.ix.2005. Leg. A.A. Korobkov (LE 06-20)
A. tournefortiana Reichenb.	Uzbekistan, Karakalpakstan: 25 km from Muynak, near the road to Nukus; banks of a channel, 100 m. 4.xi.1999. Leg. L. Kapustina, F. Khassanov, A. Susanna S-2047, J. Vallès & M. Nizamitdin (BCN 11630)

Subg. *Dracunculus* Besser	
A. scoparia Waldst. & Kit.	Uzbekistan, Karakalpakstan: Sultanuizdag mountains, near the road from Gazli to Nukus, 79 km from Nukus, dry river bed, 400 m. 3.xi.1999. Leg. L. Kapustina, F. Khassanov, A. Susanna S-2044 & J. Vallès (BCN 11628)
Subg. *Seriphidium* Besser	
A. leucodes Schrenk	Uzbekistan, Dgizak: near lake Aidarkul, 1 km from Issikul, semi-desert. 8.xi.1999. Leg. L. Kapustina, F. Khassanov, A. Susanna S-2064 & J. Vallès (BCN 11631).
Genus *Neopallasia* Poljakov	
N. pectinata (Pall.) Poljakov	People's Republic of China, Inner Mongolia, Ulanqab province, Dörnböt /Siziwang qi. 29.viii.2007 Leg. B. Liu, R. Cao & J. Vallès (BCN)
N. pectinata (Pall.) Poljakov	Mongolia, Umnu (South) Gobi aimag. 10 km S of Bulgan sum. 1.ix.2004. Leg. Sh. Dariimaa, D. Samjid, Sh. Tsooj & J. Vallès (BCN Mong. 54)

Fluorescent in situ *hybridization*

Chromosome preparation – Root tips were obtained from achenes germinating in wet filter paper in Petri dishes and subsequently pre-treated in 0.002 M 8-hidroxiquinoline at room temperature during 2 h 15 min After this first step, root tips were fixed in 3:1 absolute ethanol and glacial acetic acid for 4 h, transferred in a solution of ethanol 70% and stored at 4°C.

The root tips were washed in distilled water during 10 min and prepared following the method described in Leitch and Heslop-Harrison (1993) with minor modifications. The tips were incubated in a microcentrifuge tube containing 200 µl of an enzymatic solution [3% cellulase Onozuka-RS (Yakult Honsha) and 0.5% pectolyase Y-23 (Kikkoman)] during 40-50 min, depending on species, at 37°C. They were washed in distilled water for a 5 min, placed in a clean slide, crushed into a drop of fixative and air-dried. Staining of the slides with 0.01% acetic orcein was necessary to search the metaphase plates. The slides were frozen for 2 h at -80°C and washed in order to remove the acetic orcein, first with 45% of acetic acid and subsequently twice with distilled water. The air-dried slides were stored in the freezer.

Probes preparation and fluorescent in situ *hybridization* – For 5S rDNA probe, total genomic DNA of *Artemisia princeps* Pamp. was extracted following the method of Doyle and Doyle (1987) modified by Cullings (1992) from young leaves. 5S rDNA probe was amplified by PCR using the primers described in Hoshi et al. (2006) and with the following conditions: one cycle of 5 min at

94.2ºC, 35 cycles of (30 sec at 94.2ºC, 30 sec at 55.5ºC and 30 sec at 72.2ºC) and one cycle for final extension at 72.2ºC during 7 min. The PCR reaction mixture (final volume of 50 µl) contained 60-100 ng of DNA template, 200 pmol of each primer, 0.1 mM of each dNTP, 10 mM of Tris-HCl pH 8.3 buffer, 50 mM of KCl, 1.5 mM of $MgCl_2$ and 1 unit of Taq polymerase (Tanaka). The 5S and 18S probes were labelled with random primed DNA labelling with digoxigenin-dUTP (Roche Diagnostics) and with Avidin-FITC BioNick labelling system (Invitrogen), respectively, following the manufacturer's instructions. Hybridization mixtures contained 50% formamide, 10% dextran sulfate, and each probe (concentration of 4 ng/µl in 2xSSC) was subsequently denatured during 10 min at 95ºC.

Slide preparations were incubated in 100 µg/ml DNase-free RNase in 2xSSC for 1 h at 37ºC in a wet chamber, washed once in 2xSSC (pH 7) for 10 min with slow shaking and then 10 min in 1xPBS (pH 7); treated with 4% paraformaldehyde in 1xPBS during 10 min, denatured at 72ºC with 70% deionized formamide in 2xSSC 1.5 min, and dehydrated through an ethanol series (70º, 90º and 100º) and air dried. After denaturation of probe mixtures, approximately 15-20 µl of probe were loaded on the slide and covered with coverslips. The preparations were then denatured during 5 min at 75ºC, and transferred down to 37ºC overnight for hybridization in a wet chamber. Posthybridization stringency washes were done with agitation as follows: two washes in 4xSSC at 42ºC for 10 min followed by a wash in 2xSSC (with 0.2% Triton-100) at room temperature. For 5S signal detection, the slides were treated with 1% (w/v) bovine serum albumine (BSA) in 2xSSC with 0.2% Triton-100 for 45 min at 37ºC, and then incubated for 1.5 h at 37ºC in 20 µg/ml Anti-digoxigenin-rhodamine Fab fragments (Roche Diagnostics) in the same buffer. Slides were washed two times for 10 min in 2xSSC with 0.2% Triton-100 at 42ºC, once in 2xSSC at room temperature 5 min, once in distilled water at room temperature for 5 min, and finally dehydrated (70º, 90º and 100º ethanol). Counterstaining was done with Vectashield (Vector Laboratories, Burlingame), a mounting medium containing 500 ng/ml DAPI (4',6-diamidino-2-phenylindole). FISH preparations were observed with an epifluorescence Nikon Eclipse E600

microscope using the following filters: UV-1A (365/410), B-2A (450/490), G-2A (510/560), Dia-ill. Hybridization signals were analysed and photographed using a CCD camera (Pixera, pentium 600CL), and an image analyser software (Metavue, version 4.6, Molecular Devices Corporation).

Phylogenetic analyses

Procedures of PCR amplification and sequencing for the newly studied species *Artemisia anethifolia* Weber ex Stechm, *Artemisia anethoides* Mattf., *Artemisia blepharolepis* Bunge, *Artemisia jacutica* Drob., *Artemisia leucodes* Schrenk., *Artemisia macrocephala* Jacq. ex Bess and *Artemisia tournefortiana* Reichenb.and matrix editing were as in Sanz et al. (2008). Genbank accessions and provenance for both formerly published and newly generated sequences are listed in Appendix 1.

Bayesian Inference – Bayesian inference (BI) was carried out with MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). The most appropriate nucleotide substitution models were chosen with MrModeltest version 2.3 (Nylander 2004). Separated analyses were carried out on both markers resulting of GTR + I + G model (hLRT and AIC criteria) for the ITS region, HKY + G (AIC) and HKY + I + G (hLRT) models for the ETS, and GTR + I + G (hLRT and AIC) for the combined ITS + ETS. Four Markov chains were run simultaneously for $6x10^6$ generations, and these were sampled every 100 generations. Data from the first 6,000 generations were discarded as the *burn-in* period, after confirming that likelihood values were stabilized prior to the $6{,}000^{th}$ generation. The 50% majority rule consensus trees and posterior probability (PP) of nodes were calculated from the pooled samples.

Parsimony analyses – Searches for most parsimonious trees were undertaken with PAUP* 4.0b10 (Swofford 2003), using a heuristic search of 1,000 random sequence addition replicates, holding one tree at each step during the stepwise addition and no more than 500 trees of length (L) ≥1 on each sequence addition replicate [following a similar approach than Schneeweiss et al. (2003) and Nelsen and Gargas (2008), suggested in Giribet and Wheeler (1999)]. Starting trees were obtained via stepwise addition. All characters were unordered

and equally weighted, and gaps were treated as missing data. Uninformative sites were excluded. To ensure that no shorter trees exist, but also that the strict consensus tree obtained reflects all most parsimonious trees, a second search was then performed using the strict consensus as a constraint in a search of 10000 random addition replicates, saving one tree of L≥1 on each replicate, and holding only not compatible trees (search strategy described in Catalán et al. 1997).

Table 2. Karyological, C values and cytogenetic features in the studied species.

Taxa	Chrom. number (2*n*)[1]	2C(SD)[2]	2C(Mbp)[3]	1Cx(pg)[4]	rDNA signals[5] 45S	5S
Subg. *Absinthium*						
A. anethifolia	16	4.18(0.02)	4088.04	2.09	6	6
A. anethoides	16	3.29(0.05)	3217.62	1.64	6	6
A. jacutica	18	4.86(0.05)	4753.08	2.43	6	6
A. jacutica	18	4.82(0.07)	4713.96	2.41	-	-
A. macrocephala	18	5.04(0.06)	4929.12	2.52	6	6
A. sieversiana	18	6.17(0.07)	6034.26	3.08	-	-
A. sieversiana	18	6.12(0.03)	5985.36	3.06	4	4
Subg. *Artemisia*						
A. biennis	18	6.25(0.08)	6112.50	3.12	-	-
A. biennis	18	6.50(0.06)	6357.00	3.25	2	2
A. blepharolepis	18	9.98(0.14)	9760.44	4.99	2	2
A. palustris	18	5.16(0.11)	5046.48	2.58	4	4
A. palustris	18	5.25(0.07)	5134.50	2.62	-	-
A. tournefortiana	18	6.69(0.12)*	6542.82	3.34	4	4
Subg. *Dracunculus*						
A. scoparia	16	3.54(0.05)*	3462.12	1.77	10	10
Subg. *Seriphidium*						
A. leucodes	18	15.39(0.43)*	15051.42	7.69	8	8
Genus *Neopallasia*						
N. pectinata	18	4.98(0.08)	4870.44	2.49	-	-
N. pectinata	18	4.93(0.05)	4821.54	2.46	2	2
N. pectinata	36	10.56(0.21)*	10327.68	2.64	-	-

Note: [1]Chromosome counts from Vallès et al. (2001) and Garcia et al. (2004), confirmed in the present paper; [2]Nuclear DNA content [* indicate data from previous works (Torrell *et al*. 2001; Garcia *et al*. 2004; Pellicer *et al*. 2009)]; [3]Nuclear DNA content, 1pg = 978 Mbp (Doležel *et al.*, 2003); [4]Monoploid genome size; [5]Number of ribosomal loci 45S and 5S. The species are listed in the same order than in Table 1.

Congruence of ETS and ITS regions – The congruence of the ETS and ITS datasets was addressed by conducting a partition homogeneity test (incongruence length

difference "ILD", Farris et al. 1994) as implemented by PAUP*, using 1,000 homogeneity replicates and an heuristic search of 100 random sequence addition replicates, saving one tree at each step during the stepwise addition and no more than 1 tree of L≥1 on each sequence addition replicate. The ILD test depends on the length of the most parsimonious trees and not on recovering all of them (Berry et al. 2005), and therefore we adopted here a strategy that privilegiates the increased number of ILD replicates and thus a lower variance in ILD P value. Uninformative sites were excluded from the ILD test (Lee 2001). Separate and combined phylogenetical analyses of each region partition were used to detect localized incongruence among tree topologies.

Detection of recombination - We investigated possible recombination events in ETS and ITS sequences using split decomposition analysis as implemented by SplitsTree4 (Hudson and Bryant 2006) with default settings (uncorrected P method) and 1,000 bootstrap replicates together with Phi test for recombination. In addition, statistical tests implemented by RDP3 (Martin et al. 2005) were also used: GENCONV, RDP and MacChi. Because the emphasis of this study pertains to annual taxa, we did not consider localized incongruences/recombinations restricted to more terminal clades not directly related to annual species, provided that they do not affect deepen nodes of the phylogeny.

RESULTS

Cytogenetic data

The chromosome plates with the 45S and 5S signals obtained are presented in Figure 1, and genome size values plus data from fluorescent *in situ* hybridization are summarized in Table 2, complemented with chromosome counts performed by our team in the same populations (Vallès et al. 2001; Garcia et al. 2004). Mean genome size values have been used in those species either with more than one population studied or with previously published C values. All FISH signals are located in the distal ends or in satellites of chromosomes, as reported in previous FISH-based studies in the genus (Torrell et al. 2001b, 2003; Garcia et al. 2007, 2009; Pellicer et al. 2008).

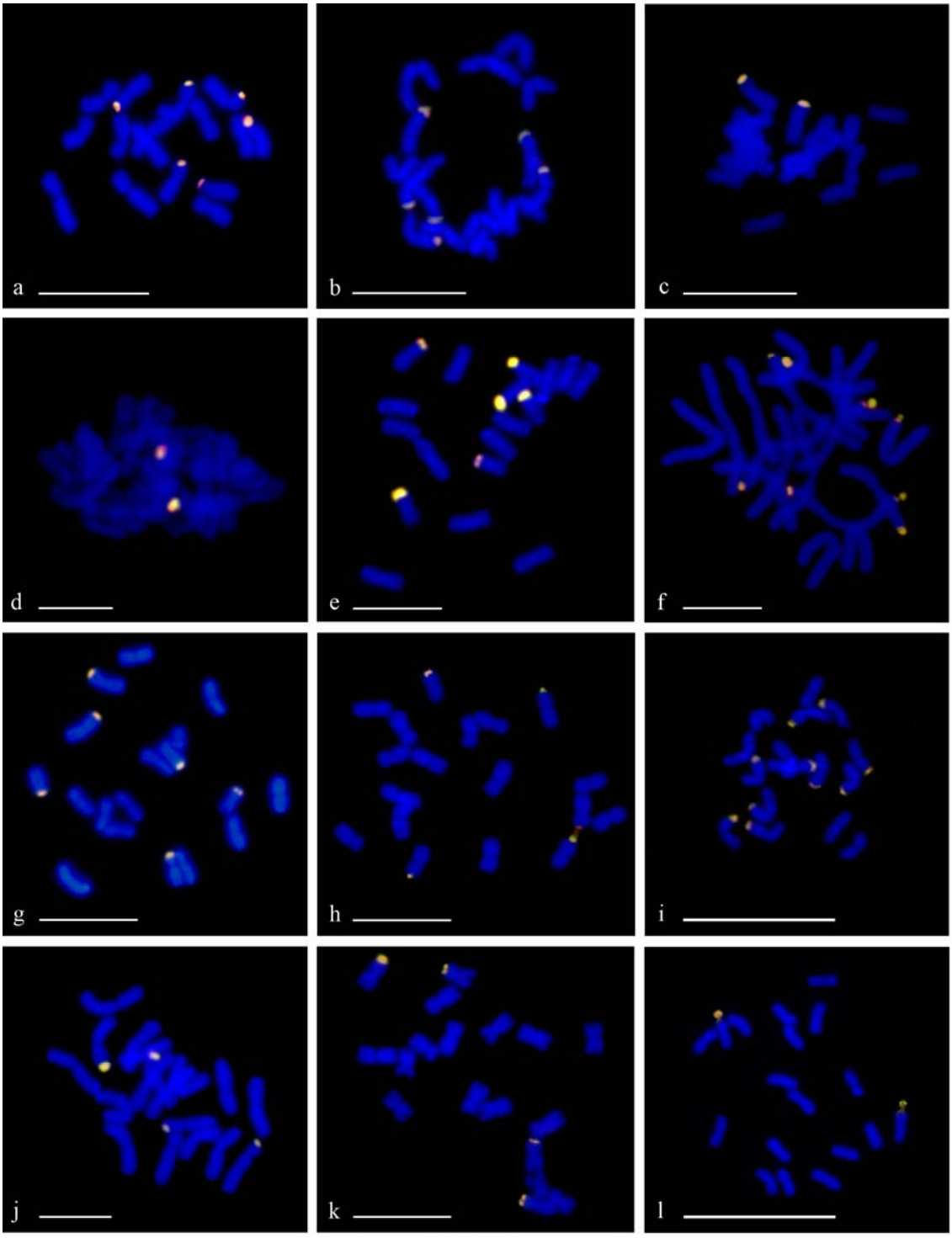

Figure 1: Somatic metaphase protoplasts of *A. anethifolia* (a), *A. anethoides* (b), *A. biennis* (c), *A. blepharolepis* (d), *A. jacutica* (e), *A. leucodes* (f), *A. macrocephala* (g), *A. palustris* (h), *A. scoparia* (i), *A. sierversiana* (j), *A. tournefortiana* (k), *N. pectinata* (l). The images of ribosomal DNA 5S (digoxigenin-labelled) and 18S-5.8S-26S (fluorescein-labelled) are presented merged. Scale bar = 10 μm

Phylogenetic analyses

The tree resulting from the phylogenetic analyses is presented in Figure 2.

Parsimony analyses – The ETS analysis resulted in 32,800 trees of 135 steps, with CI=0.6000, RI=0.9269, RC=0.5562. The ITS analysis stopped prior to completion of the initial heuristic search at 192 addition-sequence replicates, due to memory limit. It resulted in >90,000 trees of 298 steps, with CI=0.4732, RI=0.8596, RC=0.4076. The combined analysis also stopped prematurely at 235 addition-sequence replicates. It resulted in >90,000 trees of 451 steps, with CI=0.4922, RI=0.8767, RC=0.4315. The search of trees shorter or incompatible with the strict consensus obtained did not result in the finding of any tree for ETS, ITS, nor the combined dataset.

Congruence of the regions ETS and ITS – The partition homogeneity test indicates that the null hypothesis of congruence among the two datasets can not be rejected at the 99% confidence level (P=0.0440), however the P is mainly significant at the 95% level. The topologies of the trees obtained with the ETS and ITS are congruent for significantly statistically supported nodes, with the only exception of the placement of *A. stelleriana*. This species groups with *A. rubripes* and *A. vulgaris* in ETS tree (PP=96%), whereas it forms a monophyletic group with *A. koidzumii* and *A. princeps* in the ITS reconstruction (PP=99%). The combined tree shows *A. stelleriana* in a polytomy along with all these species (Fig. 2). The partition homogeneity test excluding *A. stelleriana* conduces to an increased P value, from 0.0440 to 0.1300, which confirms the strong implication of this species in the discrepancy of the datasets. Bayesian analysis of the combined dataset excluding *A. stelleriana* logically restablishes the grouping of *A. koidzumii* and *A. princeps* (PP=100%). More surprisingly, the support of the apparently not closely related clade of subgenus *Seriphidium* increases from PP=84% to 99% in this analysis, indicating that inconsistencies introduced by *A. stelleriana* could be more extended than previously thought. For this reason, we excluded this species for the further statistical analyses taking the phylogeny into consideration.

The relatively goof compatibility of the ETS and ITS datasets is also expressed in terms of branch supports on the trees, as 18% of the 22 significantly supported branches occur only in combined analysis (32% are present in ETS, ITS and combined trees, 27% in only ITS and combined trees, and 23% in only ETS

and combined trees, Fig. 2). Apart of *A. stelleriana*, the only other case of support loss through data combination concerns the grouping of *A. cretacea* and *A. fragrans* in ITS reconstruction (PP=96%), which gives rise to a polytomy in the combined tree (Fig. 2).

Detection of recombination - No evidence of recombination has been established with statistical support by the tool used.

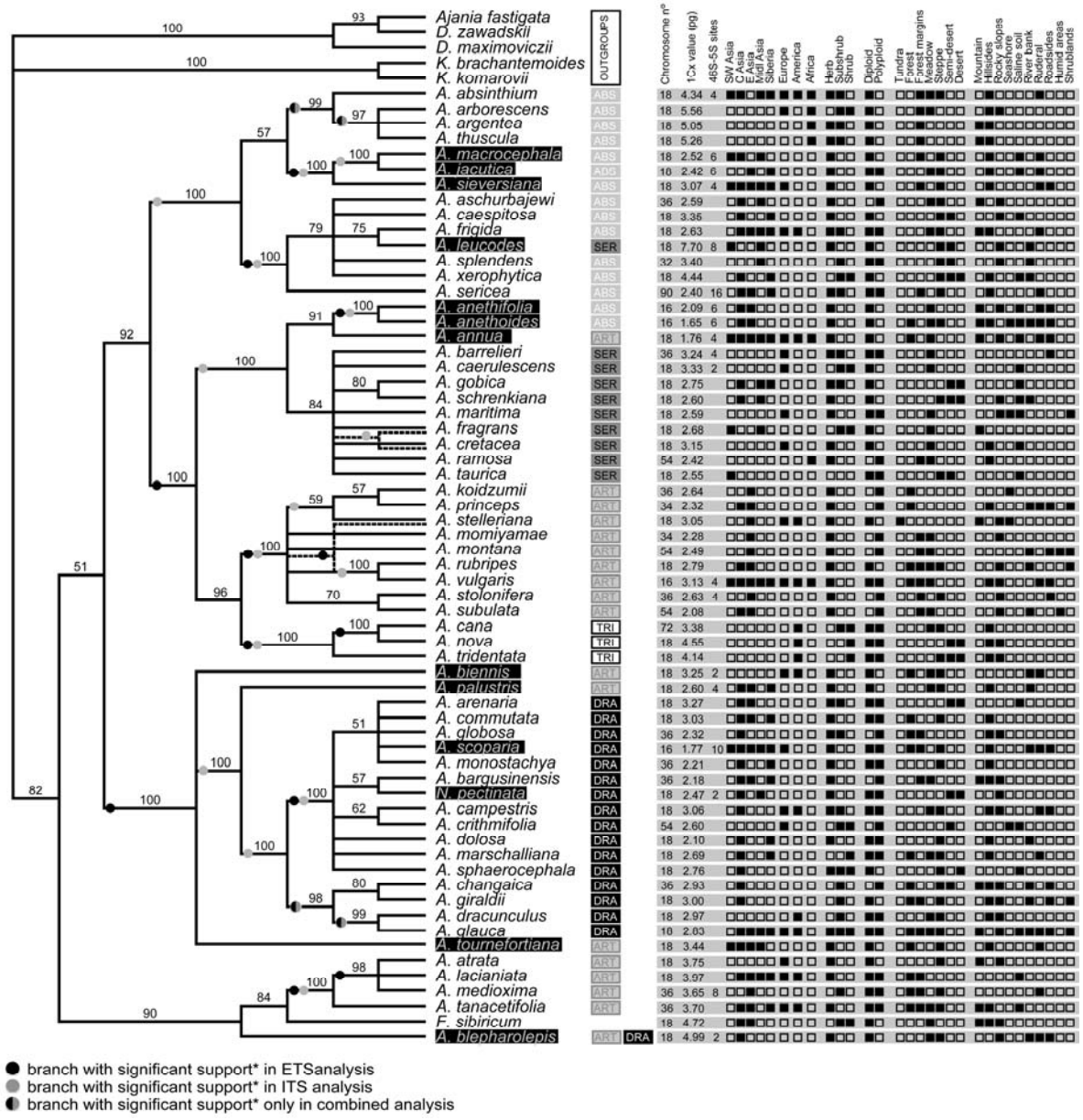

Figure 2: Phylogenetic circumscription of the annual species studied with data for their subgeneric classification, chromosome numbers, genome size (1Cx values) and distribution and ecological preferences. *(PP ≥ 95%).

Geographic and ecologic traits

Figure 2 (table entries) shows in columns the general distribution, growth forms, polyploidy incidence, ecosystem and habitat preferences for the species studied along the phylogeny. Most of the taxa are perennial, and the annual species seem to have been originated in at least five different episodes. There is no general rule concerning the origin and distribution of the perennial progenitors with their related annuals. That is, there exist annual species with reduced distribution which proceeded from worldwide distributed perennial and vice versa. Nevertheless, the fact that all annuals are herbs indicates that when a shift of plant form took place was always from shrubs/subshrubs to herbs. The annual species and their perennial relatives tend to share preferences about ecological requirements related with the vegetation ecosystems, but sometimes occupying different microhabitats. Thus, while some annuals have revealed themselves as specialists for extreme conditions (e.g. salt stress, aridity, altitude), other have more cosmopolite behaviours becoming ruderal herbs growing in anthropic places.

DISCUSSION

Variation in rDNA sites in the different subgenera

Given the inter- and intraspecific similarity of chromosome conformation of this genus, it is difficult to clarify and justify the systematic relationships using only karyotype morphometry (Hoshi et al. 2003 and references therein), although Matoba et al. (2007) proposed, on the basis of the numerical analysis of the karyotype of 12 species belonging to three subgenera, that subgenus *Artemisia* is more advanced than others in relation to chromosomal evolutionary processes. A very symmetrical karyotype is a common trait in the genus (Vallès and Garnatje 2005; and references therein) and in the whole tribe of Anthemideae (Schweizer and Ehrendorfer 1983). The number and distribution of signals may be used as a tool to elaborate and differentiate karyotypes in a genus with such chromosomal morphometrical homogeneity, i.e., a role of systematic marker may be attributed to FISH marks. From this point of view, the location of FISH signals in terminal position agrees with that of other species of the genus (Torrell

et al. 2003; Garcia et al. 2007), as well as with the general banding style defined for the Anthemideae (Schweizer and Ehrendorfer 1983).

Two basic chromosome numbers, $x = 8$ and $x = 9$, are present in the subgenus *Absinthium* and the nuclear DNA amount, slightly bigger in those species showing $2n = 18$ chromosomes, seems to be in agreement with this number. The species *A. jacutica* plus both *Artemisia macrocephala* and *Artemisia sieversiana* Nakai are embedded into a robust clade (PP=100%), showing positive correspondence between their traditional and molecular systematic placement (Fig. 2). In this case, the number of positive ribosomal signals is four in *A. sieversiana* (Fig. 1j), and six in *A. jacutica* and *A macrocephala*. (Fig. 1e, g). The perennial species of this subgenus previously studied (Pellicer et al. 2008), *A. absinthium* L., showed four bands colocalized as elsewhere in the genus (Torrell et al. 2003; Garcia et al. 2007, 2009). An explanation for the different loci patterns between those three related species might be looked in the fact that while *A. jacutica* and *A. macrocephala* are known as annual herbs, *A. sieversiana* has been considered of annual, biennial and even of perennial behaviour. In this sense, Sanz et al. (2008) pointed through a narrow relationship between *A. absinthium* and *A. sieversiana* species that where clustered in sister groups. Another interesting point to comment is the different signal intensity reported for many of the species studied. In the case of *A. jacutica* we have found four significantly more intense FISH signals than the remaining two, which are weaker. This feature has been also found in other species of *Artemisia* (Garcia et al. in press) as well as in other plant groups (Cerbah et al. 1998; Datson and Murray 2006; Srisuwan et al. 2006), and reflects the semi-quantitative value of FISH, the intensity of signals depending of the gene copy number (Srisuwan et al. 2006 and references therein). Differences on signal intensity may be related to gene copy loss processes, basically in polyploid species (Leitch et al. 2008), hence possibly the number of copies may have fallen below the detection threshold for *in situ* hybridization (Jiang and Grill 1994).

Artemisia anethifolia and *A. anethoides*, are placed out from the subgenus *Abshinthium* clade, joint to *Artemisia annua* L., as sister group of the perennial species belonging to subgenus *Seriphidium* (Fig. 2). The unexpected phylogenetic

position of *A. annua*, belonging to the subgenus *Artemisia*, was previously reported by Vallès et al. (2003) and Sanz et al. (2008). In these two $x = 8$-based species we found six positive signals for both ribosomal DNA, while in *A. annua* ($x = 9$) four FISH signals have been previously reported (Torrell et al. 2003). Thus, the number of rDNA sites does not show a clear relationship with the chromosome number and genome size between these taxa, being the last trait similar in those three species. Even so, *A. annua* shares the number of ribosomal loci with the perennial *Seriphidium* species studied by Torrell et al. (2003).

The results found in the annual species included traditionally in the subgenus *Artemisia* are similar, but *A. blepharolepis* and *Artemisia biennis* Willd. (Table 2). In this case, all the studied species exhibit the same chromosome number ($2n = 18$) but a quite different DNA amount and loci number among some of them. The number of 45S and 5S sites is two in *A. biennis* and *A. blepharolepis*, and four in *Artemisia palustris* L. and *A. tournefortiana* (Fig. 1c, d, h, k respectively). As explained before, *A. annua* L., and the perennial species included in this subgenus, *Artemisia chamaemelifolia* Vill., *Artemisia stolonifera* (Maxim.) V.L. Komarov and *Artemisia vulgaris* L., present also four signals (Torrell et al. 2003; Hoshi et al. 2006; Pellicer et al. 2008). Again, one of the most patent divergences between annuals and perennials, in the case of subgenus *Artemisia*, is that while perennials appear clearly embedded in two of the main clades of the subgenus, the annuals are widely distributed along the phylogeny following uncertain systematic criteria, sometimes showing conflictive phylogenetic placements for different DNA regions.

The case of *A. blepharolepis* is still more interessant, and also its systematic position has been largely argued. While Yu-chuan (1993) and Ling et al. (2006) support the inclusion of this taxon in the subgenus *Dracunculus*, several authors have denied this hypothesis proposing its inclusion in the subgenus *Artemisia* on the basis of morphological characters (Grubov 1982; Darijma 1989). The phylogenetic reconstruction presented this species misplaced from the subgenus *Dracunculus*, embedded without statistical support to one of the subclades of the subgenus *Artemisia*. Furthemore, genome size of this species

is significantly larger than the annual related species (2C = 9.98 pg). The very low number of FISH marks (only two) is conclusive neither for *A. blepharolepis* nor for *A. biennis* regarding the systematic placement, because the subgenus *Artemisia* and the basal taxa of *Dracunculus* contain mostly taxa with four FISH signals.

Artemisia scoparia is the only studied species included in the subgenus *Dracunculus* [with the exception of the related species *Neopallasia pectinata* (Pall.) Poljakov], and also it is placed within the subgenus (Fig. 2). This taxon exhibits a very low DNA amount (Table 2) and a high number of rDNA bands (10) in its 16 chromosomes (Fig. 1i) comparing with the eight signals displayed for the diploid perennial species from this group, being its basic chromosome number of *x* = 9, and with higher genome sizes (Pellicer et al. unpublished results). The unique species (*x* = 9-based) that seems to share a phylogenetic (Pellicer et al. unpublished) and cytogenetic (Hoshi et al. 2006) behaviour with *A. scoparia* is *Artemisia capillaris* Thunb. The species *N. pectinata* (originally described as *Artemisia pectinata* Pall.) is clearly included into the subgenus *Dracunculus*. With a single ribosomal locus, clearly located at satellite position, it has been revealed as the third annual species with such a scarce organization of ribosomal sites, only found in one perennial *Artemisia* (Torrell et al. 2003). Polyploid occurrence in the annual plants evolution seems not to be a phenomenon of great incidence (Hodgron 1987). In fact, only a couple of annual species have been reported to account for polyploid populations [e.g. *A. jacutica* (Agapova et al. 1990); *A. scoparia* (Asano 1960; Kawatani and Ohno 1964)], and also in *N. pectinata*, but the lack of cytogenetic data for these populations makes any deduction about ribosomal DNA changes in these plants impossible.

Finally, one species from the subgenus *Seriphidium* is included in the present work, *A. leucodes*. Although its morphological traits point through the inclusion of this species into the *Seriphidium* group, the phylogenetic analysis revealed it embedded as sister taxon of one of the two clades in which subgenus *Absinthium* is divided (Fig. 2), as previously pointed Vallès et al. (2003). This species also shows an exceptionally high genome size for an annual species (see

Table 2) and eight rDNA bands have been revealed in its chromosomes (Fig 1f). This is a considerably higher number of signals than that observed in the perennial *Seriphidium -Artemisia barrelieri* Besser, *Artemisia caerulescens* L., *Artemisia herba-alba* Asso-, which show two and four bands for the ribosomal DNA being the last number the most frequent (Torrell et al. 2003). Similar cases have been found in the literature even in the Asteraceae family, such as the annual species *Siebera pungens* J. Gay (Cardueae) with $2n = 20$ chromosomes and 19.68 pg of DNA (Garnatje et al. 2004). The abnormal number of FISH marks in *A. leucodes* as compared with the other members of its subgenus, and also for the subgenus *Absinthum*, agrees with its phylogenetic position: with data of ITS sequence analysis, this taxon is not included in the *Seriphidium* clade (Vallès et al. 2003), what could be the effect of the long branch attraction, common in annuals.

Hoshi et al. (2006) have determined the number of 5S and 45S rDNA loci in four *Artemisia* species. They have proposed that the number of rDNA loci is constant in the subgenera *Artemisia* and *Dracunculus*. Our results, considering a much larger number of taxa, from present and previous papers, do not agree with these statements, and also present a greater heterogenic pattern of ribosomal FISH signals when annuals are taken into account. The current results indicate that mainly, no relationship exists among the number of rDNA sites and the systematic situation (inclusion in the subgenus, position in the phylogenetic tree). On the one hand, this would suggest that some genome restructuring has taken place several times across the genus evolution. This can be also influenced by the fact that a satisfactory, natural classification of the genus *Artemisa* has not yet been achieved, some subgenera, particularly *Absinthium* and *Artemisia*, being split in several clades in the molecular phylogenetic approaches obtained to date (Sanz et al. 2008; Tkach et al. 2008). On the other hand, a number of rDNA sites more variable in the annuals that in the perennial species is in agreement with the statement that a bigger cytogenetic variation correlates with the fact that dysploidy, polyploidy, autogamy, and saltative evolution have been suggested as major phenomena responsible for evolutionary patterns in annual plants (Raven and Axelrod, 1995).

Relationships with life cycle and ecology

The present results show a general increase of rDNA sites from perennials to annuals, a previously reported tendency (Hidalgo et al. 2008). Other cytogenetic changes, such as reductions in chromosome number and total chromosome length, and the increases in mean chromosome length, chromosome length heterogeneity and karyotypic asymmetry were found to be correlated with the change in habit from perennial to annual. Lower chromosome numbers are mostly found in short-living annuals which grow in ephemeral habitats in semi-desert regions (Watanabe et al. 1999). All the reductions in chromosome number have been observed in plant groups growing in unstable habitats comprising semidesert regions or seasonally xeric areas, and these reductions have been associated with the evolution to annual habit (Watanabe et al. 1999). The population of the species *A. scoparia*, included in the present work, inhabits an intermittently dried river bed, fact that is in concordance with a faster life cycle completed before the seasonal rainfalls. The studied species of *Artemisia* included in the present work occur in steppes, sandy places and saline soils -with a special mention to *A. anethifolia* which has been classified as a halophyte (Zhao 1998)-, some of them being annual (*A macrocephala*, *A. palustris* and *A. tournefortiana*), others biennial and *A. scoparia* and *A. sieversiana* usually appearing as annual or biennial but the latter also considered as perennial by some authors (Poljakov 1961; Ling 2005). An annual behaviour could contribute to complete the life cycle in conditions allowing only a short cycle, as happens in flooding places or in those with a short wet season. The transition to annual life histories in *Nemesia* (Scrophulariaceae) may have been a response to climate and environmental changes. This genus has a significant proportion (ca. 75%) of annuals (Datson et al. 2008). Other annuals, such as *Brachyscome*, that grows generally in an unstable xeric habitat and its local populations are subjected to large fluctuations in size and frequent local extinction due to seasonal flooding, drought and fire (Watanabe et al. 1999). Drift can result either in an increase or a decrease chromosome number. A tendency toward a reduction of the genome size is observed in annuals and perennials, but structural changes involving the deletion of large chromosome segments are difficult to fix in perennials due the

large effective population size (Watanabe et al. 1999). Thus, under environments favouring a perennial life cycle, a larger and more competitive polyploid state may have been selected for these plants.

Annual life cycle, although is not the only factor responsible to the changes produced in the genome, could play an important role in these rearrangements. The emergence of the annual forms took place several times in the evolutionary history of the genus *Artemisia* as the present (Fig. 2) and former (Sanz et al. 2008; Tkach et al. 2008) phylogenetic studies suggest; these events seem to have occurred independently in each subgenus except in *Tridentatae*. Multiple origins of annual species have been found in several genera that contain both annual and perennial herbs, for example *Houstonia* (Church 2003) and *Sidalcea* (Andreasen and Baldwin 2001). Fiz et al. (2002) have also observed in *Bellis* the same independent origin of the annual life forms from perennials. In this genus the annuals occur in areas with marked summer drought in contrast with the perennials that colonize wetter areas, sometimes occurred during to dry environments, specially in the Asteraceae where the annual forms seems to be favoured in dry environments.

Hidalgo et al. (2008) observed that some species have evolved from perennial state, extreme habitats, high genome size, and four 45S and two 5S signals (*Myopordon*) to the annual autogamous habit and moderate habitats (*Oligochaeta*) with lower genome size, twelve 45S and two 5S signals. It seems to be clear that a main tendency, but not the unique, is the increase of the rDNA sites associated to the annuality, as a consequence of the chromosomal restructuring accompanying the genome downsizing. In other words, stronger cytogenetic changes are expected for taxa having inherited characteristics far away from the optimums for the new life cycle and ecological conditions. Given that the process of genome size downsizing seems to be one trigger of chromosome restructuring, and consequent rDNA sites breaking and spreading (Hidalgo et al. 2008), a relation between C-value variation and rDNA site number is expected. That rule occurred in most of the annual *Artemisia* species, so genome downsizing accompanied of a gain in rDNA loci with respect to their perennial relatives has been detected in *A. anethifolia*, *A. anethoides*, *A. jacutica*,

A. macrocephala and *A. scoparia* (Fig. 2, Table 2). However, the different origins of the annual species might determine the direction of genome size and rDNA changes, as seen in the previously commented species, but pointing through other minor directions (Fig. 2, Table 2), which makes difficult to conclude about the existence of a general annual behaviour.

Despite this, it seems that annual species coming from perennial species groups with high genome size can adjust their C-DNA value by downsizing, and that relative high genome size is not a total obstacle for a species to become annual. At the contrary, polyploidy could be prohibitive for making an annual in *Artemisia*: in fact, although polyploids are very frequent in the genus (Sánchez-Jiménez et al. 2009 and references therein), there is no evidence of annual species generated by a polyploid ancestor. Thus, annual species with known tetraploid populations (*A. jacutica, A. scoparia* and *N. pectinata*) have also diploid ones, meaning that polyploidy is secondary in these taxa. Therefore, diploidy could be a favouring a possible shift to annuality in *Artemisia* and related genera.

The case of *A. blepharolepis*, a steppic annual taxon, which can share the habitat with perennial congeneric taxa, is the species with the lowest number of FISH marks, only two, but has a very high nuclear DNA amount for an annual species. We cannot appreciate a clear correlation between habitat and FISH marks, but it appears as clear that annual plants are more able to colonize several habitats and that the big amount of divisions produced in these species could favour chromosomal restructuring and a more flexible genome as compared with perennial species.

CONCLUDING REMARKS

A tendency towards the increase of the number of rDNA sites in annual as compared with perennial *Artemisia* species is indicated by the results of the present work. This trend is clear, but has some exceptions and, although we have considered more than half the annual taxa in the genus, further studies will be necessary to confirm the relationship, if any, between this cytogenetic trait and life cycle, which is also related with other cytogenetic (such as genome size) and ecological factors. According to the data obtained, the *Artemisia* subgenera

cannot be characterized by specific FISH patterns, at least based on the annual ones, seen their heterogeneous systematic placement. This is partly due to the fact that classical subgeneric classification is probably not natural, as suggested by molecular analyses. The species of some clades of the phylogenetic reconstructions provided to date share the number of rDNA loci. Also further studies are needed both in molecular phylogeny and molecular cytogenetics to define the relationships between genome organization, systematics and evolution in the genus.

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Appendix 1. Genbank numbers of the species included in the phylogenetic analysis. Bold names indicate anual species. For the species sequenced for the first time, voucher references and/or collection numbers are indicated, the already published can be consulted on the referred papers.

TAXON	Herbarium collection n° or Bibl. Ref.	ITS1	ITS2	ITS1-5.8S-ITS2	Herbarium collection n°, Bibl. Ref.	3'-ETS
A. absinthium	Torrell et al. 1999	AF045394	AF079946		Sanz et al. 2008	DQ028850
A. anethifolia	LE-Korobkov 06-22	XXXXXX	XXXXXX		LE-Korobkov 06-22	XXXXXX
A. anethoides	BCN 23790	XXXXXX	XXXXXX		BCN 23790	XXXXXX
A. annua	Tkach et al. 2008			AM398847	Tkach et al. 2008	AM397956
A. arborescens	Torrell et al. 1999 (unpub.)	AF045393	AF079945		Pellicer et al. 2009	FJ642934
A. arenaria	Sanz et al. 2008	EF063639	EF063640		Sanz et al. 2008	DQ028897
A. argentea	Pellicer et al. 2009	FJ642971	FJ643007		Pellicer et al. 2009	FJ642935
A. aschurbajewi	Torrell et al. 1999	AF504170	AF504143		Sanz et al. 2008	DQ028838
A. atrata	Pellicer et al. 2009	FJ642973	FJ643009		Pellicer et al. 2009	FJ642937
A. barrelieri	Torrell et al. 1999	AF045410	AF79961		Sanz et al. 2008	DQ028875
A. biennis	Tkach et al. 2008			AM398851	Tkach et al. 2008	AM398032
A. blepharolepis	BCN 34490	XXXXXX	XXXXXX		BCN 34490	XXXXXX
A. caerulescens	Torrell et al. 1999	AF045409	AF079960		Sanz et al. 2008	DQ28872
A. caespitosa	Tkach et al. 2008			AM398855	Tkach et al. 2008	AM397957
A. campestris	Torrell et al. 1999	AF045398	AF079950		Sanz et al. 2008	DQ028854
A. cana	Torrell et al. 1999	AF045413	AF079965		Sanz et al. 2008	DQ028882
A. changaica	Tkach et al. 2008			AM398858	Tkach et al. 2008	AM397965
A. commutata	Tkach et al. 2008			AM398860	Tkach et al. 2008	AM397967
A. cretacea	Pellicer et al. 2009	FJ642975	FJ643011		Pellicer et al. 2009	FJ642939
A. crithmifolia	Torrell et al. 1999	AF045399	AF079962		Sanz et al. 2008	DQ028856
A. dolosa	Tkach et al. 2008			AM398864	Tkach et al. 2008	AM397971
A. dracunculus	Torrell et al. 1999	AF504172	AF504145		Sanz et al. 2008	DQ028859
A. fragrans	Torrell et el. 1999	AF045406	AF079957		Sanz et al. 2008	DQ028871
A. freyniana	Tkach et al. 2008			AM398868	Tkach et al. 2008	AM397975
A. giraldii	Pellicer et al. 2009	FJ642979	FJ643015		Pellicer et al. 2009	FJ642943
A. glauca	Tkach et al. 2008			AM398871	Tkach et al. 2008	AM397978

A. globosa	Pellicer et al. 2009	FJ642980	FJ643016		Pellicer et al. 2009	FJ642944
A. gobica	Tkach et al. 2008			AM398876	Tkach et al. 2008	AM397983
A. jacutica	Korobkov-LE 06-31	XXXXXX	XXXXXX		Korobkov-LE 06-31	XXXXXX
A. koidzumii	Tkach et al. 2008			AM398884	Tkach et al. 2008	AM308035
A. laciniata	Tkach et al. 2008			AM398886	Tkach et al. 2008	AM397991
A. leucodes	BCN 11631	XXXXXX	XXXXXX		BCN 11631	XXXXXX
A. macrocephala	BCN 23801	XXXXXX	XXXXXX		BCN 23801	XXXXXX
A. maritima	Pellicer et al. 2009	FJ642987	FJ643023		Pellicer et al. 2009	FJ642951
A. marschalliana	Torrell et al. 1999	AF505177	AF504150		Sanz et al. 2008	DQ028858
A. medioxima	Pellicer et al. 2009	FJ642988	FJ643024		Pellicer et al. 2009	FJ642952
A. momiyamae	Pellicer et al. 2009	FJ642989	FJ643025		Pellicer et al. 2009	FJ642953
A. monostachya	Tkach et al. 2008			AM398896	Tkach et al. 2008	AM398000
A. montana	Pellicer et al. 2009	FJ642991	FJ643027		Pellicer et al. 2009	FJ642955
A. nova	Torrell et al. 1999	AF045412	AF060462		Sanz et al. 2008	DQ028883
A. palustris	Tkach et al. 2008			AM398902	Tkach et al. 2008	AM398006
A. princeps	Tkach et al. 2008			AM398905	Tkach et al. 2008	AM398009
A. ramosa	Pellicer et al. 2009	FJ642994	FJ643030		Pellicer et al. 2009	FJ642958
A. rubripes	Pellicer et al. 2009	FJ642995	FJ643031		Pellicer et al. 2009	FJ642959
A. schrenkiana	Pellicer et al. 2009	FJ642997	FJ643033		Pellicer et al. 2009	FJ642961
A. scoparia	Tkach et al. 2008			AM398914	Tkach et al. 2008	AM398017
A. sericea	Tkach et al. 2008			AM398916	Tkach et al. 2008	AM398019
A. sieversiana	Tkach et al. 2008			AM398917	Tkach et al. 2008	AM398020
A. sphaerocephala	Tkach et al. 2008			AM398918	Tkach et al. 2008	AM398021
A. stelleriana	Sanz et al. 2008	DQ028918	DQ028905		Sanz et al. 2008	DQ028896
A. stolonifera	Pellicer et al. 2009	FJ643000	FJ643036		Pellicer et al. 2009	FJ642964
A. subulata	Pellicer et al. 2009	FJ643001	FJ643037		Pellicer et al. 2009	FJ642965
A. tanacetifolia	Tkach et al. 2008			AM398923	Tkach et al. 2008	AM398026
A. taurica	Pellicer et al. 2009	FJ643003	FJ643039		Pellicer et al. 2009	FJ642967
A. thuscula	Tkach et al. 2008			AM398924	Tkach et al. 2008	AM39027
A. tournefortiana	BCN 11630	XXXXXX	XXXXXX		BCN 11630	XXXXXX
A. tridentata	Torrell et al. 1999	AF045411	AF079963		Sanz et al. 2008	DQ028884
A. umbrosa	Pellicer et al. 2009	FJ643004	FJ643040		Pellicer et al. 2009	FJ642968

A. vallesiaca	Pellicer et al. 2009	FJ643005	FJ643041		Pellicer et al. 2009	FJ642969
A. vulgaris	Tkach et al. 2008			AM398927	Tkach et al. 2008	AM398029
A. xerophytica	Tkach et al. 2008			AM398929	Tkach et al. 2008	AM398031
Ajania fastigata	Torrell et el. 1999	AF504169	AF504142		Sanz et al. 2008	DQ028868
Dendranthema maximoviczii	Sanz et al. 2008	DQ028923	DQ028910		Sanz et al. 2008	DQ028899
Dendranthema zawadskii	Sanz et al. 2008	DQ028924	DQ028911		Sanz et al. 2008	DQ028901
Filifolium sibiricum	Pellicer et al. 2009	FJ643006	FJ643042		Pellicer et al. 2009	FJ642970
Kaschgaria brachantemoides	Torrell et al. 1999	AF504189	AF504162		Sanz et al. 2008	DQ028865
Kaschgaria komarovii	Sanz et al. 2008	DQ028925	DQ028912		Sanz et al. 2008	DQ028902
Neopallasia pectinata	Sanz et al. 2008	DQ028927	DQ028927		Sanz et al. 2008	DQ028914

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Recomptes cromosòmics en espècies asiàtiques d'*Artemisia* L. (*Asteraceae*): des dels diploides fins al primer report del poliploide més elevat del gènere

Botanical Journal of the Linnean Society 153: 301-310 (2007)

Jaume Pellicer, Sònia Garcia, Teresa Garnatje, Oriane Hidalgo, Aleksandr A. Korobvov, Shagdar Dariimaa i Joan Vallès

Aquest treball aporta dades de nombres cromosòmics en 24 espècies d'*Artemisia* L. Deu de les espècies estudiades s'inclouen en el subgènere *Dracunculus* (Besser) Rydb. i la resta pertanyen a altres subgèneres. Set recomptes són inèdits, 14 mostren resultats en concordança amb dades prèvies ja publicades, i tres contribueixen amb nous nivells de ploïdia. El recompte cromosòmic dut a terme a l'espècie *Artemisia medioxima* ha revelat el nivell de ploïdia més elevat mai comptat al gènere (16*x*). S'ha trobat solament una espècie que presenta el nombre cromosòmic bàsic $x = 8$. La resta d'espècies estudiades tenen el nombre bàsic $x = 9$, amb nivells de ploïdia que van des del diploide ($2n = 18$) fins a l'hexaidecaploide ($2n = 144$), fet que il·lustra el paper fonamental de la poliploïdia en l'evolució del gènere.

