Unitat de Botànica
Departament de Biologia
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Facultat de Ciències

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Evolució, sistemàtica i biogeografia de *Campanula* L. i relacions filogenètiques amb gèneres afins de Campanulàcies

TESI DOCTORAL

Cristina Roquet Ruiz

Barcelona 2008

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Memòria presentada per:

Cristina Roquet Ruiz

Per optar al títol de Doctora per la Universitat Autònoma de Barcelona
Programa de Doctorat en Biologia

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El temps no es perd ni es guanya,
transcorre i el vivim, amb vents propicis
de vegades; d'altres cops amb angoixa.

Tot és incert i, alhora, necessari,
i mai no se sap bé què hi ha rera les dunes
del gran esforç de créixer i de comprendre.

Transcorre el temps;
ningú no el perd ni el guanya.
Transcorre el temps i transcorrem nosaltres.

Miquel Martí i Pol

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Introducció

1. INTRODUCCIÓ

1.1 LA FAMÍLIA *CAMPANULACEAE* JUSSIEU

La família *Campanulaceae* Jussieu, de distribució subcosmopolita, comprèn 5 subfamílies [*Campanuloideae* Burnett, *Cyphioideae* (A. DC.) Schönland, *Cyphocarpoideae* Miers, *Lobelioideae* Schönland i *Nemacladoideae* Lammers] segons Stevens (2006). Les subfamílies *Campanuloideae* i *Lobelioideae* són les que concentren un major nombre de gèneres i espècies. Mentre les *Campanuloideae* apareixen majoritàriament a les zones temperades de l'hemisferi nord, les *Lobeliodeae* les reemplacen a les regions tropicals i subtropicals, especialment a l'hemisferi sud.

La subfamília *Campanuloideae* agrupa prop de 50 gèneres i 1000 espècies, la majoria de les quals es troben a les zones temperades d'Euràsia i Àfrica (Shulkina *et al.* 2003). Aquestes espècies són majoritàriament perennes; generalment amb làtex blanc; les fulles són alternes, simples, sense estípules; la majoria presenten flors hermafrodites, actinomorfes i pentàmeres, amb calzes de 3 a 10 lòbuls i corol.la tubular o acampanada, de color blavós a blanc (tot i que també poden ser grogues o vermelles), amb 5 lòbuls més o menys profunds. Les flors presenten un ovari amb 2 a 8 carpels soldats, 5 estams amb filaments freqüentment eixamplats a la base i ciliats; amb pol.len colpat o porat; els fruits són càpsules dehiscents per porus o valves amb nombroses llavors. Les campanulàcies mostren un interessant mecanisme floral que afavoreix la pol.linització creuada: l'estil presenta pèls col·lectors de pol.len en forma d'urna (o bé l'estil és enganxós, en el cas de *Wahlenbergia* Schrad. ex Roth), i el pol.len s'allibera sobre l'estil a mesura que aquest s'allarga, de manera que, quan la flor s'obre, el pol.len apareix com un cilindre al voltant de l'estil (Kovanda 1978; Shetler 1979; Erdelska 1983). Els insectes pol.linitzadors, quan intenten accedir al nèctar situat entre l'ovari i les bases eixamplades dels filaments de les anteres, es recobreixen de grans de pol.len presents a l'entorn de l'estil (Nowicke *et al.* 1992).

1.2 ANTECEDENTS HISTÒRICS A LA SUBFAMÍLIA *CAMPANULOIDEAE*

1.2.1 Estudis morfològics

Els primers estudis sobre *Campanuloideae* (sovint considerada com *Campanulaceae* s. str.) els trobem en les obres de De Candolle (1830, 1839), Boissier (1875) i Schönland (1889). Aquests

treballs són les classificacions més seguides, però actualment es troben desfasats i resulten incomplets. Aquests, junt amb el tractament de Fedorov (1957) (que comprèn sols els gèneres presents a l'antiga Unió Soviètica, tot aportant 6 noves tribus), constitueixen la base tradicional utilitzada en la classificació de treballs posteriors d'altres autors sobre *Campanuloideae*.

Els principals tractaments florístics de *Campanuloideae* difereixen considerablement entre ells per la seva classificació (Fedorov 1957; Damboldt 1976; Fedorov & Kovanda 1976; Hong 1983). A banda dels treballs esmentats, recentment Shulkina *et al.* (2003) realitzaren un estudi del creixement dels òrgans vegetatius i dels hàbits d'espècies representatives de *Campanuloideae*. Els resultats obtinguts indiquen dues tendències evolutives que concorden amb dos tipus de pol.len (porat *vs.* colpat-colporoidat-colporat). Aquesta hipòtesi concorda amb els resultats de la filogènia molecular d'Eddie *et al.* (2003). Per altra banda, Hong (1995) establí sis grups genèrics en funció d'un conjunt de caràcters morfològics, pol.línics i cromosòmics, així com de la seva distribució. El grup de *Campanula* és el que reuneix un nombre més elevat de gèneres, incloent-hi alguns dels quals han estat considerats com a part de *Campanula* en alguns tractaments.

1.2.2 Estudis citològics

Els recomptes cromosòmics de *Campanuloideae* són nombrosos, i les compilacions existents sobre els recomptes cromosòmics duts a terme en un ampli ventall d'espècies d'aquesta família mostren la complexitat que comporta esclarir les relacions inter- i intragenèriques (Fedorov 1969; Moore 1982; Contandriopoulos 1984). Dins de *Campanuloideae* trobem nombres cromosòmics molt diversos: n= 6-21, 23-30, 32, 34-36, 40, 45, 48, 51, 52 (Lammers 1992). Sols tenint en compte *Campanula,* el gènere principal de la subfamília, trobem una gran varietat de nombres cromosòmics bàsics, des de x= 6 fins x= 17 (Fedorov 1969; Contandriopoulos 1984). Aquesta varietat de nombres bàsics també es troba en altres gèneres com *Asyneuma* Griseb. & Schenk i *Phyteuma* L. A diferència d'aquests gèneres polibàsics, el gènere *Jasione* L. és monobàsic amb x= 6. Favarger & Huynh (1980) suggeriren que els gèneres polibàsics han patit fenòmens de disploïdia fluctuant durant la seva formació.

Dins d'aquesta varietat de nombres cromosòmics, el nombre n= 17 és molt més habitual tant en el gènere *Campanula* (un 69% dels recomptes) com en gèneres propers a *Campanula* (*e.g. Adenophora* Fisch.*, Trachelium* L.), constituint el 42% dels recomptes cromosòmics realitzats dins de

la família (Contandriopoulos 1984; Lammers 1992). De totes maneres, aquest nombre cromosòmic sembla haver aparegut també en altres gèneres poc relacionats com *Canarina* L., *Nesocodon* Thulin, *Ostrowskia* Regel (Eddie *et al.* 2003).

Segons Raven (1975), el nombre cromosòmic ancestral de *Campanuloideae* seria *x*= 7, hipòtesi que concorda amb els recomptes del gènere *Cyananthus* Wall. ex Benth., considerat per alguns autors com a basal dins de la subfamília per la posició superior de l'ovari (Lammers 1992; Eddie *et al.* 2003), i del grup germà Lobeliodeae, amb un clar nombre ancestral *x*= 7 (Raven 1975; Lammers 1992). Emperò, només 12 espècies de campanulàcies presenten *n*= 7 (Lammers 1992). En canvi, Contandriopoulos (1984), en un estudi citotaxonòmic detallat del gènere *Campanula* a la regió mediterrània, suggereix com a nombre cromosòmic bàsic ancestral *x*= 8, a partir del qual, per processos d'aneuploïdia, haurien sorgit els nombres cromosòmics bàsics *x*= 5, 6 i 7; i a partir d'un procés de trisomia i posterior aneuploïdia s'hauria generat el nombre cromosòmic bàsic secundari *x*= 17, el més comú a *Campanula*, nombre que trobem en el 83,5% de recomptes del subgènere *Campanula* (Contandriopoulos 1984).

1.2.3 Palinologia

Trobem diferents tipus d'obertures a les *Campanuloideae* (Erdtman 1952; Thulin 1975). Un total de 14 gèneres presenten pol.len porat (*Adenophora, Campanula, Edraianthus* A. DC.*, Githopsis* Nutt.*, Jasione, Legousia* Durand*, Merciera* DC., *Michauxia* L'Hér., *Microcodon* DC., *Musschia* Dumort., *Phyteuma, Prismatocarpus* L'Hér., *Roella* L., *Wahlenbergia*), mentre que a la resta el pol.len és colpat, colporoidat o colporat (*e.g. Campanumoea* Blume*, Canarina*, *Codonopsis* Wall., *Cyananthus*, *Leptocodon* Lem., *Platycodon* A. DC.). Els gèneres d'obertures allargades són tropicals, la majoria dels quals es troben al sud-est d'Àsia. Aquests, per la seva distribució i morfologia pol.línica, són considerats els més primitius de la família (Dunbar 1975). Aquesta hipòtesi s'ha vist confirmada pel treball d'Eddie *et al.* (2003), ja que l'arbre filogenètic obtingut a partir de seqüències de la regió ITS de DNA ribosòmic nuclear indica que els gèneres amb pol.len colpat o colporat formen un clade basal a la resta de gèneres, amb pol.len porat. En canvi, la presència de diferents tipus d'espines es troba més relacionada amb diferents nivells de ploïdia més que no pas amb correspondències sistemàtiques (Geslot & Médus 1974).

1.2.4 Estudis carpològics

Els estudis de les llavors de campanulàcies han estat pocs i limitats (*e.g.* Thulin 1975; Geslot 1980; Shetler & Morin 1986), així com escassament utilitzats en la sistemàtica del grup (Corner 1976). Les llavors són, en general, llises, i la seva ornamentació és mínima. La morfologia de les llavors dels diferents gèneres és relativament uniforme.

Per altra banda, Kolakovsky (1986) realitzà un estudi carpològic aprofundit de les campanulàcies, en la qual estableix diferents subgrups. Posteriorment, Kolakovsky (1987) va combinar les dades carpològiques amb altres caràcters morfològics, formes d'hàbit i requeriments ecològics, donant lloc a una classificació de campanulàcies en diferents subfamílies i tribus, on cal destacar l'agrupament sota una mateixa tribu, *Campanuleae*, els gèneres *Adenophora, Astrocodon* Fed., *Campanula, Hemisphaera* Kolak., *Megalocalyx* (Damboldt) Kolak., *Roucela* Dumort., *Sicyocodon* Feer, *Symphyandra* A. DC*., Trachelium*, i l'agrupament en una altra tribu de *Neocodon* Kolak. & Serdyuk. [el qual equival a les espècies de *Campanula* subgènere *Rapunculus* (Fourr.) Kharadze] amb *Asyneuma, Brachycodonia* Fed., *Cylindrocarpa* Regel, *Favratia* Feer i *Legousia*.