Botanical Journal of the Linnean Society, 2007, **153**, 301–310. With 24 figures

Chromosome counts in Asian *Artemisia* L. (Asteraceae) species: from diploids to the first report of the highest polyploid in the genus

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This work comprises 24 reports of chromosome numbers in 24 *Artemisia* L. species from Asia. Ten are included in the subgenus *Dracunculus* Besser and the rest belong to other subgenera. Seven counts are new reports, 14 are consistent with scarce previous ones, and three contribute new ploidy levels. That carried out in *A. medioxima* reports the highest ploidy level ever counted for the genus (16*x*). There is only one species with $x = 8$ as the basic chromosome number. In the remaining $x = 9$-based species, ploidy levels range from 2*x* to 16*x*, illustrating the great role played by polyploidy in the evolution of the genus. © 2007 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2007, **153**, 301–310.

ADDITIONAL KEYWORDS: Artemisiinae – dysploidy – karyology – 16*x*.

INTRODUCTION

Artemisia L. is the largest genus of the tribe Anthemideae and one of the largest of the Asteraceae. It contains more than 500 taxa, a variable number depending on the author (Vallès & Garnatje, 2005, and references cited therein). *Artemisia* are distributed mainly in the Northern Hemisphere, especially in West and Central Asia, although some have a worldwide distribution. Many representatives of the genus have been used traditionally as medicines, food, forage, ornamentals, etc. (Pareto, 1985; Marco & Barberá, 1990; Heinrich *et al*., 1998; Wright, 2002).

Palaeontological data suggest that the origin of *Artemisia* was most probably in the mountain regions of north-western Asia in the mid-Cenozoic (Ling, 1982; Wang, 2004). The most important centre of diversity is temperate Asia, although there are dispersed foci of speciation in temperate and cold regions of Eurasia and North America (Ling, 1982). The finding of different ploidy levels in these and other Artemisiinae species in Asia also supports the hypothesis of this origin of speciation.

The infrageneric classification of *Artemisia* has been the subject of many taxonomic rearrangements. It was divided classically into four groups treated as sections or subgenera: *Absinthium* DC., *Artemisia* (originally named *Abrotanum* Besser), *Dracunculus* DC., and *Seriphidium* (Besser) Rouy (Besser, 1829, 1832, 1834, 1835; Candolle, 1837). Rydberg (1916) created a new section (*Tridentatae*), including species previously within the subgenus *Seriphidium*, which was subsequently separated as an independent subgenus (McArthur, Pope & Freeman, 1981). Afterwards, Ling (1982, 1991a, b, 1995a, b) proposed the segregation of *Seriphidium* (Besser ex Hook) Fourr. as an independent genus, which was accepted by Bremer & Humphries (1993) and Bremer (1994) in their cladistic revisions of the Anthemideae and Asteraceae, respectively. However, molecular studies based on chloro-

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plast DNA (cpDNA) restriction site variation and internal transcribed spacers (ITS) of nuclear ribosomal DNA (Kornkven, Watson & Estes, 1998; Torrell *et al*., 1999; Watson *et al*., 2002; Vallès *et al*., 2003) have refuted this separation. Several other small genera, such as *Filifolium* Kitam., *Kaschgaria* Poljakov, *Neopallasia* Poljakov, and *Turaniphytum* Poljakov, are very close to *Artemisia*, or have been segregated from it (Rydberg, 1916; Poljakov, 1961; Ling & Ling, 1978; Ghafoor, 1992; Bremer & Humphries, 1993).

The relevance of karyological and cytogenetic studies to the knowledge of the systematics and evolution of the genus was noted long ago (Weinedel-Liebau, 1928; Ehrendorfer, 1964; Korobkov, 1972). From the earliest studies to the present, numerous chromosome counts (for around 350 taxa, i.e. over 50% of the genus, including species and subspecies) and cytogenetic data have been reported (Vallès & Garnatje, 2005, and references cited therein). The genus has two basic chromosome numbers: $x = 9$, present in all the subgenera, and the less frequent $x = 8$, present in *Absinthium, Artemisia*, and *Dracunculus* (Solbrig, 1977; Schweizer & Ehrendorfer, 1983; Oliva & Vallès, 1994; McArthur & Sanderson, 1999). A centric (Robertsonian) chromosome fusion may have been the cause of this descending dysploidy, reducing the basic chromosome number from $x = 9$ to $x = 8$ (Vallès & Siljak-Yakovlev, 1997). Both basic numbers show polyploid series, with known levels up to dodecaploid for $x = 9$ and hexaploid for $x = 8$ (Ehrendorfer, 1964, 1980; Estes, 1969; Persson, 1974; McArthur & Pope, 1979; Oliva & Vallès, 1994; McArthur & Sanderson, 1999). Moreover, different studies on *Artemisia* have shown that the nuclear DNA content increases with ploidy level, although the monoploid genome size decreases with increasing polyploidy (Torrell & Vallès, 2001; Garcia *et al*., 2004). It is clear that there exists a wide knowledge of *Artemisia* from the karyological and cytogenetic viewpoint, but these chromosomal data are still limited or unavailable for many widespread species.

The present study covers most of the *Artemisia* subgenera, with a particular emphasis on the subgenus *Dracunculus*, which is distributed largely in Eurasia and North America. The main traits which differentiate this group from the other subgenera are the presence of heterogamous capitula, with outer female florets and central hermaphrodite but functionally male florets. This morphology induced Cassini (1817) to name the species of this subgenus as a new genus, *Oligosporus* Cass., which was later returned to *Artemisia* (Besser, 1829, 1832, 1834, 1835; Candolle, 1837). The inclusion of this group within *Artemisia* has been confirmed by molecular phylogenetic data (Vallès *et al*., 2003). The main objective of this work is to enlarge the chromosomal data in the genus and to shed light on the role of polyploidy in its diversification.

MATERIAL AND METHODS

Root-tip meristems were obtained from wild-collected achenes germinated on wet filter paper in Petri dishes at room temperature in the dark. Seedlings were pretreated with 0.05% aqueous colchicine at room temperature for 2.5–3 h. Material was fixed in absolute ethanol and glacial acetic acid (3 : 1) for 2–4 h at room temperature and stored in the fixative at 4 °C. Samples were hydrolysed in 1 M HCl for 5–8 min at 60 °C, stained with 1% aqueous aceto-orcein for 2–12 h, and squashed on slides in 45% acetic acid–glycerol (9 : 1). The best metaphase plates were photographed with a digital camera (AxioCam MRc5 Zeiss) mounted on a Zeiss Axioplan microscope, and images were analysed with Axio Vision Ac software version 4.2. Herbarium vouchers of most species are deposited in the herbarium of the Centre de Documentació de Biodiversitat Vegetal de la Universitat de Barcelona (BCN), and some others are in the Komarov Botanical Institute, St Petersburg (LE).

To assess the existence of previously published chromosome counts in the studied species, we used the most common indexes of plant chromosome numbers (cited in Vallès, Torrell & Garcia-Jacas, 2001a), previous publications (Vallès *et al*., 2005; Garcia *et al*., 2006; and references cited therein), and the chromosome number databases Index to Plant Chromosome Numbers (Missouri Botanical Garden, http://mobot.org/W3T/Search/ipcn.html) and Index to Chromosome Numbers in the Asteraceae (Watanabe, 2002; http://www-asteraceae.cla.kobe-u.ac.jp/index.html).

RESULTS AND DISCUSSION

Some authors have proposed different series of sections and subsections in *Artemisia* (Rydberg, 1916; Poljakov, 1961; Korobkov, 1981; Ling, 1991a, b, 1995a, b), but a global treatment of the entire genus at these levels has not yet been achieved; therefore, we consider here only the classical main subdivisions. The localities are given with the use of Russian ('krai', region; 'oblast', province; 'raion', district) and Mongolian ('aimag', province, written 'aimak' in Russian language works; 'sum', village, written 'somon' in Russian language works) administrative divisions. Seven of the chromosome counts reported here are new, 14 confirm previous ones, and three report new ploidy levels.

SUBGENUS *ABSINTHIUM* DC

Artemisia caespitosa Ledeb.

Russia, Republic of Tuva: Barun-Khemchinskii raion, plateaux, right side of the Great Ienissei, in front of the Tais outfall, grassy semi-desert with other *Artemisia* species, *A.A. Korobkov*, 11.ix.2003 (LE-Korobkov). $2n = 18$ (Fig. 1).

This is the first count for this species, an endemic of Mongolia and southern Russia. It is a diploid based on the most common basic chromosome number in the genus, $x = 9$.

Artemisia davazamczii Sh. Dariimaa & Kamelin
Mongolia, Umnu (south) Gobi aimag: Bulgan sum, East Gurvan Saikhan mountains, canyon near Brigat, rocky slopes, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 1.ix.2004 (BCN 34488). $2n = 36$ (Fig. 2).

Again we present a new count for this recently described species (Kamelin *et al*., 1992), endemic to Mongolian and Russian Altai.

Artemisia sericea Web. ex Stechm.
Mongolia, Bulgan aimag: Sansar sum, north-east slope of Khugunkhaan mountain, steppe near *Betula* and *Pinus* forest, 2000 m, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 25.viii.2004 (BCN 34486). $2n = 90$ (Fig. 3).

This is the fourth count for the species, but the first for a Mongolian population. We report a decaploid $x = 9$-based cytotype. Previous studies, all in Russian populations, have presented mostly diploid or tetraploid cytotypes (Kawatani & Ohno, 1964; Stepanov, 1994), although Krogulevich & Rostovtseva (1984) reported $2n = 88$. We consider that this last count corresponds to a hypo-aneuploid decaploid population. We do not believe that this occurrence could be a result of dysploidy as all previous counts are based on $x = 9$ and it would be illogical to consider the count to be an 11-ploid based on $x = 8$. The different ploidy levels of this species confirm the relevance of polyploidy in the genus (Vallès & Garnatje, 2005; Vallès *et al*., 2005; and references cited therein).

Artemisia xerophytica Krasch.
Russia, Republic of Tuva: Erzinskii raion, north side of Tere-Khol lake, Tsuger-Ellis, sandy dunes with rare shrubs of *Caragana, A.A. Korobkov,* 13.ix.2003 (LE-Korobkov). $2n = 18$ (Fig. 4).

According to our data, this is the first count of the chromosome number of this species, which occupies semi-desert steppes and dunes and has been traditionally used for sand stabilization and to make windbreaks.

SUBGENUS *ARTEMISIA* L.

Artemisia blepharolepis Bunge
Mongolia, Umnu (South) Gobi aimag: Bulgan sum, 1 km north of the sum, desert steppe, *Sh. Dariimaa, D. Samjid, Sh. Tsooj & J. Vallès*, 26.viii.2004 (BCN 34490). $2n = 18$ (Fig. 5).

This is the first count for this species, endemic to Mongolia and northern China. This taxon has been located in the subgenus *Artemisia* by most authors working on Mongolian flora (Grubov, 1982; Dariimaa, 1989), although Ling, Humphries & Shultz (2006) have included it in the subgenus *Dracunculus*, with which it shares some morphological characters. Further work is needed to clarify its relationships; molecular phylogenetic research on the subgenus *Dracunculus* (J. Pellicer, T. Garnatje & J. Vallès, unpubl. data) could throw light on this point in due course.

Artemisia freyniana (Pamp.) Krasch.
Russia, Primorskii krai: Khassanskii raion, cape Gamov, pass to Vityaz bay, rocky ridge, meadows with shrubs, *A.A. Korobkov*, 15.x.2004 (LE-Korobkov). $2n = 18$ (Fig. 6).

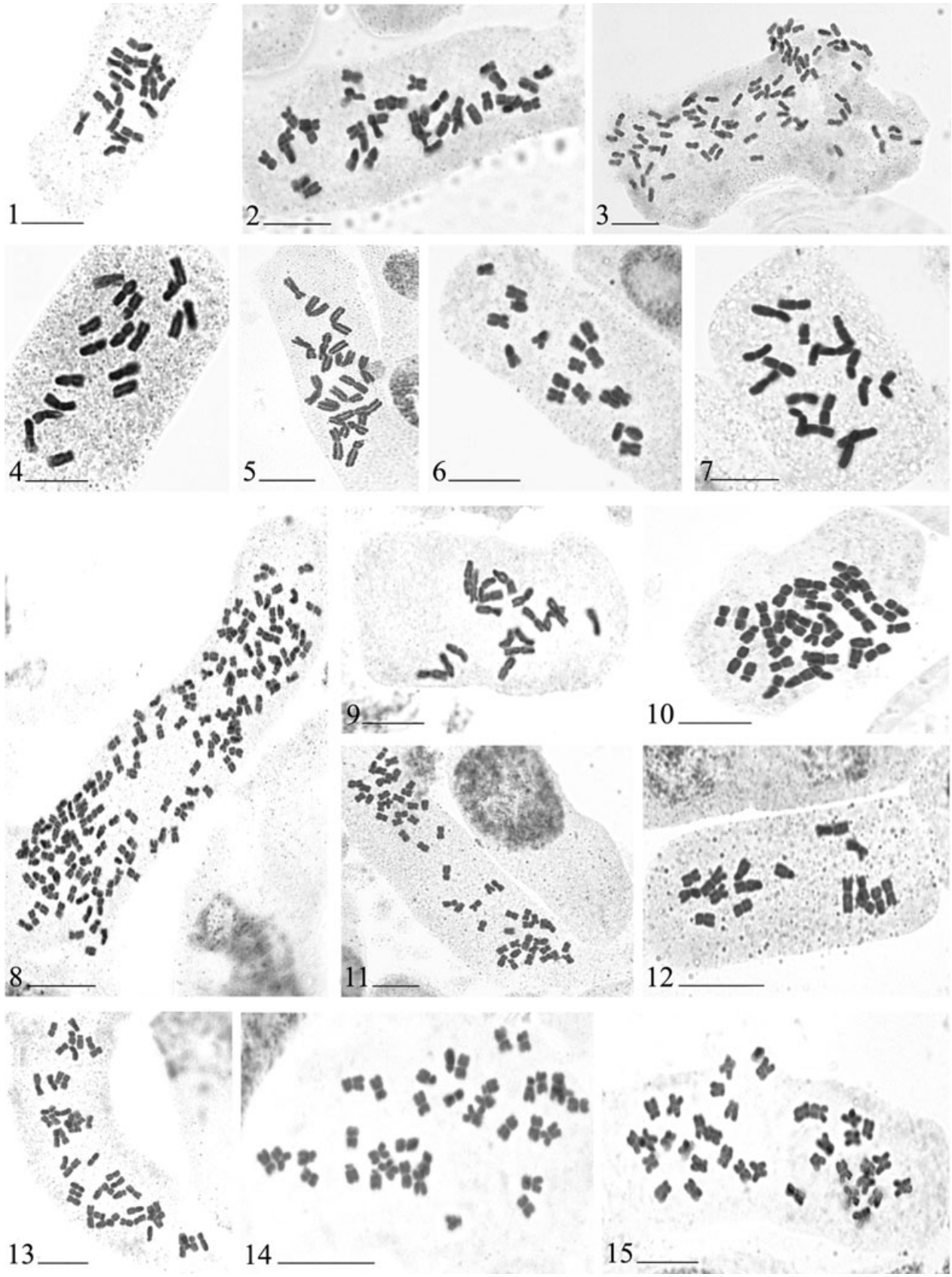
This is the first chromosome count for this endemic of Mongolia and eastern Russia.

Artemisia insulana Krasch.
Russia, Kamchatskaya oblast: Bering island, lower course of River Poludenna, windy tundra with shrubs, *V.V. Yakuvov*, 20.viii.2003 (LE-Korobkov). $2n = 18$ (Fig. 7).

This is the second report for the species, an endemic of eastern Russia. It agrees with one from the Komandorskie islands (Korobkov, 1981).

Artemisia medioxima Krasch. ex Poljakov
Russia, Khabarovskii krai: Baninskii raion, Tumnin river basin, upper Akur river valley, base of rocky slopes, *Larix* and *Betula* forest, *A.A. Korobkov*, 21.x.2004 (LE-Korobkov). $2n = 144$ (Fig. 8).

This is the second chromosome count for this species. The previous one ($2n = 4x = 36$) corresponds to a Mongolian population (Garcia *et al*., 2006). This is the first report of the 16*x* ploidy level in *Artemisia* and the subtribe Artemisiinae, and is the highest chromosome number ever counted in this plant group, which is confirmed by a high nuclear DNA content assessed by flow cytometry (J. Pellicer, T. Garnatje & J. Vallès, unpubl. data). To date, the highest ploidy level described for *Artemisia* L. was a unique case of a dodecaploid ($x = 9$, $2n = 108$) counted in a Russian population of *A. macrantha* Ledeb. (Malakhova, 1990). However, chromosome numbers up to $2n = 171$, 180, 198 have been described in the Asteraceae, in genera such as *Leucanthemum* L. and *Senecio* L. (Dowrick, 1952; Hedberg & Hedberg, 1977). Some *Artemisia* species have long polyploid series, such as, for instance, *A. dracunculus* L., with $2n = 18$, 36, 54, 72, 90 (Kawatani & Ohno, 1964; Rousi, 1969; Vallès *et al*., 2001a). Polyploidy is a relevant evolutionary mechanism in plants (Bretagnolle *et al*., 1998, and references cited therein) and is especially active in some Anthemideae groups (Solbrig, 1977; Vallès *et al*., 2001a, b; and references cited therein). The present exceptional finding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Figures 1–15. Somatic metaphases. Fig. 1. *Artemisia caespitosa* (2*n* = 18). Fig. 2. *A. davazamczii* (2*n* = 36). Fig. 3. *A. sericea* (2*n* = 90). Fig. 4. *A. xerophytica* (2*n* = 18). Fig. 5. *A. blepharolepis* (2*n* = 18). Fig. 6. *A. freyniana* (2*n* = 18). Fig. 7. *A. insulana* (2*n* = 18). Fig. 8. *A. medioxima* (2*n* = 144). Fig. 9. *A. palustris* (2*n* = 18). Fig. 10. *A. selengensis* (2*n* = 36). Fig. 11. *A. subulata* (2*n* = 54). Fig. 12. *A. sylvatica* (2*n* = 16). Fig. 13. *A. umbrosa* (2*n* = 50). Fig. 14. *A. bargusinensis* (2*n* = 36). Fig. 15. *A. changaica* (2*n* = 36). Scale bars, 10 μm.

indicates the great genetic plasticity of the genus *Artemisia*. It also reinforces the importance of polyploidy as a process contributing to the diversification of these species in their areas of expansion. In this particular case, the plants inhabit a herb-dominated community on open rocky slopes in a site being colonized after fire. The plants are clearly larger than those of other *A. medioxima* populations and present an extraordinary development of vegetative leaf rosettes. The ease of pioneer colonization might have caused a predominance of vegetative multiplication, which is often associated with a high ploidy level.

Artemisia palustris L.
Mongolia, Uvur-Khangai aimag: Khotont sum, 10 km east, margins of cultivated fields in steppe area, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 26.viii.2004 (BCN 34847). 2*n* = 18 (Fig. 9).

This is the third count for this species and confirms the diploid cytotype based on *x* = 9 found previously by Volkova & Boyko (1986) and Wang *et al*. (1998) in Russian and Chinese populations, respectively. Our report is the first carried out on a Mongolian population.

Artemisia selengensis Turcz. ex Besser
Mongolia, Selenge aimag: Shaamar sum, 3 km west of Burk Tolze hills, near river Orkhon, 700 m, *Sh. Dariimaa, Sh. Tsooj, J. Vallès & E. Yatamsuren*, 9.ix.2004 (BCN 34489). 2*n* = 36 (Fig. 10).

The chromosome number of this widely distributed species has been counted by several authors (Shimotomai, 1946; Kawatani & Ohno, 1964; Lee, 1972; Volkova & Boyko, 1986; Hoshi *et al*., 2003) in Japanese, Russian, French (in material coming from a botanical garden), and Korean populations. Nevertheless, no data on any Mongolian population have been reported until now. The tetraploid *x* = 9-based cytotype agrees with the results from other geographical areas.

Artemisia subulata Nakai
Russia, Primorskii krai: Ussuri river valley, near Kirovskii, slope of the valley of a creek, community with other *Artemisia* and herbs, *A.A. Korobkov*, 9.ix.2004 (LE-Korobkov). 2*n* = 54 (Fig. 11).

This record is the first for the species, distributed widely in Japan, Korea, and eastern Russia. This hexaploid level again supports polyploidization as an evolutionary factor contributing to speciation in the genus.

Artemisia sylvatica Maxim.
Russia, Primorskii krai: Nadezhdinskii raion, Razdolnaya river valley, near town Terekhovk, base of the coastal rocks, effusive block degradation, shrub community, *A.A. Korobkov*, 10.x.2004 (LE-Korobkov). 2*n* = 16 (Fig. 12).

Our report agrees with the two previously carried out by Volkova & Boyko (1986) and Probatova & Sokolovskaya (1988) in Russian populations. The species is a diploid based on *x* = 8, the other basic chromosome number of the genus, present in the subgenera *Absinthium, Artemisia*, and *Dracunculus* and less frequent than *x* = 9 (Solbrig, 1977; Schweizer & Ehrendorfer, 1983; Oliva & Vallès, 1994; McArthur & Sanderson, 1999).

Artemisia umbrosa Turcz. ex DC.
Russia, Primorskii krai: Khassanskii raion, cape Gamov, base of coastal rocks, community dominated by Poaceae and *Artemisia*, *A.A. Korobkov*, 16.x.2004 (LE-Korobkov). 2*n* = 50 (Fig. 13).

To our knowledge, this is the third count for the species. Two previous counts were by Hoshi *et al*. (2003) and Garcia *et al*. (2006) with different results for Russian (2*n* = 50) and Mongolian (2*n* = 54) populations, respectively. The Russian counts suggest aneuploidy from 2*n* = 6*x* = 54 (basic number *x* = 9), although both basic numbers (*x* = 8 and 9) are present in the *A. vulgaris* L. complex to which *A. umbrosa* belongs (Vallès & Garnatje, 2005). The records of 2*n* = 50 could be considered to indicate the presence of dysploidy if these changes were established widely in many populations of the species; however, we prefer the term aneuploidy because dysploidy would indicate a new basic chromosome number for the genus and, in this case, only two populations account for this number. The chromosome number 2*n* = 54 has also been reported in an eastern Russian population of *Artemisia dubia* Wall., which is another member of this complex (Volkova & Boyko, 1986).

Subgenus *Dracunculus* Besser

Artemisia bargusinensis Spreng.
Russia, Republic of Tuva: Pii-Khemskii raion, 60 km north–north-east of Turan, sloping grasslands with steppe, *V. Nikitin, V. Byalt & A. Sytin* (det. *A.A. Korobkov*), 11.viii.2002 (LE-Korobkov). 2*n* = 36 (Fig. 14).

This count agrees with the earlier counts, also on Russian populations, by Korobkov (1972) and Zhukova & Petrovsky (1987).

Artemisia changaica Krasch.
Mongolia, Arkhangai aimag: Taryat sum, Khorgo-Terkh National Park, larch forest above lake Terkhen Sagan nur, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 27.viii.2004 (BCN 34487). $2n = 36$ (Fig. 15).

This is also the first count for this species.

Artemisia depauperata Krasch.
Russia, Republic of Tuva: Erzinskii raion, calcareous mountains beside Tes-Khem river, among small stones, *A.A. Korobkov*, 18.ix.2003 (LE-Korobkov). $2n = 36$ (Fig. 16).

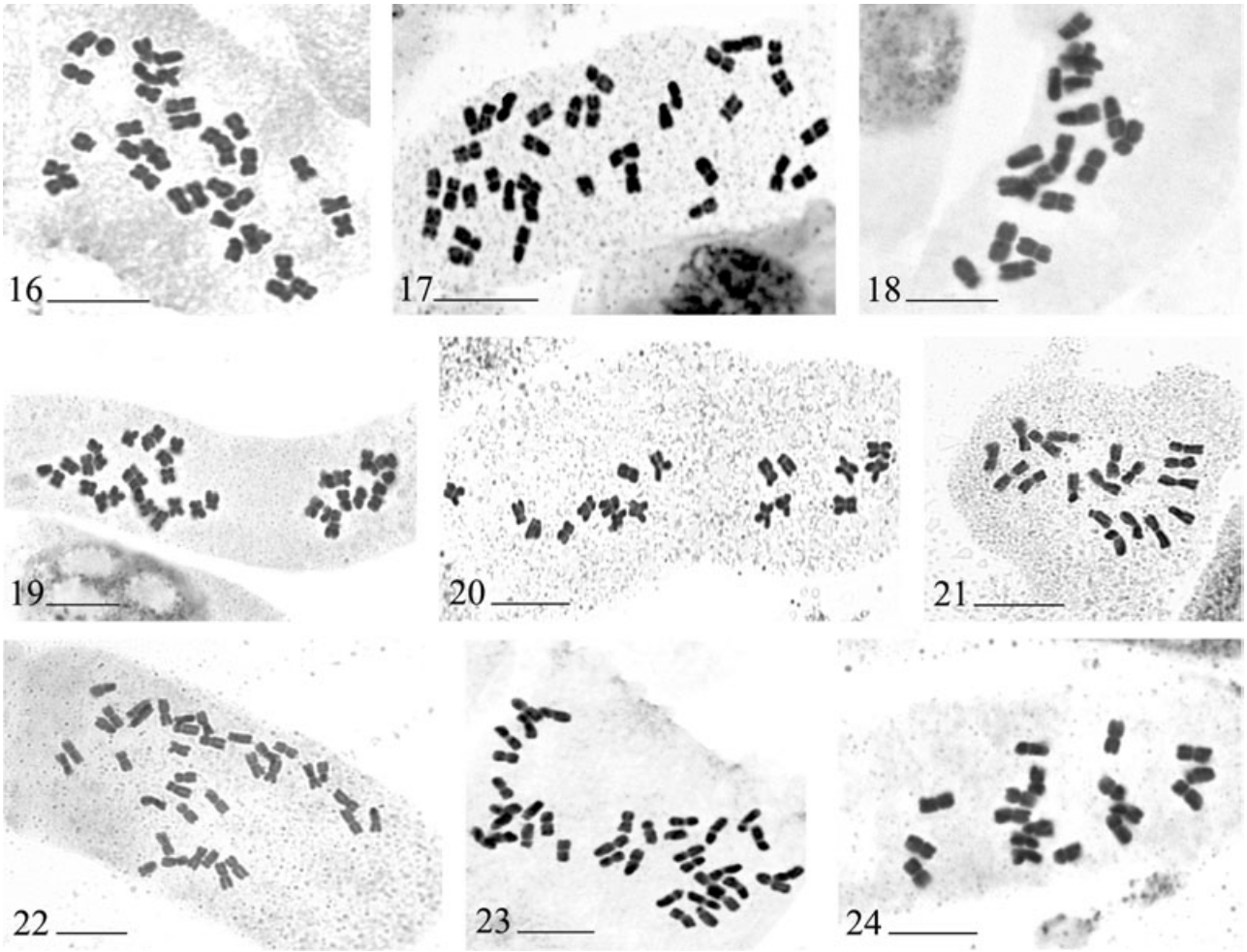
This is the third count for this species, agreeing with Krogulevich (1978) and Garcia *et al.* (2006), reporting on Russian and Mongolian populations, respectively.

Artemisia desertorum Spreng.
Russia, Primorskii krai: Nadezhdinskii raion, Razdolnaya river valley, near Terekhovk, steep rocky slope on the right side of the valley, meadows in oak forest, *A.A. Korobkov*, 10.x.2004 (LE-Korobkov). $2n = 36$ (Fig. 17).

To our knowledge, this is the second count for this species. It agrees with a count on a Russian population by Volkova & Boyko (1985).

Artemisia limosa Koidz.
Russia, Sakhalin island: Magutan volcano, north of Yuzhno-Sakhalinsk, *Y.S. Smirnov*, 20.ix.2000 (LE-Korobkov 00-03). $2n = 18$ (Fig. 18).

There is a previous count for this Sakhalin endemic (Sokolovskaya, Probatova & Rudyka, 1985), where the



Figures 16–24. Somatic metaphases. Fig. 16. *Artemisia depauperata* ($2n = 36$). Fig. 17. *A. desertorum* ($2n = 36$). Fig. 18. *A. limosa* ($2n = 18$). Fig. 19. *A. manshurica* ($2n = 36$). Fig. 20. *A. oxycephala* ($2n = 18$). Fig. 21. *A. sphaerocephala* ($2n = 18$). Fig. 22. *A. subdigitata* ($2n = 36$). Fig. 23. *A. tomentella* ($2n = 36$). Fig. 24. *A. schrenkiana* ($2n = 18$). Scale bars, 10 μm.

diploid was described. Our results agree with this previous study.

Artemisia manshurica Kom.
Russia, Primorskii krai: Muraviev-Amurskii peninsula, summit of the pass, steep slopes at the narrow crest, meadows with shrubs in a *Malus* forest, *A.A. Korobkov*, 21.x.2004 (LE-Korobkov). $2n = 36$ (Fig. 19).

To our knowledge, this is the fourth count for this species. All the earlier reports are from Chinese and Russian populations (Sokolovskaya *et al.*, 1985; Wang *et al.*, 2000; Hoshi *et al.*, 2003), and that presented here agrees with them.

Artemisia oxycephala Kitag.
Mongolia, Tuv (central) aimag: Mungunmort sum, 10 km south of the sum, *Sh. Dariimaa, Sh. Tsooj, J. Vallès & E. Yatamsuren*, 7.ix.2004 (BCN 34491). $2n = 18$ (Fig. 20).

Our result is the first on a Mongolian population of this species, showing the existence of the diploid cytotype based on $x = 9$. A previous count for this species reported the tetraploid level in plants from China (Wang, 2000; $2n = 36$).

Artemisia sphaerocephala Krasch.
Mongolia, Bulgan aimag: Dashin Shellen sum, 10 km north-west of the sum, near the Institute of Folk Medicine, steppe with *Caragana*, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 24.viii.2004 (BCN 34485). $2n = 18$ (Fig. 21).

This is the third count for this desert steppe species traditionally used for windbreaks and sand stabilization. Ours is the first for Mongolian material, the two previous counts being on plants from Chinese populations and reporting the same chromosome number (Yan *et al.*, 1989; Qiao, Yan & Zhang, 1990).

Artemisia subdigitata Matff.
Mongolia, Umnu (south) Gobi aimag: Bulgan sum, eastern Gurvan Saikhan mountains, canyon near Brigat, rocky slopes, *Sh. Dariimaa, Sh. Tsooj & J. Vallès*, 1.ix.2004 (BCN 34846). $2n = 36$ (Fig. 22).

Our count, the first for a Mongolian population, agrees with two reported from China by Yan *et al.* (1989) and Qiao *et al.* (1990). We have not found any diploid count for this taxon in the literature.

Artemisia tomentella Trautv.
Russia, Republic of Tuva: close to Erzin city, small population in a degraded field, *A.A. Korobkov*, 16.ix.2003 (LE-Korobkov). $2n = 36$ (Fig. 23).

This is the third count for this Central Asian endemic. The previous reports (Filatova, 1971; Magulaev, 1976) indicated the diploid and tetraploid cytotypes based on $x = 9$, and the present result confirms the latter.

Subgenus *Seriphidium* Besser

Artemisia schrenkiana Ledeb.
Russia, Republic of Tuva: Chegl-Kholskii raion, depression of the salt lakes Shara-Hur and Dus-Khol, 10 km west of Agar-Dag-Taiga mountain chain, saline soil on the shores of the lakes, *A.A. Korobkov*, 17.ix.2003 (LE-Korobkov) $2n = 18$ (Fig. 24).

To our knowledge, this is the second count for this species, endemic in Kazakhstan, Mongolia, and Russia. This report confirms the previous one (Filatova, 1975).

CONCLUDING REMARKS

All the species studied have chromosome numbers based on $x = 9$, except one, which is based on $x = 8$. This confirms the existence of two basic numbers in the genus (Vallès & Garnatje, 2005, and references cited therein) and further demonstrates the dominance of $x = 9$ in the genus, tribe, and family (Solbrig, 1977; Schweizer & Ehrendorfer, 1983; Oliva & Vallès, 1994; Vallès & Siljak-Yakovlev, 1997; Garcia *et al.*, 2006; and references cited therein). In this paper, we have reported many ploidy levels, ranging from $2x$ to the hitherto unknown $16x$, the highest known ploidy level in the genus, a finding which accounts for its great cytogenetic diversity. Polyploidy is considered by many authors as an important evolutionary mechanism in plants (Bretagnolle *et al.*, 1998; Soltis & Soltis, 1999; Soltis *et al.*, 2004). Most of the *Artemisia* that colonize extreme and arid habitats are polyploids. This fact supports the hypothesis that polyploids have more tolerance of extreme environmental conditions (Otto & Whitton, 2000). The high frequency of polyploids in the plants examined here (more than 50% of the studied populations) leads us to consider that *Artemisia* is continuously developing genetic and ecological evolutionary mechanisms leading to diversification and speciation, particularly in Asia, which is regarded as the main centre of diversification of the genus.

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Recomptes cromosòmics en espècies d'*Artemisia* (*Asteraceae*, *Anthemideae*) i variació de la grandària del genoma al subgènere *Dracunculus*: implicacions cariològiques, sistemàtiques i filogenètiques

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Jaume Pellicer, Sònia Garcia, Teresa Garnatje, Shagdar Dariimaa, Aleksandr A. Korobkov i Joan Vallès

Aquest treball presenta 12 recomptes cromosòmics en diferents representants d'*Artemisia* de Rússia. Els recomptes duts a terme en *A. czekanowskiana*, *A. globosa*, *A. ledebouriana*, *A. lithophila*, *A. macilenta*, *A. pycnorhiza* i *A. sosnovskyi* són inèdits. L'estudi de les espècies *A. czekanowskiana* ($2n = 10x = 90$) i *A. macrantha* ($2n = 12x = 108$) han revelat cèl·lules amb recomptes aneusòmics. La presència d'un cromosoma dicèntric i un fragment acèntric o bé un cromosoma B s'ha trobat en *A. lithophila*. A més, s'ha estimat la grandària del genoma, mitjançant citometria de flux, en 21 poblacions corresponents a 18 espècies d'*Artemisia*, representants del subgènere *Dracunculus*, principalment de Rússia, tot i que també s'ha estudiat material provinent de Mongòlia. La quantitat de DNA nuclear ha variat des de 2C = 4,21 fins a 2C = 24,85 pg, i el contingut nuclear referit al complement cromosòmic haploide (1Cx) des de 2,06 fins a 3,00 pg. S'ha avaluat la constància de la quantitat de DNA nuclear, tot concloent que existeix una pèrdua de DNA (a nivell d'1Cx) en ascendir nivells de ploïdia. Hem cercat les possibles correlacions entre grandària del genoma, trets morfològics i posició filogenètica d'algunes de les espècies estudiades.

Relacions filogenètiques i delimitació del subgènere *Dracunculus* (gènere *Artemisia*, *Asteraceae*) en base a seqüències del DNA ribosòmic i cloroplàstic

Taxon (en preparació)

Jaume Pellicer, Joan Vallès, Aleksandr A. Korobkov i Teresa Garnatje

El subgènere *Dracunculus* del gènere *Artemisia* ha estat analitzat usant seqüències del DNA nuclear (ITS, ETS) i cloroplàstic (*trn*S-*trn*C, *trn*S-*trn*fM) d'una àmplia mostra del grup. Representants de moltes de les seccions i sèries en què es divideix el subgènere en els diferents tractaments taxonòmics que se n'han fet han estat inclosos en aquest treball. Les anàlisis filogenètiques han sigut dutes a terme mitjançant criteris bayesians i de màxima parsimònia per a circumscriure el subgènere, i mitjançant inferència bayesiana per a aprofundir en les relacions entre les espècies que constitueixen el nucli del subgènere. Aquesta primera aproximació filogenètica al subgènere *Dracunculus* divideix el complex en dues grans clades, una que inclou el nucli del subgènere amb una majoria de representants eurasiàtics més unes poques espècies nord-americanes i una altra en la qual espècies endèmiques nord-americanes són situades entre els reprentants del subgènere *Tridentatae*. Una tercera clada és constituïda per les espècies *A. salsoloides* i *A. tanaitica*, que mostren, amb la seva posició separada, la parafília del subgènere. A l'interior del nucli del subgènere, discutim l'existència de diversos llinatges, com ara els complexos d'*A. dracunculus* i d'*A. campestris*, així com les posicions i relacions dels gèneres relacionats *Mausolea*, *Neopallasia* i *Turaniphytum* dins del subgènere. En aquest sentit, alguns grups que mostren discordances entre la sistemàtica molecular i els tractaments taxonòmics clàssics del subgènere han estat interpretats a la llum de processos d'hibridació que possiblement van tenir lloc en el nucli del grup. A més, la quantitat de DNA nuclear ha estat estimada per primer cop en 22 espècies i les dades d'aquesta mena de treballs nostres previs han estat combinades amb les presents per a estudiar l'evolució de la mida del genoma amb la poliploïdia en el subgènere, així com per a procurar trobar evidències que reforcin les relacions filogenètiques que hem trobat.

Phylogenetic relationships and subgeneric delimitation of subgenus *Dracunculus* (genus *Artemisia*, Asteraceae) based on ribosomal and chloroplast DNA sequences

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ABSTRACT. The subgenus *Dracunculus* of the genus *Artemisia* has been analyzed using nuclear (ITS, ETS) and plastid (*trn*S-*trn*C, *trn*S-*trn*fM) DNA sequences of a large sample of this group. Representatives of many sections and series in which the subgenus is divided in the different taxonomic treatments have been included in the present work. Phylogenetic analyses have been carried out under both Bayesian and maximum parsimony criteria to circumscribe the subgenus, and under Bayesian inference to delve into the relationships between the species embedded in the core of the subgenus. This first phylogenetic approach to the subgenus *Dracunculus* resolves the complex in three main clades, one including the core of the subgenus with most of the Eurasian representatives plus a few North American endemic species, and a second clade in which the North American endemics are nested within the subgenus *Tridentatae* representatives. A third small clade grouping *A. salsoloides* and *A. tanaitica* strengthens the paraphyly of the subgenus in its present delineation. Within the core of the subgenus, the existence of different lineages such as the *A. dracunculus* or *A. campestris* complexes, as well as the relationships of the allied genera *Mausolea*, *Neopallasia* and *Turaniphytum* within the subgenus have been discussed. In this sense, some linkages that reflect discordances between the molecular systematics and the previous taxonomic treatments of the subgenus have been interpreted in the light of possible hybridization events that took place in the core of the group. Genome size data have been newly estimated for 22 species, and data from previous studies have been combined with the present ones to understand the evolution of the DNA amount with the polyploidy in the subgenus, as well as to find further evidence in support of the phylogenetic relationships.

Keywords: Bayesian inference, Compositae, genome size, hybridization, phylogenetic reconstruction.

INTRODUCTION

Several approaches devoted to elucidate the phylogenetic relationships within the representatives of the genus *Artemisia* L. have been carried out up to the present time (Kornkven & al., 1998; Torrell & al., 1999; Watson & al., 2002; D'Andrea & al., 2003; Vallès & al., 2003; Sanz & al., 2008; Tkach & al., 2008). These studies have highlighted the infrageneric complexity of the genus, extensive to the Artemisiinae subtribe, as well as some incoherences between the traditional classification and the molecular demarcation of some groups. Since Tournefort's (1700) classification until the present day, many taxonomic rearrangements have also been carried out in the genus (see Vallès & McArthur, 2001; Vallès & Garnatje, 2005; for data compilation). The subgenus *Dracunculus* (Besser) Rydb., which is the object of the present investigation, prior to its present status was described as an independent genus, *Oligosporus*, by Cassini (1817). He selected a group of taxa to create a new genus to integrate those species with capitula structured in functionally separate sexes, with outer female florets, and central functionally male florets, because of their abortive ovaries. As the morphological traits were not enough to support the complex as an independent genus, Besser (1829, 1832, 1834, 1835), Candolle (1837) and later Rydberg (1916) proposed its reinclusion as a section/subgenus of *Artemisia*. Despite there being a general agreement about the taxonomic circumscription of *Dracunculus*, consensus is still lacking about the infrageneric relationships within the species (Poljakov, 1961; Darijma, 1989; Ling, 1992; Ling & al. 2006). In this sense, attention is to be paid to the existence of some allied genera, such as *Mausolea* Poljakov, *Neopallasia* Poljakov, and *Turaniphytum* Poljakov, previously labelled under *Artemisia* (respectively, *A. eriocarpa* Bunge, *A. pectinata* Pall. and *A. eranthema* Bunge), which have revealed a close relationship with the species of the subgenus *Dracunculus* (Vallès & al., 2003; Sanz & al., 2008). Furthermore, the absence of a solid phylogenetic framework for the subgenus does not allow an in-depth study of their particular position, and consequently the relationships within the subgenus are not completely resolved.

As currently considered, the subgenus *Dracunculus* includes about sixty to eighty taxa depending on the authors (Poljakov, 1961; Shultz, 2006; Ling & al.,

2006) mainly distributed across the Northern Hemisphere growing in the arid and semi-arid regions from Europe to Asia, and reaching North America.

DNA sequence data have been utilized widely in plants to elucidate the relationships between the species. The value of the sequences of the *trn*S-*trn*C and *trn*S-*trn*fM chloroplastic and the nuclear ITS and ETS regions has been proved in several groups of Asteraceae (McKenzie & al., 2006; Fehrer & al., 2007; Mort & al., 2008; Sanz & al., 2008 and references therein).

Previous studies regarding the systematics of *Artemisia*, although pointing through the monophyly of the Eurasian group (Sanz & al., 2008; Tkach & al., 2008), also shed light on the misplacement of some of the North American endemics (Sanz & al., 2008) included in the subgenus *Dracunculus sensu* Shultz (2006). Thus, *Artemisia filifolia* Torr. and *Picrothamnus desertorum* Nutt. (previously known as *Artemisia spinescens* D. C. Eaton), which share the floral characters with the Eurasian representatives, are nested into a separate clade together with the North American *Tridentatae*.

The integration of data resulting from phylogenetic studies with those of other disciplines, such as genome size variation, may contribute to a better understanding and interpretation of the evolutionary ways within narrow plant groups (Ohri, 1998). In fact, genome size is very variable across flowering plants, and has applications in many fields (e.g. Bennett & Leitch, 2005; and references therein) and the polyploidy directly influences changes at genome size level, this being a common process in plants (e.g. Soltis & Soltis, 2000; Wendel, 2000; Cui & al., 2006; Chen, 2007). As is frequent in the genus *Artemisia*, the subgenus *Dracunculus* presents a high rate of polyploidy among its species. Ploidy levels reported range from diploid to decaploid (Kawatani & Ohno, 1964; Rousi, 1969; Torrell & Vallès, 2001; Pellicer & al., 2007b), being essentially $x = 9$-based. The disploid basic chromosome number ($x = 8$) remains restricted to the annual species *Artemisia scoparia* Waldst. et Kitam. (Vallès & al., 2001; and references therein). Genome size studies devoted to specific complexes of *Artemisia* (Garcia & al., 2006, 2008; Pellicer & al., 2007a), as well as those with a more general viewpoint (Torrell & al., 2001; Garcia & al., 2004; Pellicer & al.,

2009), have pointed out an increased rate of DNA loss correlated to polyploidy in the genus.

The main goals of the present study are to establish a phylogenetic framework of the subgenus *Dracunculus* in order to (i) study the taxonomic circumscription of the subgenus by means of the analysis of a representative sampling, (ii) investigate the interespecific relationships within the subgenus, as well as with other related Artemisiinae genera and, (iii) analyze the evolutionary implications of the genome size and polyploidy using data previously published and new C-values obtained for the present study.

MATERIALS AND METHODS

Taxon sampling. — DNA sequences from 59 species of *Artemisia* belonging to the subgenus *Dracunculus* and three related Artemisiinae genera (*Mausolea*, *Neopallasia* and *Turaniphytum*) were newly generated (58 ITS and 3'-ETS, 57 5'-ETS, 61 *trn*S-*trn*C and *trn*S-*trn*fM sequences). Sampling of the subgenus was completed with 14 previously published ITS and ETS sequences (Torrell & al., 1999; Sanz & al., 2008, Tkach & al., 2008). Additionally, in order to perform a phylogenetic framework in which to circumscribe *Dracunculus* within the genus and the subtribe, ITS and ETS sequences of 49 formerly studied species (Torrell & al. 1999; Vallès & al., 2003; Sanz & al., 2008, Tkach & al., 2008; Pellicer & al., 2009) were used. Taxa, herbarium voucher information and EMBL/GenBank accession numbers for all the new and previously published sequences are listed in Appendix 1.

New plant material was obtained from field expeditions, from culture of achenes in the greenhouses at the Faculty of Pharmacy, University of Barcelona, and the Botanical Institute of Barcelona, and also from voucher specimens from the herbaria BCN (Centre de Documentació de Biodiversitat Vegetal CEDOC, University of Barcelona), LE (Botanicheskii Institut im. 'V. L. Komarova', Sankt Peterburg), W (Natural History Museum-Herbarium, Viena), and E (Royal Botanic Garden Edinburgh, Edinburgh).

DNA extraction, amplification and sequencing. — Total genomic DNA was extracted from silica-dried, herbarium vouchers or fresh leaves using the CTAB

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

PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, California, U.S.A.) or with DNA Clean & Concentrator-5 D4003 (Zymo Research, Orange, U.S.A). Direct sequencing of the amplified fragments was performed using Big Dye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, California, U.S.A.) using the primers ITS4, 18SETS, ETS1f, trnS^{UGA}, trnfM^{CAU} and trnC^{GCA}. Nucleotide sequencing was carried out at the Serveis Cientificotècnics (Universitat de Barcelona) using a ABI PRISM 3700 DNA Analyzer (PE Biosystems, Foster City, California, U.S.A.).

Sequence assembly, alignment and phylogenetic analyses. — Nucleotide sequences were assembled and edited using BioEdit version 7.0.9 (Hall, 1999), and trimmed of primer regions. Alignments were made separately for each

region with ClustalW (Thomson & al., 1997) using the default settings implemented in BioEdit, and gaps were manually adjusted to improve the alignments. Two sets of DNA data were prepared. A first one addressed to circumscribe the subgenus *Dracunculus* within the genus *Artemisia*, which included the ITS (without the 5.8S gene) and the 3′ end of the ETS region, and a second group of DNA matrices restricted to the subgenus *Dracunculus*, including the ITS (ITS1-5.8S-ITS2), both 5′ and 3′ ETS ends, *trn*S^{UGA}-*trn*fM^{CAU} and *trn*S^{GCU}-*trn*C^{GCA}, were also constructed. In total, seven matrices grouped in the following two sets were prepared for independent analyses prior to combination.

Set 1. *Circumscription of the subgenus* Dracunculus. (i) Bayesian inferences (BI) were carried out with MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). The most appropriate nucleotide substitution models were chosen with MrModeltest version 2.3 (Nylander, 2004). Separate analyses were carried out on both markers resulting from GTR + G model (hyerarchical Likelihood Ratio Tests, hLRT) GTR + I + G model (Akaike Information Criterion AIC) for the ITS, GTR + I + G model (hLRT and AIC) for both ETS and the combined ITS + ETS. Four Markov chains were run simultaneously for $6x10^6$ generations, and these were sampled every 100 generations. Data from the first 6000 generations were discarded as the "burn-in" period, after confirming that likelihood values were stabilized prior to the 6000^{th} generation. The 50% majority rule consensus trees and posterior probabilities (PP) of nodes were calculated from the pooled samples. (ii) On parsimony analyses (MP) a first heuristic search of the separate and combined ITS + ETS data using PAUP* version 4.0b10 (Swofford, 2003) was carried out under the maximum parsimony criterion. Standard parameters used were: Multrees, 100 random taxon additions, maxtrees = 100000 (increased automatically by 1000), tree bisection-reconnection (TBR) branch swapping, one tree held at each iteration and character states specified as unweighted. As the three analyses turned out to be blocked due to memory restrictions, posterior heuristic searches were developed with the constraint of saving no more than 1000 trees larger than/equal to the tree length [steps number: ITS (378), ETS (224), ITS + ETS (619)] which collapsed the previous analysis at the "Branch swapping options". Branch support of the tree nodes was evaluated by faststep

bootstrap implemented in PAUP* using 1000 replicates, 10 random sequence additions and no branch swapping. This is an alternative to large dataset, and it provides similar estimates, although less, to those performed with branch swapping (Mort & al., 2000).

Set 2. *Phylogenetic relationships within the subgenus* Dracunculus. Bayesian inferences (BI) following the same procedure as previously described were carried out for the different sequence matrices, i. e. separated [ITS (SYM + G), ETS (HKY + I + G), *trn*S-*trn*C (AIC: GTR + I + G; hLRT: F81+ I + G), *trn*S-*trnf*M (AIC: F81 + I; hLRT: F81+ I + G)], and the following combinations A: [ITS + ETS, *trn*S-*trn*C + *trn*S-*trnf*M (GTR + I + G)], B: [ITS + *trn*S-*trn*C + *trn*S-*trnf*M, ETS + *trn*S-*trn*C + *trn*S-*trnf*M (GTR + I + G)] and C: [ITS + ETS + *trn*S-*trn*C + *trn*S-*trnf*M (GTR + I + G)]. For the last combination, an analysis of the partitioned data set (with coded indels) was carried out. Four Markov chains were run simultaneously for $3x10^6$ generations (separated data sets), and $6x10^6$ generations (combined data sets) sampled every 100 generations. Data from the first 3000th and 6000th generations were discarded as the "burn-in" period, after confirming that likelihood values were stabilized. The 50% majority rule consensus trees and posterior probabilities (PP) of nodes were calculated.

Table 1. Numerical results of the ITS, ETS and the combined analyses from set 1. Uninformative characters were excluded from the analyses.

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

Genome size assessments. — Leaf tissue of five individuals for the 31 populations (22 species) studied was chopped in 600 µl of Galbraith's isolation

buffer (Galbraith & al., 1983) with a razor blade, together with the chosen internal standard, and supplemented with 100 µg/ml ribonuclease A (RNase A, Boehringer, Meylan, France). For each individual, two independent samples were extracted to be processed under the cytometric assessment. Samples were subsequently stained with 36 µl of propidium iodide (1mg/ml) to a final concentration of 60 µg/ml (Sigma-Aldrich Química), kept on ice for 20 min and measured in an Epics XL flow cytometer (Coulter Corporation). Measurements were made at the 'Serveis Cientificotècnics' of the Universitat de Barcelona. To ascertain that the instrument showed a linear response across the range of genome sizes studied, we performed several assays which included both internal standards and one of the populations with the highest genome size at the same time. The difference between the obtained results with respect to each standard was negligible (less than 2% of deviation) hence we can certify the linearity of the flow cytometer in this interval and the convenience of the use of the chosen internal standards. We also calculated the mean half peak coefficient of variation (HPCV) corresponding to ten samples. The HPCV obtained were 2.54% in the case of *Petunia hybrida*, 1.87% for *Pisum sativum*.

RESULTS

Bayesian analyses. — A phylogram displaying the subgeneric circumscription of *Dracunculus* within the *Artemisia* is presented in Fig. 1. A second approach has been performed to study in greater depth in the relationships of the representatives included in the main clade which clearly defines the subgenus *Dracunculus* (Fig. 1, clade A; PP = 100%, BS = 91%), which is basically composed of Eurasian representatives (Fig. 2). When the AIC and hLRT criteria presented different models that best fitted to our data sets (Table 1, see also the Material & Methods section), independent analyses were performed for each model. After checking that no inconsistencies existed between the resulting trees, we have only presented the trees extracted from the AIC model, as this has proved to be more advantageous than hLRT model selection (Posada & Buckley, 2004).

Table 2. Nuclear DNA content and karyological information of the populations studied. [1]Nuclear DNA content [2C value (standard deviation)]. [2]Nuclear DNA content; 1pg = 978 Mbp (Doležel & al., 2003). [3]Monoploid genome size. The order of species is the same as in Appendix 2.

Taxa	Chrom. nº	Ploidy level	2C (SD)[1]	2C (Mbp)[2]	1Cx (pg)[3]	Standard
A. arenaria	36	4	10.38(0.04)	10151 .64	2.60	*Pisum*
A. borealis	36	4	10.95(0.26)	10709 .10	2.74	*Pisum*
A. borealis	36	4	10.65(0.15)	10415 .70	2.66	*Pisum*
A. campestris subsp. *variabilis*	54	6	15.44(0.11)	15100 .32	2.57	*Pisum*
A. capillaris	18	2	3.25(0.03)	3178 .50	1.63	*Pisum*
A. capillaris	18	2	3.44(0.04)	3364 .32	1.72	*Pisum*
A. crithmifolia	54	6	14.79(0.09)	14464 .62	2.47	*Pisum*
A. crithmifolia	54	6	15.08(0.15)	14748 .24	2.51	*Pisum*
A. crithmifolia	54	6	15.04(0.11)	14709 .12	2.51	*Pisum*
A. crithmifolia	54	6	15.55(0.13)	15207 .90	2.59	*Pisum*
A. crithmifolia	54	6	15.04(0.08)	14709 .12	2.51	*Pisum*
A. desertorum	36	4	8.61(0.12)	8420 .58	2.15	*Petunia*
A. dolosa	18	2	4.20(0.08)	4107 .60	2.10	*Petunia*
A. dolosa	36	4	8.86(0.23)	8665 .08	2.22	*Petunia*
A. dracunculus	36	4	11.82(0.10)	11559 .96	2.96	*Pisum*
A. dracunculus	36	4	11.93(0.11)	11667 .54	2.98	*Pisum*
A. eriopoda	36	4	8.95(0.12)	8753 .10	2.24	*Petunia*
A. glauca	18	2	5.75(0.04)	5623 .50	2.88	*Petunia*
A. intramongolica	36	4	10.42(0.09)	10190 .76	2.61	*Pisum*
A. japonica	36	4	8.64(0.13)	8449 .92	2.16	*Petunia*
A. klementzae	36	4	8.47(0.13)	8283 .66	2.12	*Petunia*
A. littoricola	36	4	8.73(0.19)	8537 .94	2.18	*Petunia*
A. manshurica	36	4	8.54(0.05)	8352 .12	2.14	*Petunia*
A. ordosica	18	2	5.76(0.11)	5633 .28	2.88	*Petunia*
A. pycnocephala	18	2	6.22(0.15)	6083 .16	3.11	*Pisum*
A. salsoloides	18	2	11.40(0.04)	11149 .20	5.70	*Pisum*
A. songarica	18	2	5.51(0.15)	5388 .78	2.76	*Petunia*
A. tomentella	18	2	5.20(0.08)	5085 .60	2.60	*Petunia*
A. tomentella	36	4	8.76(0.13)	8567 .28	2.19	*Petunia*
A. xanthochroa	36	4	9.61(0.08)	9398 .58	2.40	*Pisum*
A. xylorhiza	36	4	9.26(0.15)	9056 .28	2.32	*Pisum*

Parsimony analyses. — Table 1 summarizes the numerical data related to tree in Fig. 1 (combined ITS + ETS), as well as the data for the independent analyses of both ITS and ETS matrices, which includes the data set characteristics, i.e. number of accessions, range of sequence divergence, tree lengths and data on character fitness (consistency, retention, homoplasy and rescaled consistency indexes) of the most-parsimonious trees (MPT).

Congruence between trees and conflictive species (Figs. 1, 2). — As the BI turned out to be of better resolution than parsimony, and tree topologies showed no incongruence between the significantly supported branches, only the Bayesian trees of the combined data sets have been presented (note that in Fig. 2 only BI is shown). The partition homogeneity tests indicate that the null hypothesis of congruence among nuclear and chloroplast datasets cannot be rejected ($P < 0.05$). Even so, the topologies of the trees obtained from independent and partially combined matrices (see the material and methods section) were evaluated for congruence prior to combining all the data sets, showing no general conflicts between significantly supported clades of the chloroplast and nuclear regions. Misplacements of some species between markers were detected (later discussed), and some species with an incongruent position were removed from the combined analysis. In this sense, *Artemisia crithmifolia* L., *Artemisia kuschakewiczii* Winkl., *Artemisia nanschanica* Krasch., *Artemisia pamirica* C. Winkl. and *Artemisia sosnovskyi* Krasch. were excluded from the combined analysis (subgenus *Dracunculus s. s.*; Fig. 2) because of the variable placement of these species in the trees resulting from the analyses of the combined chloroplast and the combined nuclear datasets (branch support PP > 95% between markers).

Genome size in the subgenus *Dracunculus*- Table 2 gives the 2C-, 1Cx-values and ploidy levels for the populations studied. For calculations, previously published data on genome size in the subgenus have been used (Torrell & Vallès, 2001; Garcia & al., 2004, 2008; Pellicer & al., 2007a, 2009). The 2C DNA contents [considering the clades A, D and E (Fig. 1)] vary about 7.1-fold, from 3.37 pg of *Artemisia capillaris* Thunb. ($2n = 2x = 18$) to 23.90 pg in *A. dracunculus* ($2n = 10x = 90$; Torrell & Vallès, 2001, Pellicer & al. 2007a). Monoploid genome size varies 3.4-fold, from 1Cx = 1.68 pg in *A. capillaris* to 5.70 pg in *Artemisia salsoloides* Willd., a value that is clearly reduced to 1.9-fold [from *A. capillaris* (1.68 pg) to *Artemisia arenaria* DC. ($2n = 2x$, 3.27 pg); Pellicer & al., 2009] when only the core of the subgenus is taken into consideration (Fig. 2, clade A).

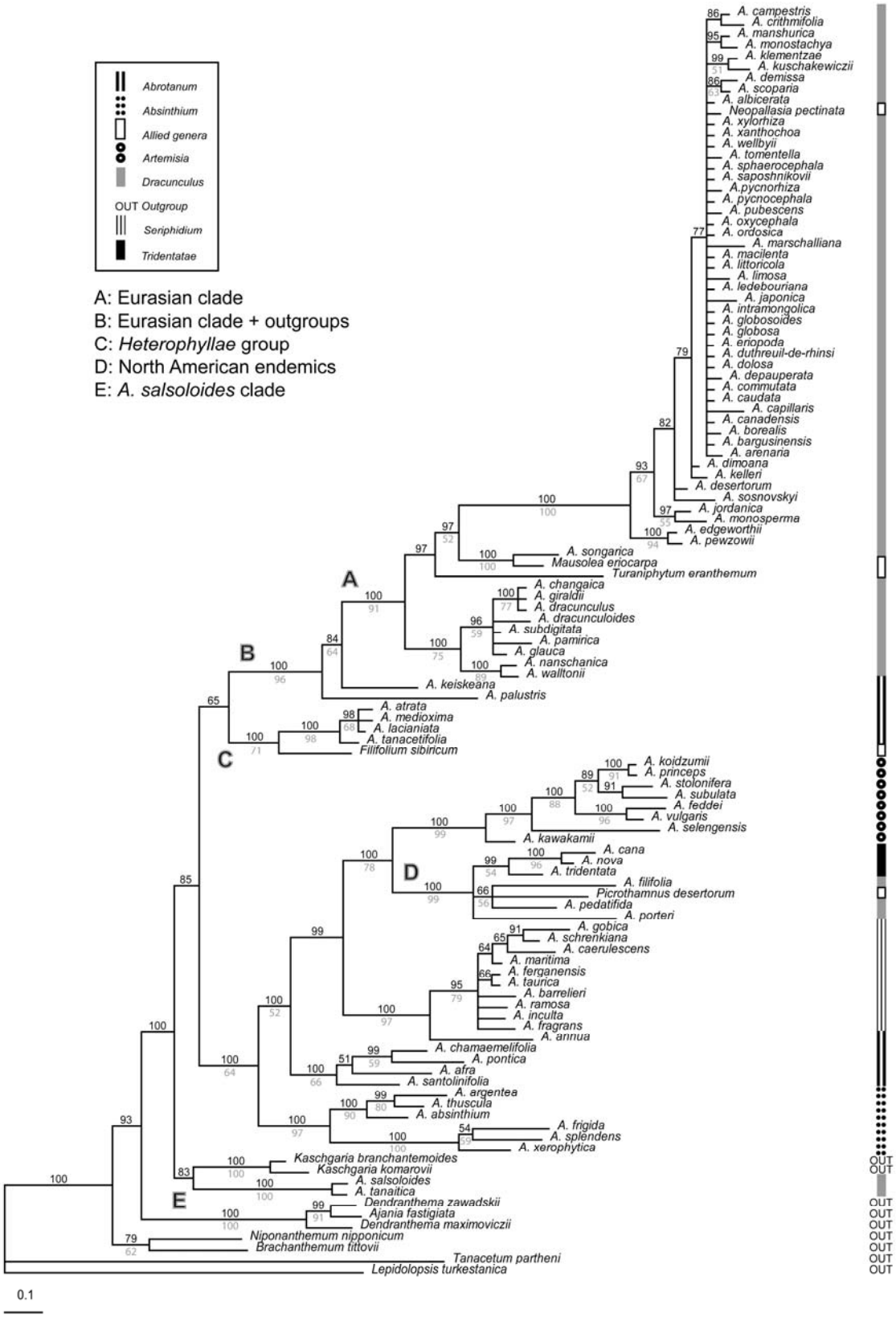

Figure 1. Phylogenetic tree from Bayesian inference (Bayesian support (PP, black)) is indicated above branches and parsimony bootstrap (BS, grey) is indicated below branches). *Artemisia* subgenera are mapped under traditional criteria. Clades discussed in the text are indicated by capital letters.

DISCUSSION

Systematic placement and delimitation of subgenus *Dracunculus* within the genus *Artemisia* (Fig. 1). — The phylogeny constructed confirms the monophyly of the main lineage of the subgenus *Dracunculus* (Fig. 1, clade A). This group has performed a very robust clade (PP = 100%; BS = 91%), and has embedded the core of the subgenus, including most of the Eurasian species (with the exclusion of *A. salsoloides* and *Artemisia tanaitica* Klokov, later discussed) plus some North American endemic representatives (*Artemisia canadensis* Michx., *Artemisia caudata* Michx. and *Artemisia pycnocephala* DC.) closely related to those from the old world (Fig. 1, clade A). A second group of *Dracunculus* species, following the criteria established by Shultz (2006) in the revision of the Flora of North America, are grouped in a separate lineage together with the North American endemic *Tridentatae* (Fig. 1, clade D), which are *A. filifolia*, *Artemisia pedatifida* Nutt., *Artemisia porteri* Cronquist and the species *Picrothamnus desertorum* (formerly *A. spinescens*). The geographical isolation of those endemic species seems to be the most plausible hypothesis to explain their linkage under molecular criteria within the *Tridentatae* (Sanz & al., 2008). Different authors have also highlighted the close relationship of this group of species with the *Tridentate* on the basis of the karyological, cytological and phytochemical similarities (Beetle, 1960; McArthur & Pope, 1979). The phylogenetic position of *A. filifolia* has been previously reported by Kornkvern & al. (1998), from chloroplast restriction site data, and also Watson & al. (2002) and Sanz & al. (2008) from ribosomal nuclear sequences. All of them pointed out the close relationship of this species with the *Tridentatae* group, despite the lacking of morphological resemblance between the species. Although the present results confirm these previous findings, and new relationships between the non *Tridentatae* North American endemics are reported, more intensive, in-depth studies will be necessary on these species to clarify the relationships among them.

As commented previously, it is also interesting to remark that the species *A. salsoloides* and *A. tanaitica* are excluded from the subgenus *Dracunculus* clade, both robustly grouped (PP = 100%; BS = 100%), even in the ITS and ETS

analyses, but with an undetermined position in the phylogeny (Fig. 1, clade E). The close relationship between these two species also correlates with the fact that Čzerepanov (1995) had considered the two to be synonyms. Leonova (1988) proposed the segregation of *A. salsoloides* from the section *Campestres* Korobkov to create the new monotypic section *Salsoloides* Leonova within the subgenus because of its characteristic leaf indumentum. Genome size data clearly differ from the 2C values found in the diploid species of the subgenus. While diploids range from 3.37 to 6.54 pg, the *A. salsoloides* ($2n=18$) estimate was of 11.40 pg, which is not only more than double that of the largest diploid found, but also larger than the amount of the majority of the tetraploid representatives of the subgenus. In order to confirm the phylogenetic position of the species, previous analyses with different specimens of *A. salsoloides* from various provenances have revealed identical DNA sequences, hence embedded into the same separated clade (data not shown). From the taxonomical viewpoint, the specimens analysed fitted well with the morphological descriptions of those plants (Poljakov, 1961), and is understood their inclusion in the subgenus because they share common morphological traits with some of the representatives of *Dracunculus*. In this sense, floral characters from *in vivo* and herbarium vouchers presented the characteristic structure for the subgenus. So, the species is still retained within *Dracunculus*, but the presence of both exclusive morphological traits and genome size data gives support to the consideration of a possible phylogenetic segregation or the acceptance of the paraphyly of the subgenus.

Phylogenetic relationships of the Eurasian clade with the sister group (Figs. 1, 2). — Previous studies placed the species *Filifolium sibiricum* (L.) Kitam. as sister taxon of the subgenus *Dracunculus* (Sanz & al., 2008). This is a comprehensible relationship if we take into account the fact that the species shares the same floral structure characteristic of the subgenus. Our phylogeny has revealed a strong relationship of this species with the subsection *Heterophyllae* (*Artemisia atrata* Lam., *Artemisia laciniata* Willd. *Artemisia medioxima* Krasch. ex Poljakov and *Artemisia tanacetifolia* L.) sensu Darijma (1989), but without statistical

support to confirm their sister position to the subgenus. In this sense, both ITS and ETS analyses showed the species *Artemisia keiskeana* Miq. and *Artemisia palustris* L. (subgenus *Artemisia*) as possible candidates to the sister position of the Eurasian lineage (Fig. 1, clade B; PP = 100%, BS = 96%). It can be difficult to understand, from the morphological viewpoint, the relationships of the subgenus *Dracunculus* with its sister taxa, also with the *Heterophyllae*. The phytochemistry of the group can shed light on the knowledge of these species and their relationships. Greger (1988) pointed out that the distribution of secondary metabolites (e. g. polyacetylenes or coumarin sesquiterpene ethers) can provide valuable criteria when studying the systematics of *Artemisia*. This author found coincidences regarding the occurrence of dehidrofalcarinone derivatives and other aromatic acetylenes specific to the subgenus *Dracunculus* in the *Heterophyllae* group (subgenus *Artemisia*), and also pointed out the dominance of this dehidrofalcarinone pathway in *A. keiskeana*. Likewise, Belenovskaja (1996) indicated a close relationship between the species *A. palustris* and the *Heterophyllae* group on the basis of their characteristic phenolic composition. Although the relationship between the *Heterophyllae* and the Eurasian clade, as previously mentioned, is not stastistically supported (Fig. 1; PP = 65%), Greger's findings make more reliable and comprehensible the basal phylogenetic relationships of the subgenus with those species.

Phylogenetic relationships within the Eurasian clade (Fig. 2). —

Taxonomic conflicts within the core of the subgenus (Clade 1). As stated before, the nucleus of the subgenus *Dracunculus* appears embedded into a large and quite homogeneous lineage (sequence divergence < 11.5%; see Table 1) which includes representatives mainly from Eurasia, as well as few from North America (Fig. 2). This group, strongly supported (PP = 100%), is basically split in three main subclades (1, 2 and 3), which are not always in agreement with the classical taxonomic treatments and with biogeographic and morphological features (Poljakov, 1961; Darijma, 1989; Ling & al., 2006). Likewise, the lack of consensus between the infrageneric treatments (see Fig. 2) makes it more difficult

to compare our results with those classifications, and provides a source of points that need to be discussed:

(i) Subclade 1a: This large lineage is basically split in two minor clades (Fig. 2, 1d and 1e) with the exception of the species *Artemisia eriopoda* Bunge, widely distributed across Central and East Asia, and *Artemisia saposhnikovii* Krasch. ex Poljakov, endemic to Kyrgyzstan and South West Xinjiang (People's Republic of China). The latter species was related by Poljakov (1961) to *Artemisia albicerata* Krasch., *A. arenaria* and *Artemisia songarica* Schrenk., among others, to perform the series *Psammophilae*, but no molecular evidences for such relationship between them has been found. Although the phylogenetic position of many representatives embraced within these two clades shows no correspondence between molecular and morphological data, some clusters highlight diverse interesting relationships. The case of the *Artemisia campestris* L. group is a good example. It is composed, apart from this species, basically of *Artemisia borealis* Pall., *Artemisia limosa* Koidz., *A. caudata*, *A. pycnocephala*, *Artemisia marschalliana* Spreng. and *Artemisia duthreuil-de-rhinsii* Krasch. (Fig. 2, PP = 100%). The last species is newly included within the complex in this study, whereas the remaining had previously been studied at a morphological level in different floras (Poljakov, 1961; Shultz, 2006). *Artemisia caudata* and *A. pycnocephala* are the unique North American endemics included in the present work whose phylogenetic position correlates with the main circumscription of the subgenus. This fact can be easily understood because of the narrow relationship of these species with the worldwide distributed *A. campestris*. In fact, these taxa have been considered as subspecies [i. e. *A. campestris* L. subsp. *pycnocephala* (Less.) H.M. Hall & Clem. and *A. campestris* L. subsp. *caudata* (Michx.) H. M. Hall & Clem.]. Similar explanations can be found for *A. borealis* and *A. marschalliana*, both formerly labelled as *A. campestris* subspecies, which appear related to the East Asian *A. limosa,* endemic to Sakhalin island (Poljakov, 1961), and to *A. campestris* respectively. Moreover, other apparently related taxa appear embedded into separate clades (Fig. 2, clades 1d, 1e) such as *Artemisia macilenta* (Maxim.) Krasch., *Artemisia pycnorhiza* Ledeb., *Artemisia oxycephala* Kitam., *Artemisia commutata* Besser or *Artemisia dolosa* Krasch., among others.

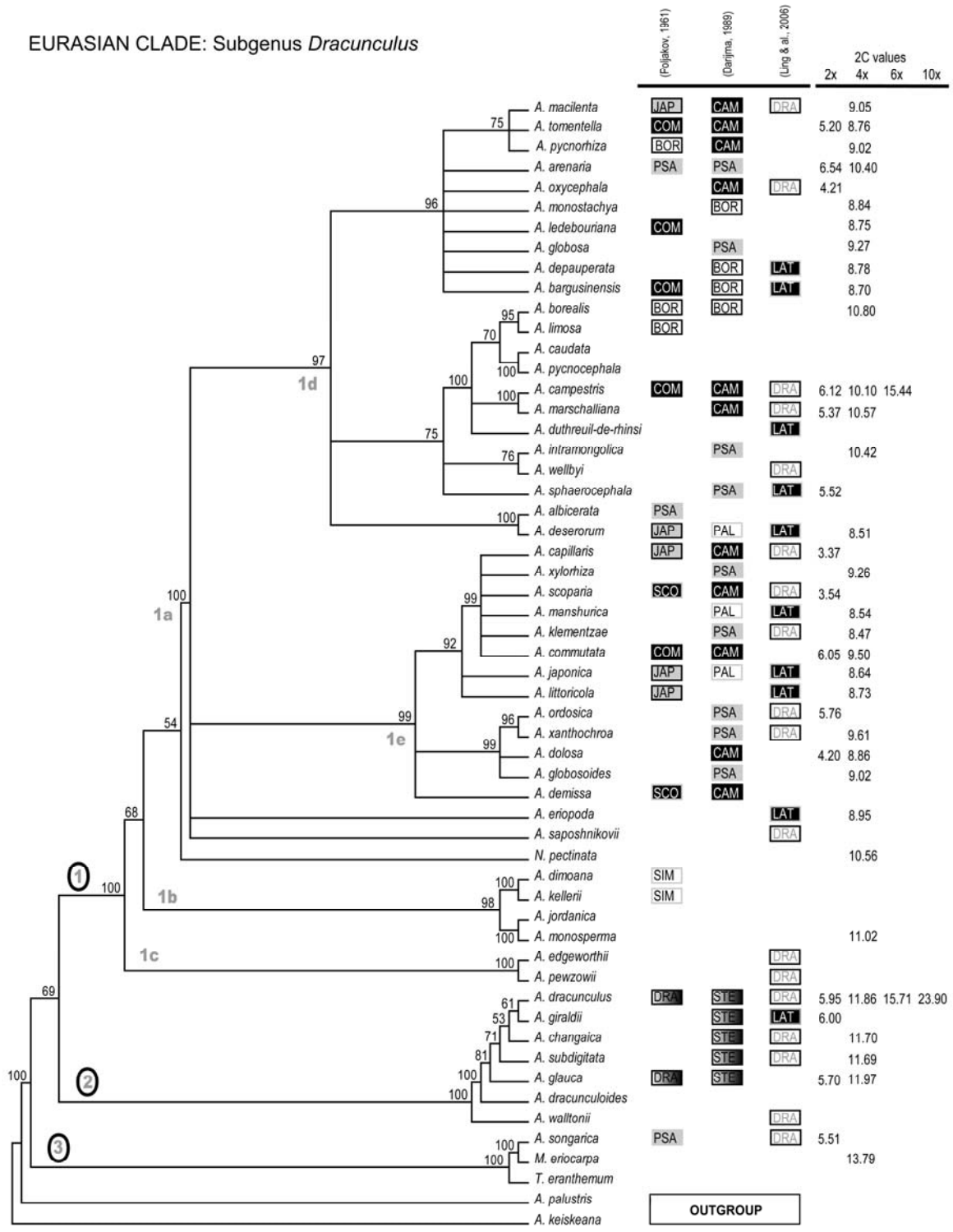

Figure 2. Phylogenetic tree obtained by Bayesian inference from the combined nuclear (ITS, ETS) and cloroplastic (*trn*S^{UGA}-*trnf*M^{CAU} and *trn*S^{GCU}-*trn*C^{GCA}) regions.

The position of these species, linked to the existence of unresolved polytomies, leads us to hypothesize the occurrence of hybridization events, not detectable at present because of sequence homogenization. Other clades seem to group species on the basis of their geographic distribution, i.e. the species *Artemisia ordosica* Krasch., *A. xanthochroa* Krasch., *A. dolosa* and *A. globosoides* Ling and Y.R. Ling, which are embedded into a well supported clade (Fig. 2, PP = 99%) that involves basically Mongolian endemics with the exception of *A. ordosica*, that also occurs in China (but restricted to Inner Mongolia and neighbouring provinces). The relationship between the annual/biennial species *A. scoparia* and *Artemisia demissa* Krasch. is not clear. While the nuclear markers (Fig. 1) embrace these two species, but without statistical support (PP = 86%; BS = 69%), in agreement with Poljakov's (1961) inclusion under the series *Scopariae,* chloroplast data place *A. scoparia* embedded into a clade with *A. capillaris* among others. Thus, our combined phylogenetic data do not confirm Poljakov's relationship (Fig. 2), but cluster the species *A. scoparia* and *A. capillaris* in the same lineage, which is complemented by their rather similar genome sizes, these being the smallest amounts reported for the subgenus (*A. capillaris* 2C = 3.37 pg; *A. scoparia* 2C = 3.54 pg).

***(ii) Subclade 1b*:** This well-supported lineage (PP = 98%) is split into two subclades which embed the species *Artemisia dimoana* M. Pop. and *Artemisia kelleri* Krasch. on one side (PP = 100%), and *Artemisia monosperma* Delile and *Artemisia jordanica* Danin (PP = 100%) on the other side. They all show a preference to inhabit sandy desert areas of Central Asia (*A. dimoana* and *A. kelleri*) and South West Asia (*A. jordanica*, *A. monosperma*). Poljakov (1961), in his revision of *Artemisia* for the Flora of the USSR, placed *A. dimoana* and *A. kelleri* into the series *Simplicifoliae* Krasch. because of the presence of simple (partly-lobed) leaves. The species *A. monosperma* and *A. jordanica* share many morphological characters, and present a high degree of resemblance (Danin, 1999). Notwithstanding, while the first one is distributed from Egypt to Israel reaching Lebanon, the second one is present in South Jordan, Saudi Arabia and the South West of Iraq, without overlapped territories, and being the unique representatives of the subgenus in the zone. Previous works regarding *Artemisia*

systematics and cytogenetics (Torrell & al., 1999 for the phylogeny; Torrell & al., 2001 for the cytogenetics) pointed out a narrow relationship between *A. monosperma* and the *A. campestris* complex also based on their morphological and ecological traits. Our findings, based on a representative sample of the subgenus, clearly disagree with these suggestions, having seen its phylogenetic position.

(iii) Subclade 1c: Both annual-biennial species *Artemisia edgeworthii* Balakr. and *Artemisia pewzowii* C. Winkl. perform a very robust clade (Fig. 2, 1c; PP = 100%). Although *A. pewzowii* has a more reduced distribution than *A. edgeworthii*, both species overlap some of their Chinese territories (e. g. provinces of Qinghai, Xinjiang and Xizang). There are no significant morphological traits supporting this clustering; indeed pollen data reflect the presence of different subtypes [(*Anomalae* for *A. edgeworthii*, *Sacrorum* for *A. pewzowii*; sensu Jiang & al., (2005)]. Nevertheless, both species share life cycle and distribution areas, which could explain such a linkage.

The *Artemisia dracunculus* complex. This complex is composed of about 10 species that differ from the rest of the members of the subgenus by the synapomorphy of basically simple, linear to linear-lanceolate, leaves. This morphological trait marks the main difference between these species and the rest of the subgenus, which present basically pinnately-sected leaves (Poljakov, 1961), with scarce exceptions such as A. *jordanica* (Danin, 1999). Polyploidy in this complex is of great incidence, as in general for the subgenus, with series of 2n = 18, 36, 54, 72, 90 chromosomes in species such as *Artemisia dracunculus* (Kreitschitz & Vallès, 2003, and references therein). The complex performs a monophyletic group (Fig. 2, clade 2; PP = 100%) which includes the worldwide distributed *A. dracunculus* and other closely related taxa such as *Artemisia giraldii* Pamp., *Artemisia changaica* Krasch., *Artemisia subdigitata* Mattf., *Artemisia glauca* Pall., *A. pamirica* (see Fig. 1 for *A. pamirica*) and *Artemisia dracunculoides* Pursh. Mainly endemic to Central Asia (with the exception of the North American endemic *A. dracunculoides*), these allied species present more discrete distributions than the type species *A. dracunculus*. The specific relationships between these taxa are not resolved at all in the tree based on all

markers (Fig. 2). From the combined ITS + ETS tree (Fig. 1) a close relationship between *A. dracunculus*, *A. changaica* and *A. giraldii* can be extracted. When both nuclear and plastid sequences are combined (Fig. 2), even maintaining the identity of the complex, the nodes of the subclades lose support, not necessarily as a result of incongruent information between markers, but induced by the irresolution of the plastid regions.

Disagreement about the taxonomic consideration of some of these plants can also be found in the literature. In the revisions of Poljakov (1961) and Darijma (1989), these taxa were considered at the species level, while Ling & al. (2006) relegated the taxonomic rank of some of them to varieties of *A. dracunculus* on the basis of their close morphological similarity. Furthermore, Ling (1987) proposed the consideration of *A. subdigitata* as a variety of *Artemisia dubia* Wall. ex Besser, traditionally included by Besser (1932) within the section *Abrotanum* (*s. l.*, incl. sect. *Artemisia*). He reviewed voucher specimens and concluded that the former could be considered as a variety of *A. dubia*, as both presented the capitula structure typical of the subgenus *Dracunculus*. The position in our tree of *A. subdigitata* within the *A. dracunculus* complex is more likely congruent with their morphological traits, rather than with those of *A. dubia*, which we sequenced from the Naturhistorisches Museum (Vienna; W), and resulted phylogenetically close to the *A. vulgaris* complex (data not shown). Sister to the *A. dracunculus* group appears the species *Artemisia waltonii* J. R. Drum. ex Pamp. (clustered with *A. nanschanica* in the tree at Fig. 1). The species is native to China and essentially differs by its pinnatisect- or partite leaves. Thus, it seems that the presence of simple leaves, characteristic of the complex, has appeared at least two times during the evolution of the subgenus in two different lineages.

***Mausolea, Turaniphytum* and *Neopallasia*: a close relationship with *Artemisia*.** The close phylogenetic relationship existing between the genus *Artemisia* and several other *Artemisiinae* genera is well known, some of them previously nested into the genus justified under molecular (Vallès & al., 2003; Oberprieler & al., 2007; Sanz & al., 2008), or geographical criteria (Kubitzki, 2007). Vallès & al. (2003) and Sanz & al. (2008) pointed out the inclusion of *Mausolea* and

Turaniphytum in the subgenus *Dracunculus*, but were unable to study in greater depth the relationships of these genera within the group. Our results confirm this fact. *Mausolea* and *Turaniphytum* are completely merged within *Artemisia* subgenus *Dracunculus* (Figs. 1 and 2). This inclusion becomes more understandable if we take into account that those genera present heterogamous capitula (functionally male central florets), characteristic of the subgenus. Not only the combined analysis (Fig. 2), but even the nuclear and plastid independent Bayesian analyses (data not shown) revealed a strong relationship between the species *A. songarica*, *M. eriocarpa* and *T. eranthemum* (Fig. 2, clade 3; PP = 100%). From the morphological standpoint, we are not able to state which are the main traits defining this lineage, should it exist, but the strong congruence found between both ribosomal and chloroplast data does make us look for other explanations for such a linkage. Those three species inhabit desert and semidesert areas of Western Asia (Afghanistan, Iran, Kazakhstan) reaching Central Asia in the cases of *Artemisia songarica* and *Mausolea*. The new relationship found between the latter two species might be better understood on the basis of a geographical effect, because of the coincidental distribution areas.

Neopallasia is another annual endemic genus from Central Asia composed of three species which was segregated from *Artemisia* by Poljakov (1955) despite the fact that its floral characters recommend its inclusion within the subgenus *Dracunculus* (Sanz & al., 2008). Our results also place the only species of this genus included in the present analysis, *N. pectinata* (Pall.) Poljak., clearly embedded into a well supported (PP = 100%) and large clade containing most representatives of the subgenus (Fig. 2, clade 1). Despite our large sampling, the phylogenetic position of this species and the relationship with the remaining species of *Dracunculus* still lacks complete resolution. This fact might be influenced mainly by the poor phylogenetic signal of the chloroplast data (trees not shown), which places the species at undetermined position.

Genome size evolution and polyploidy in the subgenus *Dracunculus*. The distribution of genome size data for tetraploid representatives seems to be consistent with some of the different lineages (Fig. 2). Thus, while the

phylogenetically closely related clades 1d and 1e account for quite similar mean 2C values (clade 1d, 2C = 9.42 pg; clade 1e, 2C = 8.95), clade 2, which is more distant and embeds the *A. dracunculus* complex, is also segregated on the basis of its mean 2C value (2C = 11.80 pg). This lineage is quite homogeneous in terms of 2C values, a fact that prevents us from finding differences between the species other than the morphological ones, and makes a conclusion about the appropriate taxonomic rank more difficult. It is also interesting to point that some of the species included in clade 1d, *A. campestris* and relatives, account for larger 2C values than the remaining tetraploid representatives (Fig. 2), although the lack of a larger sample does not permit to make statistical tests.

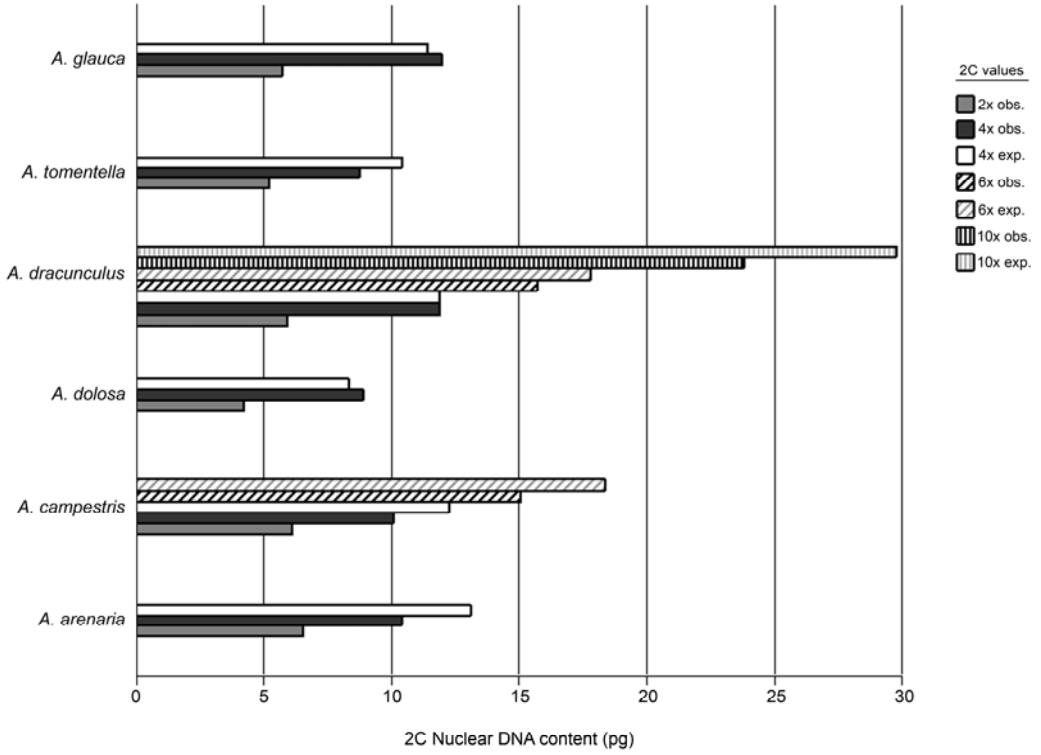

Figure 3. Evolution of nuclear DNA content between ploidy levels in some species of *Artemisia*.

Recent research addressed to study the genome size changes related to polyploidization events in *Artemisia* (Pellicer & al., 2009), has revealed an increased ratio of DNA elimination along ascending ploidy levels as a mechanism of response, possibly, to the control of genome obesity after polyploidization events (Bennetzen & al., 2005). These results can be observed in the large polyploid species *A. dracunculus* (Fig. 3). While the lowest polyploid

level (4*x*) genome size data observed and expected are similar, in ascending ploidy levels, differences become larger, demonstrating an increase in the portion of nuclear DNA which is being removed during polyploidization. Similar changes take place in the species *A. campestris*, the differences being more patent since the first polyploid levels (Fig. 3). Slight changes at low polyploid levels (4*x*) have also been detected (e.g., *A. dolosa* or *A. glauca*; Fig. 3). A heterogenic behaviour towards DNA gain or genome downsizing in polyploids of the same genus has been reported in literature (Leitch & al., 2008), as the result of different individual evolutionary histories. In the case of *Artemisia*, contrary to other plant groups (Leitch & Bennett, 2004; Leitch & al., 2008), conclusive hypotheses about the erosive effects of time after polyploidization cannot be stated because of the unknown origin of these polyploidy species. Many studies devoted to highlight the questions concerning the relative changes towards increases/decreases in DNA content with pylogenetic frameworks have been published (Bennetzen & Kellogg, 1997; Price & al., 2005; Leitch & al., 2008). Bennetzen & Kellogg (1997) proposed that DNA content evolution goes from low to high due to polyploidy and retroelement accumulation. In contrast to that, other authors have proposed a reduction in DNA content as a more common phenomenon in plants (Bennetzen & al., 2005; and references therein). Nowadays, bi-directional changes of plant genome size evolution have been stated (Soltis & al., 2003; Leitch & al., 2008).

Taxonomic implications. First steps to return some allied genera within *Artemisia*. — From the results obtained in the present work, and taking into account as well previous work of our team on the subtribe Artemiisinae (Vallès & al., 2003; Sanz & al., 2008), the classification as segregate genera of at least the monotypic genera *Mausolea* and *Filifolium*, and possibly also the oligospecific genera *Turaniphytum* and *Neopallasia* (both containing three species) should be reconsidered. Both *Mausolea* and *Filifolium* species are clearly embedded into the genus *Artemisia* in the present phylogeny, confirming the previous findings (Vallès & al., 2003; Sanz & al., 2008). Thus, they should be recognized as *Artemisia* species. For *Mausolea*, the solution is simply to come back to the

original name with which the taxon was described, *Artemisia eriocarpa* Bunge. *Filifolium sibiricum* was originally described as *Tanacetum sibiricum* L. (non Falk = *T. vulgare* L.) and afterwards combined into *Artemisia*, as *Artemisia sibirica* (L.) Maxim., a status which is consistent with the present molecular findings. Although the lack of material available for some of the species of *Neopallasia* and *Turaniphytum*, does not allow us to make fully conclusive decisions on the whole genera, we have presented new molecular insights which induced to reconsider *N. pectinata* and *T. eranthemum* as they were described, i.e. *Artemisisa pectinata* Pall. and *Artemisia eranthema* Bunge. In fact, one of the three species of *Turaniphytum* (the only one originally described within this genus) is nowadays considered as an *Artemisia* (*Artemisia kopetdaghense* (Poljakov) Y. R. Ling), and the remaining one (*T. condringtonii* (Rech. f.) Podlech.) was described as *Artemisia condringtonii* Rech. f. Similar taxonomic rearrangements concern *Neopallasia*: while only *Neopallasia tibetica* Y. R. Ling has been described under the circumscription of this genus, the species *Neopallasia yunnanensis* (Pamp.) Y. R. Ling had been described as *Artemisia pectinata* Pall. var. *yunnanensis* Pamp. All these former taxonomic arrangements, together with the present molecular frame, support the inclusion of the mentioned species under the genus *Artemisia*, implying the pass of some genera and the names of the species to them attributed to the synonymy.

CONCLUDING REMARKS

The monophyly of the subgenus *Dracunculus* of the Eurasian grade is confirmed in the phylogenetic reconstructions based on nuclear and chloroplastic DNA regions, excluding *Artemisia salsoloides* and *A. tanaitica* and including the presently considered related genera *Mausolea*, *Neopallasia* and *Turaniphytum*. *Artemisia keiskeana* and *A. palustris* are closely related to the subgenus, both being candidates to constitute the sister group of *Dracunculus*, with a close phytochemical relationship with the subgenus. Three independent lineages can be distinguished within the subgenus for the first time in the present work. Some morphological characters such as leaf shape support these results, and also the genome size information seems to be congruent with these major groups,

indicating, as is common in the genus, a reduction of monoploid genome sizes that increase in higher ploidy levels. Furthermore, the inclusion of some related genera into *Artemisia* leads us to propose taxonomic reorganizations for these taxa, many of them previously labelled under this genus.