1.2.5 Estudis de filogènia molecular

Una altra eina utilitzada recentment per esclarir les relacions filogenètiques de les campanulàcies són les dades moleculars. Cosner *et al.* (1994) seqüenciaren una regió cloroplàstica altament conservada, la regió *rbc*L, de diferents gèneres per obtenir una filogènia de l'ordre campanulals. Eddie *et al.* (2003) realitzaren un estudi filogenètic molecular de les campanulàcies *s. str.*, però la utilització d'un únic marcador molecular poc conservat (ITS) es revelà insuficient per resoldre controvèrsies a nivell intragenèric, així com per definir les relacions entre gèneres propers a *Campanula*, i tampoc s'obtingueren resultats concloents sobre la circumscripció natural de *Campanula.* El marcador molecular ITS (*Internal Transcribed Spacer*, espaiador intern transcrit) pertany al DNA ribosòmic nuclear, i és una de les regions més utilitzades en els darrers 15 anys per inferir relacions filogenètiques, especialment a nivell intergenèric i intragenèric, de diferents famílies d'angiospermes. Cosner *et al.* (2004) realitzaren un estudi basat en reordenaments genòmics en el DNA cloroplàstic de diferents gèneres de campanulàcies, en què confirmaren que els gèneres de pol.len colpat o colporat són basals dins de la família. A nivell genèric, només s'han realitzat estudis moleculars sobre el gènere *Adenophora* amb el marcador molecular ITS, amb el qual s'obtingué

poca resolució (Ge *et al.* 1997); i sobre la posició del gènere *Hanabusaya* Nakai respecte a *Adenophora* (Kim *et al.* 1999). A nivell intragenèric, Park *et al.* (2006) estudiaren les relacions filogenètiques d'un petit grup d'espècies mediterrànies de *Campanula* (subsecció *Isophylla* Damboldt) amb el marcador ITS.

1.2.6 Estudis biogeogràfics

S'han elaborat diverses hipòtesis de l'origen biogeogràfic de la família i del gènere principal, *Campanula*. Raven & Axelrod (1974) situaren l'origen de la família a Lauràsia i Àfrica. Hong (1995), a partir d'un índex de caràcters primitius establert pel mateix autor, inferí el centre de formes primitives a l'est d'Àsia, una regió estable geològicament des del Paleozoic, i establí la regió mediterrània i la regió de Sud-àfrica com a centres de diferenciació. L'autor considera que la diferenciació i dispersió de les formes primitives es produí abans del final del Cretàcic. Altres autors situen l'origen de la família al supercontinent Gondwana, ja que molts gèneres basals es troben a l'hemisferi sud (Bremer & Gustafsson 1997), o bé a l'Àfrica, donada la distribució de les *Lobelioideae* (Eddie & Cupido 2001). Per últim, Eddie *et al.* (2003) suggeriren que el centre d'especiació es troba a l'Àsia.

1.3 EL GÈNERE *CAMPANULA*

El gènere principal de la subfamília Campanuloideae, tant en nombre d'espècies com en abast biogeogràfic, és *Campanula*. Aquest gènere, amb 350-500 espècies (Fedorov 1957), presenta una sorprenent diversitat tant a nivell morfològic com cariològic (Contandriopoulos 1984), així com de requeriments ecològics i tipus d'hàbitat, des de boscos i prats fins a sòls pedregosos, estepes i deserts (Fedorov 1957).

1.3.1 Descripció morfològica

Les espècies del gènere *Campanula* són plantes herbàcies majoritàriament perennes; de tija erecta o decumbent; amb fulles alternes (rarament oposades); les flors presenten un pedicel de longitud variable; el calze és pentàmer, a vegades amb apèndixs reflexos entre els lòbuls; la corol.la és sovint acampanada o tubular, dividida en 5 lòbuls soldats; els estams són lliures i els filaments tenen la base eixamplada; l'ovari té 3-5 lòculs; l'estil és únic, pilós, amb 3-5 lòbuls estigmàtics; el fruit

és una càpsula amb 3-5 lòculs, dehiscent per porus o valves; les llavors són nombroses, planes, petites, llises i àpteres.

1.3.2 Antecedents històrics

-Estudis morfològics

Campanula és un gènere del qual molts altres gèneres propers n'han format part (*e.g. Adenophora*, *Asyneuma*, *Feeria* Buser, *Legousia*). A més, donada l'elevada variabilitat morfològica del gènere, es pot dir que és més fàcil determinar què no és *Campanula* que el que hauria de ser una espècie d'aquest gènere (Eddie *et al.* 2003). La circumscripció i el tractament sistemàtic tant subgenèric com seccional de *Campanula* han generat molta controvèrsia. Tal i com ja s'ha exposat, s'ha generat en les darreres dècades un important volum de treball sobre *Campanuloideae* i *Campanula*, però la major part d'aquest es centra únicament en aspectes florístics i en espècies d'àrees geogràfiques concretes. Un altre punt de conflicte en la classificació de *Campanuloideae* i, més concretament, en el gènere *Campanula*, ha estat la utilització de diferents conceptes de gènere per part de diferents autors, alguns dels quals no es corresponen amb grups naturals, ja que es constitueixen sobre la base de caràcters sotmesos a homoplàsia i plasticitat fenotípica, com és el cas del gènere *Symphyandra*, que es basa en la fusió permanent dels estams, mentre que existeixen estadis intermedis de fusió d'estams en flors joves d'altres espècies de *Campanula* (Damboldt 1976; Shulkina *et al.* 2003). En el treball de Shulkina *et al.* (2003) s'indica que *Campanula* és un grup molt heterogeni que caldria revisar, així com l'existència de nombrosos casos de convergència evolutiva tant en les estructures vegetatives com reproductores de Campanuloideae. Les dades obtingudes en el treball de Shulkina *et al.* (2003), basat en l'estudi morfològic i de desenvolupament de plàntules, sols permeten delimitar dos grans grups, un dels quals estaria format per *Campanula* junt amb gèneres "satèl.lit" tals com *Adenophora, Asyneuma, Azorina* Feer*, Edraianthus, Githopsis, Michauxia*, *Phyteuma* i *Symphyandra,* entre altres.

El tractament sistemàtic clàssic de *Campanula* difereix substancialment en diferents autors a causa de la diferent importància donada a determinats caràcters morfològics. Tal i com ja s'ha exposat anteriorment, els autors que van desenvolupar els treballs clàssics més importants van ser De Candolle (1830) i Boissier (1875). De Candolle (1830) establí dues seccions principals basant-se en la presència o absència d'apèndixs reflexos entre els lòbuls del calze. Boissier (1875) prioritzà el tipus de dehiscència capsular com a criteri. Emperò, cal tenir en compte que posteriorment a aquests

treballs s'han descrit més espècies que no s'ajusten a aquestes classificacions. El treball de Schönland (1894) segueix el criteri de Boissier, però inclou espècies que quedaren fora de l'àrea de distribució del treball d'aquest darrer, limitat a Grècia, Egipte i el Pròxim Orient. La classificació de Nyman (1882), així com treballs menys antics com el de Fedorov (1957) i Hayek (1931) també segueixen l'esquema bàsic de Boissier.

Entre els tractaments més moderns, destaca el de Fedorov (1957), malgrat es restringeix a les espècies presents al territori de l'antiga Unió Soviètica. El seu tractament és molt analític, resultant-ne un gran nombre de subseccions i sèries. També va definir dos nous gèneres per a espècies de *Campanula* (*Astrocodon* i *Brachycodonia*). En canvi, el tractament de *Campanula* a Flora Europaea (Fedorov & Kovanda 1976) és molt més simple i conservador, amb sols dues seccions (sect. *Rapunculus* Dumort. i sect. *Campanula* Fed.) establertes segons la dehiscència capsular lateral o basal, tot reincorporant el gènere monotípic *Brachycodonia* a *Campanula.* Un altre treball destacat és el de Damboldt (1976) el qual segueix la línia de Fedorov a la Flora de la URSS (1957) amb modificacions: algunes subseccions són tractades com a seccions, *Brachycodonia* és tractat com a subgènere de *Campanula* i es proposen noves seccions: *Alaria* Damboldt, *Megalocodon* Damboldt*, Platysperma* Damboldt i *Pterophyllum* Damboldt. També crea un nou subgènere, *Megalocalyx* Damboldt, per a les espècies anuals de la subsecció *Annuae* Boiss. amb apèndixs reflexos als calzes, i situa el gènere *Roucela* Dumort. al rang subgenèric, el qual inclou les espècies anuals de la subsecció *Annuae* Boiss. sense apèndixs al calze. D'altra banda, reincorpora les espècies de *Symphyandra* al gènere *Campanula*. D'aquesta manera, les espècies de *Campanula* presents a Turquia conformen sis subgèneres: *Brachycodonia* (Fedorov) Damboldt, *Campanula, Megalocalyx, Rapunculus* (Fourr.) Kharadze*, Roucela* (Dumort.) Damboldt i *Sicyocodon* (Feer) Damboldt. Altres autors van descriure nous gèneres per algunes espècies de *Campanula* que consideraven fora de la circumscripció d'aquest (*e.g. Azorina vidalii* Feer*, Campanulastrum americanum* Small*, Favratia zoysii* Feer). Altres treballs importants, malgrat restringits en àrees concretes, són els de Hayek (1925, Europa Central; 1931, Balcans), Oganessian (1995, Caucas), Quézel (1953, Àfrica del Nord) i Shetler (1963, Nord-Amèrica).

-Estudis citològics

A part de la morfologia, altres caràcters han estat explorats per aportar noves dades a l'estudi de la sistemàtica del gènere *Campanula.* Gadella (1964), sobre la base d'un estudi de recomptes

cromosòmics i híbrids experimentals, junt amb dades morfològiques, suggereix els següents nombres bàsics pel gènere: *x*= 8, 10, 13 i 17. A partir del darrer, *x*= 17, hauria sorgit la sèrie derivada *x*= 15. Trobem la sèrie completa de nombres cromosòmics de *Campanula* a la regió mediterrània. El nombre cromosòmic més habitual, *n*= 17, és present tant en espècies d'àmplia distribució com en espècies relictes del Taurus, i també en seccions localitzades a l'oest d'Àsia. Per explicar aquesta diversitat de nombres, Contandriopoulos (1984) parla d'inestabilitat dels nombres primaris, així com de diversificació posterior de nombres bàsics secundaris. Planteja un esquema filogenètic a partir de *x*= 8 amb tres llinatges: un primer clade amb *x*= 9 representat pel subgènere *Brachycodonia*; un segon amb el nombre cromosòmic bàsic secundari *x*= 17, generat per trisomia, que donà lloc a les series poliploides *n*= 17, 34 i 51; un tercer grup generat per disploïdia descendent que donà lloc als nombres de base *x*= 7, 6 i 5, a partir del quals es formaren series poliploides hiper- i hipoploides, relacionades amb grups ben definits com els subgèneres *Megalocalyx, Roucela* i *Sicyocodon*.