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Appendix 1. Populations studied for phylogenetic analyses.

Taxa	Localites, date, collectors and herbarium vouchers
A. albicerata Krasch.	Kazakhstan, Heptapotamia. Syr-Taukum sands. 25-X-1965. Leg. N. Filatova and L. Kurocskina (W-1969/10294)
A. arenaria DC.	Russia, Volgograd oblast. Silicic sands in hill slopes with *Artemisia*, Poaceae and grass steppe among *Betula*. 11-X-2000. Leg. A. A. Korobkov (LE-Korobkov 00-41).
A. bargusinensis Spreng.	Russia, Tyva Republic, Pi-Khem raion. 60 km N-NE of Turan, slope grasslands with steppe. 11-VIII-2002. Leg. V. Nikitin, V. Byalt and A. Sytin (LE-Korobkov).
A. borealis Pall.	Russia. Chukotka autonomous district. River Andyr basin, Tambatnsi mountains, 2 km from Tambatnsi. 1-IX-1958. Leg. S. Ikonnikov and G. Ladygina (LE)
A. campestris L.	Spain, Vilafant. 08-XII-1996. Leg. M. Torrell and J. Vallès (BCN 13132)
A. capillaris Thunb.	Japan, Tsukuba. Reseach Center for Medicinal Plant Resources. National Institute of Biomedical Innovation (Index Seminum 2006)
A. caudata Michx.	Canada, British Columbia. Peace river breaks above Bear Flat. Meadow and schrub steppe balds. 640m. 15-VII-1979. Leg. L.E. Paulick and B. Taylor (LE-49-674)
A. changaica Krasch.	Mongolia, Arkhangai aimag. Taryat sum, Khorgo-Terkh National Park, Larix sibirica forest above lake Terkhen Sagan nur. 27-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 34487).
A. commutata Besser	Mongolia, Selenge aimag. Shaamar sum, 3 km west of the sum, Buureg Tolgoi hills, near river Okhon. 700 m. 9-IX-2004. Leg. Sh. Tsooj, J. Vallès and E. Yatamsuren (BCN)
A. crithmifolia L.	Portugal, Douro Litoral. Azurara, sand dunes. 10-XII-1995. Leg. J. Poch and J. Vallès (BCN 13233)
A. demissa Krasch.	Mongolia, Umnu (South) Gobi aimag. 10 km S of Bulgan sum. 1-IX-2004. Leg. Sh. Dariimaa, D. Samjid, Sh. Tsooj and J. Vallès (BCN Mong.55)
A. depauperata Krasch.	Mongolia, Arkhangai aimag. Tsetserleg city, Sagaan-Davaa pass. 2200 m. 26-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 23804).
A. desertorum Spreng.	Russia, Primorie krai, Nadezhda raion. Near the town of Terekhovk, abrupt rocky slope on the right side of the coast, meadows in a *Quercus* forest. 10-X-2004. Leg. A.A. Korobkov (LE-Korobkov).
A. dimoana M. Pop.	Turkmenistan. Lower Karakum. N of Tedzha and Murgaty rivers. 25-X-1956. Leg. V. Leontiev (LE)
A. dolosa Krasch.	Mongolia, Uvur Khangai aimag. 20 km south of Khujirt sum, meadow steppe. 30.viii.2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 23805)

A. dracunculoides Pursh.	United States of Ameria, Arizona. Globe, Pinal mountain in ruderal path edges. 17-VIII-1997. Leg. J. Peñuelas (BCF 45562)
A. dracunculus L.	Russia, Chita oblast, Kyra raion. Near the village of Kyra, northern slope South of the village, rich steppe with herbs and bushes. 1-IX-2005. Leg. et det. A. A. Korobkov (LE 06-04).
A. duthreuil-de-rhinsi Krasch.	China, Tibet. NW shore of the lake Peku. 4640 m. 30-VIII-1991. Leg. A.A. Tishkov (LE)
A. edgeworthii Balakr.	China, Tibet. In waste ground. 4500 m. 28-VIII-1991. Leg. A.A. Tishkov (LE-2808)
A. eriopoda Bunge	China, Inner Mongolia, Ulanqab province. 50 km N of Höhhöt. 29-VII-2007. Leg. W. Chan, E.R. Cao, B. Liu and J. Vallès (BCN)
A. giraldii Pamp.	Mongolia, Bulgan aimag. Sansar sum, north-east slope of Khugunkhaan mountain, steppe near Betula and Pinus forest, 2000 m, Sh. Dariimaa, Sh. Tsooj and J. Vallès, 25-VIII-2004 (BCN 23806).
A. glauca Pall. ex Willd.	Artemisia glauca Mongolia, Tuv (Central) aimag: Baian-Sogt sum, 40 km W of Ulaan Baatar, 24.viii.2004, Sh. Dariimaa, Sh. Tsooj & J. Vallès (Mong. 2)
A. globosa Krasch.	Russia, Tyva Republic, Erzin raion. Northern shore of the lake Tere-Khol, sandy area of Tsuguer-Ellis. 13-IX-1003. Leg. A.A. Korobkov (LE 04-116).
A. globosoides Krasch.	Mongolia, Umnu (South) Gobi aimag. Bayanzag, 20 km NW of Bulgan sum. 3-IX-2004. Sh. Dariimaa, D. Samjid, Sh. Tsooj and J. Vallès (BCN Mong. 63)
A. intramongolica H.C. Fu	China, Inner Mongolia. 24 km N of Dzhasakachi. 15-VIII-1957. Leg. M. P. Petrov (LE)
A. japonica Thunb.	Japan. Yamanashi Prefecture. Fujikawaguchiko-machi. Tokyo Metropolitan Medicinal Plant Garden (Index Seminum 2006-07)
A. jordanica Danin	Jordan, Edom. Shael Amar to Aimir, gravel plain with sandy soil. 19-VII-1997. Leg. A. Danin (BC 971401)
A. keiskeana Miq.	Russia, Primorie krai, Khassan raion. Cape Gamon, forest in an abrupt rocky slope to sea. 16-X-2004. Leg. A.A. Korobkov (LE-Korobkov)
A. kellerii Krasch.	Turkmenistan. Near Akhcha-Kuima railway station. 12-IX-1913. Leg. K. Andrjusczenko (LE)
*A. klementzae*Krasch.	Mongolia, Bulgan aimag. Sansar sum, Khugunkhaan mountain, sandy steppe. 25.viii.2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 23807)
A. kuschakewiczii C. Winkl.	Tadzhikistan, Gorno-Badakhshan, Pamir. Kokuibet-su, 5 km from river Zor-Tashkots. 3620m. 1-IX-1958. Leg. S. Ikonnikov and G. Ladygina (LE)
A. ledebouriana Besser	Russia, Buryat Republic, Pribaikal raion. Shore of the lake Baikal, at 159-160 km on road from the village of Turku, sand dunes. 16-IX-2005. Leg. et det. A.A. Korobkov (LE 06-06).
A. limosa Koidz.	Russia, Sakhalin island. Magutan volcano, north of Yuzhno-Sakhalinsk. 20-IX-2000. Leg. Y.S. Smirnov (LE Korobkov 00-03)

A. littoricola Kitam.	Russia, Sakhalín Island. 5 km S of Krasnogorsk. 8-X-2000. Leg. K. Tkachenko (LE-Korobkov 00-05)
A. macilenta (Maxim.) Krasch.	Russia, Chita oblast, Kyra raion. Northern Onon-Baldzhin mountain system, southern slope, deposits of sand and stones, steppe. 8-IX-2005. Leg. et det. A. A. Korobkov (LE 06-02).
A. manshurica Thunb.	Russia, Primorskii krai: Muraviev-Amurskii peninsula, summit of the pass, steep slopes at the narrow crest, meadows with shrubs in a Malus forest. 21-X-2004. Leg. A.A. Korobkov (LE-Korobkov)
A. marschalliana Spreng.	Armenia, Krasnoselsk. Northern shore of the Sevan lake, between Shorzha and Adanish. 2000 m. 17-VIII-1995. G. Faivush, M. Gabrielian, N. Garcia-Jacas, M. Guara, M. Hovhanniesian, A. Susanna and J. Vallès (BCN-14058)
A. monosperma Delile	Egypt, Burg el Arab. desert sands. 10-IV-1995. Leg. A. Badr (BCN 14067)
A. monostachya Bunge ex Maxim.	Mongolia, Arkhangai aimag. Ikh Tamir sum, 30 km north-west of the sum, Khoer Davaa pass. 26-VIII-2004. Sh. Dariimaa, Sh. Tsooj and J. Vallès. (BCN 23808)
A. nanschanica Krasch.	China, Qinghai Province. Madoi Xian, just NE oof Yematan between Madoi and Yushu. Open gravelly slope.10-VIII-1996. Leg. H. Ting-nong, B. Bartholomew, M.G. Gilbert and M.F. Watson (1589/E00065481)
A. ordosica Krasch.	China, Inner Mongolia. Alxa province, 5 km S of Bayan Höt / Alxa Suoqi, sandy soils. 05-IX-2007. Leg. J. Vallès and S.W. Zhao (BCN)
A. oxycephala Kitam.	Mongolia, Tuv (central) aimag: Mungunmort sum,10 km south of the sum, Sh. Dariimaa, Sh. Tsooj, J. Vallès and E. Yatamsuren, 7.IX.2004 (BCN 34491)
A. palustris L.	Mongolia, Uvur-Khangai aimag. Khotont sum, 10 km east, margins of cultivated fields in steppe area. 26.VIII.2004. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 34847)
A. pamirica C. Winkl.	Natural History Muesum, Vienna (W-1969/5914)
A. pewzowii C. Winkl.	China. Qinghai Province. Nangqen Xian, Beca Xiang, SE of Becaka.Gentle slopes with degraded alpine meadow; steeper slopes with groves of Juniperus. Growing in alpine meadow. 07-IX-1996. Leg. H. Ting-nong, B. Bartholomew, M.G. Gilbert and M.F. Watson (2946/E00061484)
A. pubescens Ledeb.	Natural History Museum, Vienna (W-1958/17577)
A. pycnocephala DC.	United Kingdom, England. NCCPG *Artemisia* Collection (from U.S.A) XI-2006. J.D. Twibell (1991-03)
A. pycnorhiza Ledeb.	Russia, Tyva Republic, Erzin raion. Left shore of Tes-Khem river, 20 km NW of the city of Erzin, base of Izvestkyakov mountains, rocks. 18-IX-2003. Leg. A. A. Korobkov (LE 04-115).
A. salsoloides Willd.	Russia, Volgogradskaya oblast. Kumylissi. 15-X-2000. Leg. A.A. Korobkov (LE 00-11)
A. saposhnikovii Krasch. ex Poljakov	Kyrgystan. Central Tian-Schan. 2600 m. 20-VII-1978. Leg. G. Ladygina and S. Ikonokov (W-1995/06073)
A. scoparia Waldst et Kitam.	Mongolia, Uvur-Khangai aimag.15 km E of Kharkhorin. 25-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN Mong.17)

A. songarica Schrenk.	Kazakhstan, Heptapotamia. Syr-Taukum sands. 10-VI-1965. Leg. N. Filatova and L. Kurocskina (W-1969/10267)
A. sosnovskyi Karsch.	Russia, Daguestan Republic, Tsumand raion. Near the village of Asvali, rocky dry slopes of eastern exposition. 28-X-2005. Leg. R. N. Murtazaliev (LE 06-34).
A. sphaerocephala Krasch.	Mongolia, Bulgan aimag: Dashin Shellen sum, 10 km north-west of the sum, near the Institute of Folk Medicine, steppe with Caragana. 24.VIII.2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès, (BCN 34485)
A. subdigitata Mattf.	Mongolia, Umnu (south) Gobi aimag. Bulgan sum, eastern Gurvan Saikhan mountains, canyon near Brigat, rocky slopes. 01-IX-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 34846)
A. tanaitica Klokov.	Natural History Muesum, Vienna (W-1963/2876)
A. tomentella Trautv.	Russia, Republic of Tuva. Close to Erzin city, small population in a degraded field. 16-IX-2003. A.A. Korobkov (LE-Korobkov)
A. waltonii J.R.Drumm. ex Pamp.	China, Qinghai Province, Chindu Xian, Xiwu Xiang. Xia Saiba, E of Chumda.Growing on rocky slopes. 15-VIII-1996. Leg. H. Ting-nong, B. Bartholomew, M.G. Gilbert and M.F. Watson (1870/E00061067)
A. wellbyi Hemsl. et Pears.	Kashmir, Tsakihun Tso. Ladak (Western Himalayas), in dry ground. 4500 m. 24-VII-1931. Leg. W. Koelz (LE-2432)
A. xanthochroa Krasch.	Mongolia, Uvur-Khangai aimag. Tugrug sum, 40 km south of the sum, desert steppe with Caragana. 31-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 23810)
A. xylorhiza Krasch. ex Filat.	Mongolia, East Gobi. 30 km SE of Hubsugul, on sandstone outcrops. 27-VII-1971. Leg. E.A. Ucarenko and E.U. Parkobekar (LE)
Mausolea eriocarpa (Bunge) Poljakov	Uzbekistan. Bukhara. Road from Gazli to Nukus, sandy desert. 200 m. Leg. L. Kapustina, F. Khassanov, A. Susanna and J. Vallès (BCN-11629)
Neopallasia pectinata Pall.	Mongolia, Umnu (South) Gobi aimag. 10 km S of Bulgan sum. 1-IX-2004. Sh. Dariimaa, D. Samjid, Sh. Tsooj and J. Vallès (BCN Mong. 54)
Turaniphytum eranthemum (Bunge) Poljakov	Kazakhstan, Almatynskaya oblast. 1 km N. of the road Kapchagai-Bakanas, sandy desert. 26-VIII-2000. Leg. A.A. Ivaschenko, A. Susanna and J. Vallès (BCN-50684)

Appendix 2. Populations studied for genome size assessments

Taxa	Localites
A. arenaria DC.	Russia, district of Astrakhan. 10 km W of Nizhniy Baskunchak. 15-X-2001. Leg. I.N. Safronova (LE 00-110)
A. borealis Pall.	United Kingdom, England. NCCPG. *Artemisia* Collection, from france. XI-2006. Leg. J.D. Twibell (9098-22)
A. borealis Pall.	United Kingdom, England. NCCPG. *Artemisia* Collection, from Italy. XI-2006. Leg. J.D. Twibell (9090-27)

A. campestris L. subsp. *variabilis* Ten. (Greuter)	United Kingdom, England. NCCPG. *Artemisia* Collection, from Italy, Mt. Vesubius. Leg. J.D. Twibell (1993-23)
A. capillaris Thunb.	United Kingdom, England: NCCPG. *Artemisia* Collection, XI-2006, J.D. Twibell (Japan. Kyoto. 1996-17)
A. capillaris Thunb.	Japan. Tsukuba. Reseach Center for Medicinal Plant Resources. National Institute of Biomedical Innovation (Index Seminum 2006)
A. crithmifolia L.	United Kingdom, Wales. NCCPG. Artemisia collection, from Swansea, Crymlyn Burrows. IX-1990. Leg. John D. Twibell (1990-15)
A. crithmifolia L.	United Kingdom, England. NCCPG. *Artemisia* Collection , from France, Biscarrosse beach. Leg. J.D. Twibell (1988-11)
A. crithmifolia L.	United Kingdom, England. NCCPG. *Artemisia* Collection, from England, Crosby. Leg. J.D. Twibell (1998-5)
A. crithmifolia L.	United Kingdom, England. NCCPG. *Artemisia* Collection, from France, Notre Dame de Monts. Leg. J.D. Twibell (1958-11)
A. crithmifolia L.	United Kingdom, England. NCCPG. *Artemisia* Collection, from France, île de Rhé. Leg. J.D. Twibell (1948-16)
A. desertorum Spreng.	Russia, Primorie krai, Nadezhda raion. Near the town of Terekhovk, abrupt rocky slope on the right side of the coast, meadows in a Quercus forest. 10-X-2004. Leg. et det. A. A. Korobkov (LE-Korobkov).
A. dolosa Krasch.	Mongolia, Uvur Khangai aimag. 20 km south of Khujirt sum, meadow steppe. 30-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 23805)
A. dolosa Krasch.	Mongolia, Tuv (Central) aimag. Mungunmort sum,20 km W of the sum, W slope of Mungun mountain. 7-IX- 2004. Leg. Sh. Dariimaa, Sh. Tsooj, J. Vallès and E. Yatamsuren (BCN 23791)
A. dracunculus L.	Russia, Republic of Tyva. Near Kyzyl, 12-IX-2003. Leg. A.A. Korobkov (LE 04-113)
A. dracunculus L.	Kazakhstan, Almatynskaya oblast. Road from Almaty to the Astronlomical Observatory, 11 km from the Observatory, road edges.1500m. 21-VIII-2000. Leg. A.A. Ivaschenko, A. Susanna S-2101 and J. Vallès (BCF 50690)
A. eriopoda Bunge	Ulanqab province, Yin Shan, Da Qing Shan, 50 km N of Höhhöt, Wu Chan. 29-VIII-2007. Leg. R. Cao, B. Liu and J. Vallès X-9 (BCN)
A. glauca Pall.	Artemisia glauca Mongolia, Tuv (Central) aimag: Baian-Sogt sum, 40 km W of Ulaan Baatar, 24.viii.2004, Sh. Dariimaa, Sh. Tsooj & J. Vallès (Mong. 2)
A. intramongolica H.C. Fu	China, Inner Mongolia. 24 km N of Dzhasakachi. 15-VIII-1957. Leg. M. P. Petrov (LE)
A. japonica Thunb.	Japan. Yamanashi Prefecture. Fujikawaguchiko-machi. Tokyo Metropolitan Medicinal Plant Garden (Index Seminum 2006-07)
A. klementzae Krasch.	Mongolia, Bulgan aimag. Sansar sum, Khugunkhaan mountain, sandy steppe. 25-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 23807)
A. littoricola Kitam.	Russia, Sakhalín Island. 5 km S of Krasnogorsk. 8-X-2000. Leg. K. Tkachenko (LE-Korobkov 00-05)
A. manshurica Thunb.	Russia, Primorskii krai: Muraviev-Amurskii peninsula, summit of the pass, steep slopes at the narrow crest, meadows with shrubs in a Malus forest. 21-X-2004. Leg. A.A. Korobkov (LE-Korobkov)

A. ordosica Krasch.	China, Inner Mongolia, Alxa province. 5 km S of Bayan Höt / Alxa Suoqi, sandy soils. 5-IX-2007. Leg. J. Vallès and S.W. Zhao
A. pycnocephala DC.	United Kingdom, England. NCCPG *Artemisia* Collection (from U.S.A). XI-2006. Leg. J.D. Twibell (1991-03)
A. salsoloides Willd.	Russia, Volgogradskaya oblast. Kumylissi. 15-X-2000. Leg. A.A. Korobkov (LE 00-11)
A. songarica Schrenk.	China, Inner Mongolia, Alxa province. Oalter house, near desert dunes close to main road S of Bayan Höt / Alxa Suoqi. 5-IX-2007. Leg. J. Vallès and S.W. Zhao
A. tomentella Trautv.	Russia, Republic of Tuva. Close to Erzin city, small population in a degraded field. 16-IX-2003. A.A. Korobkov (LE-Korobkov)
A. tomentella Trautv.	Russia, Republic of Tuva. Close to Erzin city, small population in a degraded field. 16-IX-2003. A.A. Korobkov (LE-Korobkov)
A. xanthochroa Krasch.	Mongolia, Uvur-Khangai aimag. Tugrug sum, 40 km south of the sum, desert steppe with Caragana. 31-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 23810)
A. xylorhiza Krasch. ex Filat.	Mongolia, East Gobi. 30 km SE of Hubsugul, on sandstone outcrops. 27-VII-1971. Leg. E.A. Ucarenko and E.U. Parkobekar (LE)

Relacions entre espècies i sèries poliploides d'*Artemisia* subgènere *Dracunculus* (*Asteraceae*) avaluades per hibridació *in situ* fluorescent dels gens de l'rRNA 5S i 18S-5.8S-26S

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La hibridació *in situ* fluorescent del DNA ribosòmic 5S i 18S-5.8S-26S ha sigut duta a terme en 12 representants del gènere *Artemisia*, pertanyents al subgènere *Dracunculus*. *Artemisia sericea* i *Filifolium sibiricum* han sigut també estudiats per a establir comparacions. S'ha observat la colocalització d'ambdues regions, la qual cosa confirma les troballes prèvies en *Artemisia*, en què els dos tipus de DNA ribosòmic sempre ocupen la mateixa posició. El suposat model ancestral d'organització del DNA ribosòmic en el gènere *Artemisia*, que consisteix en dos *loci* -és a dir quatre senyals d'hibridació *in situ*- en el nivell diploide, es troba també en el subgènere *Dracunculus*, però diversos canvis en el genoma d'algunes de les seves espècies han sigut observats. Plantegem la hipòtesi que alguns mecanismes de rearranjaments cromosòmics, com ara la translocació, poden explicar alguns dels canvis en els nombres de *loci* de l'rDNA, però probablement s'han esdevingut també processos d'hibridació en membres de la clada més derivada del subgènere. En la llarga sèrie poliploide d'*A. dracunculus*, que va del nivell diploide al decaploide, l'increment del nombre de *loci* de l'rDNA és quasi proporcional al nivell de ploïdia, en contrast amb la pèrdua de la quantitat de DNA nuclear monoploide, que té lloc de manera feble en els nivells de ploïdia baixos, però que és força més remarcable en els alts.

Relationships between species and polyploid series of *Artemisia* subgenus *Dracunculus* (Asteraceae) assessed by fluorescent *in situ* hybridization of 5S and 18S-5.8S-26S rRNA genes

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ABSTRACT

Fluorescent *in situ* hybridization (FISH) of 5S and 18S-5.8S-26S ribosomal DNA has been carried out in twelve representatives of the genus *Artemisia*, belonging to the subgenus *Dracunculus*. *Artemisia sericea* and *Filifolium sibiricum* have been also studied in order to establish comparisons. Colocalization of these regions has been observed, confirming previous findings in *Artemisia*, in which both ribosomal DNA always occupy the same position. The putative ancestral pattern for the *Artemisia* genus, showing four rDNA sites at diploid level- is also found in the *Dracunculus* subgenus but several changes in the genome of its species have been observed. We hypothesise that the translocation could explain some of the changes in the number of rDNA loci, but probably some hybridization processes have also taken place in the most derived clade of this subgenus. In the large polyploid series of *A. dracunculus*, the increase of rDNA loci is nearly proportional to the ploidy level, contrasting with the loss of the monoploid genome size, which slightly occurs at the lower ploidy levels but is more remarkable in the higher ones.

Key words: Anthemideae, Compositae, FISH, genome size, polyploidy.

INTRODUCTION

The subgenus *Dracunculus* (Besser) Rydb. (belonging to the genus *Artemisia* L.) comprises between 60 and 80 taxa at specific and subspecific levels. This number is variable depending on the authors consulted (Poljakov, 1961; Ling *et al.*, 2006). The subgenus is largely distributed along the Northern Hemisphere, mainly occupying the arid and semi-arid zones from Europe to Asia, being the last continent its more important diversification centre, and reaching some zones of North America.

The species belonging to subgenus *Dracunculus* are shrubs, subshrubs and mainly perennial herbaceous plants reaching 2 m in height. Some annual species are also included, such as *Artemisia demissa* Krasch., *Artemisia edgeworthii* Balakr., *Artemisia pewzowii* C. Winkl. and *Artemisia scoparia* Waldst. & Kit. This subgenus encompasses several species with economic value, such as *A. dracunculus* L., the type species of the subgenus, which is largely used as a condiment in cooking, some other are used to stabilize the soils (*Artemisia sphaerocephala* Krasch., *Artemisia wudanica* Liou & W. Wang), and also in folk medicine (*Artemisia capillaris* Thunb., *Artemisia ordosica* Krasch.). For the last reason, the subgenus has been previously studied from several points of view (Belenovskaja, 1996; Greger, 1988; Vallès and Garnatje, 2005 and references therein).

The phylogenetic studies published up to now have been either centred in the North American endemic subgenus *Tridentatae* (Rydb.) McArthur (Kornkven *et al.*, 1998, 1999; McArthur *et al.*, 1998; Stanton *et al.*, 2002) or addressed to reveal the relationships between taxa within the *Artemisia* genus or the whole Artemisiinae subtribe (Torrell *et al.*, 1999; Watson *et al.*, 2002; D'Andrea *et al.*, 2003; Vallès *et al.*, 2003; Sanz *et al.*, 2008; Tkach *et al.*, 2008a, b). A new delineation of the subgenus with the exclusion of some North American endemic species, such as *Artemisia filifolia* Torr., *Artemisia pedatifida* Nutt. and *Artemisia spinescens* D.C. Eaton, which appear clearly merged in the clade of the *Tridentatae* subgenus and allied species, has been suggested in a phylogenetic study based on nuclear and chloroplast regions sequencing, also proposing the final inclusion of the genera *Mausolea* Bunge ex Poljakov, *Neopallasia* Poljakov

and *Turaniphytum* Poljakov in the subgenus *Dracunculus* (Pellicer *et al.*, unpublished).

Karyological studies have revealed the existence of both basic chromosome numbers $x = 8$ and $x = 9$, being the latter the most frequent in the subgenus (as in the genus) and the former only found up to the present time in *A. scoparia* (Kawatani and Ohno, 1964, Vallès and Siljak-Yakovlev, 1997). Polyploidy has had a great effect in this group as in most *Artemisia*, Asteraceae and angiosperm species (Stebbins 1971; Soltis and Soltis 2000; Pellicer *et al.*, 2007a and references therein; Leitch and Leitch 2008). Several polyploid series have been reported in the subgenus, reaching the decaploid level in *A. dracunculus* (Ehrendofer, 1964; Estes, 1969; Persson, 1974; McArthur and Pope, 1979; Malakhova, 1990; Oliva and Vallès, 1994; McArthur and Sanderson, 1999). For some species, such as *Artemisia klementzae* Krasch. or *Artemisia xanthochroa* Krasch. among others, only polyploid populations have been found (Garcia *et al.*, 2006). Molecular cytogenetic studies focused in different genus *Artemisia* subgenera (*Artemisia, Seriphidium* and *Tridentatae*) have been carried out (Torrell *et al.*, 2003; Garcia *et al.*, 2007, 2009a; Pellicer *et al.*, 2008), but for the subgenus *Dracunculus* the information is very scarce (Torrell *et al.*, 2001; Hoshi *et al.*, 2006).

Multiple ribosomal RNA genes encoding 18S, 5.8S and 26S rRNA (denoted as nucleolus organizing regions, NORs) and 5S (functionally related to the nucleolus but not localized in it) occur as tandem repeats on one or several pairs of chromosomes (Montijn *et al.*, 1999). Fluorescent *in situ* hybridization (FISH) provides essential information useful for the understanding of the systematic and evolutionary relationships between groups of related species. It also supplies an interesting tool for studies on genome organization (Cerbah, 1997). Different researches carried out in *Artemisia* (quoted in the precedent paragraph) have shed light about the mechanisms at chromosome and whole-genome levels that gave rise to the great diversification of the genus.

Within the framework of phylogenetic and evolutionary studies in *Artemisia*, the present work has used FISH assays to obtain the distribution patterns of 18S-5.8S-26S and 5S rDNA in the chromosomes of 16 populations

of 12 species belonging to its subgenus *Dracunculus* as well as one population of *Artemisia sericea* Weber ex Stechm., of subgenus *Absinthium*, and one of the related genus *Filifolium* Kitam. The latter has been also included in this study, because molecular phylogenetic studies (Sanz *et al.*, 2008) present it merged within the genus *Artemisia* and placed as the sister group of the subgenus *Dracunculus*. *Artemisia sericea* has been studied to compare a non-*Dracunculus* high polyploid with those belonging the subgenus constituting the core of the present work. The main objectives of this work are: i) to characterize the distribution patterns of 5S and 18S-5.8S-26S ribosomal RNA genes in the subgenus *Dracunculus*, ii) to study the evolution of ribosomal DNA loci with polyploidy, and iii) to analyse the relationships between phylogenetic, cytogenetic and genome size data.

MATERIAL AND METHODS

Plant material

The origins, vouchers (deposited in the herbarium BCN, Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona) and collectors of the species studied are listed in Table 1. The populations considered range from diploid to decaploid levels, and for two species (*A. dolosa* and *A. dracunculus*) different ploidy levels (two and four respectively) have been investigated. Root tips were obtained from achenes germinating in wet paper in Petri dishes and subsequently pretreated in 0.002 M 8-hydroxyquinoline solution at room temperature during 2 h 15 min. After this first step, the root tips were fixed in 3:1 absolute ethanol and glacial acetic acid, stored at 4°C and transferred in a solution of 70% ethanol after a few days in the fixative.

Chromosome preparation (protoplast obtention)

Root tips were washed in distilled water during 10 min and prepared following the method described in Leitch and Heslop-Harrison (1993), with minor modifications. The tips were incubated in a microcentrifuge tube containing 200 µl of an enzymatic solution [3% cellulase Onozuka-RS (Yakult Honsha) and 0.5% pectolyase Y-23 (Kikkoman)] during 40-50 min, depending on species, at 37°C. They were washed in distilled water for 5 min, placed in a clean slide, crushed

into a drop of fixative and air-dried. Staining of the slides with 0.01% acetic orcein was carried out to find the metaphase plates. The slides were frozen for 1-3 h at –80°C to make easier the coverslips removal, and, for acetic orcein distaining, they were washed with 45% acetic acid for 1 h and subsequently two times with distilled water during 10 min and air-dried.

Table 1. Origin, collectors and herbarium vouchers of the species studied.

Taxon	Origin, voucher and collectors
Artemisia changaica Krasch.	Mongolia, Arkhangai aimag. Taryat sum, Khorgo-Terkh National Park, Larix sibirica forest above lake Terkhen Sagan nur. 27-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 34487).
Artemisia commutata Besser	Mongolia, Selenge aimag. Shaamar sum, 3 km west of the sum, Buureg Tolgoi hills, near river Okhon. 700 m. 9-IX-2004. Leg. Sh. Tsooj, J. Vallès and E. Yatamsuren (BCN).
Artemisia dolosa Krasch.	Mongolia, Uvur Khangai aimag. 20 km south of Khujirt sum, meadow steppe. 30-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 23805).
Artemisia dolosa Krasch.	Mongolia, Tuv (Central) aimag. Mungunmort sum,20 km W of the sum, W slope of Mungun mountain. 7-IX- 2004. Leg. Sh. Dariimaa, Sh. Tsooj, J. Vallès & E. Yatamsuren (BCN 23791).
Artemisia dracunculus L.	Russia, Chita oblast, Kyra raion. Near the village of Kyra, northern slope South of the village, rich steppe with herbs and bushes. 1-IX-2005. Leg. et det. A.A. Korobkov (LE 06-04).
Artemisia dracunculus L.	Russia, Volgograd oblast. Left shore of Khoper river, between gypseous slopes, meadows. 15-X-2000. Leg. et det. A.A. Korobkov (LE 00-40).
Artemisia dracunculus L.	Kazakhstan, Chimkent oblast. Chokpak ornithological station, railroad edges near Chokpak railway station, 500 m. 1-IX-2000. Leg. A.A. Ivaschenko, A. Susanna S-2211 and J. Vallès, (BCF 50688).
Artemisia dracunculus L.	Poland, Lower Silesia, Wrocław (Fabryczna), in the embankment. 8-VIII-2001. Leg. A. Kreitschitz, det. A. Wąsowicz. (Herbarium A. Kreitschitz).
Artemisia giraldii Pamp.	Mongolia, Bulgan aimag. Sansar sum, north-east slope of Khugunkhaan mountain, steppe near *Betula* and *Pinus* forest, 2000 m. 25-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN 23806).
Artemisia glauca Pall.	Russia, Tyva Republic. Near the city of Kyzyl, summits of hills, groupments of *Artemisia* and Poaceae. 12-IX-2003. Leg. et det. A.A. Korobkov. (LE-Korobkov).
Artemisia ledebouriana Besser	Russia, Buryat Republic, Pribaikal raion. Shore of the lake Baikal, at 159-160 km on road from the village of Turku, sand dunes. 16-IX-2005. Leg. et det. A.A. Korobkov (LE 06-06).
Artemisia littoricola Kitam.	Russia, Sakhalín Island. 5 km S of Krasnogorsk. 8-X-2000. Leg. K. Tkachenko (LE-Korobkov 00-05).
Artemisia monostachya Bunge ex Maxim.	Russia, Chita oblast, Kyra raion. Near Kyra, southern rocky slope in the left Kyra river shore, mountain steppe among *Prunus armeniaca*. 9-IX-2005. Leg. et det. A.A. Korobkov (LE 06-07).
Artemisia pycnorhiza Ledeb.	Russia, Tyva Republic, Erzin raion. Left shore of Tes-Khem river, 20 km NW of the city of Erzin, base of Izvestkyakov mountains, rocks. 18-IX-2003. Leg. et det. A.A. Korobkov (LE 04-115).

Artemisia salsoloides Willd.	Russia, Volgogradskaya oblast. Kumylissi. 15-X-2000. Leg. A.A. Korobkov (LE 00-11).
Artemisia sericea Weber ex Stechm.	Russia, Chita oblast, Kyr raion: near the village of Kyr, Sokhodin reserve, slopes in the left shore of a small river. 9-IX-2005. Leg. A.A. Korobkov (LE-Korobkov 06-26).
Artemisia sphaerocephala Krasch.	Mongolia, Bulgan aimag: Dashin Shellen sum, 10 km north-west of the sum, near the Institute of Folk Medicine, steppe with Caragana. 24-VIII-2004. Leg. Sh. Dariimaa, Sh. Tsooj and J. Vallès. (BCN 34485).
Filifolium sibiricum (L.) Kitam.	Mongolia, Bulgan aimag: Sansar sum, north-east slope of Khugunkhaan mountain. 25-VIII-2004, Sh. Dariimaa, Sh. Tsooj and J. Vallès (BCN-Mong. 14)

Probe preparation and fluorescent *in situ* hybridization

The 18S-5.8S-26S rRNA gene of wheat, pTa71, which contained the intergenic spacer region, was used as 45S rDNA probe (Gerlach and Bedbrook, 1979). Total genomic DNA of *Artemisia princeps* Pamp. was extracted from young leaves following the method of Doyle and Doyle (1987) modified by Cullings (1992). The 5S rDNA probe was amplified by PCR using the primers described in Hoshi *et al*. (2006) and with the following PCR conditions: one cycle of 5 min at 94.2ºC, 35 cycles of (30 sec at 94.2ºC, 30 sec at 55.5ºC and 30 sec at 72.2ºC) and one cycle for final extension at 72.2ºC during 77 min. The PCR reaction mixture (final volume of 50 µl) contained 60 ng of DNA template, 200 pmol of each primer, 0.1 mM of each dNTP, 10 mM of Tris-HCl pH 8.3 buffer, 50 mM of KCl, 1.5 mM of $MgCl_2$ and 1 unit of Taq polymerase (Tanaka). The 5S and 18S probes were labelled with random primed DNA labelling with digoxigenin-dUTP (Roche Diagnostics, Mannheim, Germany) and with Avidin-FITC BioNick labelling system (Invitrogen), respectively, following the manufacturer's instructions. Hybridization mixtures containing 50% formamide, 10% dextran sulfate, and each probe (at a concentration of 4 ng/µl in 2xSSC) were subsequently denatured during 10 min at 95ºC. Slide preparations were incubated in 100 µg/ml DNase-free RNase in 2xSSC for 1 h at 37ºC in a wet chamber, washed once in 2xSSC (pH = 7) for 10 min with slow shaking and then 10 min in 1xPBS (pH 7), treated with 4% paraformaldehyde in 1xPBS during 10 min, denatured at 72ºC with 70% deionized formamide in 2xSSC 1.5 min, and dehydrated through an ethanol series (70º, 90º and 100º) and air dried. After denaturation of probe mixtures, approximately 15-20 µl of probe were

loaded on the slide and covered with coverslips. The preparations were then denatured during 5 min at 75°C, and transferred down to 37°C overnight for hybridization in a wet chamber. Posthybridization stringency washes were done with agitation as follows: two washes in 4xSSC at 42°C for 10 min followed by a wash in 2xSSC (with 0.2% Triton-100) at room temperature. For 5S signal detection, the slides were treated with 1% (w/v) bovine serum albumin (BSA) in 2xSSC with 0.2% Triton-100 for 45 min at 37°C, and then incubated for 1.5 h at 37°C in 20 μg/ml anti-digoxigenin-rhodamine Fab fragments (Roche Diagnostics) in the same buffer. Slides were washed twice for 10 min in 2xSSC with 0.2% Triton-100 at 42°C, once in 2xSSC at room temperature 5 min, once in distilled water at room temperature for 5 min, and finally 8 dehydrated (ethanol 70°, 90° and 100°). Counterstaining was done with Vectashield (Vector Laboratories, Burlingame), a mounting medium containing 500 ng/ml DAPI (4',6-diamidino-2-phenylindole). FISH preparations were observed with an epifluorescence Nikon Eclipse E600 microscope using the following filters: UV-1A (365/410), B-2A (450/490), G-2A (510/560), Dia-ill. Hybridization signals were analysed and photographed using a CCD camera (Pixera, pentium 600CL), and an image analyser software (Metavue, version 4.6, Molecular Devices Corporation).

RESULTS AND DISCUSSION

A summary of karyological and cytogenetic data of the species studied in this work and also of some of the related species previously studied is presented in Table 2, and the metaphase plates with the hybridization signals are shown in Figs. 1 and 2. The chromosome counts were prior published (Vallès *et al.*, 2005; Garcia *et al.*, 2006; Pellicer *et al.*, 2007a, b) but all of them have been confirmed in the present study by means of protoplast obtention. The genome size data also come from precedent work of our team, the original sources being indicated in Table 2. All the taxa studied are $x = 9$-based with the exception of the annual *A. scoparia* ($x = 8$). The ploidy levels found range from diploid to decaploid. The last level has only been found in *A. dracunculus* and *A. sericea*, the latter belonging to the *Absinthium* subgenus. In fact, the decaploid is the

unique level we have found in *A. sericea* (Pellicer *et al.*, 2007a) even though other levels (2*x*, 4*x*) exist for this taxon in the literature (Kawatani and Ohno, 1964; Stepanov, 1994).

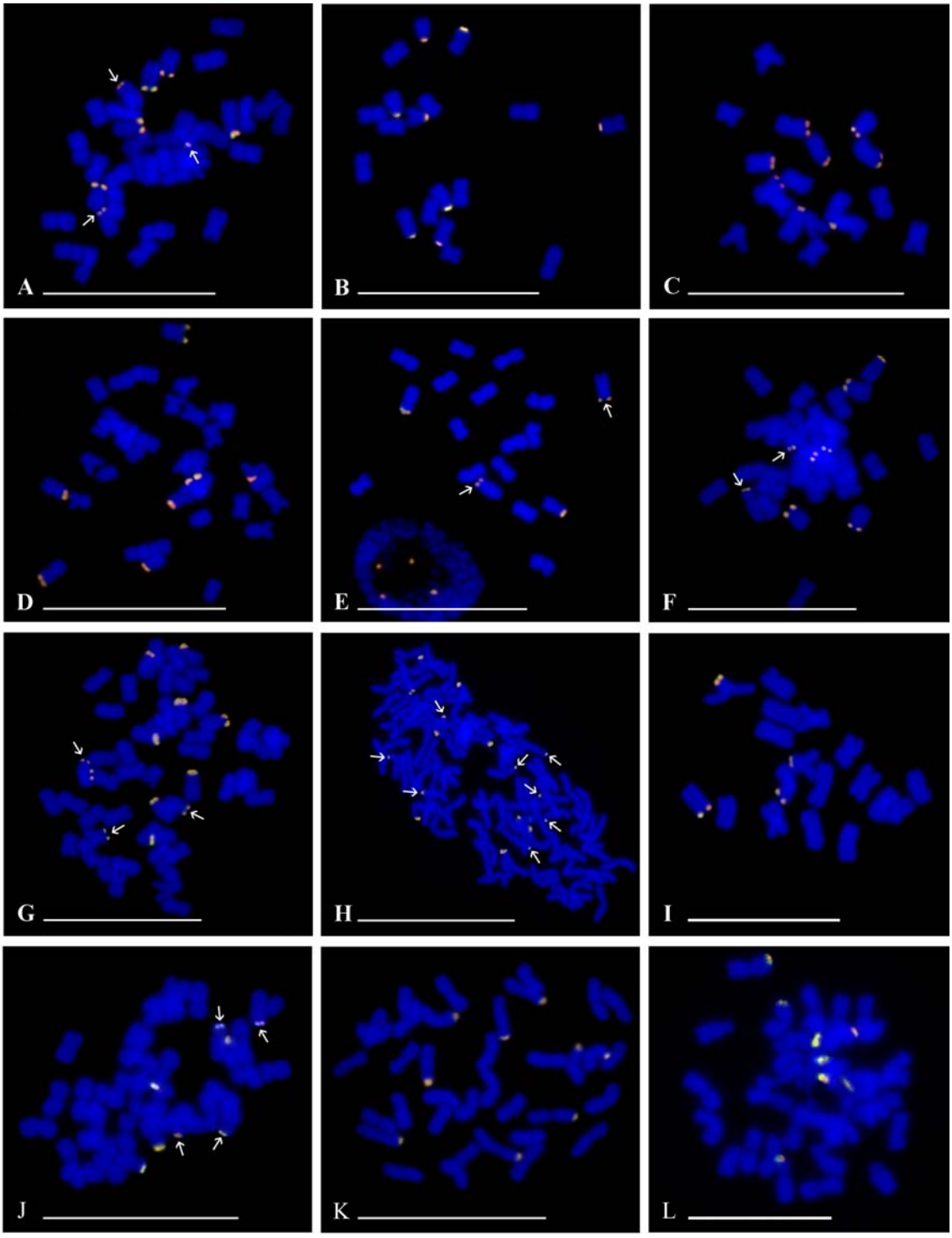

Figure 1: Somatic metaphase protoplasts of *A. changaica* (A), *A. commutata* (B), *A. dolosa* (C), *A. dolosa* (D), *A. dracunculus* (E), *A. dracunculus* (F), *A. dracunculus* (G), *A. dracunculus* (H), *A. giraldii* (I), *A. glauca* (J), *A. ledebouriana* (K), *A. littoricola* (L). The images of 5S (digoxigenin-labelled) and 18S-5.8S-26S (fluorescein-labelled) ribosomal DNA are presented merged. Scale bar = 10 μm.

Table 2. Genome size data and number of FISH signals

Taxon	2n	Ploidy level	2C value (pg)	1Cx (pg)	rDNA[1]
A. changaica	36	4x	11.70	2.93	8
*A. capillaris**	18	2x	3.37	3.37	9
A. commutata	18	2x	6.05	3.03	8
A. dolosa	18	2x	4.20	2.10	8
A. dolosa	36	4x	8.86	2.22	8
A. dracunculus	18	2x	5.94	2.98	4
A. dracunculus	36	4x	11.82	2.97	8
A. dracunculus	54	6x	15.71	2.62	12
A. dracunculus	90	10x	23.90	2.39	18-19
A. giraldii	18	2x	6.00	3.00	4
A. glauca	36	4x	11.97	2.99	8
A. ledebouriana	36	4x	8.75	2.19	9
A. littoricola	36	4x	8.73	2.18	8
A. monostachya	36	4x	8.84	2.21	8
A. pycnorhiza	36	4x	9.02	2.26	10
A. salsoloides	18	2x	11.40	5.70	4
*A. scoparia**	16	2x	3.54	3.54	10
A. sericea	90	10x	24.33	2.43	16
A. sphaerocephala	18	2x	5.52	2.76	8
F. sibiricum	18	2x	9.44	4.72	4
*N. pectinata**	18	2x	4.95	4.95	2

Note: Genome size data has been extracted from previously published and from unpublished works (Torrell et al., 2001; Garcia et al., 2004; Pellicer et al., 2009; Pellicer et al., unpublished). FISH results of *A. capillaris**, *A. scoparia** and *N. pectinata** have been obtained from Pellicer et al., (unpublished) and Hoshi et al., (2006). [1]rDNA; number of 5S and 45S sites.

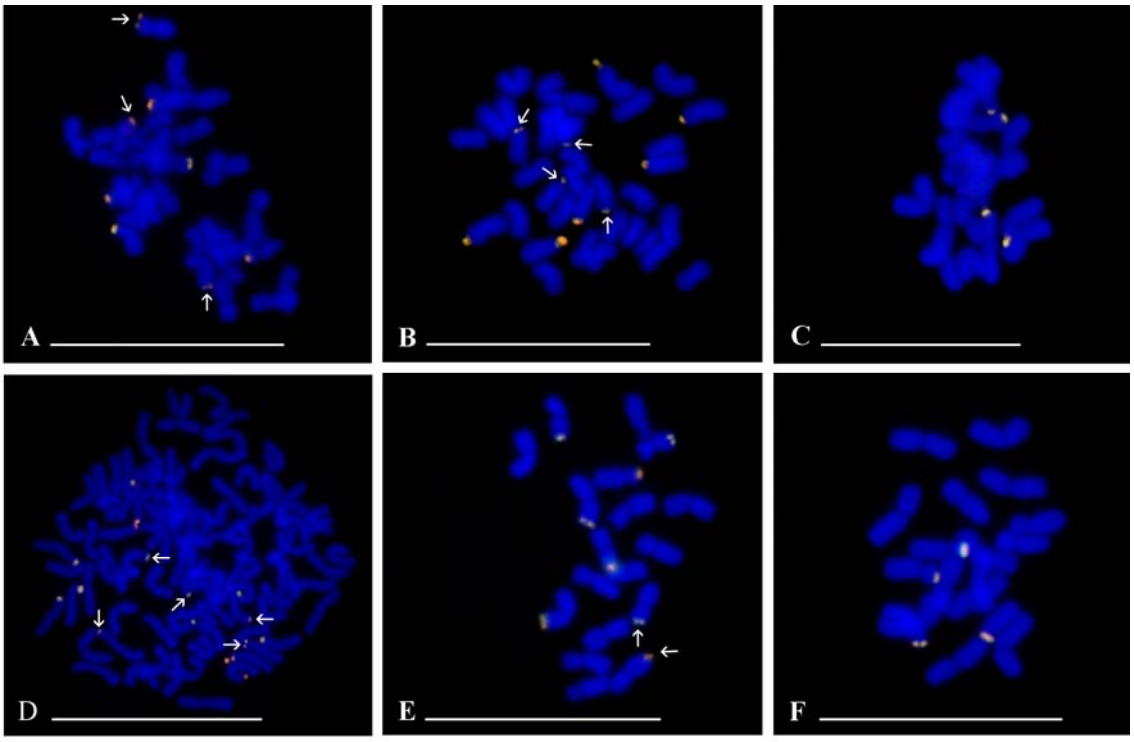

Figure 2: Somatic metaphase protoplasts of *A. monostachya* (A), *A. pycnorhiza* (B), *A. salsoloides* (C), *A. sericea* (D), *A. sphaerocephala* (E), *Filifolium sibiricum* (F). The images of 5S (digoxigenin-labelled) and 18S-5.8S-26S (fluorescein-labelled) ribosomal DNA are presented merged. Scale bar = 10 μm.

Colocalization of both 5S and 45S ribosomal DNA has been detected in the species studied, as previously stated by Torrell *et al.* (2003), Garcia *et al.* (2007, 2009a, b) and Pellicer *et al.* (2008) in *Artemisia*, some related genera such as *Filifolium* and *Neopallasia* also showing this trait (Garcia *et al.*, unpublished; Pellicer *et al.*, unpublished). All FISH signals are located in the ends of chromosomes or in satellites (Figs. 1 and 2), as reported in previous studies in the subgenus (Torrell *et al.*, 2001; Hoshi *et al.*, 2006; Pellicer *et al.*, unpublished).

FISH signal strength

Seven out of the nine FISH marks that Hoshi *et al.* (2006) observed in *A. capillaris*, a species belonging to the subgenus the present paper is centred on, were major and the remaining two minor. We have experienced also this different signal intensity or band dimension in several of the populations here studied. We can usually detect and differentiate only major and minor signals, but in the hexaploid *A. dracunculus* (Fig. 1G) population we have found three intensity levels. Fluorescent *in situ* hybridization is a semiquantitative technique in which the intensity of signal provides information about the copy number of genes (Maluszynska and Heslop-Harrison 1993). This phenomenon is rather common and has been reported in quite different plant groups, such as *Artemisia* itself (Torrell *et al.*, 2003; Pellicer *et al.*, unpublished), other Asteraceae (Cerbah *et al.*, 1998) and other families (Zoldos *et al.*, 1999; Fusheng *et al.*, 2003; Nkongolo *et al.*, 2004; Bo *et al.*, 2006; Srisuwan *et al.*, 2006).

Cytogenetic results along the phylogeny

Mapping the cytogenetic data on the phylogenetic reconstruction for the whole *Artemisia* genus (data not shown) we can hypothesise that the ancestor of the genus could be a diploid species with 18 chromosomes and two colocalized

rDNA loci [see Sanz *et al.* (2008) and Pellicer *et al.* (unpublished) for the phylogenetic trees and Torrell *et al.* (2001, 2003); Vallès and Garnatje (2005); Garcia *et al.* (2007, 2009a, b) and Pellicer *et al.* (2008) for the cytogenetic ones]. A schematic representation for the taxa included in the present study is shown in Fig. 3. In three of the *Artemisia* subgenera (*Absinthium*, *Artemisia* and *Seriphidium*), this putative ancestral pattern is present: the diploid species show four rDNA sites, i.e. two loci (Torrell *et al.*, 2003; Pellicer *et al.*, 2008). The only representative from the *Absinthium* subgenus included in this work, *A. sericea* (Fig. 2D), shows a decaploid level and it will be discussed later. The same pattern occurs in the subgenus *Artemisia* even though it does not constitute a monophyletic group in the phylogeny (Pellicer *et al.*, unpublished). The species of the section *Heterophyllae*, belonging to subgenus *Artemisia* but constituting a separated clade, share this pattern with *Filifolium sibiricum* (Fig. 2F), phylogenetically closely related with them. *Artemisia annua* L., a diploid annual subgenus *Artemisia* species, situated close to the subgenus *Seriphidium* in the molecular phylogeny (Vallès *et al.*, 2003; Sanz *et al.*, 2008), has also four FISH signals (Torrell *et al.*, 2003). The rDNA distribution is the same in subgenus *Seriphidium*, with four and eight signals (two and four loci) respectively in diploid and tetraploid taxa (Torrell *et al.*, 2003; see the text and Fig. 2 of this paper, not its Table 3, which contains errors in the numbers provided there).

Contrarily, the subgenus *Tridentatae* exhibits some variations from the putative ancestral pattern. Most of its species present six FISH signals (three loci) at the diploid level and some other changes in the rDNA distribution regarding the most common, possibly primitive model occur (Garcia *et al.*, 2007, 2009b), likely because of the hybridization events having taken place among the taxa of this North American endemic subgenus, or because of the origin of this group, the only one formed in America and one of the most derived in the genus.

Within the subgenus *Dracunculus*, the object of the present work, several changes from the putative ancestral pattern can be observed. *Artemisia salsoloides*, whose taxonomic position is controversial (Fig. 3), has four sites (two loci) at the diploid level (Fig. 2C), agreeing with the postulated ancestral model, but this fact supports neither the inclusion of this taxon within the subgenus nor

the exclusion from it, because the subgenus has not a unique pattern. Anyway, *A. salsoloides* coincides in rDNA loci number with the most basal clade within the subgenus, which follows the ancestral model, as we will discuss later. The genome size of this species (1Cx = 5.7 pg, see Table 2) is considerably higher than the average of the genome size of the remaining species belonging to the subgenus studied in the present work (mean 1Cx = 2.78 for the diploids and 1Cx = 2.59 for the whole ploidy levels) supporting its phylogenetic position, out of the *Dracunculus* subgenus, recently suggested by Pellicer *et al.* (unpublished).

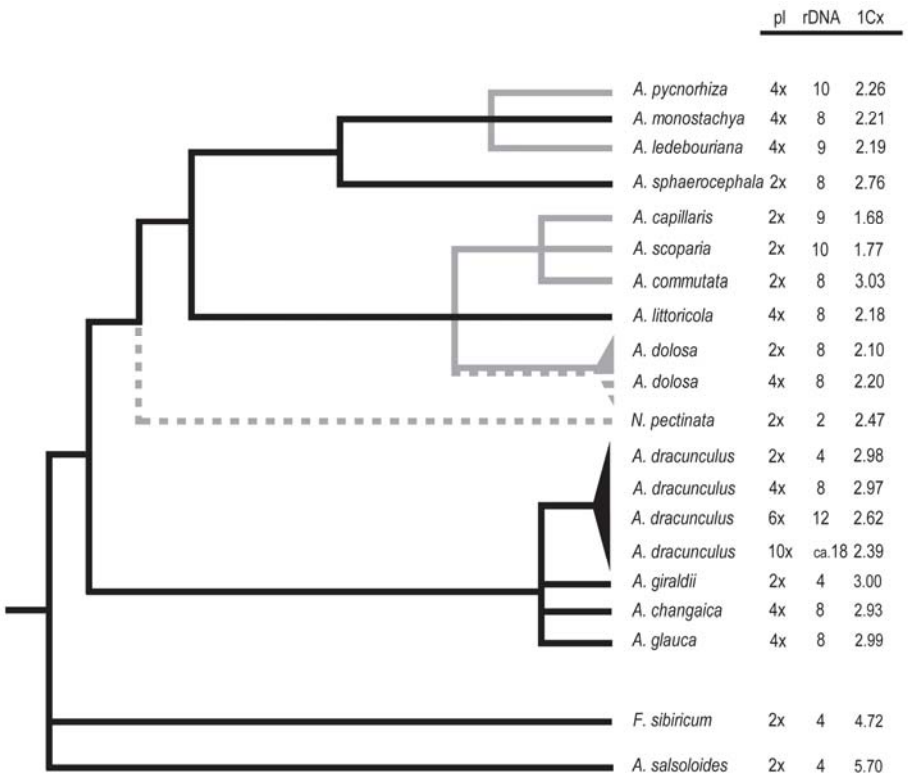

Figure 3. Phylogenetic diagram based on data from Pellicer et al., (unpublished). Black lines (ancestral pattern, 2 loci); grey lines (rDNA sites gain); grey dotted lines (rDNA sites loss).

The *Dracunculus* complex is divided in two groups clearly supported in the phylogenetic tree (Fig. 3 for the diagram; Pellicer *et al.* (unpublished) for the phylogenetic reconstructions). On the one hand, the species of the *A. drancunculus* (Fig. 1E) clade show a great constancy in their cytogenetic pattern according with the proposed ancestral model. At the diploid level, *A. dracunculus* and *A. giraldii* (Fig. 1I) have four rDNA sites, and at the tetraploid one *A.*

changaica (Fig. 1A), *A. dracunculus* (Fig. 1F) and *A. glauca* (Fig. 1J), share the same pattern with eight rDNA sites. These species also show a similar DNA amount (Table 2) and they form a well defined group in accordance with morphological characters, such as the predominance of entire leaves and the existence of similar capitula shape and dimensions. The polyploidy seems to be the only chromosomal evolution process that has had an important role in this group as we will explain in the next section. On the other hand, there is a second group, constituting a much bigger clade, which includes *Neopallasia pectinata* (Pall.) Poljakov. This species shows only two rDNA sites (one locus) at the diploid level (Pellicer *et al.*, unpublished). This loss (with regard to the ancestral pattern with four sites, two loci) could be explained by the life cycle of this species, an annual one recently reincluded in *Artemisia* (Pellicer *et al.*, unpublished) that occurs in the arid steppes of Central Asia. The other annual species of this group is *A. scoparia* which, contrariwise, presents an increased number of rDNA loci (ten sites, i.e., five loci, for the diploid level with a particularly low chromosome number, $2n = 16$; Pellicer *et al.*, unpublished). The particular ecology of this species might have influenced a very fast life cycle (Pellicer *et al.*, unpublished). The short life cycle could explain the rapid changes in the genome of *N. pectinata* and the chromosome recombinations that have occurred could have propitiated the fragmentation of the rDNA regions and their scattering in the genome in *A. scoparia*, as suggested by Hidalgo *et al.* (2008) in the genus *Oligochaeta*. The hypothesis that the lower genome sizes could favour the short life cycles has been largely discussed in the literature, and the annual cycle is generally considered to be related to low genome size (Bancheva and Greilhuber 2006) especially in autogamous species, but other annual Asteraceae, such as *Siebera pungens*, also show a high DNA amount (Garnatje *et al.*, 2004).

The remaining species of this clade, present different and sometimes contrasting rDNA loci numbers. The putative ancestral pattern occurs only in *A. littoricola* (Fig. 1L) and *A. monostachya* (Fig. 2A), both tetraploid with eight signals (four loci). Conversely, in *A. ledebouriana* (Fig. 1K) and *A. pycnorhiza* (Fig. 2B), also tetraploid, we have found nine and 10 hybridization signals, respectively. Concerning genome size, 1Cx values are considerably constant

(from 2.18 to 2.26 pg) in all tetraploid populations. Most diploid taxa (*A. commutata*, *A. dolosa* and *A. sphaerocephala*) show eight rDNA sites (four loci), deviating from the four-site/two-loci model (Fig. 1B, 1C, 2E). An exception, but also divergent from the putative ancestral pattern, is the above-commented *A. scoparia*, with 10 signals and 16 chromosomes. Furthermore, Hoshi *et al.* (2006) observed nine sites in *A. capillaris*, having the more common chromosome number, $2n = 18$. The genome sizes of the diploid taxa appear much more variable than those of the tetraploid species (from 1.68 to 3.03 pg for 1Cx). In these diploid species a duplication of the rDNA sites have taken place in absence of polyploidization events. *Artemisia ledebouriana* and *A. pycnorhiza* seem to keep the basic pattern with some chromosome reorganizations whereas *A. capillaris* has suffered a rDNA loci duplication without polyploidization -as most diploids- and also a chromosomal reorganization leading to an odd number of bands. The clade we are dealing with now includes also, among other taxa, the *Artemisia campestris* L. complex, not considered in the present paper, but for which preliminary FISH data have been provided (Torrell *et al.*, 2001) indicating also a pattern different from the typical ancestral one, with six marks (three loci) at the diploid level.

The non detection of a duplicated number of rDNA loci from diploids to tetraploids in some members of this subgenus *Dracunculus* clade may be related with the activity of such loci, since, as Maluszynska *et al.* (1998) pointed out, in species with numerous rDNA loci many of them are not active. Furthermore, several chromosome changes, including fusions, fissions, translocations and similar mechanisms, together with hybridization processes presumably having occurred in the most derived *Dracunculus* clade could explain the low resolution of the phylogenetic reconstructions (Pellicer *et al.*, unpublished).

The polyploidy effects

In the case of *A. dolosa*, in which two populations of different ploidy levels have been studied (Table 2), we have observed the same number of rDNA loci in the diploid and tetraploid cytotypes (Fig. 1C and 1D), but genome size is doubled in the tetraploid as compared with the diploid (Pellicer *et al.*, unpublished). The 1Cx

value is slightly higher in the tetraploid than in the diploid. As the ribosomal DNA loss is not reflected in a genome size decrease, a gain of another kind of DNA sequences seems to have taken place in the polyploid formation process.

The most complete polyploid series studied is that of *A. dracunculus* (Table 2). In this series, the number of rDNA sites increases proportionally to the ploidy level up to hexaploid one (4, 8 and 12 signals for 2*x*, 4*x* and 6*x* populations, respectively; the octoploid level could not be investigated). Following the same proportions, we could expect 20 sites in the decaploid level, where only 18-19 (depending on the metaphase plates) have been detected (Fig. 1H). This small loci loss is likely to be due to chromosome restructuring. *Artemisia dracunculus* is a largely cultivated species which may have suffered changes in its genome despite of the genome size follows the usual pattern observed along the *Artemisia* genus, this is, the 1Cx values remain quite constant in the lower ploidy levels and a decrease is observed in the higher ones, the whole following a saturation model in agreement with the Michaelis-Menten function (Pellicer *et al.*, 2009). *Artemisia sericea*, which belongs to the subgenus *Absinthium*, was used in the present work to compare the behaviour of a *Dracunculus* high polyploid with one of another subgenus. We have only found a decaploid level with 16 rDNA sites in this species (Table 2) indicating a much bigger loci loss than in 10*x* *A. dracunculus*, although the genome sizes are very similar in both taxa (2C values of 23.64 and 23.90 pg, Table 2), suggesting that several chromosome reorganizations have taken place in its genome from the putative ancestral pattern.

CONCLUDING REMARKS

The physical mapping of rRNA genes in a representative number of taxa of the subgenus *Dracunculus* has shown different patterns in the two main clades in which this subgenus is differentiated in the molecular phylogenies, indicating an agreement between molecular and cytogenetic data sets. The plants of the smaller, basal *A. dracunculus* complex clade show a pattern basically coincidental with what is thought to be the ancestral model in the genus, whereas the representatives of the larger, more derived clade, involving the remaining

taxa of the subgenus, exhibit different patterns deviating from the putatively ancestral one. Polyploidization and hybridization are the most common phenomena occurring in this subgenus, but several chromosomal reorganizations, such as translocations, could explain some changes in the number of rDNA sites. Deeper and larger cytogenetic studies are necessary in order to clarify the last process. The results of the present cytogenetic work, indicating that *A. salsoloides* shares the rDNA loci pattern with the most basal clade of the subgenus *Dracunculus*, do not definitely support the segregation of this species from the subgenus, suggested by molecular phylogenetic data, nor its inclusion.

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Canvis en la grandària del genoma en una distribució fragmentada: el cas d'*Artemisia crithmifolia* L. (*Asteraceae*, *Anthemideae*)

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Artemisia crithmifolia és un arbust hexaploide que habita a les dunes costaneres del litoral atlàntic a la franja oest d'Europa, des del sud de la península Ibèrica fins als Països Baixos, arribant a les illes Britàniques. Hem estimat la grandària del genoma mitjançant citometria de flux en 45 poblacions d'*A. crithmifolia*, que cobreixen tot el rang de distribució de l'espècie. Els valors 2C de DNA nuclear del tàxon estudiat han variat entre 14,27 i 15,72 pg, essent el valor mitjà de 14,98 pg. S'ha trobat una correlació negativa entre la quantitat de DNA nuclear i la latitud i, a més, diferències significatives entre dos grups, resultat de la fragmentació en l'àrea de distribució de l'espècie, han estat evidenciades.

Changes in genome size in a fragmented distribution area: the case of *Artemisia crithmifolia* L. (Asteraceae, Anthemideae).

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Abstract — *Artemisia crithmifolia* is a hexaploid shrub which inhabits the coastal Atlantic sand dunes of Western Europe, from the Southern Iberian Peninsula to the Netherlands, reaching the British Isles. Genome size data of 45 populations of *A. crithmifolia*, covering its entire distribution area, were obtained using the flow cytometry method. The 2C nuclear DNA content in this species ranged from 14.27 to 15.72 pg, the mean value being 14.98 pg. A negative correlation between nuclear DNA amount and latitude has been found, and statistically significant differences between two groups resulting from the fragmentation of the distribution area were evidenced.

Key words: 2C value, Compositae, dunes, flow cytometry, nuclear DNA amount.

INTRODUCTION

The genus *Artemisia* L. is one of the largest of the Asteraceae, with more than 500 species (Oberprieler *et al.* 2007). Different taxonomic rearrangements, based on morphological traits, have been carried out, and five large subgenera are considered at present (*Absinthium* DC., *Artemisia*, *Dracunculus* Besser, *Seriphidium* Besser and *Tridentatae* (Rydb.) McArthur). The genus is widely dispersed across the Northern Hemisphere although a few species are distributed in the Southern Hemisphere (Ling 1982; Vallès and Garnatje 2005).

Artemisia crithmifolia L. is a hexaploid shrub distributed over the Western coast of Europe, from the Southern Iberian Peninsula to the Northern Netherlands, reaching the British Isles. Two important disjunctions have been detected along its range, which are coincidental with the Northern coasts of both the Iberian Peninsula [where it seems to be extinct with the exception of one population (Aedo *et al.* 1990)] and France. This species occupies the maritime sands, principally at the back of the dunes in process of stabilization on the Northern Atlantic beaches, being part of two associations, *Corynephoretum atlanticum* and *Roseto-Ephedretum* (Kuhnzholtz-Lordat 1927), which are closely related (Vanden 1958).

Artemisia crithmifolia, a species from subgenus *Dracunculus*, presents capitula with glabrous receptacles, the outer florets female and the remaining ones functionally male, as commonly reported in the subgenus. The panicle branches are not sticky and the leaf lobes are short, fleshy, convex but not keeled beneath, according to Tutin *et al.* (1976). This species is closely related to *A. campestris* L. at morphological and molecular levels (Torrell *et al.* 1999). Its taxonomical consideration has been variable, as morphological features have been used to describe not only the species as an independent one, but also some subspecific entities subordinated to *A. campestris*. For this reason, different synonyms related to *A. crithmifolia* can be found in the literature [e.g. *A. campestris* L. subsp. *maritima* Arcangeli; *A. campestris* L. subsp. *lloydii* (Rouy) Cout., *A. gayana* Besser., *A. lloydii* (Rouy) A.W. Hill]. All these taxonomic considerations could be related to the fact that the species presents a certain degree of morphological

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variability, maybe due to different environmental conditions where it grows. This taxon shows morphological and ecological affinities with another member of the *A. campestris* complex, *A. campestris* subsp. *sericea* (Fr.) Lemke et Roth., growing in Baltic coastal sand dunes, and also with some related Asian taxa, such as *A. jordanica* Danin and *A. monosperma* Delile.

BAKKER (1976), in his study on phytogeographical aspects of the vegetation in the Atlantic province of Europe, postulated the relationship between the presence of *A. crithmifolia* (cited as *A. lloydii*) and other chamaephytes and the maintenance of a warm refuge during the glacial periods in the Southern part of this province. This author rules out that this presence can be due to lime content in the soils. In their work on general threats to plant species growing in the coastal habitats, VAN DER MAAREL & VAN DER MAAREL-VERSLUYS (1996) present *A. crithmifolia* in an uncertain status of conservation but needing attention. We agree with these authors that the development of urban-industrial-recreational facilities, especially in dunes, has caused the loss of the extensive areas where these plants used to grow. We have already experienced this fact during the sampling for the present work.

The DNA C-value for a species is the amount of nuclear DNA in the unreplicated haploid genome of a gamete (SWIFT 1950). It seems that C-value tends to be characteristic of a taxon, and quite constant within a species, the C accounting for constant. Since the C-value term was coined to date, many studies concerning the relationships between genome size and biological and ecological traits, involving aspects like polyploidy, cell physiology, biogeographic distribution, genetic plasticity or breeding system, have been carried out (e.g., BENNETT 1972; JASIENSKI and BAZZAZ 1995; MACGILLIVRAY and GRIME 1995; GRIME 1996; VINOGRADOV 2003; LEITCH and BENNETT 2004; CHASE *et al.* 2005; PRICE *et al*. 2005; BEAULIEU *et al.* 2007; GARCIA *et al.* 2008), suggesting that genome size does in fact have an evolutionary effect or actually its size is a consequence of evolution. Reports on infraspecific variation of genome size have been conducted, and the idea of a relative constancy of genome size within a species has been evidenced (BARANYI and GREILHUBER 1996; BENNETT *et al.* 2000).

The aims of the present work are: i) to contribute to the enlargement of genome size knowledge in plants, especially in the Asteraceae and in the genus *Artemisia*, on which many of our works are focused; ii) to study the constancy of the C-value parameter along the geographical distribution range in a given species; iii) to evaluate whether some morphological differences observed between populations are also reflected at genome size level; and iv) to study possible changes of genome size related to insularity.

MATERIAL AND METHODS

Table 1 shows the provenance of all the populations investigated with collectors, dates and voucher information. In order to confirm the presence or not of the studied species, different transects covering the Atlantic coast along its range of distribution were explored. In all cases, fresh young leaves were collected for flow cytometric measurements. Herbarium vouchers have been deposited in the Herbarium BCN (Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona). The internal standard used, *Petunia hybrida* Vilm. 'PxPc6' (2C = 2.85 pg; MARIE and BROWN 1993), was cultivated in the greenhouses of the Facultat de Farmàcia (Universitat de Barcelona) and the Institut Botànic de Barcelona (CSIC-ICUB). It was provided by the Institut des Sciences du Végétal, Gif-sur-Yvette (France).

DNA content assessment - Fresh young leaves from the plants studied were co-chopped using a razor blade in a plastic Petri dish with an internal standard in 600 µl of Galbraith's isolation buffer (GALBRAITH *et al.* 1983) and supplemented with 100 µl/ml ribonuclease A (RNase A, Boehringer, Meylan, France). A sample containing only the standard was first prepared and analysed to determine its peak position. Nuclei were filtered through a 30-mm nylon filter in order to eliminate cell debris before adding 36 µl of propidium iodide (Sigma-Aldrich Química, Alcobendas, Madrid, Spain) in a final concentration of 60 µg/ml. Samples were kept on ice for 20 min before measurement. Five individuals per population were analysed. Two samples from each individual were extracted and measured independently. Fluorescence analysis was carried out using an Epics XL flow cytometer (Coulter Corporation, Hialeah, Florida). The total nuclear DNA content was calculated by multiplying the known DNA content of the standard by the quotient between the 2C peak positions of the target species and the standard in the histogram of fluorescence intensities, under the assumption that there is a linear correlation between the fluorescent signals from stained nuclei of the unknown specimen, the known internal standard, and DNA

content. We also calculated the mean half peak coefficient of variation (HPCV) corresponding to ten samples.

Statistical analyses - One-way ANOVA has been carried out to test differences between the datasets. We have indeed performed analyses either considering two groups (populations growing in the Northern part of the distribution area, including France, Belgium and the Netherlands, and those at the Spanish-Portuguese coasts at the South) or three groups (those of the Iberian Peninsula, the French group and the Belgian-Dutch one). We have done it both ways because, although we think that there is a real fragmentation in the distribution area of *A. crithmifolia* between the Iberian Peninsula and France, on the Cantabrian coast (so that two groups should only be considered), we cannot be sure that another fragmentation has not occurred between the French and Belgian-Dutch populations (hence, three groups should be considered). The 2C value corresponding to Loredo (on the Cantabrian coast) was excluded, because it is isolated in the Cantabrian territory (North coast of Spain), marking the border between the Iberian and the French datasets. Also, populations from the British Isles have been excluded as they were not numerous enough to perform a single group for the statistical analysis. Correlation between 2C values and the latitude of populations was evaluated using the non-parametrical Spearman correlation test.

RESULTS AND DISCUSSION

Nuclear genome size was assessed for 45 populations of *Artemisia crithmifolia* covering its entire natural range (Table 1, Fig. 1). The mean 2C value is 14.98 pg (equivalent to 14,650.44 Mbp according to DOLEŽEL *et al.* 2003), with a standard deviation of 0.41.

Variation in DNA amount within the species - The minimum value (14.27 pg = 13,956.06 Mbp) was obtained for the only population from Belgium and the maximum (15.72 = 15,374.16 Mbp) for the population from Tróia, in Portugal, the largest interpopulational difference being of 1.45 pg, equivalent to 9.22%, that is 1.1-fold. The data presented fit well with the only previous record of DNA content in *A. crithmifolia*, from a Portuguese population (Esposende, 2C = 15.60 pg, SD = 0.27; TORRELL and VALLÈS 2001), but it is to be remarked that the nuclear DNA amount currently assessed in plants of the same population is clearly lower [14.80 pg (SD = 0.38); Table 1] than the one calculated in the precedent work. In any case, the 2C values reflect the constancy of the hexaploid level, which is the only one reported in the taxon studied ($2n = 6x = 54$; KAWATANI and OHNO 1964; PASTOR 1992; OLIVA and VALLÈS 1994; TORRELL *et al.* 2001; and references therein). These values indicate that a certain interpopulational variability exists in this species along its distribution area, even if this intraspecific variation can be considered as not very high, according to the results reported for several plant groups (DOLEŽEL and BARTOŠ 2005; GARCIA *et al.* 2006; 2008 and references therein).

Numerous data reporting intraspecific genome size variations can be found in the literature (BENNETT and LEITCH 1997; RAYBURN *et al.* 2004) but this phenomenon is controversial (GREILHUBER 1997; 1998; 2005; OBERMAYER and GREILHUBER 1999). AUCKLAND *et al.* (2001) have found constancy in the genome size of disjunct populations in *Abies fraseri* (Pursh) Noir, whereas SUDA *et al.* (2005) postulated that the variability in *Hieracium* subgenus *Pilosella*, could indicate hybridogenous lineages. It is known that ecogeographical conditions could cause intraspecific variation in plant genome size (GASMANOVÁ *et al.* 2007). The constancy of the chromosome number makes it difficult to think of hybridogenic processes within *A. crithmifolia* (although an allopolyploid origin cannot be discarded for the taxon itself). The ecological conditions in which this plant grows are rather constant. In addition, the low HPCV value (0.99) suggests the absence of secondary metabolites which could be responsible for variation in DNA content. Taking into account these arguments, we consider that the differences in genome size in *A. crithmifolia* reflect a true populational or individual specific variability.

Differences in DNA amount between groups of populations - In order to detect significant changes, at genome size level, we have carried out different tests in concordance with the distribution of the species. The one-way ANOVA indicates a statistically significant difference ($F = 8.62$, $P = 0.0054$) between the 2C value means of the populations of the Southern (Iberian Peninsula and Portugal) and the Northern (remaining) areas, 14.82 pg being the mean of the populations of the Northern part and 15.15 pg for the Southern ones. If we take into account the geographic disjunctions, three main groups of distribution can be observed, so we also performed the analysis considering these three groups and results are comparable. The 2C values still remain significantly different ($F=7.19$, $P=0.0022$), but the populations from France (mean

TABLE 1 — Origin, DNA nuclear amount and latitude of the studied populations of *Artemisia crithmifolia* L. - [1]Nuclear DNA content: mean (standard deviation) of five individuals. - [2]1 pg = 978 Mbp (DOLEŽEL *et al.* 2003).

Code	Locality, collectors and herbarium voucher	Latitude (°)	2C(SD)(pg)[1]	2C (Mbp)[2]
PI1	Spain: Huelva, Playa de Mazagón, Garnatje & Pellicer. 18.02.2008. GR-272 (BCN)	37.14	15.36(0.27)	15022.08
PI2	Spain: Huelva, Playa de la Antilla, Garnatje & Pellicer. 18.02.2008. GR-271 (BCN)	37.21	15.67(0.51)	15325.26
PI3	Portugal: Algarve, Praia do Anção, Garnatje & Pellicer. 17.02.2008. GR-270 (BCN)	37.03	15.58(0.35)	15237.24
PI4	Portugal: Algarve, Alvor, Garnatje & Pellicer. 17.02.2008. GR-269 (BCN)	37.12	15.61(0.42)	15266.58
PI5	Portugal: Leiria, Foz de Arelho, Garnatje & Pellicer. 16.02.2008. GR-258 (BCN)	39.43	15.06 (0.30)	14728.68
PI6	Portugal: Setúbal, Sines, Garnatje & Pellicer. 17.02.2008. GR-266 (BCN)	37.95	15.31 (0.41)	14973.18
PI7	Portugal: Setúbal, Tróia, Garnatje & Pellicer. 17.02.2008. GR-264 (BCN)	38.49	15.72 (0.21)	15374.16
PI8	Portugal: Setúbal, Praia da Mata, Garnatje & Pellicer. 17.02.2008. GR-259 (BCN)	38.62	15.17 (0.17)	14836.26
PI9	Portugal: Leiria, Agua de Madeiros, Garnatje & Pellicer. 15.02.2008. GR-255 (BCN)	39.74	14.92 (0.18)	14591.76
PI10	Portugal: Coimbra, Praia da Mira, Garnatje & Pellicer. 15.02.2008. GR-250 (BCN)	40.46	14.78 (0.46)	14454.84
PI11	Portugal: Aveiro, Furadouro, Garnatje & Pellicer. 15.02.2008. GR-249 (BCN)	40.88	15.09 (0.50)	14758.02
PI12	Portugal: Porto, Vila do Conde, Garnatje & Pellicer. 15.02.2008. GR-248 (BCN)	41.35	14.61 (0.53)	14288.58
PI13	Portugal: Braga, Esposende, Garnatje & Pellicer. 15.02.2008. GR-247 (BCN)	41.53	14.80 (0.38)	14474.40
PI14	Spain: Pontevedra, Praia de Armona, Garnatje & Pellicer. 14.02.2008. GR-246 (BCN)	41.87	14.92 (0.18)	14591.76
PI15	Spain: Pontevedra, Praia de Areamilla, Garnatje & Pellicer. 14.02.2008. GR-245 (BCN)	42.25	15.08 (0.25)	14748.24
PI16	Spain: Pontevedra, Praia de Lanzada, Garnatje & Pellicer. 13.02.2008. GR-244 (BCN)	42.43	15.33 (0.33)	14992.74
PI17	Spain: Pontevedra, Praia Aguieira. Garnatje & Pellicer. 13.02.2008. GR-243 (BCN)	42.47	14.79 (0.53)	14464.62
PI18	Spain: Pontevedra, Praia do Rostro. Garnatje & Pellicer. 13.02.2008. GR-242 (BCN)	42.96	15.45 (0.42)	15110.10
PI19	Spain: A Coruña, Praia de Soesto. Garnatje & Pellicer. 12.02.2008. GR-241 (BCN)	43.20	14.55 (0.40)	14229.90
PI20	Spain: Santander, Ribamontán al Mar, Loredo. Garnatje & Vallès. 30-12-2007. (BCN)	43.46	15.62 (0.26)	15276.36
F1	France: Pyrénées Atlantiques, Anglet, plage des dunes. Pellicer & Vallès. 27.02.2008. JP-1 (BCN)	43.53	14.97 (0.14)	14640.66
F2	France: Landes, Capbreton, plage de Savanne. Pellicer & Vallès. 27.02.2008. JP-2 (BCN)	43.65	14.33 (0.39)	14014.74
F3	France: Landes, Vieux-Boucau-les-Bains. Pellicer & Vallès. 27.02.2008. JP-3 (BCN)	43.79	14.66 (0.45)	14337.48
F4	France: Landes, Contis-Plage. Pellicer & Vallès. 27.02.2008. JP-4 (BCN)	44.09	14.60 (0.36)	14278.80
F5	France: Landes, Biscarrose-Plage. Pellicer & Vallès. 27.02.2008. JP-5 (BCN)	44.45	14.51 (0.35)	14190.78
F6	France: Gironde, Pyla-sur-Mer, plage de la Corniche. Pellicer & Vallès. 27.02.2008. JP-6 (BCN)	44.63	14.93 (0.36)	14601.54

Code	Locality, collectors and herbarium voucher	Latitude (º)	2C(SD)(pg)1	2C (Mbp)2
F7	France: Gironde, Le Porge, Le Porge-Océan. Pellicer & Vallès. 28.02.2008. JP-7 (BCN)	44.89	15.07 (0.32)	14738.46
F8	France: Gironde, Hourtin, Hourtin-Plage. Pellicer & Vallès. 28.02.2008. JP-9 (BCN)	45.22	14.45 (0.53)	14132.10
F9	France: Gironde, Soulac-sur-Mer, plage d'Amélie-sur-Mer. Pellicer & Vallès. 28.02.2008. JP-10 (BCN)	44.52	15.20 (0.43)	14865.60
F10	France: Charente Maritime, La Palmyre, Phare de la Coubre. Pellicer & Vallès. 28.02.2008. JP-11 (BCN)	45.70	14.91 (0.23)	14581.98
F11	France: Charente Maritime, Île d'Oléron, Saint-Denis d'Oléron, plage des Boiries. Pellicer & Vallès. 28.02.2008. JP-12 (BCN)	46.03	14.63 (0.40)	14308.14
F12	France: Charente Maritime, Île d'Oléron, Saint-Pierre d'Oléron, plage de Vert-Bois. Pellicer & Vallès. 28.02.2008. JP-13 (BCN)	45.87	14.45 (0.37)	14132.10
F13	France: Charente Maritime, plage de Saint Jean des Sables, between Rochefort and La Rochelle. Pellicer & Vallès. 29.02.2008. JP-14 (BCN)	46.10	14.69 (0.40)	14366.82
F14	France: Charente Maritime, Île de Ré, Rivedoux-Plage. Pellicer & Vallès. 29.02.2008. JP-15 (BCN)	46.16	14.39 (0.52)	14073.42
F15	France: Vendée, Bretignolles-sur-Mer, plage des dunes. Pellicer & Vallès. 29.02.2008. JP-17 (BCN)	46.61	15.03 (0.18)	14699.34
F16	France: Loire Atlantique, dunes du Collet. Pellicer & Vallès. 29.02.2008. JP-18 (BCN)	47.04	14.79 (0.58)	14464.62
F17	France: Loire Atlantique, La Turballe, way to Pen-Bron. Pellicer & Vallès. 01.03.2008. JP-19 (BCN)	47.35	14.97 (0.15)	14640.66
F18	France: Morbihan, Quiberon. Pellicer & Vallès. 01.03.2008. JP-20 (BCN)	47.48	15.19 (0.25)	14855.82
B1	Belgium: De Panne Plage, Dunnes de Perruquet. Garcia & Ibarria. 16.03.2008. SC-1 (BCN)	51.23	14.27 (0.20)	13956.06
H1	Netherlands: Veere, Koudekerke. Garcia & Ibarria. 18.03.2008. SC-3 (BCN)	51.49	15.24 (0.33)	14904.72
H2	Netherlands: Katwijk, Zuidduinen. Garcia & Ibarria. 19.03.2008. SC-5 (BCN)	52.19	14.28 (0.20)	13965.84
H3	Netherlands: Zandvoort. Garcia & Ibarria. 20.03.2008. SC-7 (BCN)	52.37	14.93 (0.34)	14601.54
H4	Netherlands: Ijmuiden. Garcia & Ibarria. 22.03.2008. SC-8 (BCN)	52.46	14.85 (0.28)	14523.30
UK1	United Kingdom, England: Crosby. Smith, Twibell, Vallès & Wilcox. 20.08.2008. (BCN)	53.62	15.52 (0.29)	15178.56
UK2	United Kingdom, Wales: Swansea/Abertawe, Crymlyn Burrows. Guest, Jones, Twibell, Vallès & Woodman. 19.08.2008. (BCN)	51.62	15.63 (0.32)	15286.14

2C value = 14.76 pg) and Belgium-Netherlands (mean 2C value = 14.71 pg) appear as homogeneous groups with non-significant differences. We excluded from the analysis the population from the Cantabrian coast (because of its intermediate, isolated geographic location), which has a mean genome size of 15.62 pg, and also the British ones because of the scarce number of representatives.

It is very likely that the distribution area of the species could have been continuous along the Atlantic coast from the Iberian Peninsula to Netherlands, but fragmentations of this area may have occurred, judging by the presence of the Loredo population on the coast of the Cantabrian sea (Northern Spain). The records of some probably recently disappeared populations from Sestao and Desierto de Bilbao (GREDILLA 1914) referenced in the South Basque Country Flora (ASEGINOLAZA *et al.* 1984), and also others, such as the one from El Sardinero, at Santander (testified at the beginning of the 20th century by herbarium vouchers and not found from the 1980's on), point to this fragmentation too. Contrary to that, neither bibliographic nor herbarium testimonies have been found concerning the existence of the species along the north coast of France to Belgium. In any case, the present results indicate that differences between the two groups created by this recent

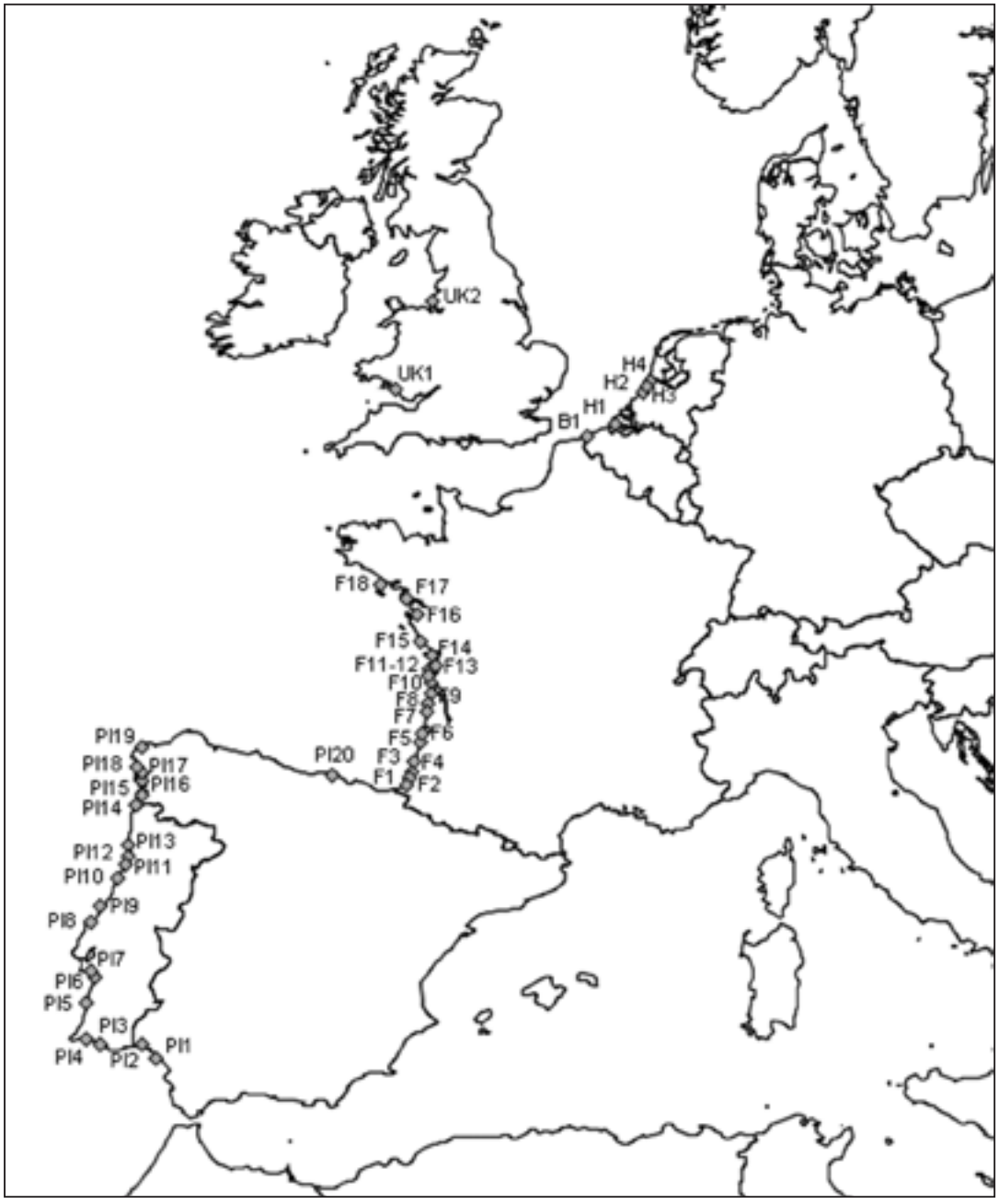

Fig. 1 — Distribution of the populations of *Artemisia crithmifolia* studied. The codes are the same as in Table 1.

fragmentation currently exist. These differences could be related to the lime-content in the soil, which is low in the southern and northern part of the Atlantic province (stretching from Portugal to Norway), with higher figures in the central part, the transitions being especially abrupt near the Gironde (France), but no pattern was found (BAKKER 1976). According to this author, one of the factors that could explain the changes in the vegetation is the number of days with frost, but no relationship has been found in our research. However, maybe the fact that some regions of the Southern part acted as refuges during the glacial periods could possibly have had some effect on the genome size of these populations.

A slightly negative correlation exists between genome size and latitude in *A. crithmifolia* (r=0.32, P=0.03). We have also found a correlation between both variables in *Cheirolophus intybaceus* (GARNATJE *et al.* in press) and closely related taxa

distributed along the Mediterranean coast of Spain and France, but in that case the correlation was positive. We postulated that genome size was smaller in drier and warmer regions of the Southern part of the Iberian Peninsula, but this pattern is not valid for the Atlantic region judging from the results which indicate a higher DNA amount in the warm and dry areas of the South. PRICE *et al.* (1981a;b; 1986) and CASTRO-JIMÉNEZ *et al.* (1989) suggested that plants growing in mesic habitats have larger C-values than those growing in drier ones and OHRI and KHOSHOO (1986) suggested the existence of a positive relationship between genome size and latitude, but conflicting reports about this topic appear in the literature. Pines have been thoroughly investigated from this point of view (see BOGUNIC *et al.* 2007, and references therein) although their results are not conclusive but our findings agree with those of GROTKOPP *et al.* (2004) who found that a Northern latitudinal limit was negatively correlated with genome size in *Pinus*.

We have observed a certain degree of variation in the indumentum in and within several populations. These differences do not have a clear geographical basis and also we did not observe any correlation between glabrescence or pilosity and genome size.

Genome size and insularity - Artemisia crithmifolia also occurs on some small isles, which are dispersed along the Atlantic littoral, close to the coast. In fact, some of them are connected to the continent by bridges (e.g. Île de Ré and Île d'Oléron), or 10-20 km distant such as the Cíes Islands (Galicia, North-West Spain) and Belle Île or Île d'Yeu (France). As expected, the results obtained in three populations of Île d'Oléron and Île de Ré do not show discordant 2C values compared to those of continental ones, indicating that apparently no genome size changes occur on islands located close to the continent.

Genome size of the two British populations is among the highest in all accessions studied, their 2C values being clearly larger than the mean (Table 1). Although no statistical tests can be performed due to the scarce number of insular populations, their relatively high nuclear DNA content is to be pointed out, as well as the fact that the number of individuals found is very small and apparently in recession (A. Jones, Ph. Smith, J.D. Twibell, pers. comm.). Studies including analyses of genome size variation at interpopulational level are scarce (GARCIA *et al.* 2006; GARNATJE *et al.* in press), and a common trend towards an increase or decrease of genome size between continental and insular populations is not detected. Despite the lack of a general trend, our results seem to be in concordance with those found in *Artemisia arborescens*, where increased 2C values were found in insular populations (GARCIA *et al.* 2006).

The origin of these two British populations is uncertain. Authors have hypothesized, at least for the population from Crosby, on the arrival of seed mixtures from the mounds of the adjacent pumping station (SMITH 2005). More studies addressed to elucidate the phylogeographic history of the species will be interesting for a better understanding of the genome size changes, and to confirm the direction towards genome expansion, if any, after island colonization.

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Estudi palinològic d'*Ajania* Poljakov i gèneres relacionats (*Asteraceae, Anthemideae*)

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S'ha dut a terme un estudi morfomètric en grans de pol·len mitjançant tècniques de microscòpia electrònica de rastreig en set gèneres de la subtribu *Artemisiinae* (*Anthemideae*). S'han considerat 46 poblacions, que representen 40 espècies, principalment del gènere *Ajania* (del qual s'han estudiat 31 poblacions de 25 espècies). A més d'aquest gènere, el treball inclou observacions relatives a *Brachanthemum*, *Cancrinia*, *Crossostephium*, *Dendranthema*, *Elachanthemum*, *Hippolytia*, *Kaschgaria*, *Poljakovia* i *Stilpnolepis*. La majoria de les dades constitueixen les primeres observacions per a algunes de les espècies i també per a algun dels gèneres relacionats (*Cancrinia* i *Poljakovia*). S'han confirmat dos models d'ornamentació de l'exina en la tribu, el tipus *Anthemis* (equinat) i el tipus *Artemisia* (equinulat), un resultat congruent amb els estudis previs. El tipus *Artemisia* és exclusiu de la subtribu, mentre que el tipus *Anthemis* és present també més enllà de les *Artemisiinae*, la qual cosa suggereix que podria tractar-se de l'estat ancestral en aquest grup. Aquests tipus pol·línics apareixen clarament diferenciats en base a la seva mida i a l'ornamentació de l'exina. Tanmateix, la seva distribució a la filogènia de les *Artemisiinae* és segregada: el tipus *Anthemis* es troba a *Dendranthema* i gèneres afins, mentre que el tipus *Artemisia* és present en *Artemisia* i gèneres estretament relacionats. Tot i això, hem trobat algunes excepcions estranyes que no segueixen aquesta tendència (p. ex. *Ajania junnanica*, *Elachanthemum* i *Stilpnolepis*), l'origen de les quals es discuteix.