-Palinologia

D'altra banda, els estudis pol.línics sobre *Campanula* són escassos. Dunbar (1975) assenyala una tendència evolutiva des d'un patró reticulat en la ultraestructura de la sexina cap a un patró d'estructures en forma de dit a la superfície dels grans de pol.len, que estaria correlacionat amb la progressiva reducció de la inflorescència. Emperò, Dunbar & Wallentinus (1976) assenyalen la manca de caràcters consistents per a separar clarament els gèneres *Adenophora, Asyneuma, Campanula, Edraianthus, Jasione, Phyteuma, Roella, Symphyandra* i *Wahlenbergia.* En alguns casos la mida del pol.len de *Campanula* es correlaciona amb el nivell de poliploïdia, en especial a la secció *Heterophylla* (Witasek) Tzvelev, tot i que existeixen diverses excepcions (Gadella 1964).

-Estudis carpològics

Com ja s'ha comentat abans, els treballs sobre l'estudi de les llavors de *Campanula* i gèneres propers són pocs i limitats. Geslot (1980) indica l'existència de diferències subtils en el tegument seminal entre les seccions *Campanulastrum* (Small) Fed., *Eucodon* DC. i *Heterophylla* (Wit.) Fed. En un estudi de les espècies de *Campanula* nord-americanes, Shetler & Morin (1986) indiquen que es poden fer alguns agrupaments en base a la morfologia de la llavor, però adverteixen de la manca de consistència d'aquestes dades per a establir una hipòtesi filogenètica sobre la base de les llavors.

Les espècies anuals presenten alguns caràcters comuns en les llavors, que s'atribueixen a possibles avantatges selectius en hàbitats secs o alterats.

1.3.3 Distribució del gènere *Campanula*

Una gran part de les espècies de *Campanula* es distribueixen a la regió mediterrània així com a la península d'Anatòlia i a la regió muntanyosa del Caucas. Concretament, sols en la regió caucasiana hi ha 67 espècies de *Campanula* (Oganessian 1995). En nombrosos casos hi ha parelles d'espècies vicariants distribuïdes una a l'Est i l'altra a l'Oest del Mediterrani. Tot i així, la seva distribució total engloba una àrea molt extensa, des de Nord-Amèrica fins a Japó, passant per Euràsia, i des de les regions àrtiques fins les àrides muntanyes d'Àfrica del Nord. A Nord-Amèrica hi ha 20 espècies, de les quals 16 són endèmiques, amb una distribució pre-Pleistocènica, ja que es troben en zones meridionals on no arribaren les glaciacions, o bé en zones de refugi aïllades (Shetler 1963). *Campanula* comprèn espècies amb una àrea de distribució molt àmplia (*e.g. C. rotundifolia* L., present a Nord-Àmerica i a àmplies regions d'Euràsia), mentre altres es troben restringides en àrees molt concretes, constituint importants endemismes (*e.g. C. creutzburgii* Greuter, endemisme de Creta) (Contandriopoulos 1984). Tot i les diferents hipòtesis sobre el centre d'especiació de la família, l'origen dels dos grans clades de *Campanula* detectats en l'estudi d'Eddie *et al.* (2003) podria situar-se a l'Est del Mediterrani o al Sud-Oest d'Àsia, on la regió d'Anatòlia presenta una flora interessant i molt diversificada, ja que és un punt de connexió de les regions biogeogràfiques eurosiberiana, mediterrània i iranoturaniana.

1.4 La Sistemàtica Molecular

Actualment, la Sistemàtica moderna integra l'estudi del màxim nombre de caràcters possibles, procedents de diferents fonts de dades i metodologies (morfologia, palinologia, citologia, biogeografia, entre altres) per aconseguir classificacions més naturals. En les darreres dècades, els avenços científico-tècnics han obert un nou camp a explorar: els caràcters moleculars. La Sistemàtica Molecular utilitza dades provinents dels àcids ribonucleics i d'altres molècules per inferir els orígens, l'evolució i les relacions de parentiu entre els organismes. La recent i constant millora de les tècniques d'anàlisi informàtica han millorat la interpretació de les dades moleculars. Cal tenir

present, emperò, que les filogènies obtingudes mitjançant tècniques moleculars han de ser interpretades tenint en compte el coneixement botànic tradicional (Judd *et al.* 2002).

L'aplicació de les dades moleculars en Sistemàtica no ha estat exempta de controvèrsia pel diferent valor que se li ha donat en l'obtenció de filogènies. El fenomen d'homoplàsia pot ocórrer en qualsevol tipus de dades, emperò, les dades moleculars presenten les següents avantatges respecte altres dades: la quantitat de caràcters moleculars disponibles és major; els estadis d'aquests caràcters són menys ambigus; evolucionen de forma més regular que els caràcters morfològics i fisiològics, facilitant la valoració de l'homologia (Graur & Li 1999). A més, les seqüències de DNA corresponent a regions poc importants funcionalment presenten l'avantatge d'evolucionar majoritàriament per mutacions neutres, no subjectes a pressió selectiva ambiental, i per tant resulten més adequades per evitar el fenomen d'homoplàsia (Doyle & Gaut 2000).

En cas d'aparèixer incongruències importants entre les dades moleculars i altres dades, o bé entre diferents tipus de dades moleculars, aquestes poden ser degudes al fet que els caràcters morfològics hagin sorgit paral·lelament en diferents llinatges i no reflecteixin per tant l'evolució del grup; a una diferent taxa evolutiva entre les dades morfològiques i moleculars, ja que canvis genètics puntuals poden provocar transformacions morfològiques profundes (Wendel & Doyle 1998); a la introgressió de gens entre espècies (Doyle 1992); o a diferents factors inductors d'error, com poden ser l'ús erroni de gens paràlegs (derivats de duplicació gènica) en lloc d'ortòlegs (derivats de fenòmens d'especiació), taxa d'evolució de la molècula analitzada inadequada, elecció de grups externs excessivament distants, o errors estadístics deguts a un insuficient mostreig de tàxons i de caràcters derivat d'una elecció errònia de la regió d'estudi. (Sanderson & Shaffer 2002).

1.4.1 Marcadors moleculars utilitzats per a la filogènia de *Campanula*.

- DNA nuclear ribosòmic: la regió ITS

La regió ITS (*Internal Transcribed Spacer*) es compon de la subunitat 5.8S més els dos espaiadors interns que són transcrits, l'ITS-1 i ITS-2, situats entre les regions altament conservades de les subunitats 18S i 26S de DNA nuclear ribosòmic. Aquests espaiadors interns no són codificants, per tant, es troben sotmesos a una baixa pressió selectiva. Aquesta regió ha estat molt utilitzada per inferir les relacions filogenètiques en diferents famílies d'angiospermes, especialment a nivell inter- i intragenèric, pels següents avantatges que presenta: la taxa d'evolució sol ser

apropiada a estudis intragenèrics, aportant suficients caràcters per a la reconstrucció filogenètica; està flanquejada per regions altament conservades que faciliten l'amplificació i seqüenciació amb encebadors universals (White *et al.* 1990); i és filogenèticament interpretable ja que les diferències de longitud solen ser petites dins d'un mateix gènere. Aquesta regió presenta milers de còpies al genoma nuclear que no evolucionen de manera independent, sinó que es troben sota una evolució concertada que homogeneïtza les unitats repetitives a nivell intraespecífic. Tot i això, Álvarez & Wendel (2003) van detectar una manca d'homogeneïtat de la regió ITS que podria ser deguda a estadis de transició en l'evolució concertada, a taxes de mutació elevades, a hibridació interespecífica, o al desenvolupament de pseudogens.

-DNA cloroplàstic: *trn*L-F

La regió no codificant *trn*L-F correspon a l'intró del gen *trn*L, d'uns 500 parells de bases (pb), i la regió intergènica situada entre l'exó 3' del gen *trn*L i el gen *trn*F, de longitud variable segons l'espècie (Taberlet *et al.* 1991), situada al DNA cloroplàstic. Aquesta regió té una mida moderada (aproximadament 1000 pb) i es troba situada entre dues regions molt conservades: el gen que transcriu l'àcid ribonucleic de transferència (RNAt) per a la leucina i el RNAt per a la fenilalanina. Aquest marcador té una taxa de canvi més lenta que l'ITS però tot i això permet obtenir caràcters informatius per comparar gèneres diferents i fins i tot espècies d'un mateix gènere, depenent de la família en que es centri l'estudi (Gielly & Taberlet 1994; Salatino *et al.* 2001).

-DNA cloroplàstic: *rbc*L

La regió *rbc*L codifica per a la subunitat gran de l'enzim RuBisCO, i ha estat una de les regions més utilitzades per a filogènies d'angiospermes a nivell intra- i, sobretot, interfamilial. Les avantatges que presenta aquest gen són les següents: és pràcticament universal en totes les plantes (excepte a les paràsites), és prou llarg (1428 pb), no presenta problemes d'alineament, i presenta nombroses còpies en cada cèl.lula. Una de las limitacions del *rbcL* com a marcador filogenètic és la seva baixa taxa de canvi, que en algunes famílies la fa poc útil per a inferir relacions filogenètiques intragenèriques.

- DNA cloroplàstic: *trn*G

Els encebadors que més s'utilitzen per aquesta regió amplifiquen només l'intró del gen *trn*G

que transcriu RNAt. Aquest intró té aproximadament 763 pb, i és una de les regions cloroplàstiques amb més variabilitat intragenèrica, encara que fins ara s'ha utilitzat relativament en pocs estudis (Shaw *et al.* 2005). Per exemple, a l'estudi de Pacak & Szweykoswska-Kulinska (2000), l'intró *trn*G proveïa caràcters moleculars informatius en grups on l'intró *trn*L era invariable, i a l'estudi de Pedersen & Hedenäs (2003), proveïa el doble de caràcters informatius que la regió *trn*L-F.

1.4.2 Datació de filogènies moleculars

Fins fa prop de 50 anys, la ciència només comptava amb una única eina, els fòssils, per inferir el marc temporal de l'evolució d'un o més tàxons, mitjançant la comparació de trets morfològics entre aquests i les espècies actuals. En un estudi comparatiu d'hemoglobines provinents de diferents espècies, Zuckerkandl & Pauling (1962) postularen que l'acumulació de diferències entre proteïnes de diferents espècies és proporcional al temps transcorregut des que aquestes espècies es van separar a partir del seu darrer ancestre comú. Més tard Kimura (1968) exposà que la majoria de mutacions degudes a errors de replicació del DNA són neutres a efectes de la selecció natural, i que la seva acumulació pot ser utilitzada per mesurar el temps de separació entre llinatges.

En un primer moment es va assumir que la taxa de mutació era constant en el temps, així com en les diferents regions del genoma i en diferents espècies. Emperò, estudis posteriors han demostrat que en molts casos el suposat "rellotge molecular" en sentit estricte no existeix, sinó que hi ha una considerable heterogeneïtat de taxes de canvi tant entre diferents regions de DNA com entre diferents llinatges d'un mateix grup taxonòmic (*e.g.* Arbogast *et al.* 2002). Les causes de la heterogeneïtat són diverses: no totes les mutacions són neutres; les transicions són més probables que les transversions; la heterogeneïtat de les taxes de canvi està lligada al nombre de generacions (i per tant, a les taxes reproductives) més que no pas al nombre d'anys; entre altres.