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Palynological study of *Ajania* and related genera (Asteraceae, Anthemideae)

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A morphometrical study of pollen grains using scanning electron microscopy was performed in seven genera belonging to subtribe Artemisiinae (Anthemideae). Forty-six populations representing 40 species were considered, mainly from the genus *Ajania* (31 populations studied of 25 species). This work also includes observations on the genera *Brachanthemum*, *Cancrinia*, *Crossostephium*, *Dendranthema*, *Elachanthemum*, *Hippolytia*, *Kaschgaria*, *Poljakovia* and *Stilpnolepis*. Most data presented here constitute the first pollen observation for some species and genera (*Cancrinia* and *Poljakovia*). Two different pollen exine ornamentations are confirmed for the tribe, *Anthemis*-type (echinate) and *Artemisia*-type (microechinate), a result consistent with previous studies. The *Artemisia*-type is exclusive to the subtribe, whereas the *Anthemis*-type is found present outside Artemisiinae, suggesting that it may represent the ancestral character state for the group. These pollen types appear to be clearly differentiated on the basis of their size and exine ornamentation. Their phylogenetic distribution in Artemisiinae also generally segregates them: the *Anthemis*-type is found in *Dendranthema* and allied genera, whereas the *Artemisia*-type occurs in *Artemisia* and closely related genera. However, we found some very rare exceptions to this trend (e.g. *Ajania junnanica*, *Elachanthemum* and *Stilpnolepis*), the possible origins of which are discussed.

ADDITIONAL KEYWORDS: Artemisiinae – Compositae – *Elachanthemum* – exine ornamentation – molecular systematics – pollen type – *Stilpnolepis*.

INTRODUCTION

Pollen forms and structures in Asteraceae show great variation, as reflected in the numerous pollen types described for the family (Jeffrey, 2007). Pollen characters provide much taxonomically valuable information and have been commonly used as phylogenetic markers. This is the case in subtribe Artemisiinae Less., in which two pollen types were described on the basis of the exine ornamentation (Stix, 1960): the *Anthemis*-type, with obvious spines (echinate), and the *Artemisia-type*, with spinules (microechinate). The exine ultrastructure has been studied for both pollen types (e.g. Skvarla & Turner, 1971 for *Anthemis* L.; Rowley, Claugher & Skvarla, 1999 and references therein, for *Artemisia* L.). These characters are useful, as a complement to external morphology, to separate some groups of genera (Skvarla *et al.*, 1977). However, because the ultrastructure is essentially uniform (Skvarla & Larson, 1965; Skvarla & Turner, 1966; Heywood & Humphries, 1977), the most distinctive trait is the surface ornamentation, i.e. the

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occurrence of spines or spinules (Skvarla & Turner, 1966). Microechinate pollen was first reported by Wodehouse (1926) as restricted to a group of genera including *Artemisia* and some close relatives, which has been confirmed in many studies dealing with *Artemisia* spp. from different geographical origins (e.g. Monoszon, 1948, 1950a, b; Straka, 1952; Stix, 1960; Skvarla & Larson, 1965; Singh & Joshi, 1969; Praglowski, 1971; Vallès, Suárez & Seoane, 1987; Martín, Torrell & Vallès, 2001; Martín *et al.*, 2003; Grigoreva, Korobkov & Tokarev, 2009). Several further studies on pollen exine ornamentation and molecular phylogeny confirmed that each pollen type characterizes one of the main groups of Artemisiinae: *Dendranthema* (DC.) Des Moul. and relatives have the *Anthemis*-type, whereas *Artemisia* and allies show the *Artemisia*-type (Chen & Zhang, 1991; Rowley *et al.*, 1999; Martín *et al.*, 2001, 2003 for the pollen studies; Vallès *et al.*, 2003; Sanz *et al.*, 2008) for the phylogenetic analyses). Exceptions to this trend have been generally considered to be the result of taxonomic misplacement (Martín *et al.*, 2001, 2003). In this sense, pollen type has been used to confirm or justify the segregation of several genera from *Artemisia* and their placement in the *Dendranthema* group and vice versa. One such genus is *Ajania* Poljakov.

The Asian genus *Ajania* comprises *c*. 30–40 species, depending on the authors (Bremer & Humphries, 1993; Bremer, 1994; Kubitzki, 2007), with a large number of representatives in China and Japan and some in Afghanistan, Kazakhstan, Kyrgyzstan, Mongolia, Northern India, Russia and Tadzhikistan. This genus was segregated from *Artemisia* by Poljakov (1955). Tzvelev (1961), in the *Flora of the USSR*, accepted the genus *Ajania* with *c*. 25 species, nine of which grow in the USSR, but considered, using pollen ornamentation as one of the main arguments, that Poljakov (1955) had erroneously combined into *Ajania* some species that should be maintained in *Artemisia*. Tzvelev (1961) pointed out that *Ajania* had evolved from ancestral taxa more closely related to *Dendranthema* and that the adaptation of *Ajania* to Middle Asian steppes and deserts gave rise to a strong resemblance to the representatives of *Artemisia* occupying these areas. In order to explain the similarities between the three genera, Bremer & Humphries (1993) assumed that independent lines have evolved from the same dendranthemoid ancestor. Three species were removed from *Ajania* to constitute a separate new genus, *Phaeostigma* Muldashev (Muldashev, 1982, 1983). This author justified this change based on pollen characters (microechinate pollen), among others, and also pointed out the affinities of this genus with *Artemisia*. He also used palynological features for proposing the combination of *Ajania junnanica* Poljakov within *Artemisia*, because its pollen has 'very small spines' (Muldashev, 1983). Two species of *Ajania* and one of *Phaeostigma* have been recently studied from the palynological point of view (Martín *et al.*, 2001 for *Ajania fastigiata* (C.Winkl.) Poljakov and *A. fruticulosa* (Ledeb.) Poljakov; Martín *et al.*, 2003 for *Phaeostigma salicifolium* (Mattf.) Muldashev). Species of *Ajania* were found to have *Anthemis*-type pollen, which confirms their placement in the *Dendranthema* group, also supported by molecular phylogenetic analyses (Y. Masuda & K. Kondo, pers. comm.; Sanz *et al.* (2008). The representative of *Phaeostigma* included in the study had *Artemisia*-type pollen, a result congruent with the hypothesis of its close relationship with *Artemisia*. Because of their complex taxonomic history, with numerous relocations of species between the two main groups of Artemisiinae, *Ajania* and segregate genera represent a good group for addressing pollen studies in the subtribe.

The present paper aims to provide new pollen data for *Ajania* and some other representatives of Artemisiinae, including *Brachanthemum* DC., *Cancrinia* Kar. & Kir., *Crossostephium* Less., *Dendranthema*, *Elachanthemum* Y.Ling & Y.R.Ling, *Hippolytia* Poljakov, *Kaschgaria* Poljakov, *Poljakovia* Grubov & Filatova and *Stilpnolepis* Krasch. The specific objectives of this study are: (1) to increase the number of palynological data for Artemisiinae; (2) to improve the understanding of the characterization of the two pollen types found in the subtribe through the analysis of new and previous data from our team; (3) to discuss these findings in a phylogenetic framework with a view to contributing to a resolution of questions related to the systematic and phylogenetic relationships within the subtribe; and (4) to consider possible cause(s) for the transition from one pollen type to another.

MATERIAL AND METHODS

Plant material

Pollen grains from dried specimens of plants collected in the field and deposited in BCN (Universitat de Barcelona), HIMC (Inner Mongolia University, Hohhot) and LE (Botanicheskii Institut im. V.L. Komarova, Saint Petersburg) were used to carry out the study (Table 1). Observations using optical and scanning electron microscopy (SEM) were carried out in 46 populations of 40 species of the genera *Ajania* (31 populations of 25 species), *Brachanthemum* (four species), *Cancrinia* (two species), *Crossostephium* (one species), *Dendranthema* (two species), *Elachanthe-*

Table 1. Origin of the populations studied, with the indications of the herbaria where the voucher specimens are deposited

Taxa	Populations
Ajania achilleoides (Turcz.) Poljakov ex Grubov*	Mongolia, Ubsunur, 60 km SW Under-Khangai, Kheltguin-Ula mountains, 16.VIII.1979, Z. Kapamysheva (LE)
A. achilleoides (Turcz.) Poljakov ex Grubov*	Mongolia, Central Gobi, 16 km NE Erdene-Dalai, 4.IX.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
A. achilleoides (Turcz.) Poljakov ex Grubov*	Mongolia, Central Gobi, 46 km NE Erdene-Dalai, 4.IX.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
A. aureoglobosa (W.W.Sm. & Farr.) Muldashev	China, province of Gansu, near Liang Shui, 18.X.1914, E.N. Meyer (LE)
A. fastigiata (Winkl.) Poljakov*	China, autonomous region of Xingian-Uigur, Kashgar, 25 km SW Kiushisha, 1400 m, 19.X.1959, M. Petrov (LE)
A. fruticulosa (Ledeb.) Poljakov*	Kyrgyzstan, mountain pass in the Kurutag mountains, 16.XI.1957, A. Yunatov (LE)
A. fruticulosa (Ledeb.) Poljakov*	Mongolia, Southern Gobi, 10 km S Bulgan, Sh. Dariimaa, Sh. Tsooj, J. Vallès, E. Yatamsuren, 2.IX.2004 (BCN)
A. fruticulosa (Ledeb.) Poljakov*	Mongolia, Southern Gobi, 20 km SW Mandal Oboo, 4.IX.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
A. gracilis (Hook.f. & Thomson) Poljakov ex Tzvelev	Tadzhikistan, Pamiro-Alai, near Kirakul, 5.VIII.?, A. Kushakevich (LE)
A. grubovii Muldashev	Mongolia, Dzhungar Gobi, Mongolian Altai, 17.VIII.1979, V. Grubov (LE)
A. junnanica Poljakov	China, Northern Yunnan, Pe-Cong-Ching, 3200 m, 1909–1911, R. Maire (LE)
A. khartensis (Dunn) C.Shih	China, Gansu, 100 km SW Dunkhun, 2.VIII.1958, M. Petrov (LE)
A. kokanica (Krasch.) Tzvelev	Kyrgyzstan, Northern Alai, high river Shakhimaruan river, 12.VIII.1938, A. Mukhamedzhanov (LE)
A. myriantha (Franch.) Y.R.Ling ex C.Shih	China, Northern and Central Yunnan, mountains near Liao-Do, 2000 m, XI.1910, R. Maire (LE)
A. nana (Krasch.) Muldashev	China, Northern Szetschuan, between Epor and Kanguang, 19.X.1885, G.N. Potanin (LE)
A. nematoloba (Hand.-Mazz.) Ling ex C.Shih	Mongolia, Alaschan mountain, VIII.1880, N.M. Przewalski (LE)
A. nubigena (Wall.) C.Shih	Nepal, Bagmati zone, Kasuwa district, below Khanyyin, 3650 m, 22.IX.1966, D. Nicholson (LE)
A. pacifica (Nakai) K.Bremer & Humphries	Japan, Honshu prefecture, Chiba, 10 m, 1.XII.1973, M. Togashi (LE)
A. pallasiana (Fisch. ex Besser) Poljakov	China, Kheiluntszyn province, Yaohe district, Hualatszy, 10.IX.1950, Chang Kiang-Cheng (LE)
Ajania parviflora (Grun.) Ling	China, Inner Mongolia, Alxa province, road S128, km 102, near Suhait, sandy and stony soils, 6.IX.2007, J. Vallès, S.W. Zhao (BCN)
A. potaninii (Krasch.) Poljakov	China, Gansu, Fin-Ten-Lin mountain pass, 1885, G.N. Potanin (LE)
A. przewalskii Poljakov	Mongolia, Alaschan, 9.VIII.1880, N.M. Przewalski (LE)
A. purpurea C.Shih	China, Tibet, Yan-Uzi-Uzyan basin, Nru-Chu canyon, 25.VII.1900, V. Ladyguin (LE)

Table 1. *Continued*

Taxa	Populations
A. remotipinna (Hand.-Mazz.) Y.Ling & C.Shih	Mongolia, near Kalgans, 1870, A. Lomonossov (LE)
A. roborowskii Muldashev	China, Gansu, 25 km S Lanchisou, 12.VIII.1958, M. Petrov (LE)
A. rupestris (Matsum. & Koidz.) Muldashev*	Japan, Sirano-Asamajama, Happu-Giku, IX.1889, Tschonoski (LE)
A. rupestris (Matsum. & Koidz.) Muldashev*	Japan, Happu-Giku, IX.1889, Tschonoski (LE)
A. scharnhorstii (Regel & Schmalh.) Tzvelev*	China, Tian-Shan, Bogdo-Ola mountains, near Urumqi, 26.VIII.1908, G. Merzbacher (LE)
A. scharnhorstii (Regel & Schmalh.) Tzvelev*	China, Tian-Shan, Bogdo-Ola mountains, 29.VIII.1908, G. Merzbacher (LE)
A. tibetica (Hook.f. & Thomson) Tzvelev	China, Tibet, Peku lake, 4.650 m, 31.VIII.1991 (LE)
A. trilobata Poljakov	Kazakhstan, Semirschen region, Przhevalski district, canyon of river Karakol, 22.VII.1913, V. Saposhnikov (LE)
Brachanthemum gobicum Krasch.	Mongolia, Ubur-Khangai, Arms Bogd mountains, 31.VIII.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
Brachanthemum kirghisorum Krasch.	Kyrgyzstan, Alatau mountains, Issik-Kul lake basin, 15 km W of Kyzylty, 1650 m, 20.VII.1970, N.N. Izmailova, S.S. Ikonnikov, D.M. Ladugina (HIMC)
Brachanthemum mongolorum Grubov	Mongolia, Northern region, 15 km W Barun-Matad-Ula, 12.VIII.1989. Ch. Sanchir, V. Khramtsov (LE)
Brachanthemum pulvinatum (Hand.-Mazz.) C.Shih	China, 4.IX.1990 (HIMC)
Cancrinia discoidea (Ledeb.) Poljakov ex Tzvelev	Mongolia, Southern Gobi, 17 km NE Bulgan, 5.IX.1995, A. Bayandzag (BCN)
Cancrinia maximowiczii C.Winkl.	China, 21.VII.1980 (HIMC)
Crossostephium chinense (L.) Makino	China, Chzhchi province, Beijing surroundings, Pokhuashan mountains, 1850–1858, S.M. Vazilievskii (LE)
Dendranthema mongolicum (Y.R.Ling) Tzvelev	Mongolia, Arkhangai, mountain pass Sagan-Davaa, near Tsetserleg, 2200 m, 25.VIII.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
Dendranthema zawadskii (Herbich) Tzvelev	Mongolia, Bulgan, Khugunkhaan mountains, 2000 m, 25.VIII.2004, Sh. Dariimaa, Sh. Tsooj, J. Vallès (BCN)
Elachanthemum intricatum (Franch.) Y.Ling & Y.R.Ling	Mongolia, Suothern Gobi, Gobi Altai, near Gurvan Tes, 5.IX.1979, V.I, Grubov, A. Muldashev, Sh. Dariimaa (BCN)
Hippolytia alashanensis (Ling) C.Shih	China, Inner Mongolia, Alxa province, SW slopes of Helan Shan, Tonguan, 5.IX.2007, J. Vallès, S.W. Zhao (BCN)
Hippolytia trifida (Turcz.) Poljakov	China, 11.VIII.1994 (HIMC)
Kaschgaria komarovii (Krasch. & Rubtzov) Poljakov	Mongolia, Dzhungar Gobi, near Bulgan, 29.VII.1988, I.A. Gubanov, Sh. Dariimaa, R.V. Kamelin (BCN)
Poljakovia falcatolobata (Krasch.) Grubov & Filatova	China, Burkhan-Budda mountains, Khatu canyon, 25.VII.1911, V.N. Ladyguin (LE)
Stilpnolepis centiflora (Maxim.) Krasch.	China, 15.IX.1963 (HIMC)

Asterisks (*) indicate different populations of the same species studied.
BCN, Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona; HIMC, Faculty of Life Sciences, Inner Mongolia University, Hohhot; LE, Botanicheskii Institut im. V.L. Komarova, Saint Petersburg.

mum (one species), *Hippolytia* (two species), *Kaschgaria* (one species), *Poljakovia* (one species) and *Stilpnolepis* (one species).

Pollen observations and measurements

Pollen was obtained by dissecting dehydrated anthers in 96% ethanol. Samples were examined with a scanning electron microscope after acetolysis following Avetissian's (1950) micro-method. Observations were then carried out after coating with gold using a diode sputtering and a Hitachi 52300 scanning microscope at l5 kV. For biometrical measurements, pollen samples were acetolysed following the same method, mounted on glycerogelatine and sealed. Measurements were made using a Visopan apparatus (Reichert, Austria). For each specimen, 15 fully developed grains were measured, except in *Ajania aureoglobosa* (W.W.Sm. & Farr.) Muldashev (seven grains), *A. fruticulosa* (Ledeb.) Poljakov sample 57 (eight grains), *A. junnanica* Poljakov (10 grains) and *Crossostephium chinense* Merr. (five grains). The parameters considered, following Erdtman (1969), Faegri & Iversen (1975) and Reitsma (1970), were: polar diameter (P), equatorial diameter (E) and sphericity (P/E). For each, the arithmetic mean and standard deviation were calculated. In the case of pollen grains with spiny ornamentation, the height of the spine was also measured from the tip to the start of the multiperforate basement. The density of supratectal spines/spinules was calculated in the mesocolpium area by counting the number of spines/spinules in 25 μm^2 of the pollen surface. We also calculated an approximate pollen volume [V, calculated using the ellipsoid formula: $V = 4/3\Pi(1/2P)(1/2E)^2$] and counted the number of spines/spinules in 25 μm^2 of the pollen surface. The pollen terminology used follows Reitsma (1970).

Statistical analyses

A database grouping present and previous results (Martín *et al.*, 2001, 2003) in the whole subtribe Artemisiinae was constructed for comparative purposes between the different morphological traits of each pollen type (e.g. volume, spine height and spine density; Appendix). The pollen size measures being dependent on the preparation method (Reitsma, 1969), we restricted this database to the species processed with an identical protocol for allowing comparisons. StatGraphics Plus 5.1 (Statistical Graphic Corp.) was used to carry out the Kruskal–Wallis contrasts. This is a non-parametric test that does not involve any assumption about the frequency of distribution of the variables and therefore fits our data better. Some of the 76 representatives of Anthemideae listed are not currently classified in Artemisiinae (as redefined by Oberprieler, Himmelreich & Vogt, 2007) and we have not included them for the statistical analyses.

Molecular phylogeny

Sequences for external (ETS) and internal (ITS) transcribed spacers from GenBank were analysed to provide a phylogenetic framework for discussing pollen type distribution and evolution in Artemisiinae. Representatives of the genera *Achillea*, *Lepidolopsis* and *Tanacetum* were chosen as outgroups for Artemisiinae on the basis of the analyses of Anthemideae of Oberprieler *et al.* (2007). Sequences were edited with BioEdit v7.0.9 (Ton Hall, Ibis Biosciences). The alignment was first performed using T-COFFEE as implemented by BioX 1.1b1 [E. Lagercrantz (http://www.lagercrantz.name/software/*biox/*)] and then this was manually revised in MacClade 4.08 (Maddison & Maddison, 2005). MrModeltest 2.2 (Nylander, 2004) was used to select the best-fit models of nucleotide substitution for our datasets. Bayesian inference analyses performed with MrBayes 3.1.1. (Huelsenbeck & Ronquist, 2001) were initiated with random starting trees and run for 10^6 generations. Four Markov chains were run simultaneously and trees were sampled every 100 generations, which resulted in 10 000 sampled trees. To ensure the Markov chains had become stable, log-likelihood values for sampling trees were plotted against generation time and those before stationarity were discarded as 'burn-in'. A majority-rule consensus tree was obtained with PAUP version 4.0b4a (Swofford, 1999). Posterior probability support (PP) $\geq$ 95% was considered statistically significant. We carried out separate and combined ETS and ITS analyses, restricting the dataset to individuals with both regions sequenced [ITS of *Phaeostigma salicifolium* AM774423 and EF577281, *P. variifolium* EF577283, and *Stilpnolepis centiflora* (Maxim.) Krasch. AY127695, AY127696 were consequently removed]. Clones of ETS for the same individual that grouped together in the separate analysis were combined in a consensus sequence and, if this was not the case, they were introduced separately in the combined dataset. In the same way, ETS and ITS sequences of inconsistent positioning in separate analyses were treated independently in the combined analysis. We also carried out independent ETS and ITS analyses involving the restricted taxonomic sampling of the combined dataset.

RESULTS

Pollen traits of the studied taxa are shown in Table 2, Fig. 1A–X and the Appendix. Results from statistical

Table 2. Pollen characteristics of the taxa studied

Taxa	P (μm)	E (μm)	P/E (μm)	Pollen type	Spine height (μm)
*Ajania achilleoides**	22.91–27.08 X = 24.92 (1.33)	20.83–27.08 X = 23.74 (1.63)	1.04	*Anthemis*	3.24–4.28 X = 3.60 (0.40)
*A. achilleoides**	20.83–27.08 X = 22.91 (1.92)	17.70–25.00 X = 22.14 (1.69)	1.03	*Anthemis*	2.75–3.18 X = 2.92 (0.16)
*A. achilleoides**	20.83–25.00 X = 22.91 (1.36)	18.75–22.91 X = 21.93 (1.27)	1.04	*Anthemis*	2.75–3.10 X = 2.85 (0.15)
A. aureoglobosa	19.79–25.00 X = 22.46 (1.88)	20.83–22.91 X = 21.72 (1.11)	1.03	*Anthemis*	2.75–3.10 X = 2.96 (0.17)
A. fastigiata	22.91–31.25 X = 26.31 (2.17)	22.91–31.25 X = 25.41 (1.92)	1.03	*Anthemis*	3.63–4.41 X = 3.83 (0.33)
*A. fruticulosa**	20.83–33.33 X = 25.64 (4.41)	20.83–28.12 X = 23.34 (2.89)	1.09	*Anthemis*	3.76–4.15 X = 3.91 (0.14)
*A. fruticulosa**	22.91–27.08 X = 25.27 (1.07)	22.91–29.16 X = 25.20 (1.58)	1.00	*Anthemis*	3.27–4.31 X = 3.75 (0.41)
*A. fruticulosa**	20.83–29.16 X = 25.41 (2.18)	18.75–26.04 X = 23.81 (1.96)	1.06	*Anthemis*	2.59–4.41 X = 3.57 (0.74)
A. gracilis	21.87–28.12 X = 24.38 (1.73)	20.83–25.00 X = 22.65 (1.42)	1.07	*Anthemis*	1.98–2.84 X = 2.30 (0.34)
A. grubovii	25.00–29.16 X = 26.94 (1.51)	22.91–29.16 X = 25.20 (1.67)	1.06	*Anthemis*	1.72–2.06 X = 1.92 (0.12)
A. junnanica	14.58–20.83 X = 18.33 (2.31)	12.5–18.75 X = 16.45 (2.68)	1.11	*Artemisia*	–
A. khartensis	22.91–29.16 X = 24.85 (1.61)	20.83–27.08 X = 24.30 (1.79)	1.02	*Anthemis*	2.75–3.10 X = 2.92 (0.12)
A. kokanica	25.00–31.25 X = 26.73 (1.87)	22.91–27.08 X = 25.55 (1.17)	1.04	*Anthemis*	3.37–4.15 X = 3.68 (0.28)
A. myriantha	20.83–27.08 X = 23.88 (1.49)	20.83–26.04 X = 23.67 (1.39)	1.00	*Anthemis*	2.15–2.32 X = 2.21 (0.09)
A. nana	20.83–31.25 X = 24.51 (2.66)	20.83–26.04 X = 23.39 (1.46)	1.04	*Anthemis*	3.11–3.89 X = 3.39 (0.39)
A. nematoloba	14.58–23.95 X = 21.03 (2.36)	14.58–21.87 X = 19.64 (2.29)	1.07	*Anthemis*	2.06–2.15 X = 2.13 (0.04)
A. nubigena	25.00–29.16 X = 27.77 (1.28)	25.00–29.16 X = 27.14 (1.59)	1.02	*Anthemis*	3.10–4.31 X = 3.54 (0.45)
A. pacifica	35.41–41.66 X = 38.39 (2.11)	33.33–40.62 X = 34.05 (8.37)	1.12	*Anthemis*	3.89–4.93 X = 4.43 (0.43)
A. pallasiana	20.83–33.33 X = 27.56 (4.07)	20.83–33.33 X = 26.80 (3.57)	1.02	*Anthemis*	3.62–4.48 X = 4.03 (0.35)
A. parviflora	16.00–24.00 X = 19.72 (2.09)	18.00–22.00 X = 19.60 (1.20)	1.00	*Anthemis*	3.28–3.88 X = 3.67 (0.22)
A. potaninii	21.87–25.00 X = 23.18 (1.21)	18.75–25.00 X = 21.94 (2.06)	1.05	*Anthemis*	2.58–3.01 X = 2.84 (0.17)
A. przewalskii	22.91–25.00 X = 23.60 (0.85)	17.70–25.00 X = 22.28 (1.79)	1.05	*Anthemis*	3.11–3.89 X = 3.26 (0.34)
A. purpurea	25.00–29.16 X = 25.62 (1.23)	22.91–29.16 X = 24.64 (1.70)	1.03	*Anthemis*	2.58–3.10 X = 2.87 (0.22)
A. remotipinna	22.91–29.16 X = 25.62 (1.56)	20.83–28.12 X = 24.99 (1.71)	1.02	*Anthemis*	3.76–4.15 X = 3.96 (0.17)

Table 2. *Continued*

Taxa	P (μm)	E (μm)	P/E (μm)	Pollen type	Spine height (μm)
A. roborowskii	22.91–31.25 X = 26.31 (2.85)	18.75–31.25 X = 24.16 (3.03)	1.08	*Anthemis*	2.58–2.75 X = 2.63 (0.07)
*A. rupestris**	22.91–28.12 X = 24.37 (1.61)	22.91–27.08 X = 24.02 (1.44)	1.01	*Anthemis*	3.01–3.62 X = 3.30 (0.29)
*A. rupestris**	22.91–29.16 X = 26.31 (1.98)	20.83–29.16 X = 24.85 (2.29)	1.05	*Anthemis*	3.10–3.62 X = 3.37 (0.23)
*A. scharnhorstii**	22.91–35.41 X = 26.38 (3.09)	20.83–27.08 X = 24.16 (2.16)	1.09	*Anthemis*	2.59–3.63 X = 3.21 (0.43)
*A. scharnhorstii**	22.91–31.25 X = 26.31 (2.37)	20.83–29.16 X = 23.95 (2.55)	1.09	*Anthemis*	2.84–3.62 X = 3.08 (0.31)
A. tibetica	25.00–29.16 X = 25.69 (1.28)	20.83–27.08 X = 23.60 (1.70)	1.08	*Anthemis*	3.89–4.67 X = 4.30 (0.29)
A. trilobata	22.91–27.0 X = 25.20 (1.37)	22.91–26.04 X = 24.65 (1.01)	1.02	*Anthemis*	3.11–3.63 X = 3.31 (0.21)
Brachanthemum gobicum	33.33–37.5 X = 33.81 (1.17)	32.29–35.41 X = 33.67 (1.01)	1.00	*Anthemis*	3.62–4.56 X = 4.03 (0.41)
B. kirghisorum	24.80–34.00 X = 29.16 (2.55)	24.80–30.00 X = 27.56 (1.70)	1.05	*Anthemis*	3.2–4.02 X = 3.65 (0.32)
B. mongolorum	22.91–33.33 X = 29.02 (3.47)	22.91–31.25 X = 28.33 (3.03)	1.02	*Anthemis*	2.93–4.13 X = 3.56 (0.49)
B. pulvinatum	24.00–26.00 X = 24.9 (0.55)	22.00–25.33 X = 23.27 (0.84)	1.07	*Anthemis*	2.83–3.2 X = 2.99 (0.13)
Cancrinia discoidea	22.91–27.08 X = 24.16 (1.53)	20.83–25.00 X = 22.63 (1.54)	1.06	*Anthemis*	2.75–3.18 X = 3.01 (0.17)
C. maximowiczii	23.60–28.65 X = 25.98 (2.62)	20.00–25.07 X = 23.52 (1.56)	1.10	*Anthemis*	4.02–4.62 X = 4.26 (0.22)
Crossostephium chinense	19.48–27.08 X = 24.13 (3.18)	20.83–27.08 X = 22.87 (2.58)	1.05	*Artemisia*	–
Dendranthema mongolicum	31.25–35.41 X = 33.60 (0.99)	29.16–35.41 X = 32.01 (2.24)	1.04	*Anthemis*	4.15–5.71 X = 4.72 (0.64)
D. zawadskii	29.16–35.41 X = 32.42 (2.11)	27.08–33.33 X = 31.45 (2.01)	1.03	*Anthemis*	4.93–6.49 X = 5.50 (0.59)
Elachanthemum intricatum	22.91–25.00 X = 23.32 (0.95)	20.83–23.95 X = 22.63 (0.91)	1.03	*Artemisia*	–
Hippolytia alashanensis	24.00–28.80 X = 26.96 (1.55)	23.20–30.00 X = 26.6 (1.83)	1.01	*Anthemis*	3.73–4.17 X = 3.97 (0.16)
H. trifida	27.20–34.00 X = 30.94 (1.74)	24.80–34.00 X = 29.82 (2.41)	1.03	*Anthemis*	3.58–4.44 X = 4.07 (0.39)
Kaschgaria komarovii	20.83–22.91 X = 21.80 (0.99)	20.83–25.00 X = 21.94 (1.27)	0.99	*Artemisia*	–
Poljakovia falcatolobata	25.00–33.33 X = 29.64 (2.11)	20.83–33.33 X = 27.42 (3.96)	1.08	*Anthemis*	3.27–4.31 X = 3.75 (0.37)
Stilpnolepis centiflora	24.00–28.00 X = 26.00 (0.89)	22.00–26.00 X = 24.00 (1.26)	1.08	*Anthemis*	2.38–2.83 X = 2.58 (0.21)

Asterisks (*) indicate different populations of the same species studied (presented in the same order as in Table 1). P, polar axis [range; X, mean values (standard deviation)]; E, equatorial axis [range; X, mean values (standard deviation)]; P/E, sphericity. Spine height: range; X, mean values (standard deviation).

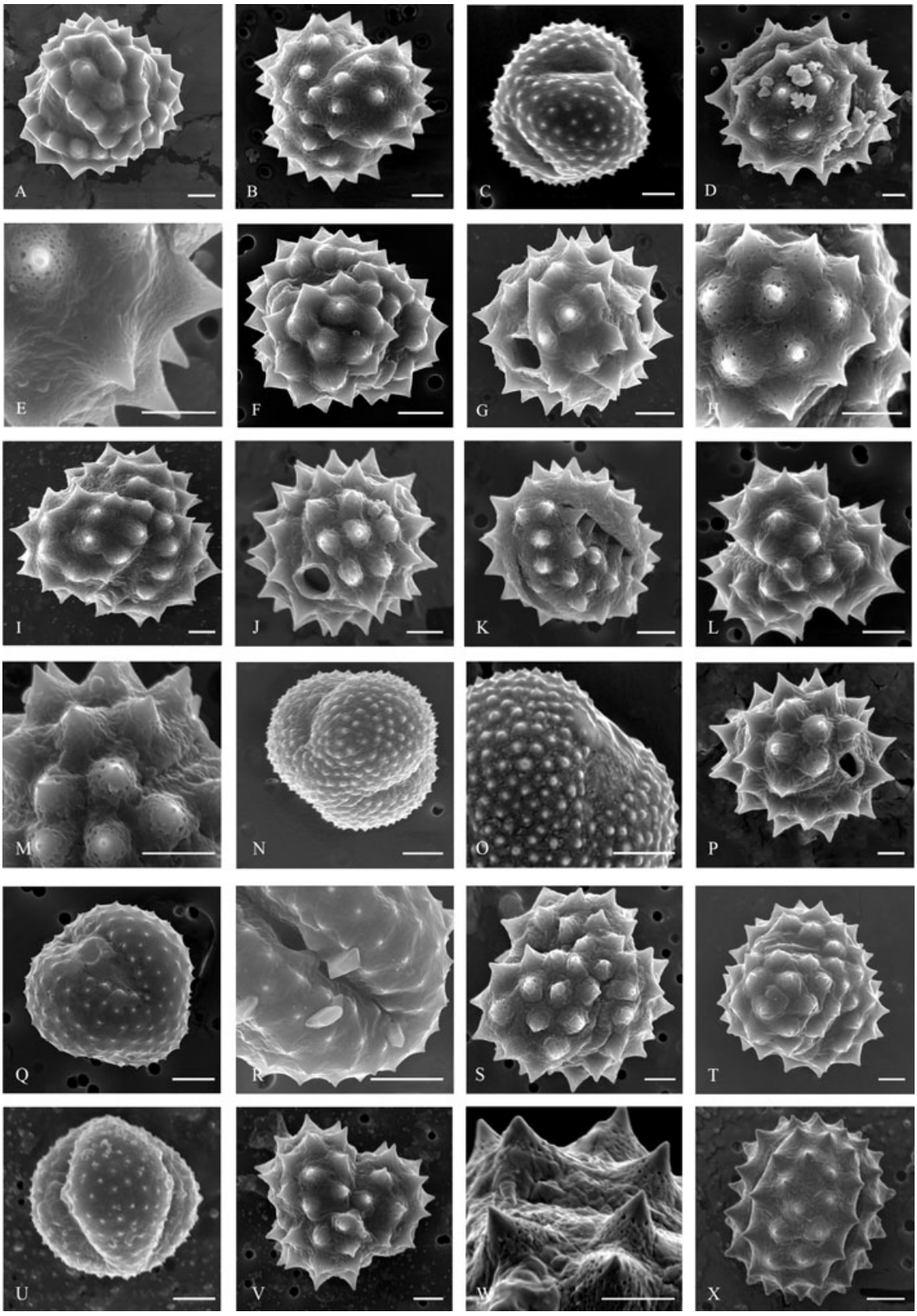
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Figure 1. Pollen grains of some of the taxa studied at scanning electron microscopy (SEM). A, *Ajania fruticulosa*. B, *A. grubovii*. C, *A. junnanica*. D, *A. pacifica*. E, *A. pacifica* (exine detail from mesocolpium). F, *A. nematoloba*. G, *A. nubigena*. H, *A. roborowskii* (apocolpium). I, *Brachanthemum gobicum*. J, *B. gobicum* (exine detail from mesocolpium). K, *B. kirghisorum*. L, *B. pulvinatum*. M, *Cancrinia discoidea*. N, *C. maximowiczii* (exine detail from apocolpium). O, *Crossostephium chinense*. P, *C. chinense* (exine detail from apocolpium). Q, *Dendranthema zawadskii*. R, *Elachanthemum intricatum*. S, *E. intricatum* (exine detail, colpus). T, *Hippolytia alashanensis*. U, *H. trifida*. V, *Kaschgaria komarovii*. W, *Poljakovia falcatolobata*. X, *Stilpnolepis centiflora*. Scale bar, 5 μm.

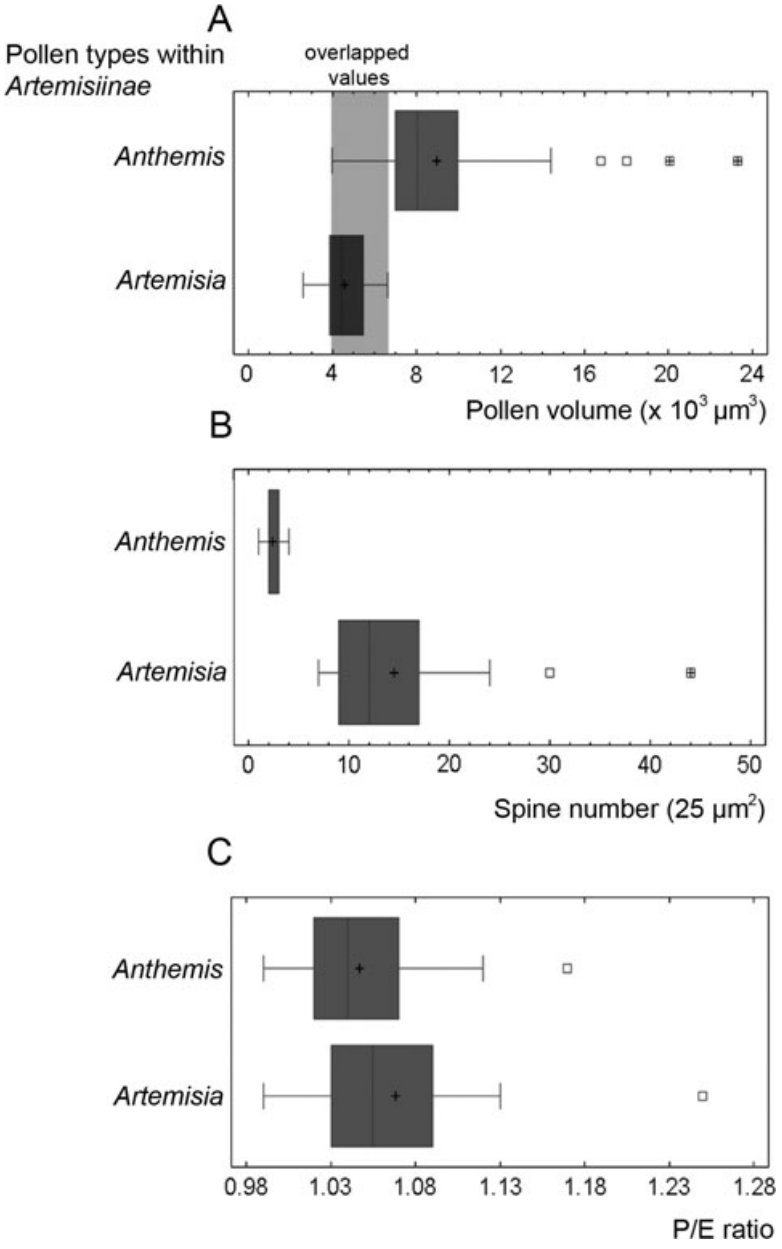
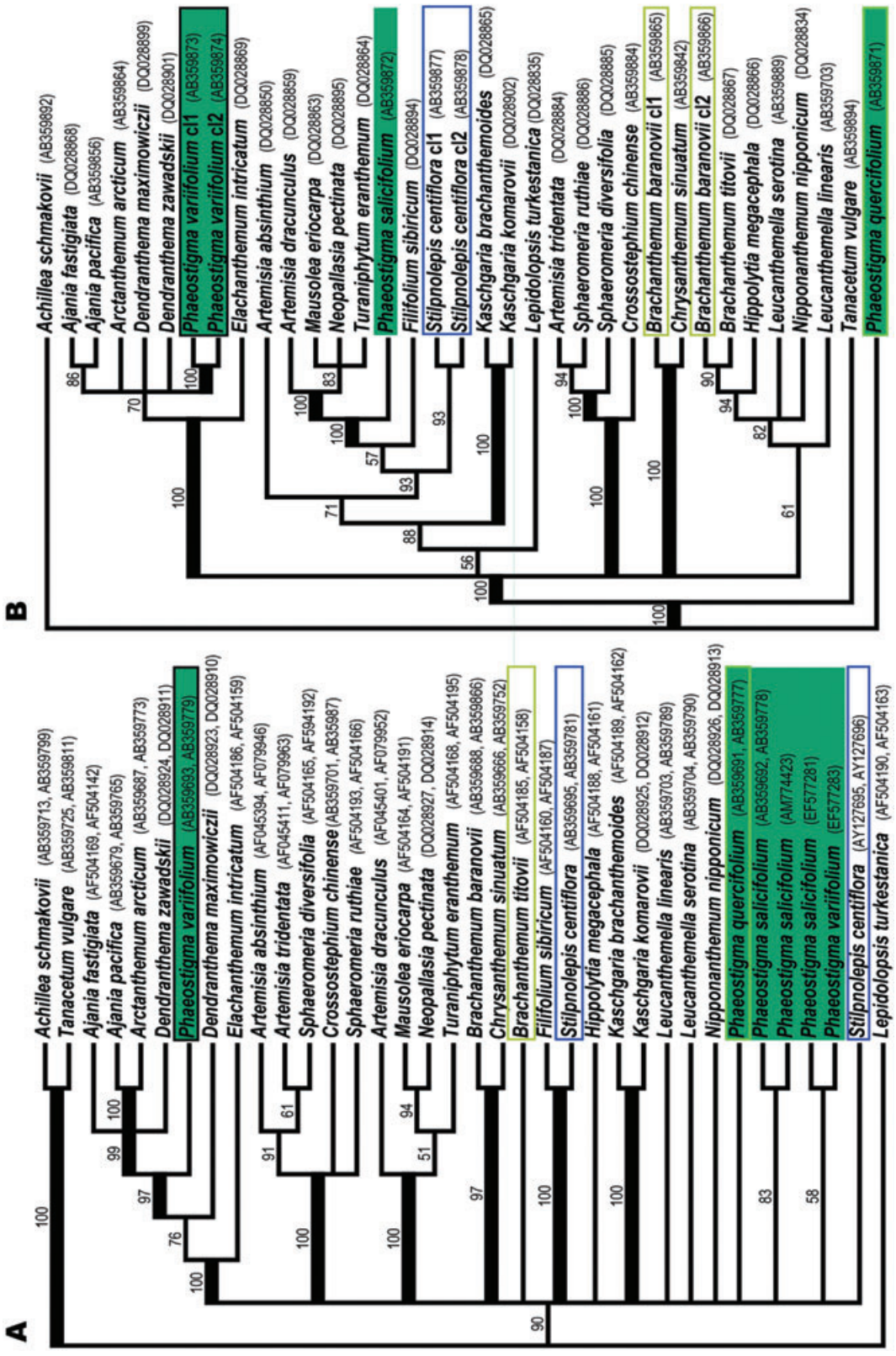


Figure 2. Box-and-whisker plots from statistical analyses of some pollen traits vs. pollen types. A, pollen volume. B, spine number. C, polar diameter/equatorial diameter (P/E ratio).

analyses are presented in Fig. 2A–C and from phylogenetic analyses in Fig. 3A–C. The studied pollen grains of Artemisiinae share the following features: they are 3-zonocolporate, isopolar and have radial symmetry. The surface ornamentation is composed of supra-tectal spines or spinules. Consistently with the previous palynological works (Chen & Zhang, 1991; Martín *et al.*, 2001, 2003 and references therein), the


A
Achillea schmakovii (AB359713, AB359799)
Tanacetum vulgare (AB359725, AB359811)
Ajania fastigiata (AF504169, AF504142)
Ajania pacifica (AB359679, AB359765)
Arctanthemum arcticum (AB359687, AB359773)
Dendranthema zawadskii (DQ028924, DQ028911)
Phaeostigma variifolium (AB359693, AB359779)
Dendranthema maximowiczii (DQ028923, DQ028910)
Elachanthemum intricatum (AF504186, AF504159)
Artemisia absinthium (AF045394, AF079946)
Artemisia tridentata (AF045411, AF079963)
Sphaeromeria diversifolia (AF504165, AF594192)
Crossostephium chinense (AB359701, AB35987)
Sphaeromeria ruthiae (AF504193, AF504166)
Artemisia dracunculus (AF045401, AF079952)
Mausolea eriocarpa (AF504164, AF504191)
Neopallasia pectinata (DQ028927, DQ028914)
Turaniphytum eranthemum (AF504168, AF504195)
Brachanthemum baranovii (AB359688, AB359866)
Chrysanthemum sinuatum (AB359666, AB359752)
Brachanthemum titovii (AF504185, AF504158)
Filifolium sibiricum (AF504160, AF504187)
Stilpnolepis centiflora (AB359695, AB359781)
Hippolytia megacephala (AF504188, AF504161)
Kaschgaria brachanthemoides (AF504189, AF504162)
Kaschgaria komarovii (DQ028925, DQ028912)
Leucanthemella linearis (AB359703, AB359789)
Leucanthemella serotina (AB359704, AB359790)
Nipponanthemum nipponicum (DQ028926, DQ028913)
Phaeostigma quercifolium (AB359691, AB359777)
Phaeostigma salicifolium (AB359692, AB359778)
Phaeostigma salicifolium (AM774423)
Phaeostigma salicifolium (EF577281)
Phaeostigma variifolium (EF577283)
Stilpnolepis centiflora (AY127695, AY127696)
Lepidolopsis turkestanica (AF504190, AF504163)
B
Achillea schmakovii (AB359892)
Ajania fastigiata (DQ028868)
Ajania pacifica (AB359856)
Arctanthemum arcticum (AB359864)
Dendranthema maximowiczii (DQ028899)
Dendranthema zawadskii (DQ028901)
Phaeostigma variifolium cl1 (AB359873)
Phaeostigma variifolium cl2 (AB359874)
Elachanthemum intricatum (DQ028869)
Artemisia absinthium (DQ028850)
Artemisia dracunculus (DQ028859)
Mausolea eriocarpa (DQ028863)
Neopallasia pectinata (DQ028895)
Turaniphytum eranthemum (DQ028864)
Phaeostigma salicifolium (AB359872)
Filifolium sibiricum (DQ028894)
Stilpnolepis centiflora cl1 (AB359877)
Stilpnolepis centiflora cl2 (AB359878)
Kaschgaria brachanthemoides (DQ028865)
Kaschgaria komarovii (DQ028902)
Lepidolopsis turkestanica (DQ028835)
Artemisia tridentata (DQ028884)
Sphaeromeria ruthiae (DQ028886)
Sphaeromeria diversifolia (DQ028885)
Crossostephium chinense (AB359884)
Brachanthemum baranovii cl1 (AB359865)
Chrysanthemum sinuatum (AB359842)
Brachanthemum baranovii cl2 (AB359866)
Brachanthemum titovii (DQ028867)
Hippolytia megacephala (DQ028866)
Leucanthemella serotina (AB359889)
Nipponanthemum nipponicum (DQ028834)
Leucanthemella linearis (AB359703)
Tanacetum vulgare (AB359894)
Phaeostigma quercifolium (AB359871)

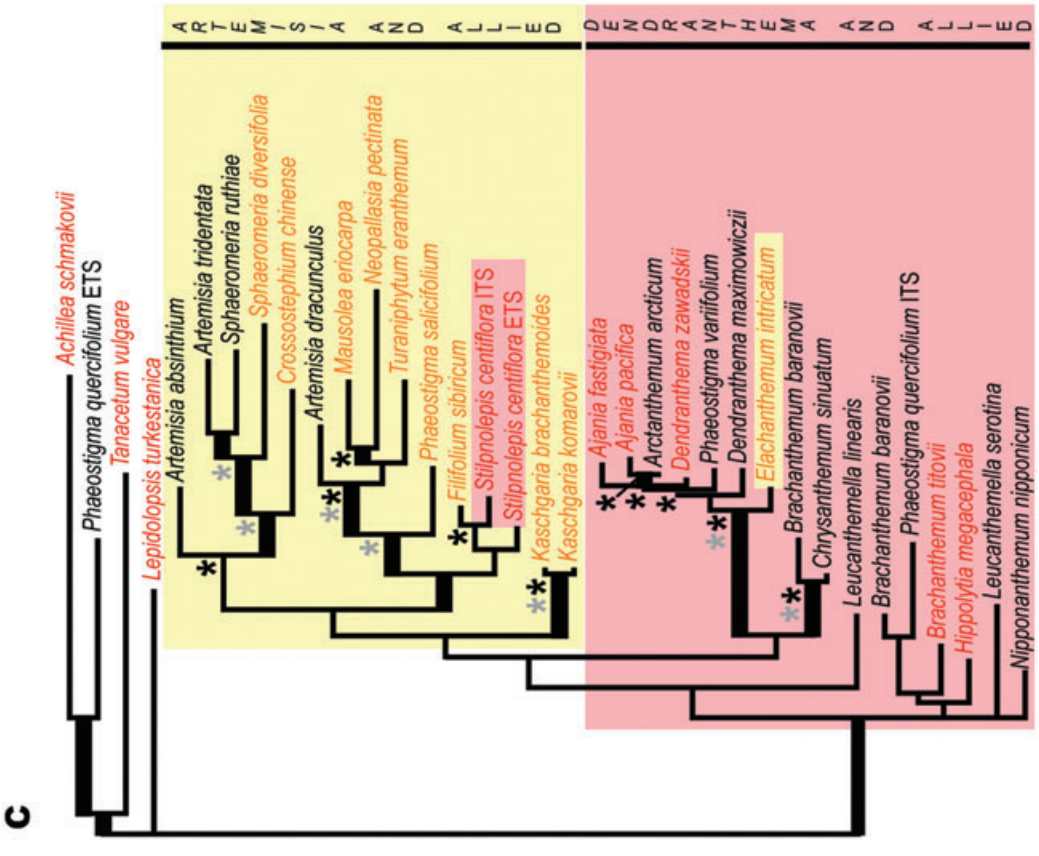


Figure 3. Bayesian phylogenetic inferences. Supported branches (PP ≥ 95%) are indicated in bold. PP values and GenBank accessions are provided for the first two trees. A, ITS majority rule consensus with SYM+G model (the GTR+G model was also selected by MrModeltest and gives similar results; data not shown). B, ETS majority rule consensus with GTR+G model. C, Combined ITS and ETS phylogram with GTR+I+G model (the GTR+G model, also selected by MrModeltest, gives comparable results; data not shown). Branches independently supported (PP ≥ 95%) by single ETS and ITS analyses involving the restricted taxonomic sampling of combined dataset are indicated on the combined tree with * (grey) for ETS and * (black) for ITS. Taxa with known pollen type are written in yellow (pale grey in the print version) for *Artemisia* and in red (dark grey in the print version) for *Anthemis*-type. ETS, external transcribed spacer; ITS, internal transcribed spacer; PP, posterior probability.

pollen observed in the present study can be assigned either to *Anthemis*-type or the *Artemisia*-type.