En la darrera dècada s'han proposat diferents mètodes per inferir els temps de divergència dins un grup d'espècies a partir de la reconstrucció filogenètica basada en molècules de DNA o proteïnes. Aquests mètodes es poden classificar en dos tipus segons si corregeixen o incorporen la heterogeneïtat de la taxa de canvi (Welch & Bromham 2005; Rutschmann 2006). Aquestes metodologies treballen amb el concepte de "rellotges moleculars relaxats". Els mètodes més utilitzats en els darrers temps són aquells que incorporen diferents taxes de canvi, tot basant-se en l'assumpció *a priori* que els llinatges més propers tindran taxes de canvi més similars (autocorrelació

temporal), és a dir, que les taxes de canvi varien gradualment al llarg de l'evolució del grup.

Aquests mètodes fan una estimació de la llargada de les branques, i modelen la distribució dels temps de divergència i de les taxes de canvi minimitzant les discrepàncies entre la longitud de les branques i les taxes de canvi al llarg de les branques. Bàsicament trobem dos grups de mètodes segons el tipus d'estadística en què es basin: màxima versemblança (*e.g. Penalized Likelihood*, desenvolupat per Sanderson 2002), i inferència bayesiana (*e.g. Multidivtime*, de Thorne *et al.* 1998), segons Welch & Bromham 2005.

Penalized Likelihood és una tècnica semiparamètrica, basada en una funció de versemblança que penalitza grans canvis de taxes de mutació entre branques properes. *Multidivtime* utilitza un model probabilístic i paramètric per calcular els canvis en la taxa d'evolució al llarg del temps mitjançant l'algoritme *Markov Chain Monte Carlo* (MCMC). Tanmateix, sigui quin sigui el mètode utilitzat, caldrà informació externa a les molècules per calibrar l'arbre filogenètic, ja siguin dades fòssils o esdeveniments paleogeològics, i transformar així les dades relatives del filograma en temps absolut.

L'interès per la datació de filogènies moleculars ha anat creixent en la darrera dècada, ja que aporta informació valuosa per investigar els processos evolutius i les hipòtesis biogeogràfiques de dispersió i vicariança. Els estudis de temps de divergència en plantes són nombrosos a diferents nivells taxonòmics, alguns exemples són: origen de les angiospermes (Magallón & Sanderson 2001; Sanderson & Doyle 2001; Wikström *et al.* 2001; Bell *et al.* 2005); astèrides (Bremer *et al.* 2004); dipsacals (Bell & Donoghue 2005); cripteroniàcies (Conti *et al.* 2002); *Fuchsia* L. (Berry *et al.* 2004). Les úniques dades de datació disponibles fins ara sobre campanulàcies provenen de l'estudi sobre l'origen de les Angiospermes de Wikström *et al.* (2001), que incloïa una espècie de *Campanula* i una de *Codonopsis* Wall. (un gènere basal dins la família). Aquest estudi concloïa que el darrer ancestre comú d'aquests dos gèneres data aproximadament de 41 milions d'anys.

1.4.3 Reconstrucció biogeogràfica a partir d'una filogènia molecular

La biogeografia cladista assumeix una correspondència entre les relacions taxonòmiques i les relacions d'àrees d'un grup de tàxons propers. Fins fa uns 30 anys, l'interès d'aquesta disciplina es va centrar principalment en la recerca de patrons biogeogràfics generals, en detriment de l'estudi històric biogeogràfic de grups taxonòmics concrets. Posteriorment, s'han desenvolupat diferents

metodologies per tal d'inferir la història biogeogràfica de llinatges concrets, centrant-se en la reconstrucció de les distribucions dels ancestres (*e.g.* Bremer 1992; Ronquist 1994; 1995; 1996; 1997; Hausdorf 1998). D'aquests mètodes, el més àmpliament utilitzat en estudis de biogeografia històrica és l'anàlisi de dispersió-vicariança (DIVA) desenvolupat per Ronquist (1996; 1997).

El programa DIVA minimitza el nombre d'esdeveniments històrics (vicariança, dispersió i extinció) per tal d'explicar la distribució geogràfica dels tàxons existents. Concretament, DIVA considera que el tipus d'especiació per defecte és la vicariança, però també considera que pot donar-se dispersió a una o més àrees, així com l'extinció en una o més àrees ancestrals. És per això que estableix que la vicariança té un cost zero, mentre que la dispersió i l'extinció tenen un cost d'u. L'anàlisi es du a terme amb el criteri d'optimització basat en la parsimònia, per tant la solució que obtindrem serà aquella que té un menor cost per explicar l'actual distribució dels tàxons estudiats (Ronquist 1997).

El programa DIVA és especialment adequat per a grups de tàxons en què no hi ha una hipòtesi general prèvia sobre les relacions d'àrees, ja que no assumeix *a priori* cap tipus de patró biogeogràfic. A més, permet que les relacions d'àrees siguin reticulades i no obligatòriament jeràrquiques. Això és important ja que les barreres de dispersió poden aparèixer i desaparèixer repetidament al llarg de la història evolutiva d'un grup (Ronquist 1997). Per una altra banda, una de les limitacions de DIVA és que no considera la incertesa filogenètica, ja que cal proporcionar-li un arbre completament resolt (sense cap politomia). És per això que la millor opció és proporcionar-li un dels arbres més parsimoniosos compatible amb el consens o bé l'arbre amb probabilitat més elevada (en el cas que haguem dut a terme els anàlisis filogenètics mitjançant màxima versemblança o inferència bayesiana).

1.5 ESTRUCTURA DE LA TESI DOCTORAL

Aquesta tesi doctoral s'ha organitzat en 3 capítols. El format de tots 3 capítols es correspon al d'un manuscrit científic, i per tant, es poden llegir per separat o en diferent ordre de lectura al que s'exposa aquí, doncs són independents els uns dels altres. Han estat escrits en llengua anglesa perquè, per bé o per mal, aquesta és la llengua vehicular més emprada per la comunitat científica actualment. El primer capítol ha estat acceptat formalment per la revista científica *Systematic Botany*

per a la seva publicació propera. Els capítols 2 i 3 es troben en diferents fases de preparació per a la seva publicació en revistes científiques d'impacte.

El capítol 1 és un extens treball que pretén estudiar les relacions filogenètiques del gènere *Campanula*, la circumscripció i classificació infragenèrica del qual és altament controvertida. Per a tal propòsit, es varen seqüenciar dues regions, una de DNA ribosòmic nuclear i una cloroplàstica per a ser analitzades independentment i de forma combinada mitjançant els mètodes de Parsimònia i Inferència Bayesiana, per tal de dilucidar les relacions filogenètiques de *Campanula* i gèneres propers, així com explorar els processos biològics que van ocórrer durant l'evolució d'aquest gènere. Es va realitzar un ampli mostreig que inclogués les principals seccions i subgèneres de *Campanula*, així com gèneres propers. Es varen mapejar sobre els arbres filogenètics resultants les següents dades per cercar patrons evolutius: nombre cromosòmic, tipus de corol.la, hàbit, així com el tipus de dehiscència de la càpsula. Donat que els resultats obtinguts apuntaven cap a una elevada convergència en caràcters morfològics relatius a les flors i les càpsules, es va intentar valorar el paper que hi podrien haver jugat els pol.linitzadors, analitzant dades obtingudes al camp i a partir de la literatura científica disponible. Part dels resultats i conclusions obtingudes en aquest estudi van ser presentats en el congrés *XII OPTIMA Meeting*, que realitzà l'Organització per l'Estudi Fito-Taxonòmic de la Regió Mediterrània el mes de Setembre del 2007.

El capítol 2 és un aprofundiment en la comprensió de l'evolució del gènere *Campanula* mitjançant la datació molecular i la reconstrucció de les distribucions ancestrals així com dels esdeveniments de vicariança i dispersió que han donat peu a la distribució actual del gènere *Campanula* i altres gèneres propers. En la darrera dècada, aquestes metodologies han esdevingut una eina important per ampliar la comprensió dels processos evolutius. En aquest treball hem ampliat les dades moleculars seqüenciant la regió cloroplàstica altament conservada *rbc*L, i hem utilitzat dos mètodes de datació que tracten de diferent manera de modelar la taxa de variació entre llinatges, tot calibrant les dades amb una dada fòssil. La reconstrucció biogeogràfica s'ha dut a terme amb el mètode d'anàlisis de dispersió i vicariança de Ronquist (1997). Part dels resultats i conclusions obtingudes en aquest estudi van ser presentats en el congrés *Origin and Evolution of Biota in Mediterranean Climate Zones* organitzat per l'Institut de Botànica Sistemàtica de la Universitat de Zurich el mes de Juliol del 2007.

El capítol 3 és un treball més detallat de filogènia molecular i reconstrucció històrica i biogeogràfica de *Campanula* subgènere *Roucela* mitjançant l'anàlisi de tres marcadors moleculars no codificants, dues regions de DNA cloroplàstic i una regió de DNA ribosòmic nuclear, amb l'objectiu d'aportar noves dades que ajudin a esclarir la delimitació i les relacions evolutives dins d'aquest subgènere controvertit taxonòmicament. A més, l'anàlisi de la història biogeogràfica va permetre inferir el seu origen ancestral així com els esdeveniments de vicariança i dispersió que expliquen la seva actual distribució d'espècies, per tal d'aprofundir en coneixement i comprensió de l'evolució d'aquest subgènere. Aquest treball, sumat a la revisió taxonòmica realitzada per Polo (2007), formarà part d'un manuscrit científic molt complet sobre el subgènere *Roucela* tant a nivell taxonòmic com sistemàtic, que serà properament enviat a una revista d'impacte per a la seva publicació.

1.6 OBJECTIUS GENERALS

1. Determinar la utilitat de les regions ITS, *rbc*L i *trn*L-F per resoldre problemes sistemàtics del gènere *Campanula.*

2. Estudiar el caràcter monofilètic i la circumscripció del gènere *Campanula* i estimar les relacions filogenètiques subgenèriques i seccionals, així com la posició d'aquest respecte a altres gèneres estretament relacionats, mitjançant l'aportació de dades moleculars.

3. Comparar les dades filogenètiques moleculars obtingudes amb caràcters morfològics i citològics per tal de cercar patrons evolutius en el gènere *Campanula*.

4. Aportar, des d'una perspectiva filogenètica, dades sobre l'origen, la història biogeogràfica i el temps de divergència dels diferents llinatges de *Campanula* i gèneres propers, i comparar les dades obtingudes amb esdeveniments paleoclimàtics i paleogeogràfics referits en la literatura científica, per tal de valorar la validesa de vàries hipòtesis elaborades per diferents autors sobre l'origen geogràfic de les campanulàcies i el seu principal gènere, *Campanula*.

5. Determinar la utilitat de les regions ITS, *trn*L-F i *trn*G per estudiar les relacions filogenètiques de *Campanula* subgènere *Roucela*.

6. Comprovar el caràcter monofilètic del subgènere *Roucela* i aportar dades moleculars que permetin establir relacions filogenètiques entre les espècies que el conformen, centrant-nos especialment en el complex *Campanula drabifolia.*

7. Estudiar la història biogeogràfica del subgènere *Roucela.*

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Capítol 1

Natural Delineation, Molecular Phylogeny and Floral Evolution in *Campanula* L.

RESUM. La circumscripció i la classificació intragenèrica de *Campanula* és altament controvertida. Les dades obtingudes a partir de seqüències nuclears i cloroplàstiques van ser analitzades independentment i de forma combinada mitjançant els mètodes de màxima parsimònia i inferència Bayesiana, per tal de dilucidar les relacions filogenètiques de *Campanula* i gèneres aliats, així com explorar els processos biològics que van ocórrer durant l'evolució d'aquest gènere. Es va realitzar un ampli mostreig que inclogués les principals seccions i subgèneres de *Campanula*, així com gèneres propers. Es mapejaren en els arbres filogenètics resultants les següents dades per cercar patrons evolutius: nombre cromosòmic, tipus de corol.la, hàbit, així com el tipus de dehiscència de la càpsula. Les anàlisis filogenètiques revelaren que *Campanula*, en la seva circumscripció actual, no és un gènere monofilètic. Aquest gènere es divideix en dos clades principals: en primer lloc, un ampli clade format per la majoria d'espècies de *Campanula* incloent gèneres propers (*Adenophora, Asyneuma, Azorina, Campanulastrum, Diosphaera, Edraianthus, Githopsis, Hanabusaya, Heterocodon, Legousia, Michauxia, Petromarula, Physoplexis, Phyteuma*, *Trachelium* and *Triodanis*), i en segon lloc, un clade constituit per *Musschia* més dos espècies de *Campanula*. L'ampli clade de *Campanula* es compon de dos grups, un tipus *Rapunculus* i un tipus *Campanula*. Tant l'anàlisi bayesiana com la de parsimònia indiquen que els principals caràcters morfològics utilitzats en les classificacions tals com la forma de la flor i la dehiscència de la càpsula han aparegut paral.lelament. Per explicar la convergència floral es suggereixen fortes pressions selectives per part dels pol.linitzadors. Plantegem dues propostes diferents per tal d'obtenir una classificació de *Campanula* que reflecteixi més acuradament l'evolució d'aquest gènere.

ABSTRACT. The circumscription and intrageneric classification of *Campanula* is highly controversial. Independent and combined data from nuclear and chloroplast sequences (*trn*L–*trn*F, ITS) were analyzed with Bayesian inference and maximum parsimony methods to elucidate the phylogenetic relationships of *Campanula* and allied genera, and explore the biological processes that occurred during the evolution of this genus. An extensive sampling of the main sections and subgenera of *Campanula* and related genera was done. Chromosome numbers, corolla types, habit and capsule dehiscence were mapped on the trees to search for evolutionary patterns. The phylogenetic analyses revealed that *Campanula*, as currently circumscribed, is not monophyletic. This genus is divided into two main clades: a large core of *Campanula* species that includes related genera (*Adenophora, Asyneuma, Azorina, Campanulastrum, Diosphaera, Edraianthus, Githopsis, Hanabusaya, Heterocodon, Legousia,*

Michauxia, Petromarula, Physoplexis, Phyteuma, *Trachelium* and *Triodanis*), and a clade constituted by *Musschia* plus two *Campanula* species. The large core of *Campanula* is divided into two main groups, a rapunculoid and a campanuloid group. Both Bayesian and Parsimony analyses indicate that the main morphological characters used in classifications such as flower shape and capsule dehiscence have arisen in parallel. Strong selective pressures from pollinators are suggested to explain floral convergence. We put forward two different proposals in order to accomplish a classification of *Campanula* that more accurately reflects the evolution of this genus.

2. NATURAL DELINEATION, MOLECULAR PHYLOGENY AND FLORAL EVOLUTION IN *CAMPANULA* L.

2.1 INTRODUCTION

Campanula L. is the largest genus of the Campanulaceae with *c.* 350-500 species inhabiting a wide range of habitats, including meadows, woodland-edges, moorlands and cliffs, as well as steppe and mountainous habitats in the Northern Hemisphere (Fedorov 1957; Kovacic 2004). It belongs to tribe Campanuleae according to Kovanda (1978) that differs from Wahlenbergiae and Platycodoneae in that the ovary is mostly inferior and the capsule dehisces at the sides (indehiscent in few cases).

This subcosmopolitan genus presents a large morphological, carpological (Kolakovsky 1986), palynological (Dunbar 1975; Dunbar & Wallentinus 1976) and karyological variability (Gadella 1964; Contandriopoulos 1984). Most of the representatives of *Campanula* and related genera are herbs with pentamerous flowers. The corolla is often campanulate or infundibuliform, tubular, rotate or with several other peculiar forms. The anthers are free or occasionally connate around the style (*Hanabusaya* Nakai and *Symphyandra* A. DC.). The filaments have generally expanded bases (triangular) that form a dome over the nectariferous disk. However, sometimes the flower bases produce little or no nectar (*Legousia* Durand), or there is a conspicuous tubular nectary (*Adenophora* Fisch.). *Campanula* has a characteristic stylar type of secondary pollen presentation, well described and discussed in the literature (Shetler 1979; Yeo 1993). The ovary is usually tri- or pentalocular, with stigmatic lobes of the same number. The capsule dehisces by pores or valves; it is rarely indehiscent, in all cases with numerous seeds.

The circumscription of *Campanula* is difficult, and the infrageneric classification has been highly controversial. Many approaches to *Campanula* taxonomy have been geographically limited and usually based on a few morphological characters. Different treatments of controversial campanuloid genera and subgenera are shown in Table 1. Sectional classification is also difficult and several authors elaborated rather divergent ordinations (*e. g.* Fedorov 1957; Damboldt 1978). The main early treatments of *Campanula* were the works of De Candolle (1830; 1839) and Boissier (1875), which resulted in quite different classifications. De Candolle considered the calyx appendages as the most important character, and in second place, the type of fruit dehiscence. In contrast, Boissier studied mainly the capsule dehiscence, the number of locules, the plant habit, the inflorescence type, and at the end, the calyx appendages. Fedorov's work was restricted to former USSR (1957) and Damboldt' s work included only Europe and Turkey (1976; 1978), but

TABLE 1. Treatment given by different authors to main possible genera in tribe Campanuloideae included in this work.

Main possible genera in Campanuloideae	De Candolle (1830, 1839)	Damboldt (1976)	Fedorov (1957)	Kolakovsky (1987)	Small (1903)	Tutin (1976)	Species included
Adenophora Fischer	*Adenophora*	*Adenophora*	*Adenophora*	*Adenophora*	-	*Adenophora*	*Adenophora divaricata* *A. himalayana* *A. japonica* *A. lobophylla* *A. morrisonensis* *A. paniculata* *A. petiolata* *A. potaninii* *A. remotiflora* *A. stenanthina* *A. stricta* *A. triphylla*
Asyneuma Griseb. & Schenk	In *Phyteuma* as section III	*Asyneuma*	*Asyneuma*	*Asyneuma*	-	*Asyneuma*	*Asyneuma limonifolium* *A. lobelioides*
Azorina Feer	-	-	-	*Azorina*	-	*Azorina*	*Azorina vidalii*
Brachycodon Fed.	In *Campanula*	In *Campanula* as subgenus	*Brachycodon*	*Brachycodonia*	-	In *Campanula* as subgenus	*Campanula fastigiata*
Campanulastrum Small	In *Campanula*	-	-	-	*Campanulastrum*	-	*Campanulastrum americanum*
Diosphaera Feer	-	In *Campanula* as section *Tracheliopsis*	-	-	-	In *Trachelium*	*Diosphaera rumeliana*
Echinocodon Kolak	In *Campanula*	In *Campanula* as section *Pterophyllum*	-	*Echinocodon*	-	In *Campanula*	*Campanula primulifolia*
Edraianthus DC.	*Edraianthus*	-	*Edraianthus*	*Edraianthus*	-	*Edraianthus*	*Edraianthus graminifolius* *E. pumilio* *E. tenuifolius*
Gadellia Shulkina	In *Campanula*	In *Campanula*	In *Campanula*	*Gadellia*	-	In *Campanula*	*Gadellia lactiflora*
Githopsis Nutt.	-	-	-	-	*Githopsis*	-	*Githopsis diffusa*
Hanabusaya Nakai	-	-	-	*Hanabusaya*	-	-	*Hanabusaya asiatica*
Hemisphaera Kolak.	In *Campanula*	In *Campanula* as subgenus *Scapiflorae*	In *Campanula*	*Hemisphaera*	-	In *Campanula*	*Campanula armazica* *C. aucheri* *C. bellidifolia* *C. saxifraga* *C. sosnowsky* *C. tridentata*

Legousia Durand	*Specularia* (= *Legousia*)	*Specularia* (= *Legousia*)	*Legousia*	*Legousia*	*Specularia* (=*Legousia*)	*Specularia* (= *Legousia*)	*Legousia falcata* *L. hybrida* *L. speculum-veneris*
Megalocalyx (Damboldt) Kolak.	In *Campanula*	In *Campanula* as subgenus	In *Campanula*	*Megalocalyx*	-	In *Campanula*	*Campanula balfourii* *C. dichotoma* *C. propinqua* *C. semisecta*
Michauxia L´Hér.	*Michauxia*	*Michauxia*	*Michauxia*	*Michauxia*	-	*Michauxia*	*Michauxia tchihatchewii*
Neocodon Kolak. & Serdyuk.	In *Campanula*	In *Campanula* as subgenus *Rapunculus*	In *Campanula* as section *Rapunculus*	*Neocodon*	-	In *Campanula* as section *Rapunculus*	*Campanula abietina* *C. carpatica* *C. olympica* *C. persicifolia* *C. rapunculus* *C. stevenii*
Petromarula Vent. ex R. Hedw.	*Petromarula*	-	-	*Petromarula*	-	*Petromarula*	*Petromarula pinnata*
Physoplexis (Endl.) Schur	In *Phyteuma*	-	-	*Physoplexis*	-	*Physoplexis*	*Physoplexis comosa*
Phyteuma L.	*Phyteuma*	-	*Phyteuma*	*Phyteuma*	*Phyteuma*	*Phyteuma*	*Phyteuma orbiculare* *P. spicatum*
Rapunculus Mill.	In *Campanula*	In *Campanula* as subgenus *Rapunculus*	In *Campanula* as section *Rapunculus*	*Neocodon*	-	In *Campanula* as section *Rapunculus*	*Campanula haradjanii* *C. olympica* *C. persicifolia* *C. rapunculus* *C stevenii*
Roucela Dumort.	In *Campanula*	In *Campanula* as subgenus *Roucela*	In *Campanula* as subsection *Annuae*	*Roucela*	-	In *Campanula*	*Campanula creutzburgii* *C. drabifolia* *C. erinus* *C. pinatzii*
Sicyocodon Feer	-	In *Campanula* as subgenus	-	*Sicyocodon*	-	-	*Campanula macrostyla*
Specularia A. DC. (the correct name is *Legousia*)	*Specularia*	*Specularia*	*Legousia*	*Legousia*	*Specularia*	*Specularia*	*Legousia falcata* *L. hybrida* *L. speculum-veneris*
Symphyandra A. D.C.	*Symphyandra*	*Symphyandra* and in *Campanula* as section *Symphyandriformes*	*Symphyandra* and in *Campanula* as section *Symphyandriformes*	*Symphyandra*	-	In *Campanula*	*Campanula armena* *C. betulifolia* *C. hofmanii* *C. ossetica* *C. pendula*

Theodorovia Kolak. ex Ogan.	-	-	In *Campanula*	*Theodorovia*	-	-	*Campanula karakuschensis*
Trachelium L.	*Trachelium*	-	-	*Trachelium*	-	*Trachelium*	*Trachelium caeruleum*
Tracheliopsis Buser	-	In *Campanula* as section	-	*Tracheliopsis*	-	-	*Campanula fruticulosa* *C. postii* *C. pubicalyx*
Triodanis Raf.	In *Specularia*	-	-	-	-	-	*Triodanis leptocarpa*

both intended to expand the current treatments and obtain a more natural classification. Their works resulted in rather divergent systems, especially at the sectional level. Other important works were done by Hayek (1925, 1931, Balkans), Quézel (1953, North Africa), Shetler (1963, North America) and Oganessian (1995, Caucasus), but all of them were limited by a narrow geographical scope.