Anthemis pollen type (Fig. 1A, B, D–N, Q, T, U, W, X)

The shape is mainly spherical, but frequently slightly prolate and slightly oblate in some cases. Mean spine length ranges from 1.92 ± 0.12 μm (*Ajania grubovii* Muldashev) to 5.50 ± 0.50 μm (*Dendranthema zawadskii* (Herbich) Tzvelev), which corresponds to an echinate pollen. The spines are conical, with convex sides, gradually tapering into pointed tips. *Dendranthema* has larger spines than other genera of the group (4.5–5.5 μm), whereas the maximum spine length found in the remaining genera is 4.4 μm. From one [e.g. *Ajania khartensis* (Dunn) C.Shih, *A. pacifica* (Nakai) K.Bremer & Humphries, *Brachanthemum gobicum* Krasch., *Dendranthema* spp.] to four [e.g. *Ajania aureoglobosa* (W.W.Sm. & Farr.) Muldashev, *A. fastigiata, A. fruticulosa*] ornamental elements (spines) are found per 25 μm^2 of pollen surface. Volumes vary between 3966.6 [*Ajania parviflora* (Grun.) Ling] and 23 305.1 μm^3 [*Ajania pacifica* (Nakai) K.Bremer & Humphries].

Artemisia pollen type (Fig. 1C, O, P, R, S, V)

The shape is spherical, although in some cases slightly prolate or oblate. The exine is microechinate, with spinules measuring < 1 μm in height. The spinules are conical, approximately as long as wide (at the base), with blunt tips. Density of ornamental elements range from seven [*Elachanthemum intricatum* (Franch.) Y.Ling & Y.R.Ling, *Phaeostigma salicifolium* (Appendix)] to 44 [*Vesicarpa potentilloides* Rydb. (Appendix)] per 25 μm^2 of pollen surface. Four species show a particularly high density of spinules (≥ 24 spinules per 25 μm^2 of pollen surface): *Ajaniopsis penicilliformis* C.Shih, *Chamartemisia compacta* Rydb., *Sphaeromeria diversifolia* Rydb. and *Vesicarpa potentilloides* (Appendix). Volumes vary between 2597.1 (*Ajania junnanica*) to 6608.3 μm^3 (*Crossostephium chinense*).

Genus *Ajania* (Fig. 1A–G)

This genus has the general morphological traits described for the *Anthemis* pollen-type [with the exception of *A. junnanica* (Fig. 1C)]. The pollen shape is spheroidal, slightly prolate in most cases and sometimes slightly oblate. Sometimes perforations of the exine appear between the spines (= ornamental elements). The same structures were reported in *Artemisia* (Praglowski, 1971; Vallès *et al.*, 1987) and they correspond to the microchannels described by Rowley & Dahl (1977), Rowley, Dahl & Rowley (1981) and Rowley *et al.* (1999) in their ultrastructural study of the exines of *A. vulgaris* L. Pollen volumes vary 5.8-fold from 3966.6 μm^3 (*A. parviflora*) to 23 305.1 μm^3 (*A. pacifica*), but the shape is quite constant in the species of this genus studied (P/E ratio ranges from 1.00 to 1.12).

DISCUSSION

The comparison of the two pollen types highlights some strong differences. The mean of the *Anthemis* pollen-type volume (V_1) is significantly larger (almost twice) than that of the *Artemisia*-type (V_2) ($V_1 = 8961.2\ \mu m^3$; $V_2 = 4574.6\ \mu m^3$; $P < 0.05$), with overlapping values between the volumes 3966.6 and 6608.3 μm^3 (Fig. 2A). The exine surface sculpture also clearly discriminates between these pollen types, the *Artemisia*-type having much smaller ornamentation elements than the *Anthemis*-type (Appendix) and significantly more abundant (the mean of ornamental elements found per 25 μm^2 of pollen surface is 2.36 for *Anthemis* and 14.52 for *Artemisia* pollen types; $P < 0.05$; Fig. 2B). Both size and density of exine ornamentation thus show exclusive values for each pollen type. No difference was found in P/E ratio ($P > 0.05$; Fig. 2C) and the shape of both pollen types is thus quite similar (the mean of P/E values is 1.04 for *Anthemis*, and 1.06 for *Artemisia* pollen types).

Distribution of the pollen types throughout Artemisiinae: the segregation mostly maintained

As expected, according to previous work (Sanz *et al.*, 2008), pollen types are segregated in the phylogenetic trees and characterize the two main groups of Artemisiinae, the *Artemisia* and *Dentranthema* groups (Fig. 3C). This confirms their value as phylogenetic markers in the tribe. However, some exceptions to this trend were found.

The molecular evidence places *Elachanthemum intricatum* in the *Dendranthema* group and *Stilpnolepis centiflora* in the *Artemisia* group, whereas these species both have the pollen type of the other group (Fig. 3C). Both belong to monotypic genera, segregated from *Artemisia* (Krascheninnikov, 1946 for *Stilpnolepis*; Ling & Ling, 1978 for *Elachanthemum*). Shih (1985) combined *Elachanthemum* spp. with *Stilpnolepis*. Ling (1987) argued against this, exine ornamentation being one of the most important differential traits; Kubitzki (2007) followed the same criterion as Ling and kept the genera separate, treating pollen type as a good taxonomic character. Apart from these systematic considerations, the lack of agreement of pollen type with phylogenetic placement

in those two genera could constitute the two first cases of reversal in pollen type reported for Artemisiinae. Nevertheless, to confirm the reversal event(s), it would be necessary to discard the hypothesis of pollen-type inheritance through hybridization for these species. The case of *Stilpnolepis* raises particular suspicion, because of its undetermined placement in previous ITS analyses (Watson *et al.*, 2002; Oberprieler *et al.*, 2007; Fig. 3A based on the same accessions AY127695, AY127696). This contrasts with the result involving different ITS accessions (AB359695, AB359781), which shows *Stilpnolepis* as sister to the genus *Filifolium* Kitam. with strong support (PP = 100%, Fig. 3A). Regarding *Elachanthemum*, ETS and ITS data do not provide any evidence of hybrid origin. Both regions strongly support the grouping of this taxon with *Dendranthema* and relatives (100% PP, Fig. 3A, B) in a clade with exclusively *Anthemis*-pollen species, *Elachanthemum* being the only exception (Fig. 3C). However, *Elachanthemum* shows an rDNA organization that is different from the rest of Artemisiinae. Most Artemisiinae have a linked rDNA type with 5S and 35S in the same unit (Garcia *et al.*, 2007, 2009), but the only confirmed exception found to this linkage in the subtribe is the case of *Elachanthemum* with the typical, separate arrangement of 5S and 35S found in most angiosperms. Other genera belonging to the same clade, such as *Ajania* or *Brachanthemum*, show the linked arrangement as found in *Artemisia*; nevertheless, results are still not conclusive for *Dendranthema*, in which it seems that linked and unlinked units may coexist in some species (Abd El-Twab & Kondo, 2006).

Our results also confirm the findings of Muldashev (1983) of an *Artemisia*-type pollen to *Ajania junnanica*, whereas the remaining species of *Ajania* have *Anthemis*-type pollen (Table 2, Appendix). In the molecular phylogenetic analysis, the sequenced *Ajania* group with *Dendranthema* and relatives, in accordance with their pollen affinities (Fig. 3C). No sequence data are available for *A. junnanica* and therefore the phylogenetic placement of this species has not yet been confirmed on a molecular basis. In fact, Muldashev (1983) suggested *A. junnanica* was certainly misplaced in *Ajania* and combined the species in the genus *Artemisia*, stating that it was 'absolutely clear' that, because of its pollen type, this taxon could not remain in *Ajania*. Not considering this trait, Ohashi & Yonekura (2004) combined *Ajania junnanica* in *Chrysanthemum* L., a genus with *Anthemis*-type pollen; those authors merged the complete genera *Ajania*, *Arctanthemum* (Tzvelev) Tzvelev, *Dendranthema* and *Phaeostigma* within *Chrysanthemum*. Bremer & Humphries (1993) also opted for the misplacement of *A. junnanica*, although in a slightly different way than Muldashev (1983). They considered *Ajania*, or part of the genus, as the sister group of *Artemisia* and allies (those having smooth or short-spined pollen).

Ajania shows some variability in pollen traits; it exhibits both pollen types (although the *Artemisia*-type is found only in one species of questioned taxonomic assignment to the genus), the greatest range of spine density (for the *Anthemis*-type) and the smallest and the largest pollen in the tribe. However, the other genera of Artemisiinae have not been as extensively sampled as *Ajania* in the present study and, consequently, we do not know if such diversity is exceptional or the rule in the tribe, or if it reflects the taxonomic heterogeneity of *Ajania*. It can, however, be stated that *Ajania* is basically a genus with *Anthemis*-type pollen grains.

Similar to *Ajania* in some morphological features, but with microechinate pollen grains as a distinctive trait, Shih (1978) described the monospecific genus *Ajaniopsis* and *Artemisia*-type pollen was confirmed in this taxon by Martín *et al.* (2001, 2003). Oberprieler *et al.* (2007) did not assign *Ajaniopsis* to a subtribe within Anthemideae because a molecular framework was lacking for this species, but suggested, on the basis of the results from Martín *et al.* (2003), that its pollen features clearly point to its inclusion in Artemisiinae.

This study also shows up several inconsistencies concerning the genus *Phaeostigma* (as stated in the Introduction, a new genus described in 1981 by Muldashev, made up of three species previously located in *Ajania*). Analysis of the ETS region groups *Phaeostigma quercifolium* (W.W.Sm.) Muldashev with *Achillea schmakovii* Kupr. (Fig. 3B, C) and the ITS region groups it among the early branching genera of Artemisiinae (Fig. 3A). Such a result may suggest a possible hybrid origin for this species, from two species belonging to different subtribes of Anthemideae. ETS firmly locates *Phaeostigma salicifolium* in a clade of the *Artemisia* group (PP = 100%, Fig. 3), in accordance with its *Artemisia*-type pollen (Martín *et al.*, 2003). These results for *P. salicifolium* agree with the assumption of a close relationship between *Phaeostigma* and *Artemisia* (Muldashev, 1982). However, this hypothesis is contradicted by the placement of *Phaeostigma varifolium* (Chang) Muldashev within the *Dendranthema* group, which is supported by both ETS and ITS markers (PP = 100%, Fig. 3). Therefore, the phylogenetic affinities of *Phaeostigma* remain unresolved and, furthermore, the monophyly of the genus could be questionable.

Our results highlight some inconsistencies between pollen types and taxonomic groups. However, evidence for possible pollen type reversals in Artemisiinae is still lacking, none of the cases considered above establishing such an event beyond doubt.

Evolutionary trends on pollen features in Artemisiinae

The unsupported basal-most nodes of the ingroup impede the determination of the ancestral character state for the Artemisiinae pollen type (Fig. 3). Nevertheless, the fact that the species of the outgroup (and most of the tribe) show the *Anthemis* pollen type makes this the most likely option for the ancestral state, an assumption also supported by the palaeogeological record (Wang, 2004). According to this hypothesis, the main tendency in the subtribe would be toward the reduction of global size and size ornamentation of pollen.

Several factors implicated in pollen downsizing events are found in the literature, such as a shift to an annual life cycle (or more generally to shorter growth cycles), autogamy or an adaptation to extreme environmental conditions (Hidalgo *et al.*, 2008a, b and references therein). However, none of these factors seems to account for the pollen type distribution pattern observed in Artemisiinae. In fact, the characteristics shown by the two Artemisiinae pollen types fit with the two main pollination syndromes: (1) larger, heavily ornamented pollen grains, such as the *Anthemis*-type, with much pollenkitt making the pollen sticky, being more likely related to entomophily; and (2) smaller (with also reduced size range variation), less ornamented pollen, as in the *Artemisia*-type, with almost no pollenkitt making the pollen dry, more likely related to anemophily (Wodehouse, 1935; Friedman & Barrett, 2009). The pollination syndrome is also expressed in terms of floral and inflorescence features, with larger, showy structures found in insect pollinated plants and smaller, non-showy structures in wind pollinated plants (Friedman & Barrett, 2009). This trend agrees well in Artemisiinae with small, greenish or whitish capitula generally displayed by taxa with *Artemisia*-type pollen and radiate capitula (e.g. *Dendranthema*), coloured capitula (e.g. *Ajania pacifica*) or corymbose capitula (e.g. *Stilpnolepis*) in taxa showing *Anthemis*-type pollen. Therefore, a shift in pollination, from entomophily to anemophily may account for the change from *Anthemis* to *Artemisia* pollen type. Following this assumption, insect pollination would probably be the ancestral state in Artemisiinae, as it is for the whole of Asteraceae. The main apomorphy of the family, the capitulum, is basically designed to draw attention to the display, by making the flowers more noticeable to the pollinator. Asteraceae are mostly pollinated by animals and the few wind-pollinated representatives of the family are exceptions. These are the *Artemisia* group of our present study, the genus *Ambrosia* (Heliantheae) and some species of *Espeletia* (Millerieae/Heliantheae *s.l.*; Jeffrey, 2007).

There is, however, one species of Artemisiinae which has pollen and inflorescence characters pointing to different pollination syndromes, *Ajaniopsis penicilliformis*, with *Artemisia* pollen and showy capitula. In addition, several taxa have inflorescences not clearly attributable to one pollination type: *Crossostephium* and *Filifolium* (both with *Artemisia* pollen) and *Brachanthemum* and *Stilpnolepis* (both with *Anthemis* pollen). Such a pattern could indicate mixed pollination. Frequent insect visits have also been reported in different species of *Artemisia*, suggesting that entomophily could be involved to a certain degree, even in species showing the anemophilous syndrome (Garnock-Jones, 1986; Vallès, 1989). Some of these cases of incongruent pollen and inflorescence trends may also indicate that secondary shifts in pollination types are ongoing processes. This occurred in the genus *Espeletia*, another member of Asteraceae in which a shift from animal to wind pollination has taken place. In this case, the typical reduction of the spine size accompanying anemophily did not immediately follow the shift in pollination type, and was only observed in the more derived species (Rundel, Smith & Meinzer, 1994 and references therein).

Some other tendencies have been described in the group. One concerns polyploidy, occurring in *Ajania* and considered as one of the main evolutionary factors in plants (Otto & Whitton, 2000 and references therein). This maybe relevant in interpreting the data set analysed here, in that ploidy may express itself directly through pollen size (Muller, 1979; Julià & Martín, 1994). This trend cannot be confirmed, however, in *Ajania*. In fact, the largest and the smallest pollen were found in high polyploid species [*A. pacifica*, $2n = 90$, and *A. nematoloba* (Hand.-Mazz.) Ling ex C.Shih, $2n = 72$, respectively]. Nevertheless, the relationship between pollen size and ploidy is known to be easily overridden by other factors and is evolutionarily short-lived (Muller, 1979; Tate & Simpson, 2004). Another point concerns the group of taxa with the *Artemisia*-type pollen that have a particular high density of ornamental elements (see Results), which are all distributed in North America with the exception of *Ajaniopsis*. In fact, some of the species with large numbers of spinules (e.g. *Chamartemisia compacta*, *Vesicarpa potentilloides*) are nowadays labelled under a single genus, *Sphaeromeria*. Thus, the presence of a high density of spinules in the group might more likely reflect a close relationship rather than a parallel adaptation to particular environmental conditions. Additionally, pollen size, spine length and spine density are probably linked characters. As observed, the *Anthemis* pollen type is larger, with larger spines at a lower density than the *Artemisia* type and Wodehouse (1935)

already pointed out this relationship. In a recent article by Schols *et al.* (2005), a similar linkage was found between perforation size, perforation density and pollen size in *Dioscorea*: a high perforation density (restricted to some specific groups in this genus) was related to a smaller size and small and dense perforations.

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APPENDIX

Species	Chrom. number	P min. (μm)	P max. (μm)	P (μm)	E min. (μm)	E max. (μm)	E (μm)	P/E	Pollen type	Spine height (μm)	No. of spines (25 μm²)	V (μm³)
Ajania achilleoides	18	22.91	27.08	24.90 ± 1.33	20.83	27.08	23.74 ± 1.63	1.04	*Anthemis*	3.60 ± 0.40	3	7 347.83
Ajania achilleoides 75	18	20.83	27.08	22.90 ± 1.92	17.70	25.00	19.64 ± 2.29	1.03	*Anthemis*	2.90 ± 0.16	3	4 625.06
Ajania achilleoides 76	18	20.83	25.00	22.91 ± 1.30	18.75	22.91	21.93 ± 1.20	1.04	*Anthemis*	2.80 ± 0.15	3	5 769.01
Ajania aureoglobosa		17.79	25.00	22.46 ± 1.88	20.83	22.91	21.70 ± 1.11	1.03	*Anthemis*	2.96 ± 0.10	4	5 537.68
Ajania fastigiata	18	22.91	31.25	26.31 ± 2.17	22.91	31.25	25.41 ± 1.92	1.03	*Anthemis*	3.83 ± 0.33	4	8 894.65
Ajania fastigiata	18	**20.00**	**25.00**	**22.60 ± 1.34**	**20.00**	**26.00**	**22.2 ± 1.46**	**1.02**	***Anthemis***	**2.90 ± 0.20**	**3**	**5 831.94**
Ajania fruticulosa	36	20.83	29.16	25.41 ± 2.18	18.75	26.04	23.81 ± 1.96	1.06	*Anthemis*	3.57 ± 0.70	2	7 542.62
Ajania fruticulosa 57	36	20.83	33.33	25.64 ± 4.41	20.83	28.12	23.34 ± 2.89	1.09	*Anthemis*	3.91 ± 0.10	4	7 313.38
Ajania fruticulosa 68	36	22.91	27.01	25.27 ± 1.07	22.91	29.16	25.20 ± 1.58	1.00	*Anthemis*	3.75 ± 0.41	2	8 202.43
Ajania fruticulosa	36	**20.00**	**25.00**	**22.6 ± 1.34**	**14.00**	**24.00**	**20.13 ± 2.62**	**1.12**	***Anthemis***	**2.90 ± 0.18**	**4**	**4 795.07**
Ajania gracilis		21.87	28.12	24.38 ± 1.73	20.83	25.00	22.65 ± 1.42	1.07	*Anthemis*	2.30 ± 0.34	3	6 548.91
Ajania grubovii		25	29.16	26.94 ± 1.51	22.91	29.16	25.20 ± 1.60	1.06	*Anthemis*	1.92 ± 0.12	3	8 957.72
Ajania junnanica		14.58	20.83	18.33 ± 2.31	12.50	18.75	16.45 ± 2.68	1.11	*Artemisia*		11	2 597.13
Ajania khartensis	54	22.91	29.16	24.85 ± 1.61	20.83	27.08	24.30 ± 1.79	1.02	*Anthemis*	2.92 ± 0.12	1	7 683.12
Ajania kokanica		25	31.25	26.73 ± 1.87	22.91	27.08	25.55 ± 1.17	1.04	*Anthemis*	3.68 ± 0.28	2	9 136.49
Ajania myriantha	18, 36	20.83	27.08	23.88 ± 1.40	20.83	26.04	23.67 ± 1.39	1.00	*Anthemis*	2.20 ± 0.09	3	7 005.34
Ajania nana		20.83	31.25	24.51 ± 2.66	20.83	26.04	23.39 ± 1.46	1.04	*Anthemis*	3.39 ± 0.39	3	7 021.05
Ajania nematoloba	72	14.58	23.95	21.03 ± 2.36	14.58	21.87	19.64 ± 2.29	1.07	*Anthemis*	2.10 ± 0.04	3	4 247.38
Ajania nubigena		25	29.16	27.77 ± 1.28	25.00	29.16	27.14 ± 1.59	1.02	*Anthemis*	3.54 ± 0.45	2	10 710.12
Ajania pacifica	90	35.41	41.66	38.39 ± 2.11	33.33	40.62	34.05 ± 8.37	1.12	*Anthemis*	4.40 ± 0.43	1	23 305.10
Ajania pallasiana	36	20.83	33.33	27.56 ± 4.07	20.83	33.33	26.80 ± 3.57	1.02	*Anthemis*	4.03 ± 0.35	2	10 364.48
Ajania parviflora 47		16	24.00	19.72 ± 2.09	18.00	22.00	19.6 ± 1.20	1	*Anthemis*	3.67 ± 0.22		3 966.59
Ajania potaninii		21.87	25.00	23.18 ± 1.21	18.75	25.00	21.94 ± 2.06	1.05	*Anthemis*	2.84 ± 0.17	2	5 842.32
Ajania przewalskii	18, 36	22.91	25.00	23.60 ± 0.85	17.70	25.00	22.28 ± 1.79	1.05	*Anthemis*	3.26 ± 0.34	3	6 133.96
Ajania cf. purpurea		25	29.16	25.62 ± 1.23	22.91	29.16	24.64 ± 1.69	1.03	*Anthemis*	2.87 ± 0.22	2	8 144.40
Ajania remotipinna		22.91	29.16	25.62 ± 1.56	20.83	28.12	24.99 ± 1.71	1.02	*Anthemis*	3.96 ± 0.17	2	8 377.42
Ajania roborowskii		22.91	31.25	26.31 ± 2.85	18.75	31.25	24.16 ± 3.03	1.08	*Anthemis*	2.63 ± 0.07	3	8 041.06
Ajania rupestris HBB	18	22.91	28.12	24.37 ± 1.61	22.91	27.08	24.02 ± 1.44	1.01	*Anthemis*	3.30 ± 0.29		7 362.07
Ajania rupestris RUS	18	22.91	29.16	26.31 ± 1.98	20.83	29.16	24.85 ± 2.29	1.05	*Anthemis*	3.3 ± 0.23		8 506.92
Ajania scharnhorstii HBB	18	22.91	35.41	26.38 ± 3.09	20.83	27.08	24.16 ± 2.16	1.09	*Anthemis*	3.21 ± 0.43		8 062.45
Ajania scharnhorstii RUS	18	22.91	31.25	26.31 ± 2.37	20.83	29.16	23.95 ± 2.55	1.09	*Anthemis*	3.08 ± 0.31		7 901.88
Ajania tibetica	18	25	29.16	25.69 ± 1.28	20.83	27.08	23.60 ± 1.70	1.08	*Anthemis*	4.30 ± 0.29	2	7 941.81
Ajania trilobata		22.91	27.08	25.20 ± 1.37	22.91	26.04	24.65 ± 1.01	1.02	*Anthemis*	3.31 ± 0.21	3	8 017.39
Ajaniopsis penicilliformis		**19.00**	**26.00**	**22.88 ± 2.43**	**18.00**	**24.00**	**21.28 ± 1.74**	**1.04**	***Artemisia***		**39**	**5 424.98**
Arctanthemum hultenii	**18**	**24.00**	**34.00**	**29.32 ± 2.36**	**22.00**	**33.00**	**27.76 ± 2.54**	**1.05**	***Anthemis***	**3.80 ± 0.42**	**2**	**11 830.46**
Artemisia vulgaris	**16, 32**	**18.60**	**25.80**	**22.80 ± 0.49**	**21.40**	**25.80**	**21.40 ± 1.05**	**1.07**	***Artemisia***			**5 467.15**
Artemisia incana	**16**	**20.00**	**28.00**	**22.86 ± 1.96**	**19.00**	**25.00**	**21.66 ± 1.73**	**1.05**	***Artemisia***		**12**	**5 615.54**
Artemisiastrum palmerii	**18**	**18.00**	**24.00**	**20.84 ± 1.40**	**16.00**	**24.00**	**20.12 ± 1.14**	**1.03**	***Artemisia***		**17**	**4 417.25**

Artemisiella stracheyi	**18**	**20.00**	**28.00**	**24.04 ± 2.40**	**19.00**	**28.00**	**22.08 ± 2.27**	**1.09**	***Artemisia***		**8**	**6 136.65**
Brachanthemum gobicum		33.33	37.50	33.81 ± 1.17	32.29	35.41	33.67 ± 1.01	1.00	*Anthemis*	4.03 ± 0.41	1	20 069.20
Brachanthemum kirghissorum		24.80	34.00	29.16 ± 2.55	24.80	30.00	27.56 ± 1.70	1.05	*Anthemis*	3.65 ± 0.32	2	11 596.97
Brachanthemum mongolorum		22.91	33.33	29.02 ± 3.47	22.91	31.25	28.33 ± 3.03	1.02	*Anthemis*	3.56 ± 0.49	2	12 195.21
Brachanthemum pulvinatum	18	24.00	26.00	24.90 ± 0.55	22.00	25.33	23.27 ± 0.84	1.07	*Anthemis*	2.99 ± 0.13	3	7 059.77
Brachantemum titovii	**18**	**24.00**	**30.00**	**26.46 ± 1.92**	**22.00**	**28.00**	**25.26 ± 1.94**	**1.04**	***Anthemis***	**2.90 ± 0.23**	**2**	**8 840.06**
Cancrinia discoidea	14	22.91	27.08	24.16 ± 1.53	20.83	25.00	22.63 ± 1.54	1.06	*Anthemis*	3.01 ± 0.17	2	6 478.35
Cancrinia maximoviczii		23.60	28.65	25.98 ± 2.62	20.00	25.07	23.52 ± 1.56	1.1	*Anthemis*	4.26 ± 0.22	2	7 525.10
Chamartemisia compacta		**18.00**	**24.00**	**19.88 ± 2.14**	**17.00**	**24.00**	**19.28 ± 1.84**	**1.03**	***Artemisia***		**30**	**3 869.27**
Crossostephium artemisioides		**17.00**	**24.00**	**19.92 ± 1.29**	**16.00**	**22.00**	**18.56 ± 1.55**	**1.07**	***Artemisia***		**15**	**3 592.89**
Crossostephium chinense	18	19.48	27.08	24.13 ± 3.18	20.83	27.08	22.87 ± 2.58	1.05	*Artemisia*		13	6 608.28
Dendranthema indicum	**18, 36, 54**	**24.00**	**28.00**	**26.66 ± 2.30**	**23.00**	**26.00**	**24.33 ± 2.59**	**1.05**	***Anthemis***	**4.50 ± 0.25**	**1**	**8 263.10**
Dendranthema mongolicum	ca. 72	31.25	35.41	33.60 ± 0.99	29.16	35.41	32.01 ± 2.24	1.04	*Anthemis*	4.72 ± 0.64	1	18 026.41
Dendranthema zawadskii	54	29.16	35.41	32.42 ± 2.11	27.08	33.33	31.45 ± 2.01	0.99	*Anthemis*	5.50 ± 0.59	1	16 790.09
Elachanthemum imtricatum		**16.00**	**22.00**	**19.84 ± 1.66**	**13.00**	**20.00**	**19.69 ± 2.09**	**1.12**	***Artemisia***		**10**	**4 027.46**
Elacanthemum imbricatum		22.91	25.00.	23.32 ± 0.95	20.83	23.95	22.63 ± 0.91	1.03	*Artemisia*		7	6 253.11
Filifolium sibiricum	**18**	**20.00**	**24.00**	**22.04 ± 1.58**	**16.00**	**24.00**	**20.48 ± 1.98**	**1.03**	***Artemisia***		**9**	**4 840.28**
Hippolytia alashanensis		24.00	28.80	26.96 ± 1.55	23.20	30.00	26.60 ± 1.83	1.01	*Anthemis*	3.97 ± 0.16	2	9 988.07
Hippolytia trifida		27.20	34.00	30.94 ± 1.74	24.80	34.00	29.82 ± 2.41	1.03	*Anthemis*	4.07 ± 0.39	1	14 405.69
Hippolytia megacephala		**21.00**	**26.00**	**23.20 ± 1.25**	**16.00**	**24.00**	**19.80 ± 2.16**	**1.17**	***Anthemis***	**2.9 ± 0.34**	**2**	**4 762.30**
Hulteniella integrifolia		**24.00**	**34.00**	**20.78 ± 2.68**	**22.00**	**28.00**	**25.00 ± 1.49**	**1.11**	***Anthemis***	**3.00 ± 1.40**	**2**	**6 800.24**
Kaschgaria komarovii		20.00	26.00	**22.28 ± 1.61**	**18.00**	**24.00**	**21.40 ± 1.35**	**1.04**	***Artemisia***		**10**	5 342.46
Kaschgaria komarovii		20.83	22.91	21.80 ± 0.99	20.83	35.00	21.94 ± 1.27	0.99	*Artemisia*		8	5 494.50
Kashgaria brachantemoides	**36**	**20.00**	**26.00**	**23.46 ± 1.45**	**18.00**	**23.00**	**20.60 ± 1.49**	**1.13**	***Artemisia***		**9**	**5 212.68**
Lepidolopsis turkestanica		**20.00**	**24.40**	**22.76 ± 1.21**	**17.00**	**24.00**	**20.93 ± 1.80**	**1.08**	***Anthemis***	**3.00 ± 0.92**	**3**	**5 220.47**
Mausolea eriocarpa	**36**	**22.00**	**29.00**	**24.26 ± 1.76**	**14.00**	**23.00**	**19.40 ± 2.47**	**1.25**	***Artemisia***		**15**	**4 780.72**
Neopallasia pectinata	**18, 36**	**18.00**	**22.00**	**20.01 ± 1.95**	**17.00**	**21.00**	**20.00 ± 1.36**	**1.00**	***Artemisia***		**9**	**4 190.88**
Phaeostigma salicifolium		**16.00**	**24.00**	**20.56 ± 1.83**	**13.00**	**22.00**	**19.12 ± 2.23**	**1.07**	***Artemisia***		**7**	**3 935.48**
Picrothamnus desertorum		**18.00**	**24.00**	**21.20 ± 1.78**	**17.00**	**25.00**	**19.60 ± 1.57**	**1.08**	***Artemisia***		**17**	**4 264.29**
Poljakovia falcatolobata		25.00	33.33	29.64 ± 2.11	20.83	33.33	27.42 ± 3.96	1.08	*Anthemis*	3.75 ± 0.37		11 668.41
Sphaeromeria diversifolia		**17.30**	**19.40**	**18.82 ± 0.84**	**21.40**	**25.80**	**17.60 ± 1.17**	**1.06**	***Artemisia***		**24**	**3 052.41**
Stilpnolepis centiflora		**27.00**	**34.00**	**29.28 ± 2.68**	**25.00**	**30.00**	**27.28 ± 1.52**	**1.07**	***Anthemis***	**3.80 ± 0.36**	**2**	**11 409.28**
Stilpnolepis centiflora		24.00	28.00	26.00 ± 0.89	22.00	26.00	24.00 ± 1.26	1.08	*Anthemis*	2.58 ± 0.21	3	7 841.41
Tanacetum vulgare	**18**	**20.00**	**28.00**	**24.33 ± 2.14**	**18.00**	**26.00**	**22.13 ± 2.21**	**1.09**	***Anthemis***	**3.30 ± 0.37**		**6 238.84**
Tridactilyna kirilovii		**24.00**	**30.00**	**26.08 ± 1.62**	**22.00**	**30.00**	**24.40 ± 1.85**	**1.06**	***Anthemis***	**3.70 ± 0.38**	**2**	**8 129.91**
Turaniphytum condrigtoni		**18.00**	**23.00**	**19.92 ± 1.16**	**17.00**	**22.00**	**18.80 ± 1.74**	**1.05**	***Artemisia***		**13**	**3 686.41**
Turaniphytum eranthemum	**18**	**16.00**	**22.00**	**18.80 ± 1.64**	**14.00**	**20.00**	**16.93 ± 1.98**	**1.11**	***Artemisia***		**17**	**2 821.44**
Vesicarpa potentilloides		**19.00**	**22.00**	**20.84 ± 1.12**	**17.00**	**22.00**	**20.16 ± 1.59**	**1.04**	***Artemisia***		**44**	**4 434.83**

Data from species in bold have been extracted from Martín *et al.* (2001, 2003).

SÍNTESI

ARTEMISIA SISTEMÁTICA

SÍNTESI

Seguidament es presenta un resum dels resultats més rellevants que s'han obtingut durant la realització d'aquest treball de tesi, així com de la discussió dels mateixos.

1. Estudis centrats en el gènere *Artemisia s. l.*

Amb els diferents treballs realitzats s'han aportat dades de quantitat de DNA nuclear per a 82 espècies (106 poblacions) del gènere *Artemisia*, la majoria d'elles desconegudes fins ara des d'aquest punt de vista, així com dades per als gèneres afins *Crossostephium* (una espècie) i *Neopallasia* (una espècie). S'han obtingut noves seqüències del DNA ribosòmic nuclear, corresponents a les regions ITS i 3'-ETS, per a 46 espècies d'*Artemisia*, així com també s'han realitzat hibridacions *in situ* fluorescents en 19 espècies. S'ha aportat, doncs, un bon nombre de dades amb la finalitat de conèixer millor els processos evolutius que tenen lloc al gènere.

1.1 Dinàmica de la grandària del genoma en el gènere *Artemisia*

En aquest treball s'ha presentat una aproximació bastant àmplia a l'estudi de la quantitat de DNA en *Artemisia* mitjançant la tècnica de citometria de flux, que completa els treballs anteriors publicats per TORRELL *et al.* (2001) i GARCIA *et al.* (2004) en aquest gènere, aportant una nova visió sobre la dinàmica d'aquest paràmetre amb el suport d'un marc filogenètic robust. S'han obtingut dades de nombroses espècies del gènere *Artemisia*, que representen tots els diferents nivells de ploïdia que es coneixen fins al moment. Aquest fet ens ha permès, a més d'analitzar la variabilitat interespecífica i intraespecífica, estudiar l'evolució d'aquest paràmetre en relació amb la poliploïdia, en una mostra representativa. D'entre els resultats més destacables, hem trobat que s'estableix una clara correspondència entre l'heterogeneïtat del valor C i aquelles clades de la filogènia que inclouen els representants dels subgèneres més conflictius -en el

sentit de poc homogenis a la filogènia- del gènere, *Artemisia* i *Absinthium*. Així doncs, hem trobat que els grups més estables i ben definits filogenèticament, també mostren els rangs de variabilitat del valor C més estrets, és a dir, que la variació interespecífica es redueix en aquells grups on les relacions filogenètiques són clares, p. ex. *Dracunculus* i *Seriphidium*, així com el subgènere *Tridentatae*, com ja havien apuntat GARCIA *et al.* (2008). Així mateix, tot i que la quantitat de DNA en individus diploides és coherent amb els grups trobats a la filogènia, aquest paràmetre no discrimina entre grups pel fet que hi ha un cert grau de superposició dels rangs de variació d'aquest valor entre algunes clades. En treballs anteriors tant de filogènia (VALLÈS *et al.* 2003; SANZ *et al.* 2008; TKACH *et al.* 2008), com de l'estudi de la grandària del genoma (TORRELL *et al.* 2001; GARCIA *et al.* 2004) s'ha observat l'estreta relació que hi ha entre *Artemisia* i alguns dels gèneres de les *Artemisiinae* que n'han estat segregats, com és el cas de *Filifolium*, *Mausolea* i *Neopallasia*, entre d'altres. En el present treball s'ha aportat una nova dada per a l'espècie *Crossostephium chinense*, que s'ha revelat com el valor C més baix trobat a la subtribu.

L'existència de dos nombres cromosòmics de base ($x = 9$ i $x = 8$) al gènere ens va dur a plantejar que podrien existir diferències entre la quantitat de DNA de les espècies (diploides) amb diferents nombres bàsics de cromosomes. No obstant, les diferències trobades no foren significatives ($P>0,05$). S'obtingueren resultats semblants en les comparacions realitzades per a comprovar l'existència de diferències significatives entre els poliploides estrictes, és a dir, d'aquells per als quals no s'ha reportat mai una població diploide, i dels poliploides amb poblacions diploides conegudes. Trobàrem diferències significatives entre les dues modalitats de poliploïdia (espècies estrictament poliploides, i espècies poliploides que també presenten poblacions diploides) en el nivell tetraploide, i en l'hexaploide en trobàrem sols amb el test t, no amb els basats en la distribució filogenètica, PGLS). Això ens podria indicar que l'origen d'aquests poliploides actua com un factor de constricció en la grandària del seu genoma. També hem comprovat que hi ha una reducció de la quantitat de DNA per genoma haploide (valor 1Cx; GREILHUBER *et al.* 2005), és a dir, aquest paràmetre disminueix a mesura que ascendim en nivells de ploïdia, confirmant

el que ja havien suggerit GARCIA *et al.* (2004) en *Artemisia*, i LEITCH I BENNETT (2004) en altres grups de plantes. Tot i això, aquestes diferències entre els valors observats i esperats si l'increment de DNA fos proporcional al nivell de ploïdia són més òbvies per al valor 2C. Un exemple molt clarificador de la pèrdua d'una determinada fracció de DNA durant els processos de poliploïdització el trobem en l'espècie *Artemisia medioxima*. Vàrem estimar els valors 2C d'una població tetraploide i d'una d'hexaidecaploide i vàrem veure que el valor estimat per a la segona població fou aproximadament un 47% més menut del que cabria esperar si l'increment de DNA entre ambdós nivells de ploïdia hagués estat proporcional. Una explicació plausible per a aquesta pèrdua tan substancial la podríem trobar en l'activació de mecanismes cel·lulars que afavorisquen la constricció genòmica, sobretot en elevats nivells de ploïdia, per tal de fer metabòlicament assimilable el procés de duplicació cromosòmica. Tot i aquesta pèrdua, hem vist que si ens fixem en el nivell de les espècies, hi ha diferents tendències evolutives que indiquen guany proporcional o pèrdues menors en nivells de ploïdia baixos, tot i que aquesta pèrdua es fa més patent paral·lelament a l'increment de ploïdia. Aquestes diferències de comportament de les espècies, creiem que segur que estan relacionades amb l'origen i antiguitat de les mateixes, com s'ha reportat per a altres gèneres com *Nicotiana* (LEITCH *et al*. 2008), però el fet de no tenir cap indici ni informació sobre l'origen d'aquestes espècies fa difícil proposar hipòtesis concloents al respecte.

Com que disposàvem d'un gran nombre de dades tant de poblacions diploides com d'altres de poliploides, i vista la tendència general cap a la pèrdua d'una fracció de DNA amb la poliploïdització, ens plantejàrem el repte d'establir l'existència d'un límit teòric per a la quantitat de DNA nuclear en *Artemisia*. Per a tal efecte, ajustàrem diferents funcions matemàtiques al nostre conjunt de dades. Amb la funció quadràtica vàrem confirmar que no existia una relació lineal entre els nostres valors ($P<0,05$), però aquesta funció biològicament no tenia cap sentit, ja que representava arribar a un valor màxim amb la poliploïdia seguit d'una posterior reducció, així que hi ajustàrem el model de saturació proposat per Michaelis-Menten. Aquest model s'ajustà satisfactòriament a les nostres dades ($P<0,001$) i tenia una explicació biològica

convincent. Així doncs, podem esperar que amb l'increment del nivell de ploïdia, la grandària del genoma s'aproxime de forma asimptòtica a un màxim teòric, com seguint una corba de saturació. Segons això, podríem predir -de forma teòrica, ja que els límits biològics no els podrem conèixer amb exactitud- el nivell de ploïdia màxim que podria arribar a assolir *Artemisia*, així com la quantitat de DNA corresponent.

1.2 Origen i evolució de les artemísies endèmiques d'Amèrica del Sud

Per tal de donar resposta a qüestions com les que ens plantegem en aquest treball, com quin és l'origen de les artemísies endèmiques d' Amèrica del Sud, així com quins són els seus patrons d'evolució citogenètica, hem seqüenciat les regions del DNA ribosòmic nuclear ITS i ETS, i hem realitzat hibridacions *in situ* fluorescents (FISH) per a detectar els *loci* del DNA ribosòmic i bandatge amb cromomicina A_3 en les espècies del complex; a més, hem estimat la grandària del seu genoma. El primer resultat que hem trobat ha estat que les espècies endèmiques del continent sud-americà formen un complex poliploide. Dels sis tàxons estudiats n'hem trobat un de diploide ($2n = 18$; *Artemisia magellanica*), un d'hexaploide ($2n = 54$; *Artemisia mendozana* var. *paramilloensis*), dos d'octoploides ($2n = 72$; *Artemisia echegarayi* i *Artemisia mendozana* var. *mendozana*) i un de dodecaploide ($2n = 108$; *Artemisia copa*). Totes les dades obtingudes són inèdites tret dels recomptes en *A. magellanica*, per a la qual n'hi havia un de previ (MOORE 1981), que indicava una població diploide amb 22 cromosomes, i de l'espècie *A. mendozana*, amb un report previ de KAWATANI I OHNO (1964) d'una població amb 72 cromosomes, però sense cap referència a la varietat estudiada. Aquestes dades ens situen en un escenari bastant actiu des del punt de vista de l'evolució on, partint de la base que aquestes espècies són d'origen al·lopoliploide, sense descartar l'autopoliploïdia, s'ha de suposar que fenòmens com la hibridació han tingut un paper important en la diversificació i l'especiació d'aquestes plantes.

Els resultats de la seqüenciació i l'anàlisi de les dades en aquest marc filogenètic del gènere mostren una relació molt estreta entre les artemísies

endèmiques d'Amèrica del Sud i les nord-americanes, en aquest cas amb les del subgènere *Tridentatae*, amb l'excepció d'*A. magellanica*, que és l'únic representant sud-americà que queda exclòs d'aquest gran llinatge americà i s'emparella amb *Artemisia biennis*, una herbàcia biennal d'àmplia distribució a Amèrica del Nord i que també es troba a Europa i Àsia. Les relacions filogenètiques entre el nucli del complex sud-americà i el nord-americà han de ser enteses amb criteris d'especiació biogeogràfica, més que no pas amb criteris de proximitat taxonòmica o morfològica. De fet, mentre que les *Tridentatae* presenten capítols amb totes les flors hermafrodites (homògams), a les endèmiques del continent sud-americà hem vist que els capítols son heterògams, amb unes flors hermafrodites i d'altres de femenines, més típic del subgènere *Artemisia,* d'igual forma que s'ha reportat en altres artemísies endèmiques no *Tridentatae* a Amèrica del Nord. Així doncs, i vist que el grup germà de les artemísies americanes és constituït pels representants del complex d'*A. vulgaris* (subgènere *Artemisia*), pren força la hipòtesi de MCARTHUR I PLUMMER (1978), que apuntaven que possiblement un ancestre del subgènere *Artemisia* hauria arribat a Amèrica del Nord via l'estret de Bering, i un cop allà s'hauria diferenciat amb la subseqüent aparició de les *Tridentatae*, tot i que altres hipòtesis s'haurien de tenir en consideració (Ling 1991, 1995). D'altra banda, la posició filogenètica d'*A. magellanica* ens pot indicar que segurament hi ha hagut més d'un episodi de colonització al continent sud-americà. Aquesta espècie i *A. biennis* s'han revelat estretament relacionades en la filogènia, tant que comparteixen el fet de presentar de forma paral·lela, un posicionament filogenètic variable segons la regió utilitzada. Aquesta relació, ja fou apuntada prèviament per LING (1995), qui ja les considerà com a espècies sinònimes per la seva semblança morfològica, tot i que alguns altres caràcters no son compartits (fragància, aspectes del cicle vital). Uns altres resultats que també donen suport a la relació entre *A. biennis* i *A. magellanica* són les dades citogenètiques obtingudes. Tant la quantitat de DNA nuclear (pràcticament la mateixa), com la presència d'un sol *locus* de DNA ribosòmic i de dues bandes positives d'heterocromatina rica en bases GC en ambdues espècies justifiquen que puguem estar pensant que es

tracta de la mateixa espècie o, si més no, de dues que comparteixen un origen molt pròxim.