Other aspects apart from morphological characters have been explored to investigate the relationships in the genus. Gadella (1964) and Contandriopoulos (1984) attempted to infer phylogenetic relationships combining cytology and morphology. However, *Campanula* presents a great variety of basic numbers, even within the taxa of the Mediterranean basin alone. The most common number, *x*= 17, has been found in some Campanulaceae not closely related to *Campanula*, such as *Canarina* L., *Nesocodon* M. Thulin and *Ostrowskia* Regel. Several numbers have been suggested as ancestral for the genus or the family (*x*= 7 by Raven 1975; *x*= 8 by Contandriopoulos 1984).

Pollen studies in *Campanula* made by Dunbar (1975) suggested a relationship between the change in pollen ornamentation from ridges to finger-like structures, and the reduction of the inflorescence. Dunbar & Wallentinus (1976) indicated the insufficiency of these characters to separate the allied genera *Adenophora, Asyneuma* Griseb. & Schenk*, Campanula, Edraianthus* A. DC.*, Jasione* L.*, Phyteuma* L.*, Roella* L.*, Symphyandra* and *Wahlenbergia* Schrad. ex Roth. Carpological studies by Kolakovsky (1986) did not serve to clarify the relationships between these taxa. Works dealing with the seeds (Geslot 1980; Shetler & Morin 1986) also found high similarity among them. Finally, Shulkina *et al.* (2003), in a work of growth and seedling morphology, suggested that *Campanula* is a heterogeneous group that should be revised, and that many of related genera should be merged in *Campanula* because they are distinguished by only a few homoplasic characters. This work also stated that similarities in Campanulaceae due to convergent evolution occur in reproductive and vegetative structures.

Many studies have also remarked on the role of reproductive systems and pollinator service and behavior in the plasticity or evolution of flower shape (Shetler 1982; McCall & Primack 1992; Maad & Armbruster 2005; Maad *et al.* 2006; Pérez *et al.* 2006). We studied from our data and the literature whether the pollinator composition may be related to the corolla shape. The available data obtained from literature suggests that rotate corollas are more visited by unspecialized insects such as Diptera (Syrphidae and Muscidae), small bees and *Xylocopa*; broad and deep-campanulate corollas are mainly visited by more specialized taxa such as

bumblebees and large solitary bees (McCall & Primack 1992; Bingham & Orthner 1998; Blionis & Vokou 2001; Al-Zein & Musselmann 2004; Schlindwein *et al.* 2005). Species with high autogamy rates (*i. e. Githopsis* Nutt., *Legousia*) have usually rotate flowers, but these are smaller (Trent 1940; Morin 1983).

Recently, phylogenetic relationships within the family have been explored by means of analysis of ITS-DNA sequences (Eddie 1997; Eddie *et al.* 2003) and cpDNA rearrangements (Cosner *et al.* 2004). These results suggest that the family is divided into two groups: the taxa related to *Campanula* with porate pollen grains and the rest of genera that have colporate or colpate grains (*Campanumoea* Blume*, Canarina, Codonopsis* Wall.*, Cyananthus* Wall. ex Benth.*, Leptocodon* Lem. and *Platycodon* A. DC.). Molecular data (ITS sequences) of the genera *Adenophora* and *Hanabusaya* have also been analysed (Ge *et al.* 1997; Kim *et al.* 1999), but very low divergence was obtained. Park *et al.* (2006) recently studied the phylogenetic relationships of a small central Mediterranean group of the genus *Campanula* (subsection *Isophylla* Damboldt) by means of ITS data, suggesting lack of concordance between sectional classification and molecular data.

2.2 Main Objectives

The complexity of *Campanula* and the lack of definitive results about its phylogenetic relationships led us to explore them by means of a combined analysis of plastid and nuclear sequences: the chloroplast DNA region *trn*L-*trn*F (*trn*L-F) and the internal transcribed spacer (ITS) of nuclear ribosomal DNA. The sequences studied by Eddie *et al.* (2003) were joined to our molecular data of ITS and *trn*L-F using a wide set of taxa. Our goals in the present work are: (1) to ameliorate the understanding of the phylogenetic relationships of *Campanula* and allied genera, (2) to test and compare the phylogenetic utility of *trn*L-F and ITS within the genus *Campanula*, (3) to achieve information about the biological processes that occurred during the evolution of this genus, and (4) to study the role of pollinators in the floral evolution of *Campanula* and related genera.

2.3 MATERIALS AND METHODS

2.3.1 Plant material

The selection of taxa was done to represent the main sections and subgenera considered in the main treatments of *Campanula* (De Candolle 1830; Boissier 1875; Fedorov 1957; Damboldt 1976). Samples of the genera *Adenophora, Asyneuma, Azorina* Feer, *Diosphaera* Feer*, Edraianthus, Feeria* Buser*, Gadellia* Schulkina*, Githopsis, Hanabusaya, Heterocodon* Nutt.*, Legousia, Michauxia* L'Hér.*, Musschia* Dumort.*, Petromarula* Vent. ex R. Hedw., *Physoplexis* Schur*, Phyteuma, Trachelium* L., and *Triodanis* Raf. were analysed.

A total of 41 new sequences of ITS region of the subfamily Campanuloideae were produced, and 82 were obtained from GenBank. The species added in this work were selected to study the relationships of subgenera (*e. g. Megalocalyx* Damboldt) and sections [*e. g. Tracheliopsis* (Buser) Damboldt] of *Campanula* not sampled in Eddie *et al.* (2003), and also to increase the sampling of some sections [*e. g. Rapunculus* (Fourr.) Boiss.], subgenera [*e. g. Roucela* (Feer) Damboldt] of *Campanula* and other genera (*Asyneuma*, *Edraianthus, Legousia*) that were poorly represented. For the *trn*L-F region, a total of 105 sequences of the subfamily Campanuloideae were included, plus one taxon belonging to Lobelioideae (*Solenopsis laurentia* C. Presl.), another subfamily of Campanulaceae (Stevens 2006). All the *trn*L-F sequences were newly produced but one that was obtained from GenBank (*Campanula elatines* L.). Sources of material and location of vouchers are in Table 2. The difference between the number of sequences used in *trn*L-F, ITS and combined data lays mainly in taxa that were available in GenBank, as in some cases it was impossible to sequence one of the regions because of lack of material, or old herbarium material. We used as outgroups *Solenopsis* C. Presl.*, Wahlenbergia*, *Roella*, *Craterocapsa* Hilliard & B. L. Burtt, *Jasione*, *Platycodon* and *Canarina* (the last two genera present a different pollen type, similar to Lobelioideae). *Solenopsis*, *Platycodon* and *Canarina* were used as outgroups only for the more conserved region *trn*L-*trn*F, and not for the ITS data because of high ambiguity in the alignment.

2.3.2 DNA extraction, amplification and sequencing

Total DNA was extracted from herbarium material or, in some cases, from silica gel-dried plant tissue following the CTAB method (Doyle & Doyle 1987) with the modifications suggested by Culling (1992). For difficult material we used the kit "DNeasy® Mini Kit" (Qiagen Inc., Valencia, CA), according to manufacturer's instructions.

PCR amplifications were performed with the thermocycler PTC-100™ Programmable Thermal Controller (MJ Research, Inc.). The complete ITS region was amplified with primers 1406F (Nickrent *et al.* 1994) and ITS4 (White *et al.* 1990). In some cases we substituted 1406F by ITS1 (White *et al.* 1990). The PCR profile included 2 minutes at 94ºC; 5 minutes at 80ºC, while DNA-polymerase (Ecotaq, Ecogen S. R. L., Barcelona, Spain) was added; 30 cycles of 1 minute denaturing at 94°C, 2 minutes annealing at 55°C, and 3 minutes of extension at 72°C; with final extension of 15 minutes at 72°C. The *trn*L-F region was amplified using external primers "c" and "f" and internal primers "d" and "e" (Taberlet *et al.* 1991), amplifying the *trn*L (UAA) intron and the intergenic spacer between the *trn*L (UAA) 3' exon and the *trn*F (GAA) 5' exon. The PCR profile consisted of 1 minute and 35 seconds at 95ºC; 5 minutes at 80ºC, while DNA-polymerase (Ecotaq, Ecogen S. R. L.) was added; 34 cycles of 1 minute denaturing at 93ºC, 1 minute annealing at 50ºC, 2 minutes of extension at 72ºC; and final extension of 10 minutes at 72ºC.

PCR products were cleaned using the "QIAQuick® DNA cleanup system" (Qiagen Inc., Valencia, CA) according to manufacturer's instructions and sequenced with ITS4 and 1406F primers for ITS region, and with the *trn*L-F c and *trn*L-F f primers for the *trn*L-F region. DNA sequencing of PCR-purified templates was done using reactions based on chemistry of "Big Dye® Terminator v3.1" (PE Biosystems, Foster City, California) following the protocol recommended by the manufacturer.

The products obtained were analyzed on an ABI Prism® 3730 PE Biosystems/Hitachi automated sequencer in the "Serveis Cientificotècnics de la Universitat de Barcelona", and the resulting chromatograms were edited with Chromas 2.0 (Technelysium Pty Ltd, Tewantin, Australia).

2.3.3 Phylogenetic analyses

Sequences were aligned independently and manually using the text editor TextPad® 4.7.3. Alignments for ITS and *trn*L-F regions were also produced with the program MAFFT v. 5.667 (Katoh *et al.* 2002, 2005), considering E-INS-I strategy and standard gap penalties, and were used to correct the manual ones. For the ITS sequence alignment, the highly conserved 5.8 subunit was not included in phylogenetic analyses as it was not available for all taxa. The 3' end of the ITS2 region close to the 26S subunit was deleted at 205 bases downstream from the start of ITS2 region because of its high ambiguity. For the *trn*L-F alignment, bases 1-36 that formed a primer-binding region were excluded to reduce missing data, and bases 268-316 were deleted to avoid ambiguity.

TABLE 2. Origin of the materials, herbaria where the vouchers are deposited and GenBank accession numbers (new sequences indicated by bold type).

Species	Voucher (ITS)	Voucher (*trn*L-F)	ITS accession	*trn*L-F accession
Adenophora divaricata Franch. & Sav. (1)	Eddie *et al.* (2003)		AY322005, AY331418	
Adenophora divaricata Franch. & Sav. (2)	Eddie *et al.* (2003)		AF090710, AF09071	
Adenophora himalayana Feer	Eddie *et al.* (2003)		AF090716, AF09071	
Adenophora lobophylla D. Y. Hong	Eddie *et al.* (2003)		AF090706, AF09070	
Adenophora morrisonensis Hayata	Eddie *et al.* (2003)		AF090718, AF09071	
Adenophora paniculata Nannf.	Eddie *et al.* (2003)		AF090714, AF09071	
Adenophora petiolata Pax & Hoffm.	Eddie *et al.* (2003)		AF090700, AF09070	
Adenophora potaninii Korsh.	Eddie *et al.* (2003)		AF090704, AF09070	
Adenophora remotiflora (Sich. & Zucc.) Miq.	Eddie *et al.* (2003)	Japan, Kawasaki: Honshu, *Estebánez 1511* (MA *s. n.*)	AY322006, AY331419	EF088693
Adenophora stenanthina (Ledeb.) Kitagawa	Eddie *et al.* (2003)		AF090708, AF09070	
Adenophora stricta Miq.	Eddie *et al.* (2003)		AF090712, AF09071	
Adenophora triphylla (Thunb.) DC.	Eddie *et al.* (2003)		AY548193, AY548194	
Adenophora wawreana Zahlbr.	Eddie *et al.* (2003)		AF090702, AF09070	
Asyneuma limonifolium Bornm.	Turkey: Erzurum, *Nisa 1006* (MA *689405*)	Turkey: Erzurum, *Nisa 1006* (MA *689405*)	EF090520, EF090561	**EF088694**
Asyneuma lobelioides Hand.-Mazz.		Turkey: Ermenek, *Aldasoro 9157 et al.* (MA *s. n.*)		EF088695
Azorina vidalii (Wats.) Feer	Eddie *et al.* (2003)	Portugal: Açores, *Sequeira 4493* (MA *s. n.*)	AY32207, AY331420	EF088696
Campanula abietina Griseb. & Schenk		Turkey: Zongüldak, *Aedo 6469b* (MA *688196*)		EF088697
Campanula affinis Roem. & Schult.		Spain, Barcelona: Montserrat, *Roquet V-2004* (BC *s. n.*)		EF088698
Campanula alliarifolia Willd.	Eddie *et al.* (2003)	Cultivated at Botanical Garden of Madrid (MA *688448*)	AY322008, AY331421	EF088700
Campanula andrewsii DC.		Greece, Peloponnese: Achaia, *Burri et al. 2-VII-1996* (LE *s. n.*)		EF088701
Campanula aparinoides Pursh	Finland, Palkane: Lake Tykolanjawi, *Nunmi s. n.* (MA *451610*)	Finland, Palkane: Lake Tykolanjawi, *Nunmi s. n.* (MA *451610*)		**EF088702**
Campanula argaea Boiss. & Bal.		Turkey, Kayseri: Erciyas Dag, *Alpinar et al. 2-VII-1994* (ISTE *s. n.*)		EF088703
Campanula armazica Kharadze	Eddie *et al.* (2003)		AY322009, AY331422	
Campanula armena Stev.	Armenia, Ashtarak: Mt. Arailer, *Vasak 15-VII- 1975* (MA *642322*)	Armenia, Ashtarak: Mt. Arailer, *Vasak 15-VII-1975* (MA *642322*)	**EF090521, EF090562**	EF088704
Campanula arvatica Lag.	Eddie *et al.* (2003)		AY322010, AY331423	
Campanula balfourii Wagner & Vierh.	Yemen, Socotra: Qalansiyah, *Thulin 8712 et al.* (UPS *82575*)	Yemen, Socotra: Qalansiyah, *Thulin 8712 et al.* (UPS *82575*)	**EF090522, EF090563**	EF088705

Campanula barbata L.	Eddie *et al.* (2003)		AY322011, AY331424	
Campanula bellidifolia Adams (1)	Eddie *et al.* (2003)		AY322012, AY331425	
Campanula bellidifolia Adams (2)	Cultivated at Botanical Garden of Madrid, *Alarcón 230* (MA *s. n.*)	Cultivated at Botanical Garden of Madrid, *Alarcón 230* (MA *s. n.*)	**EF090523, EF090564**	EF088706
Campanula betulifolia K. Koch	Turkey, Gümüshane: Tirebolu-Kürtün, *Herrero 1180* (MA *689193*)	Turkey, Gümüshane: Tirebolu-Kürtün, *Herrero 1180* (MA *689193*)	**EF090524, EF090565**	EF088707
Campanula carpatica Jacq.	Eddie *et al.* (2003)		AY322013, AY331426	
Campanula chamissonis Fed.		Japan: Honshu, *Estebánez 1478* (MA *s. n.*)		EF088709
Campanula cochlearifolia Lam.		Spain, Huesca: Bielsa, *Roquet 12-X-2004* (BC *s. n.*)		**EF088710**
Campanula collina M. Bieb.		Georgia, Javakhati: Mt. Taushan-Tagan, *Ketzkoveli 22-VII-80* (MA *575569*)		EF088711
Campanula conferta DC.	Turkey, Sakaltutan Gecidi: Erzincan, *Aldasoro 2647* (MA *689787*)	Turkey, Sakaltutan Gecidi: Erzincan, *Aldasoro 2647* (MA *689787*)	**EF090525, EF090566**	**EF088712**
Campanula coriacea Boiss. & Kotschy		Armenia, Arna: Ejevi Azor, *Oganessian 3-VIII-63* (MA *560762*)		**EF088713**
Campanula creutzburgii Greuter		Greece, Kreta: Dia, *Alpinar* (ISTE *s. n.*)		EF088714
Campanula cymbalaria Sibth & Sm.		Turkey, Kayseri: Erciyas Dag, *Alpinar et al. 23-VII-94* (ISTE *62303*)		EF088715
Campanula decumbens DC.	Spain, Cuenca: Barajas de Melo, *Arán 30-V-98 et al.* (MA *623787*)	Spain, Cuenca: Barajas de Melo, *Arán et al. 30-V-98* (MA *623787*)	**EF090526, EF090567**	**EF088716**
Campanula dichotoma L.	Italy: Sicily (MA *645874*)	Italy: Sicily (MA *645874*)	**EF090527, EF090568**	EF088717
Campanula dimorphanta Schweinf.		Taiwan, Hualien: Hisiulin Hsiang, *Chih-Chia Wang 1353* (LE *s. n.*)		**EF088708**
Campanula divaricata Michx.	Eddie *et al.* (2003)	USA, Waterville: Pigeon River (MA *391570*)	AY322014, AY331427	**EF088718**
Campanula drabifolia Sibth. & Sm.	Greece, Peloponnese: Tolon, Argolida, *Buggenhourt 18481* (MA *625645*)	Greece, Peloponnese: Tolon, Argolida, *Buggenhourt 18481* (MA *625645*)	**EF090528, EF090569**	**EF088719**
Campanula edulis Forssk.	Eddie *et al.* (2003)		AY322015, AY331428	
Campanula elatines Bout. ex Willk. & Lange		Bremer *et al.* (2002)		AJ430970
Campanula erinus L.	Eddie *et al.* (2003)	Spain, Mallorca: Cova Negra, *Sáez 6135* (BCB)	AY322016, AY331429	EF088720
Campanula fastigiata Dufour ex Schult.	Spain: Albacete, *Aedo 3937* (MA *591308*)	Spain: Albacete, *Aedo 3937* (MA *591308*)	**EF090529, EF090570**	**EF088721**
Campanula filicaulis Dur.	Morocco, Middle Atlas: Midelt, *Jury 17866* (MA *616923*)	Morocco, Middle Atlas: Midelt, *Jury 17866* (MA *616923*)	**EF090530, EF090571**	EF088722
Campanula foliosa Ten.		Italy: Mt. Vigula, *Snogerup 15903* (UPS *s. n.*)		**EF088723**
Campanula fruticulosa (O. Schwarz & Davis) Damboldt	Turkey, Burdur, Dirmil: Masda Dagi, *Dumar 6279* (ISTE *s. n.*)	Turkey, Burdur, Dirmil: Masda Dagi, *Dumar 6279* (ISTE *s. n.*)	**EF090531, EF090572**	**EF088724**
Campanula garganica Ten.	Italy: Foggia, *Aldobrandi et al. 12-VII-96* (MA *625685*)	Italy: Foggia, *Aldobrandi et al. 12-VII-96* (MA *625685*)	**EF090532, EF090573**	**EF088725**
Campanula glomerata L.	Eddie *et al.* (2003)		AY322017, AY331430	