Els resultats de la caracterització citogenètica i de la grandària del genoma en aquestes espècies ens ha mostrat una dinàmica totalment oposada a la que s'havia trobat en *Artemisia*. Fins ara, així com als resultats del treball presentat en aquesta síntesi, sempre s'havia trobat que amb la poliploïdització hi havia una porció de DNA nuclear que s'eliminava, fent decréixer el valor 1Cx en poliploides (GARCIA *et al.* 2004, 2008). Així mateix, el nombre de *loci* de DNA ribosòmic corresponent als gens 5S i 45S tampoc no s'incrementava, generalment, de forma proporcional a l'ascens en ploïdia, especialment a partir dels tetraploides, indicant que hi havia una eliminació relacionada amb els processos de reorganització cromosòmica posteriors a la fusió de dos genomes en *Artemisia* (GARCIA *et al.* 2009). Així doncs, tot i que els resultats s'han de prendre amb precaució en alguns casos, ja que d'algunes de les espècies sols es coneix un nivell de ploïdia, hem comprovat que tant el nombre de bandes positives per a la cromomicina, com el nombre de *loci* del DNA ribosòmic 5S i 45S, així com els valors 2C han augmentat de forma proporcional durant la poliploïdització, mantenint-se quasi constant el valor 1Cx, que ha mostrat una oscil·lació molt lleu cap a la reducció de la mida del genoma. Aquest fenomen, estrany en el gènere que ens ocupa, s'ha trobat en altres asteràcies com el gèneres *Carthamus* (GARNATJE *et al.* 2006) i *Tragopogon* (PIRES *et al.* 2004), i en altres famílies com les solanàcies (*Nicotiana*, MATYÁSEK *et al.* 2003).

1.3 Caracterització citogenètica d'alguns representants dels subgèneres *Absinthium* i *Artemisia*

Amb aquest treball hem pretès dur a terme la descripció citogenètica de tres espècies pertanyents als subgèneres *Artemisia* (*Artemisia chamaemelifolia* i *Artemisia vulgaris*) i *Absinthium* (*Artemisia absinthium*), en un intent d'aportar noves dades al coneixement citogenètic del subgènere *Artemisia*, a partir de les aproximacions prèvies dels treballs de TORRELL *et al.* (2003) i HOSHI *et al.* (2006),

i abordar per primer cop la distribució dels *loci* ribosòmics al subgènere *Absinthium*.

Les tres espècies presenten un cariotip bastant simètric i semblant quant a morfologia, amb algunes diferències menors. També s'ha detectat la presència de cromosomes B en *A. chamaemelifolia*. La funció exacta d'aquests cromosomes no és clara (TRIVERS *et al.* 2004), i la seva incidència és variable. En les tres espècies estudiades també hem trobat un nombre variable de bandes positives per a la cromomicina A_3 (zones del DNA riques en bases GC), sempre situades a les parts terminals dels cromosomes, en alguns casos en satèl·lits, excepte en *A. chamaemelifolia* i *A. vulgaris*, on també n'hem trobat de centromèriques i en els cromosomes B de la primera.

Pel que fa al DNA ribosòmic, els resultats d'aquest treball indiquen que en les espècies del subgènere *Artemisia* amb 16 o 18 cromosomes hi ha dos *loci* ribosòmics (quatre senyals), confirmant els resultats de treballs previs (TORRELL *et al.* 2003; HOSHI *et al.* 2006). Així mateix, també s'han trobat quatre senyals per als DNA ribosòmics 5S i 45S en *A. absinthium*. En tots els casos ambdues famílies de gens ribosòmics són colocalitzades, un tret poc característic a les angiospermes, però molt comú en algunes asteràcies i sempre present en *Artemisia* (TORRELL *et al.* 2003; GARCIA *et al.* 2007, 2009). Els senyals positius per a la cromomicina s'han trobat colocalitzats amb els *loci* ribosòmics a *A. absinthium*. En canvi, en les dues espècies del subgènere *Artemisia*, tot i trobar-ne de colocalitzats, el nombre de senyals de cromomicina ha estat superior al de *loci* del DNA ribosòmic. Les diferències trobades en el patró de distribució de l'heterocromatina rica en bases GC entre els dos subgèneres estudiats pot reflectir diferents històries evolutives per a cada grup.

1.4 Requereixen els poliploides proporcionalment menys *loci* d'rDNA que els perennes relacionats?

S'ha avaluat la distribució dels rDNA 5S i 45S en dues espècies *d'Artemisia* en poblacions d'alt nivell de ploïdia, *Artemisia lagocephala* (subgènere *Absinthium*) i *Artemisia medioxima* (subgènere *Artemisia*). Ambdues espècies presenten el

nombre bàsic de cromosomes $x = 9$. S'ha detectat que la població diploide d'*A. lagocephala* ($2n = 18$) presenta tres *loci* i l'hexaploide ($2n = 54$) sis. Així mateix, en *A. medioxima* el nombre de *loci* de l'rDNA no s'incrementa proporcionalment al nivell de ploïdia. En aquesta espècie, la població diploide ($2n = 18$) presenta dos *loci* d'rDNA, la tetraploide ($2n = 36$) en mostra quatre i l'hexaidecaploide ($2n = 144$) en té al voltant de 10. Aquests resultats evidencien una pèrdua relativa de *loci* de l'rDNA (congruent amb la disminució de la quantitat de DNA monoploide, 1Cx). Pèrdues en el nombre de *loci* ribosòmics en poblacions poliploides han estat citades a la bibliografia (MISHIMA *et al*. 2002; LI *et al*. 2004) i han estat observades també en altres espècies d'*Artemisia* (PELLICER *et al*. en preparació), tot i que en alguns gèneres l'increment del nombre de *loci* s'ha observat que és proporcional (PIRES *et al*. 2004). Els resultats de detecció de l'heterocromatina mitjançant DAPI (després del FISH) són variables. Mentre que en *A. medioxima* sembla que hi ha una reducció de senyals en els poliploides, en *A. lagocephala* el patró de bandes de DAPI no segueix aquesta tendència i hi hem trobat un increment espectacular de bandes d'heterocromatina al nivell hexaploide. Aquests resultats ens fan pensar en l'activació de fenòmens com reestructuracions (MEYER 2000) i mecanismes de silenciament gènic (CHEN 2007), que poden tenir lloc durant la poliploïdització i, més freqüentment, durant la formació d'al·lopoliploides.

1.5 Cicle vital *versus* posició sistemàtica: implicacions filogenètiques

El comportament sistemàtic i evolutiu d'algunes plantes anuals ha estat de gran interès des de temps enrere. Sembla clar que la durada del cicle de vida de les plantes té una influència fonamental en la seva biologia, fet que es tradueix principalment en canvis a nivell del genoma. El treball que ens hem plantejat tracta d'analitzar quins canvis a nivell filogenètic i del DNA ribosòmic ocorren a les artemísies anuals, per tal d'entendre millor l'evolució d'aquestes plantes.

Hem estudiat onze espècies anuals d'*Artemisia*, de les prop de vint que n'hi ha, al gènere. També hem estudiat una espècie del gènere *Neopallasia*, per la seva proximitat al gènere *Artemisia*. S'han obtingut resultats per a la

distribució de les regions 5S i 45S del DNA ribosòmic als cromosomes, així com seqüències dels espaiadors transcrits intern i extern (ITS i ETS) del DNA ribosòmic nuclear, a més de dades de la quantitat de DNA nuclear en les espècies estudiades.

Tots els senyals d'hibridació del DNA ribosòmic s'han trobat en posició terminal o en satèl·lits, com ja s'havia reportat en treballs anteriors (TORRELL *et al.* 2003; GARCIA *et al.* 2007, 2008; PELLICER *et al.* 2008). En l'estudi filogenètic, els resultats dels test ILD de congruència no són estadísticament significatius, és a dir, no existeix incongruència entre les dades d'ambdues regions un cop hem suprimit de l'anàlisi les espècies *Artemisia cretacea* i *Artemisia stelleriana*. També hem observat que no hi ha cap llinatge estrictament anual a la filogènia, sinó que aquestes espècies apareixen disperses arreu de l'arbre, indicant que com a mínim hi ha hagut cinc episodis de reversió cap a l'anualitat. S'ha observat que les anuals i les perennes relacionades tendeixen a compartir preferències ecològiques, tot i que solen ocupar microhàbitats diferents, essent les anuals més adaptades a viure en condicions extremes que les perennes relacionades. Així doncs, mentre que algunes espècies s'han convertit en supervivents a condicions adverses, d'altres mostren un comportament eurioic, de vegades relacionat amb l'activitat humana.

Els resultats d'aquest treball indiquen que en algunes espècies dels subgèneres *Absinthium* (*Artemisia anethifolia*, *Artemisia anethoides*, *Artemisia jacutica* i *Artemisia macrocephala*) *Seriphidium* (*Artemisia leucodes*) i en l'espècie estudiada dels subgèneres *Dracunculus* (*Artemisia scoparia*; PELLICER *et al.* en prep.), hi ha una tendència general cap a l'increment del nombre de *loci* ribosòmics en les espècies anuals (tret de *N. pectinata* on es redueix a un *locus* i en *Artemisia sieversiana*, on es manté el mateix nombre de *loci*), comparant amb els resultats de què disposem per a les perennes. Aquest increment del nombre de *loci* va acompanyat d'una certa reducció de la grandària del genoma, en algunes espècies més acusada que en d'altres, tot i que en *A. leucodes* l'increment del valor C és molt elevat respecte dels diploides perennes d'*Artemisia* (GARCIA *et al.* 2004). A les espècies del subgènere *Artemisia* podem observar diferents resultats. Hi ha espècies amb el mateix nombre de *loci*

ribosòmics que a les perennes del subgènere (*Artemisia annua*, *Artemisia palustris* i *Artemisia tournefortiana*), així com altres on hi ha una reducció del nombre de senyals, de quatre a les perennes a dos en *Artemisia biennis* i *Artemisia blepharolepis*. Tot i aquestes dues tendències clares, els canvis en la grandària del genoma (el valor C) no són unidireccionals. Hi ha espècies on s'ha observat un increment de DNA nuclear molt elevat (*A. blepharolepis*), altres on s'ha reduït aquest valor [*A. annua* (TORRELL I VALLÈS 2001; PELLICER *et al.* 2009) i *A. palustris*], i d'altres on les oscil·lacions d'aquest semblen estar dintre dels rangs de variabilitat del subgènere (*A. biennis*, *A. tournefortiana*). També hem comprovat que la posició filogenètica de bon nombre d'espècies anuals no es correspon amb els tractaments clàssics d'aquestes espècies. Així, les espècies *A. anethifolia*, *A. anethoides*, *A. annua*, *A. biennis*, *A. blepharolepis*, *A. leucodes*, *A. palustris* i *A. tournefortiana* no s'insereixen dintre de les clades que defineixen els subgèneres als quals pertanyen.

Aquests resultats semblen suggerir, d'una banda, que al genoma de les plantes anuals hi tenen lloc tota una sèrie de reestructuracions que influeixen directament en l'evolució d'aquestes plantes, tot i que una classificació inadequada (o inclompleta) d'alguns grups, que no es correspon amb la sistemàtica natural del gènere, podria influenciar els resultats (SANZ *et al.* 2008; TKACH *et al.* 2008). D'altra banda, com ja s'ha apuntat anteriorment, en el cas d'*Artemisia* no hem vist cap clada estrictament anual; al contrari, sembla que el canvi de cicle s'ha esdevingut a partir de les espècies perennes diverses vegades al llarg de la història evolutiva del gènere. Altres casos d'anuals amb múltiples orígens han estat descrits en gèneres com *Houstonia* (CHURCH 2003), *Sidalcea* (ANDREASEN I BALDWIN 2001) o *Bellis* (FIZ *et al.* 2002). Aquest fet podria explicar el nombre de *loci* ribosòmics tan variable que hem trobat, és a dir, l'elevada plasticitat citogenètica de les plantes anuals. Altres fenòmens com la disploïdia, la poliploïdia, l'autogàmia i l'evolució mitjançant salts poden influir també en la diferenciació d'aquestes plantes (RAVEN I AXELROD 1995).

2. Estudis centrats en el subgènere *Dracunculus* (Besser) Rydb.

Aquest apartat agrupa un seguit de treballs que s'han plantejat amb la finalitat de conèixer més a fons l'evolució i els patrons de diversificació del subgènere *Dracunculus*. En alguns d'aquests treballs hem inclòs altres espècies que ens han semblat interessants d'estudiar. Així doncs, s'han aportat dades de recomptes cromosòmics per a un total de 48 espècies d'*Artemisia*, a les quals hem d'afegir els recomptes realitzats per a la majoria de les espècies la quantitat de DNA nuclear de les quals s'ha estimat, i que han estat 39 (52 poblacions). A banda, per a *Artemisia crithmifolia* s'han obtingut valors 2C en 45 poblacions. També s'han obtingut seqüències de les regions de DNA nuclears ITS i ETS (59 espècies) i cloroplàstics *trn*S-*trn*C i *trn*S-*trn*fM (61 espècies) i s'han realitzat hibridacions *in situ* fluorescents en 13 espècies d'*Artemisia*, a més de *Filifolium sibiricum*.

2.1 Cariologia: recomptes cromosòmics en el gènere *Artemisia*

Els resultats obtinguts en els dos treballs que inclouen recomptes de cromosomes posen de manifest l'existència dels dos nombres cromosòmics bàsics d'*Artemisia* (VALLÈS I GARNATJE 2005), tot i que també s'ha de destacar l'elevada incidència d'espècies que presenten $x = 9$ com a nombre bàsic, tret bastant comú a tot el gènere (SOLBRIG 1977; SCHWEIZER I EHRENDORFER 1983; OLIVA I VALLÈS 1994; VALLÈS I SILJAK-YAKOVLEV 1997). De fet, de les 36 espècies que han estat estudiades en dos treballs, sols una (*Artemisia sylvatica*) té $x = 8$ com a nombre bàsic. Ben probablement, aquest segon nombre prové de l'anterior per disploïdia descendent, un mecanisme detectat prèviament en altres compostes (FERNÁNDEZ CASAS I SUSANNA 1986; GARCIA-JACAS *et al.* 1996; SILJAK-YAKOVLEV 1996; VALLÈS *et al.* 2001a, b) i que es considera un dels factors evolutius més importants en aquest grup. Un altre dels mecanismes evolutius més importants en plantes és la poliploïdia, i aquests estudis il·lustren que és especialment activa en el gènere *Artemisia*. De les espècies estudiades (48), 32 tàxons són poliploides, dels quals 24 són tetraploides, quatre són hexaploides, dos són decaploides, un és dodecaploide i un és hexaidecaploide, que significa el nivell de ploïdia més alt

descrit per al gènere fins al moment. Un altre fet remarcable l'hem trobat a l'espècie tipus del subgènere *Dracunculus*, *Artemisia dracunculus*, per a la qual hem reportat poblacions diploides, tetraploides, hexaploides i decaploides, fet que exemplifica clarament la plasticitat genòmica d'aquestes plantes.

En el gènere *Artemisia* s'ha vist que moltes de les espècies que poblen hàbitats extremadament àrids són poliploides, la qual cosa dóna suport a les hipòtesis que connecten la tolerància ecològica amb la poliploïdia (OTTO I WHITTON 2000). Finalment, l'abundància de poliploides (més del 50% de les espècies estudiades) i l'existència d'espècies amb els dos nombres cromosòmics bàsics trobades en aquests treballs i en d'altres de previs, conjuntament amb el gran nombre d'espècies d'aquest gènere i d'aquesta subtribu que habiten l'àrea d'Àsia central, confirmen que aquesta representa un centre d'especiació i diversificació principal per al gènere.

2.2 Circumscripció i relacions filogenètiques en el subgènere *Dracunculus*

Amb aquest treball s'ha pretés obtenir un marc filogenètic sòlid amb la finalitat de delimitar el subgènere *Dracunculus* i aprofundir en les relacions filogenètiques existents entre els seus representants, a més de millorar el coneixement sobre les relacions taxonòmiques entre *Artemisia* i alguns gèneres de les *Artemisiinae* (*Mausolea*, *Neopallasia* i *Turaniphytum*), que treballs previs (VALLÈS *et al.* 2003; SANZ *et al.* 2008) havien inclòs dins del subgènere. Amb això, pretenem donar a conèixer la història evolutiva d'aquest grup. S'han seqüenciat dues regions nuclears (ITS i 3'-ETS), que ens han servit per a circumscriure el subgènere. Un cop marcats els límits taxonòmics, i per tal de poder abordar una anàlisi en profunditat, s'hi han afegit dues regions cloroplàstiques (*trn*S-*trn*C i *trn*S-*trn*fM) així com l'extrem 5' de la regió ETS.

Els resultats del test ILD de congruència entre els diferents marcadors nuclears i cloroplàstics ens indiquen un cert nivell d'incongruència entre les dades ($P<0,05$). Tot i això, les topologies dels arbres construïts en cada anàlisi (separats per marcadors i parcialment combinats) no mostren conflictes entre la posició de les espècies en branques que mostren un suport significatiu, excepte

en les espècies *Artemisia crithmifolia*, *Artemisia kuschakewiczii*, *Artemisia nanschanica*, *Artemisia pamirica* i *Artemisia sosnovskyi*, que finalment han estat excloses de l'anàlisi combinada.

Els resultats de la primera aproximació-delimitació del subgènere han confirmat la monofília del llinatge principal, que es correspon biogeogràficament amb la zona eurasiàtica, tot incloent alguns representants nord-americans (*Artemisia canadensis*, *Artemisia caudata* i *Artemisia pycnocephala*) estretament relacionats amb *Artemisia campestris*, una espècie d'àmplia distribució mundial. Un altre fet rellevant és la segregació de les espècies *Artemisia salsoloides* i *Artemisia tanaitica*, unides formant una clada en una posició indeterminada, però clarament fora del subgènere. Aquestes dues espècies han estat considerades amb anterioritat sinònimes (ČZEREPANOV 1995). En aquest sentit, LEONOVA (1988) també va proposar amb anterioritat la segregació de la primera (*A. salsoloides*) de la secció *Campestres* i en va crear una de nova (monotípica) que portaria el nom de l'epítet específic del tàxon en qüestió. Els nostres resultats donen suport a aquestes reorganitzacions taxonòmiques, tot indicant que aquestes espècies han anat divergint a nivell molecular del subgènere *Dracunculus*. Els representants del subgènere endèmics d'Amèrica del Nord, segons el criteris establerts per SHULTZ (2006), que són *A. filifolia*, *Artemisia pedatifida*, *Artemisia porteri* i *Picrothamnus desertorum* (també conegut com *Artemisia spinescens*), també apareixen segregats formant una clada ben suportada junt a la resta de representants d'Amèrica del Nord (subgènere *Tridentatae*). Aquest fet és comprensible si entenem que l'aïllament geogràfic d'aquest grup d'espècies i el seu origen (probablement comú) poden ser les causes més plausibles de l'agrupació entre les espècies (SANZ *et al.* 2008).

El nucli del gran grup eurasiàtic queda subdividit en tres grups principals d'espècies, les relacions filogenètiques dels quals no sempre són coincidents amb els tractaments taxonòmics del grup. La clada principal d'aquest grup es divideix en llinatges menors que inclouen espècies de diferents seccions, de vegades més relacionades per qüestions biogeogràfiques que morfològiques. Un d'aquests llinatges que ens ha semblat interessant és el del complex d'*Artemisia*

campestris, que agrupa un conjunt de tàxons semblants morfològicament, i inclou també els representants nord-americans, que en alguna flora han estat considerats com a subespècies d'*A. campestris* (SHULTZ 2006). La resta de clades presenten, en alguns casos, irresolucions que poden estar influenciades per episodis d'hibridació, que actualment seria difícil de detectar a causa de l'homogeneïtzació de les seqüències. Altres agrupacions filogenètiques que es poden observar en aquest grup eurasiàtic són, d'una banda, les que relacionen les espècies que habiten en els deserts d'Àsia Central (*Artemisia dimoana* i *Artemisia kelleri*) i del sud-oest asiàtic (*Artemisia monosperma* i *Artemisia jordanica*) i, d'altra banda, la unió de dues espècies anuals (*Artemisia edgeworthii* i *Artemisia pewzowii*) amb àrees de distribució solapades. Sembla que l'habilitat de les primeres per a desenvolupar-se en deserts arenosos i àrids, així com la distribució i el cicle vital de les segones, podria justificar-ne aquesta relació.

Les espècies del complex d'*Artemisia dracunculus*, que comparteixen el fet de presentar les fulles bàsicament enteres i linears (tret d'alguns casos), també apareixen agrupades en una clada ben suportada estadísticament, tot i que no suficientment a nivell de les relacions interespecífiques. Diferents punts de vista a l'hora de considerar el rang taxonòmic d'algunes de les espècies del complex es poden trobar a la literatura. De fet, mentre POLJAKOV (1961) i DARIJMA (1989) consideren els tàxons que hem estudiat a nivell específic, LING *et al.* (2006) rebaixen aquest nivell a varietats d'*A. dracunculus* en base a la proximitat taxonòmica d'aquestes espècies.

Un altre resultat interessant que podem extraure d'aquest treball és que, tot i que en alguns casos les relacions filogenètiques no es corresponen clarament amb els tractaments taxonòmics de forma general, sí que hi ha un cert suport a aquests agrupaments que ve donat per les dades de contingut de DNA nuclear. Bons exemples d'això els podem trobar al complex d'*A. dracunculus*, que presenta un valor 2C mitjà més gran que la resta de clades, o en el grup d'*A. campestris*, que, tot i ser menor, també sembla desviar-se de la mitjana dels grups veïns.

Com ja s'ha comentat als primers paràgrafs d'aquest apartat, un dels objectius d'aquest treball ha estat aprofundir en el coneixement de les relacions filogenètiques dels gèneres *Filifolium*, *Mausolea*, *Neopallasia* i *Turaniphytum*, després de les primeres aproximacions de VALLÈS *et al.* (2003) i SANZ *et al.* (2008). *Neopallasia pectinata* apareix embeguda dintre del gran grup eurasiàtic, mentre *Mausoela eriocarpa* i *Turaniphytum eranthemum* s'agrupen junt a *Artemisia songaria* en una clada independent dintre del subgènere. La congruència tant dels marcadors nuclears com dels cloroplàstics, especialment en el cas de *Mausolea* i *Turaniphytum*, dóna un suport a la inclusió dintre del subgènere. La posició de l'espècie *Filifolium sibiricum* dintre del gènere *Artemisia* no queda clara. Mentre SANZ *et al.* (2008) determinaren aquesta espècie com a grup germà del subgènere *Dracunculus*, en el present treball hi apareix com a espècie germana de la secció *Heterophyllae* (subgènere *Artemisia*), tot i que continua mostrant una certa ambigüitat per la irresolució de les anàlisis individuals. Així doncs, proposem que aquestes espècies, que temps enrere ja havien estat reconegudes dintre d'*Artemisia*, tornen a ser incorporades en aquest gènere.

2.3 Caracterització citogenètica (FISH) en el subgènere *Dracunculus*

La hibridació *in situ* fluorescent (FISH) ha estat la tècnica emprada per a l'estudi de la distribució de les regions 5S i 45S del DNA ribosòmic en el genoma d'algunes espècies del subgènere *Dracunculus*. S'han estudiat 13 espècies (17 poblacions) pertanyents al subgènere a més de *Filifolium sibiricum*, que a les filogènies apareix situat com a grup germà del subgènere *Dracunculus*, i d'*A. sericea*, que pertany al subgènere *Absinthium* i que volíem fer servir per a comparar un alt poliploide de *Dracunculus* amb un que no ho fos. Alguns resultats anteriors publicats pel nostre grup (TORRELL *et al.* 2001) o per altres (HOSHI *et al.* 2006) s'han utilitzat per a completar l'estudi, els resultats del qual s'han discutit en un marc filogenètic recentment obtingut (PELLICER *et al.* en preparació). Entre aquests resultats destaquen el fet d'haver completat l'estudi de l'rDNA per a tots els subgèneres d'*Artemisia*, la qual cosa ens ha permès veure

l'existència d'un possible patró citogenètic ancestral per a aquestes regions en el gènere *Artemisia* i d'establir algunes hipòtesis sobre l'evolució del genoma en el subgènere. Aquest patró correspondria a un diploide de 18 cromosomes amb dos *loci* (quatre senyals) d'rDNA [com ja hem citat anteriorment, la colocalització de les dues regions és una característica del gènere (TORRELL *et al*. 2003; GARCIA *et al*. 2007, 2009)] que hauria sofert duplicacions del nombre de marcatges acompanyades de la duplicació del nombre de cromosomes en alguns casos o sense aquestes en d'altres, reducció dels *loci*, diverses reorganitzacions cromosòmiques i processos d'hibridació, per donar lloc a altres patrons que s'interpreten a la llum de filogènia, de les sèries poliploides estudiades i de la mida del genoma.

El mapatge físic dels gens de l'rRNA en aquest nombre significatiu de membres del subgènere *Dracunculus* ha mostrat diferents models en les dues clades principals en què es diferencia el subgènere a les filogènies moleculars, indicant un acord entre els conjunts de dades molecular i citogenètic. Les plantes del complex d'*A. dracunculus* mostren un model coincident amb el que hem descrit com a ancestral, mentre que els representants de la clada més gran i derivada amb la resta de tàxons del subgènere es desvien d'aquest model. La poliploïdització i la hibridació són fenòmens molt freqüents al gènere, però algunes reorganitzacions cromosòmiques, com ara translocacions, podrien explicar alguns canvis en el nombre de *loci* de l'rDNA. Els resultats d'aquest treball indiquen que *Artemisia salsoloides* té el model de la clada basal del subgènere *Dracunculus*, però no donen suport ni a la seva inclusió en el subgènere, ni a la seva segregació, suggerida per la filogènia molecular.

2.4 Canvis en la mida del genoma al llarg d'una distribució fragmentada: el cas d'*Artemisia crithmifolia*

Hem estimat la quantitat de DNA nuclear en 45 poblacions de l'espècie hexaploide *Artemisia crithmifolia*, cobrint tot el rang de distribució de l'espècie. El valor 2C mitjà ha estat de 14,98 pg, amb diferències entre les poblacions d'1,1 vegades, essent el valor mínim el de la població belga de De Panne,

14,27 pg i el més gran el de la població de Tróia (Portugal) amb 15,72 pg. Els valors 2C trobats es corresponen amb el nivell hexaploide descrit per a aquesta espècie (KAWATANI I OHNO 1964; OLIVA I VALLÈS 1994; TORRELL *et al.* 2001 i referències que conté). Les variacions que hem observat indiquen un cert nivell de variabilitat interpoblacional a llarg de la distribució. La constància del valor C al llarg de la distribució d'una determinada espècie s'ha manifestat com un paràmetre variable segons el grup estudiats. AUCKLAND *et al.* (2001) varen considerar que la mida del genoma en poblacions disjuntes d'*Abies fraseri* es mantenia constant, mentre SUDA *et al.* (2005) trobaren el cas contrari en *Hieracium*, apuntant a la hibridogènesi com a possible causa. La constància del nombre de cromosomes d'aquesta espècie en les diferents poblacions fa difícil pensar en processos d'hibridació dintre d'*A. crithmifolia* tot i que no es pot descartar un origen al·lopoliploide d'aquesta espècie.

Les comparacions que hem realitzat amb la finalitat de determinar si la quantitat de DNA nuclear entre les diferents àrees determinades per les disjuncions geogràfiques eren significativament diferents ens indiquen que, d'una banda, els valors 2C de les poblacions de la península Ibèrica i les de França-Països Baixos són significativament diferents ($P = 0,0005$), essent major a la península Ibèrica. D'altra banda, hem obtingut resultats similars en la comparació dels valors 2C considerant com a grups independents la península Ibèrica, França i els Països Baixos, tot i que els dos darrers s'han considerat homogenis. També hem detectat una lleugera correlació negativa entre la mida del genoma i la latitud en *A. crithmifolia* ($r = -0,32$, $P = 0,03$). Aquest tipus de correlació també s'ha trobat en altres gèneres com *Cheirolophus* (GARNATJE *et al.* 2009), tot i que positiva. En aquest cas es postulava que possiblement la mida del genoma era menor en aquelles zones de la Mediterrània més seques i càlides, però sembla que aquest patró no és aplicable a l'Atlàntic segons els resultats que indiquen un major contingut de DNA en les zones més càlides i seques del sud. Dinàmiques semblants a les que hem trobat aquí s'han trobat en el gènere *Pinus* (vegeu GROTKOPP *et al.* 2004; BOGUNIC *et al.* 2007). Hem observat un cert grau de variabilitat en l'indument d'aquestes plantes. Aquestes diferències no semblen tenir una base geogràfica clara i, d'acord amb això,

tampoc no hem detectat cap tipus de correlació entre la glabrescència o la pubescència i la mida del genoma.

En el mateix gènere *Artemisia*, s'ha trobat que per a una mateixa espècie, les poblacions que habiten a les illes poden presentar una mida del genoma superior a aquells relatius continentals, com és el cas d'*Artemisia arborescens* (GARCIA et al. 2006). Tot i que a nivell d'espècie no s'ha trobat aquesta tendència en *Cheirolophus intybaceus* (GARNATJE *et al.* 2009), si que s'ha observat un increment de la quantitat de DNA nuclear a nivell de gènere en les espècies insulars (GARNATJE *et al.* 2006). En el nostre cas no podem comprovar aquesta tendència, ja que, tot i que les poblacions de les illes Britàniques presenten els valors més elevats, l'escàs nombre de poblacions que hi ha en aquell territori (dues), no permet realitzar comparacions estadístiques.

3. Treballs centrats en gèneres afins

3.1 Estudi palinològic en *Ajania* i altres *Artemisiinae*

Amb la intenció de completar els treballs previs de MARTÍN *et al.* (2001, 2003), on es caracteritzen els diferents tipus pol·línics per a un gran nombre d'*Artemisiinae*, hem dut a terme un treball que se centra en l'estudi morfològic del pol·len en 25 espècies del gènere *Ajania*, tot aportant, a més, noves dades per als gèneres *Brachanthemum*, *Cancrinia*, *Crossostephium*, *Dendranthema*, *Elachanthemum*, *Hippolytia*, *Kaschgaria*, *Poljakovia* i *Stilpnolepis*. Els grans de pol·len estudiats de les *Artemisiinae* citades al paràgraf anterior comparteixen les següents característiques morfològiques: tots són 3-zonocolporats, isopolars i presenten simetria radial. La superfície del gra de pol·len presenta espines o espínules supratectals. Tots aquests trets es corresponen amb els que altres autors han trobat amb anterioritat (CHEN I ZHANG 1991; MARTÍN *et al.* 2001, 2003, i referències que conté), essent dividits en dos tipus principals coneguts com tipus *Anthemis* (equinat) i tipus *Artemisia* (equinulat). Les principals diferències que hem trobat entre ambdós tipus pol·línics han estat el volum (quasi el doble en pol·len del tipus *Anthemis* que *Artemisia*) i el nombre

d'elements estructurals (major en els pòl·lens del tipus *Artemisia* que en els del tipus *Anthemis*). D'altra banda, la forma és manté bastant constant en ambdós tipus pol·línics.

Totes les espècies estudiades del gènere *Ajania* han presentat el tipus pol·línic *Anthemis*, amb l'excepció d'*Ajania junnanica* que és l'única amb pol·len del tipus *Artemisia*. Aquest tret ja va ser il·lustrat per MULDASHEV (1983), que va apuntar la possibilitat que aquesta espècie hagués estat inclosa erròniament dintre d'*Ajania*, i que realment podria pertànyer a *Artemisia*. Les altres *Artemisiinae* amb pol·len del tipus *Anthemis* han estat *Brachanthemum*, *Cancrinia*, *Dendranthema*, *Hippolytia*, *Poljakovia* i *Stilpnolepis*. A la resta (*Crossostephium*, *Elachanthemum* i *Kaschgaria*), hem confirmat que presenten grans de pol·len del tipus *Artemisia*.

Tal i com esperàvem, i confirmant estudis previs (SANZ *et al.* 2008), ambdós tipus pol·línics apareixen segregats i caracteritzen els dos grans grups de la filogènia de les *Artemisiinae*, els grups *Artemisia* i *Dendranthema*. Això hi confirma el gran valor filogenètic d'aquest caràcter en la tribu, però tot i això hem trobat algunes excepcions. L'anàlisi filogenètica posiciona *Elachanthemum intricatum* dintre del grup *Dendranthema* i *Stilpnolepis centiflora* dintre del grup *Artemisia*, tot i que ambdues espècies presenten el tipus pol·línic alternatiu al del lloc on estan situades. Com que la taxonomia d'aquestes espècies s'ha revelat conflictiva al llarg del temps, per tal de poder confirmar un esdeveniment de reversió pol·línica en aquests tàxons abans seria necessari descartar la hipòtesi de l'herència del tipus pol·línic a través de processos d'hibridació.

Aquesta hipòtesi pot prendre força en el cas d'*Stilpnolepis*, ja que diferents adquisicions de la regió ITS d'aquesta espècie extretes dels treballs de WATSON *et al.* (2002) i OBERPRIELER *et al.* (2007) la posicionen en diferents clades de la filogènia. Pel que fa a *Elachanthemum*, l'ITS i ETS no proveeixen cap evidència que ens faça pensar en un possible origen híbrid, ja que ambdues regions inclouen aquesta espècie dintre del grup *Dendranthema*. Un altre gènere que es mostra conflictiu pel que fa a tipus pol·línic i posició sistemàtica és *Phaeostigma*. La monofília d'aquest gènere és dubtosa vist que la mateixa regió ETS situa en diferents punts de la filogènia dues espècies d'aquest gènere, a més

de suggerir possibles fenòmens d'hibridació, ja que la resolució de les regions ITS i ETS no sembla congruent.

Com que la majoria dels nodes basals de la filogènia de les *Artemisiinae* no presenten suficient suport estadístic, és difícil afirmar amb claredat quin dels dos tipus pol·línics representa el caràcter ancestral. Tot i això, el fet que la majoria de grups externs presenten el pol·len tipus *Anthemis* fa suposar que aquest tipus representa l'estat ancestral, com també ho suporten dades paleontològiques (WANG 2004). Segons això, la reducció tant en el volum del gra de pol·len com de la mida de l'ornamentació hauria estat la tendència evolutiva principal. En les *Artemisiinae*, aquesta reducció també podria estar correlacionada amb un canvi en la síndrome de pol·linització, d'entomòfila (pòl·lens grans, pesants, força ornamentats i enganxosos, com els del tipus *Anthemis*), a anemòfila (pòl·lens menuts, menys ornamentats i secs, com els del tipus *Artemisia*), segons WODEHOUSE (1935) i FRIEDMAN I BARRETT (2009). En relació amb aquests trets sobre la pol·linització també s'han reportat canvis en l'estructura floral, essent més vistosa i gran en plantes amb pol·linització entomòfila, i més discreta a les anemòfiles (FRIEDMAN I BARRETT 2009). En el nostre cas, les *Artemisiinae* amb pol·len del tipus *Artemisia* presenten capítols menys vistosos mentre que les que presenten pol·len del tipus *Anthemis* tenen capítols més grans, radiats i de colors vistosos. Tot i això sempre trobem algunes excepcions, com són *Crossostephium* o *Filifolium* (pol·len del tipus *Artemisia*), i *Brachanthemum* i *Stilpnolepis* (pol·len del tipus *Anthemis*) que presenten una morfologia floral difícilment atribuïble a un dels dos tipus de pol·linització en concret. Tret d'aquestes excepcions, un canvi d'entomofília a anemofília podria haver tingut lloc paral·lelament a una transició de pol·len del tipus *Anthemis* al del tipus *Artemisia* en les *Artemisiinae*.

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CONCLUSIONS

■CONCLUSIONS

1. Existeixen diferències significatives de valor 2C entre els llinatges filogenètics del gènere *Artemisia*, i aquests valors es mostren més heterogenis en els subgèneres on les relacions filogenètiques no són clares. Significant differences of 2C values exist among the phylogenetic lineages of the genus *Artemisia*, and these values are more heterogeneous in the subgenera in which the phylogenetic relationships are not clear.
2. Es confirma que l'increment de DNA en cada nivell de ploïdia no és proporcional, la qual cosa significa que hi ha una reducció de la quantitat de DNA monoploide (1Cx) en els poliploides. A non-proportional DNA increase in each ploidy level is confirmed, meaning a reduction in monoploid (1Cx) genome size in the polyploid species.
3. Aquesta quantitat tendeix a un màxim, seguint una corba de saturació en concordança amb el model de Michaelis-Menten, el qual permet suggerir un límit superior teòric per al contingut de DNA en el gènere. This amount tend towards a maximum, following a saturation function, in agreement with the Michaelis-Menten model, which permits to suggest a theoretical upper limit for nuclear DNA content in the genus.
4. Es proposen dues colonitzacions d'Amèrica del Sud a partir d'espècies nord-americanes: mentre que *A. magellanica* es troba filogenèticament relacionada amb *A. biennis*, la resta d'espècies sud-americanes formen una clada monofilètica que s'insereix en la formada per les espècies endèmiques nord-americanes. Two colonisation events of South America from North American species are proposed: *A. magellanica* is phylogentically related to *A. biennis*, whereas the remaining South American species perform a monophyletic clade nested in the one formed by the North American endemic taxa.
5. Les espècies d'Amèrica del Sud presenten un patró citogenètic particular: la quantitat de DNA monoploide hi roman quasi constant al llarg dels nivells de ploïdia i s'hi observa un increment proporcional dels *loci* del

DNA ribosòmic. The South American species present a particular cytogenetic pattern: monoploid DNA amount is almost constant along the ploidy levels and a proportional increase of ribosomal DNA loci is observed.

6. Es confirma el patró de dos *loci* d'rDNA en els diploides perennes per als subgèneres *Absinthium* i *Artemisia*. The pattern consisting in two rDNA loci is confirmed in diploid perennials of subgenera *Absinthium* and *Artemisia*.
7. En els nivells alts de ploïdia es produeix una pèrdua de *loci* d'rDNA i en alguns casos d'heterocromatina, mentre que en altres se'n detecta un augment, suggerint l'existència de possibles reestructuracions i mecanismes de silenciament gènic que poden tenir lloc durant la formació dels al·lopoliploides. In the high ploidy levels a loss of rDNA loci and, in some cases of heterochromatin also occurs, whereas in other levels an increase is detected, suggesting the existence of possible restructuring and gene silencing mechanisms that may take place during allopolyploid formation.
8. S'ha detectat un guany de *loci* d'rDNA en moltes de les espècies anuals a causa de les reorganitzacions del cariotip que possiblement han tingut lloc durant la seva evolució. An increase in rDNA loci has been detected in many annual species due to the karyotype reorganizations having possibly taken place during their evolution.
9. L'absència de llinatges estrictament anuals indica que hi ha hagut diferents episodis independents de reversió cap a l'anualitat en tots els subgèneres d'*Artemisia* tret de *Tridentatae*, i reflecteix l'adaptabilitat d'aquestes espècies a un ventall ampli de condicions ambientals. The absence of strictly annual lineages indicates that several independent events of reversion to annuality have occurred in all *Artemisia* subgenera but *Tridentatae*, and reflects the adaptability of these species to very different ambiental conditions.
10. La posició filogenètica d'algunes de les espècies anuals s'ha revelat conflictiva, possiblement a causa de l'elevada taxa de mutació que

presenten. The phylogenetic position of some annual species has revealed itself conflictive, probably because of their high mutatiton rate.

11. Els estudis duts a terme confirmen la baixa incidència d'espècies amb *x* = 8 com a nombre cromosòmic de base, i es corrobora la poliploïdia com a una de els principals forces evolutives en el gènere. The studies performed confirm the low incidence of species with *x* = 8 as base chromosome number and confirm polyploidy as one of the main evolutionary forces in the genus.
12. D'entre els nombrosos, i sovint nous, recomptes cromosòmics duts a terme, moltes dades corresponen a poliploides; hi destaquem la presència d'alts nivells de ploïdia com 2*n* = 10*x* = 90 per a *A. czekanowskiana, A. dracunculus* i *A. sericea*, i 2*n* = 12*x* = 108 per a *A. macrantha*, així com la presència de cromosomes B i fragments acèntrics en *A. litophila*. Among the numerous, and often unreported to date, chromosome counts carried out, many data refer to polyploids; the presence of high ploidy levels is remarkable (2*n* = 10*x* = 90 in *A. czekanowskiana, A. dracunculus* and *A. sericea*, and 2*n* = 12*x* = 108 in *A. macrantha*), as well as B chromosomes and acentric fragments in *A. litophila*.
13. El recompte cromosòmic de 2*n* = 16*x* = 144 en *A. medioxima* és el més alt trobat en el gènere fins al moment present. The chromosome count 2*n* = 16*x* = 144 in *A. medioxima* is the highest ever found in the genus to date.
14. Les reconstruccions filogenètiques suggereixen la divisió del subgènere *Dracunculus* en dos grans grups d'espècies, donant suport a les diferències morfològiques existents entre elles. Phylogenetic reconstructions suggest the division of the subgenus *Dracunculus* in two large species groups, supporting the morphological differences between them.
15. Es confirma la segregació filogenètica dels representants del subgènere *Dracunculus* endèmics d'Amèrica del Nord, així com de les espècies *A. salsoloides* i *A. tanaitica*. The phylogenetic segregation of the North

American representatives of the subgenus *Dracunculus* is confirmed, as well as those of *A. salsoloides* and *A. tanaitica*.

16. Es proposa la reinclusió en el subgènere *Dracunculus*, i per tant en el gènere *Artemisia*, dels gèneres *Mausolea*, *Neopallasia* i *Turaniphytum*. The reinclusion of the genera *Mausolea*, *Neopallasia* and *Turaniphytum* in the subgenus *Dracunculus* is proposed.
17. Es proposa que *Filifolium sibiricum*, originàriament descrit com *Tanacetum sibiricum* L. i posteriorment combinat com *A. sibirica* (L.) Maxim., torni a aquest darrer estatus. *Filifolium sibiricum*, originarily described as *Tanacetum sibiricum* L. an then combined as *A. sibirica* (L.) Maxim., is proposed to regain the latter status.
18. Es confirma la colocalització de les regions 5S i 45S del DNA ribosòmic per a totes les espècies estudiades del subgènere *Dracunculus* i també per a *Filifolium sibiricum*, i la seva posició terminal o en els satèl·lits dels cromosomes, amb comptades excepcions. The colocalization of 5S and 45S rDNA regions is confirmed in all the studied species of subgenus *Dracunculus* and also in *Filifolium sibiricum*; their position in terminal regions of the chromosomes or in satellites is also confirmed with a very few exceptions.
19. Es proposa un possible model ancestral de distribució de l'rDNA per a tot el gènere *Artemisia*, que correspon a un diploide de 18 cromosomes amb dos *loci*, és a dir quatre senyals d'hibridació *in situ*. A putative ancestral model of rDNA distribution in the whole genus *Artemisia* is proposed, corresponding to a diploid with 18 chromosomes and two loci, i.e. four *in situ* hybridization marks.
20. Les variacions respecte d'aquest model ancestral que tenen lloc en el subgènere *Dracunculus* es poden entendre com el resultat de reorganitzacions cromosòmiques i de possibles fenòmens hibridació. The variations with respect of this ancestral model occurring in the subgenus *Dracunculus* may be interpreted as the result of chromosomal reorganizations and possible hybridization phenomena.

21. En la sèrie poliploide d'*Artemisia dracunculus*, l'increment de *loci* es manté quasi proporcional al nivell de ploïdia, en contrast amb la pèrdua de DNA nuclear monoploide, que és més alta en els nivells de ploïdia elevats. In the *Artemisia dracunculus* polyploid series, the loci increase is almost proportional to ploidy level, in contrast with the loss of monoploid DNA amount, which is larger in the highest ploidy levels.
22. S'ha trobat una lleugera correlació negativa entre la latitud i la quantitat de DNA nuclear en *A. crithmifolia* i s'han observat diferències significatives per a aquest paràmetre entre grups de poblacions com a resultat de la fragmentació de l'àrea de distribució d'aquesta espècie. A slight negative correlation has been found between the latitude and the nuclear DNA amount in *A. crithmifolia*, and significant differences have been observed for this parameter as a result of the fragmentation of its distribution area.
23. Es confirmen dos models d'ornamentació de l'exina en els grans de pol·len de la tribu *Anthemideae*. Mentre que el tipus *Artemisia* (equinulat) és exclusiu de la subtribu, el tipus *Anthemis* (equinat) és present més enllà de les *Artemisiinae*. Two models of exine ornamentation are confirmed in the pollen grains of the tribe Anthemideae. The *Artemisia* type (microechinate) is exclusive of the subtribe, whereas the *Anthemis* type (echinate) is present beyond the Artemisiinae.
24. *Ajania junnanica*, *Elachanthemum* i *Stilpnolepis* presenten el tipus de pol·len *Artemisia*, contituint tres excepcions al model general. *Ajania junnanica*, *Elachanthemum* and *Stilpnolepis* have the *Artemisia*-type pollen, constituting three exceptions to the general pattern.
25. Tot i que la resolució filogenètica no permet establir conclusions fermes, el pol·len de tipus *Anthemis* pot representar el caràcter ancestral i el de tipus *Artemisia* el derivat. Although the phylogenetic resolution does not permit to establish definite conclusions, the *Anthemis* pollen type may represent the ancestral character and the *Artemisia* pollen type the derived one.

26. Les característiques dels grans de pol·len i de les inflorescències dels tàxons estudiats apunten cap a la possibilitat, en molts casos, d'un canvi en la síndrome de pol·linització, de l'entomofília a l'anemofília. Pollen grain and inflorescence characters of the studied taxa point toward the possibility, in many cases, of a shift in the pollination syndrome, from entomophyly to anemophyly.