Campanula grossheimii Kharadze	Eddie *et al.* (2003)		AY322018, AY331431	
Campanula haradjanii Rech. f.		Turkey, Gümüshane: Tirebolu-Kürtün, *Herrero 1234* (MA *688153*)		EF088726
Campanula hawkinsiana Hausskn. & Heldreich	Eddie *et al.* (2003)		AY322019, AY331432	
Campanula herminii Hoffmans. & Link.	Eddie *et al.* (2003)		AY322020, AY331432	
Campanula hofmannii (Pant.) Greuter & Burdet	Bosnia-Herzegovina: Torlokovac, *Frost-Olsen 4953* (MA *464670*)	Bosnia-Herzegovina: Torlokovac, *Frost-Olsen 4953* (MA *464670*)	**EF090533, EF090574**	**EF088727**
Campanula hohenackeri Fisch. & C. A. Mey.	Eddie *et al.* (2003)		AY322021, AY331434	
Campanula incurva Aucher ex DC.	Cultivated at Botanic Institute of Barcelona, *Roquet s. n.* (BC *s. n.*)	Cultivated at Botanic Institute of Barcelona, *Roquet s. n.* (BC *s. n.*)	**EF090534, EF090575**	EF088728
Campanula involucrata Aucher ex DC.	Turkey, Gümüshane: Yagmürdere, *Herrero 1453* (MA *687604*)	Turkey, Gümüshane: Yagmürdere, *Herrero 1453* (MA *687604*)	**EF090535, EF090576**	**EF088729**
Campanula karakuschensis Grossh.	Iran: Ghogeh Dag, *Rechinger 44029* (MA 417801)	Iran: Ghogeh Dag, *Rechinger 44029* (MA 417801)	**EF090536, EF090577**	**EF088730**
Campanula kolenatiana C. A. Mey.	Eddie *et al.* (2003)		AY322022, AY331435	
Campanula lanata Friv.	Eddie *et al.* (2003)	Bulgary, Rila: Kostenec, *Frost-Olsen 484* (MA *463958*)	AY322023, AY331436	EF088731
Campanula latifolia L.	Eddie *et al.* (2003)	Turkey, Trabzon: Sumelas, *Valcarcel 379* (MA *689767*)	AY322024, AY331437	**EF088732**
Campanula lusitanica Loefl.	Eddie *et al.* (2003)	Spain, A Corunya: Carnota, *Louzan 1-VI-96* (MA *581374*)	AY322025, AY331438	**EF088733**
Campanula lyrata Lam.		Greece, Lesbos: Plomari, *Julin 22-IV-82* (UPS *s. n.*)		EF088734
Campanula macrochlamys Boiss. & Huet		Turkey, Artvin: Lomasen Üstü, *Baytop 18-IV-82* (ISTE *48574*)		EF088735
Campanula macrostachya Willd.		Turkey, Kirkareli: Pinarhisar arasi, *Baytop 17-VI-72* (ISTE *22508*)		EF088736
Campanula macrostyla Boiss. & Heldreich		Turkey: Ermenek, *Aldasoro 9135 et al.* (MA *s. n.*)		EF088737
Campanula medium L.	Cultivated at Botanical Garden of Madrid, *MLA0183* (MA *s. n.*)	Cultivated at Botanical Garden of Madrid, *MLA0183* (MA *s. n.*)	**EF090537, EF090578**	**EF088738**
Campanula mirabilis Albov	Eddie *et al.* (2003)		AY322026, AY331439	
Campanula mollis L.	Eddie *et al.* (2003)	Spain, Almería: Gádor, Borja, *Navarro 1303* (MA *545932*)	AY322027, AY331440	EF088739
Campanula moravica (Spitzn.) Kovanda		Cultivated at Institut Botànic de Barcelona, *Roquet 5-V-2004* (BC *s. n.*)		EF088740
Campanula olympica Boiss.		Turkey, Çamlik: Rize, *Nisa 772* (MA *s. n.*)		**EF088741**
Campanula ossetica M. Bieb.	Eddie *et al.* (2003)		AY322028, AY331441	
Campanula peregrina L.	Eddie *et al.* (2003)	Turkey, Alanya: Antalya, *Baytop 26-VII-57* (ISTE *5437*)	AY322029, AY331442	EF088742
Campanula persicifolia L.	Eddie *et al.* (2003)	Cultivated at Botanical Garden of Madrid, *MLA0179* (MA)	AY322030, AY331443	**EF088743**
Campanula petraea L.	Eddie *et al.* (2003)		AY322031, AY331444	
Campanula pinatzii Greuter & Phitos	Greece, Dhodhekanisos: Kastello, *Raus 9666* (MA *464542*)	Greece, Dhodhekanisos: Kastello, *Raus 9666* (MA *464542*)	EF090538, EF090579	EF088744

Campanula pinnatifida Hub.-Mor.		Turkey: Gurun-Sivas, *Nydegger 16893* (MA *367632*)		**EF088745**
Campanula polyclada Rech. f. & Schiman-Czieka	Afghanistan, Panjao: Waras, *Rechinger 36562* (MA *416822*)	Afghanistan, Panjao: Waras, *Rechinger 36562* (MA *416822*)	**EF090539, EF090580**	EF088746
Campanula poscharskyana Degen		Cultivated at Botanical Garden of Madrid, *Alarcón 178* (MA)		EF088747
Campanula prenanthoides Durand		USA, California: Yosemite Park (MA *460216*)		EF088748
Campanula primulifolia Brot.	Eddie *et al.* (2003)	Portugal, Algarve: Foia, *Julin 26-VI-1974* (UPS *s. n.*)	AY322032, AY331445	EF088699
Campanula propinqua Fisch. & C. A. Mey. (1)	Turkey, Gumushane: Kurtun-Torul, *Herrero 1287* (MA *688027*)	Turkey, Gumushane: Kurtun-Torul, *Herrero 1287* (MA *688027*)	EF090540, EF090581	**EF088749**
Campanula propinqua Fisch. & C. A. Mey. (2)		Armenia, Eghegnadsor: Egheg, *Oganessian 18-VI-04* (ERE *154863*)		**EF088794**
Campanula ptarmicifolia Lam. (1)	Turkey: Tunceli, *Davis 31233 et al.* (ISTE *43633*)	Turkey: Tunceli, *Davis 31233 et al.* (ISTE *43633*)	EF090541, EF090582	EF088750
Campanula ptarmicifolia Lam. (2)	Turkey: Erzincam, *Aedo 2593* (MA *690039*)	Turkey: Erzincam, *Aedo 2593* (MA *690039*)	EF090555, EF090596	**EF088775**
Campanula pterocaula Hausskn.	Turkey: Bolu, *Nydegger 19005* (MA *367633*)	Turkey: Bolu, *Nydegger 19005* (MA *367633*)	EF090542, EF090583	EF088751
Campanula pubicalyx (Davis) Damboldt	Turkey, Konya: Ermenek, *Davis 16244* (ISTE *43630*)	Turkey, Konya: Ermenek, *Davis 16244* (ISTE *43630*)	**EF090543, EF090584**	**EF088752**
Campanula punctata Lam.	Eddie *et al.* (2003)	Japan: Honshu, *Estebánez 1508* (MA *s. n.*)	AY322033, AY331446	**EF088753**
Campanula pyramidalis L.	Eddie *et al.* (2003)	Croatia: Rijeka, *Vitek 99440* (MA *641379*)	AY322034, AY331447	**EF088754**
Campanula quercetorum Hub.-Mor. & C. Simon		Turkey, Evciler: Bayramiç, *Castroviejo 15236* (MA *644286*)		**EF088755**
Campanula raddeana Trautv.	Eddie *et al.* (2003)		AY322035, AY331448	
Campanula radula Fisch.	Turkey: Hakkari, *Archibald 8340* (ISTE *s. n.*)	Turkey: Hakkari, *Archibald 8340* (ISTE *s. n.*)	EF090544, EF090585	EF088756
Campanula rapunculoides L.	Turkey: Rize, *Nisa 763* (MA *689073*)	Turkey: Rize, *Nisa 763* (MA *689073*)	EF090545, EF090586	**EF088757**
Campanula rapunculus L.	Spain, Barcelona: Viladrau, *Sáez 6121* (BCB *s. n.*)	Spain, Barcelona: Viladrau, *Sáez 6121* (BCB *s. n.*)	EF090546, EF090587	**EF088758**
Campanula reverchonii A. Gray	Eddie *et al.* (2003)		AY322036, AY331449	
Campanula rotundifolia L.	Eddie *et al.* (2003)	Andorra *Sáez 6134* (BCB *s. n.*)	AY322037, AY331450	EF088759
Campanula sarmatica Ker-Gawl.	Eddie *et al.* (2003)		AY322038, AY331451	
Campanula savalanica Fed.		Iran: Shahbil *Herrero* s. n. (MA *s. n.*)		**EF088760**
Campanula saxifraga M. Bieb. subsp. *aucheri* (DC.) Ogan. (1)	Armenia, Akhurian: Krashen, *Oganessian 26-VI-2004* (ERE *154864*)	Armenia, Akhurian: Krashen, *Oganessian 26-VI-2004* (ERE *154864*)	EF090547, EF090588	EF088761
Campanula saxifraga M. Bieb. subsp. *aucheri* (DC.) Ogan (2)		Turkey, Kars: Agri Dagh, Serdarbulah *Baytop 14-VII-76 et al.* (ISTE *42896*)		**EF088795**
Campanula scheuchzeri A. Gray		Spain, Huesca: Bielsa, *Roquet 12-X-2004*(BC *s. n.*)		EF088762
Campanula sclerotricha Boiss.	Turkey, Van: Bahçesaray, *Baytop 19-IX-1978* (ISTE *30991*)	Turkey, Van: Bahçesaray, *Baytop 19-IX-1978* (ISTE *30991*)	EF090548, EF090589	**EF088763**
Campanula scoparia (Boiss. & Hausskn.) Damboldt	Turkey: Hakkari, *Duncan 71 et al.* (ISTE *s. n.*)	Turkey: Hakkari, *Duncan 71 et al.* (ISTE *s. n.*)	**EF090549, EF090590**	EF088764
Campanula scutellata Griseb.		Macedonia: Veles (MA *555269*)		EF088765

Campanula semisecta Murb.		Spain: Cazorla, *Muñoz-Garmendia et al. 16-VI-76* (MA *456218*)		**EF088766**
Campanula sibirica L.	Russia, Altai: Artishtu-Karatsu, *Castroviejo 14132* (MA *613903*)	Russia, Altai: Artishtu-Karatsu, *Castroviejo 14132* (MA *613903*)	**EF090550, EF090591**	EF088767
Campanula siegizmundi Fed.	Eddie *et al.* (2003)		AY322039, AY331452	
Campanula sosnowskyi Kharadze	Eddie *et al.* (2003)		AY322040, AY331453	
Campanula speciosa Pourr.	France: Ariège, Mijanes, *Montserrat et al. 8-VI-1983*(MA *256533*)	France: Ariège, Mijanes, *Montserrat et al. 8-VI-1983* (MA *256533*)	**EF090551, EF090592**	**EF088768**
Campanula spicata L.		Italy, Teramo: Fondo de la Salsa, *Navarro 4323* (MA *699308*)		EF088769
Campanula stevenii subsp. *stevenii* M. Bieb.	Eddie *et al.* (2003)	Armenia: Vayk, *Oganessian* (ERE *154865*)	AY322041, AY331454	**EF088770**
Campanula stricta Labill.	Iran: Chadil Kuh, *Renz 48987* (MA *420241*)	Iran: Chadil Kuh, *Renz 48987* (MA *420241*)	**EF090552, EF090593**	**EF088771**
Campanula strigosa Banks & Sol.		Turkey, Nemrut: Kahta, *Sorger 4-V-1980* (W *54340*)		**EF088772**
Campanula subcapitata Popov	Turkey, Erzurum: Pasinler, *Herrero 1831* (MA *687545*)	Turkey, Erzurum: Pasinler, *Herrero 1831* (MA *687545*)	**EF090553, EF090594**	EF088773
Campanula thyrsoides L.	Eddie *et al.* (2003)		AY322042, AY331455	
Campanula trachelium L.	Spain, *Sáez 6133* (BCB *s. n.*)	Spain, *Sáez 6133* (BCB *s. n.*)	**EF090554, EF090595**	**EF088774**
Campanula tridentata Schreb.	Eddie *et al.* (2003)		AY322043, AY331456	
Campanula tymphaea Hausskn.		Greece, Pindos: Kataras Pass, *Frost-Olsen 3685* (MA *544610*)		**EF088796**
Campanulastrum americanum (L.) Small	Eddie *et al.* (2003)	USA, Nebraska: Seward Co., *Nieto-Feliner 2063* (MA *459958*)	AY322044, AY331457	EF088776
Canarina canariensis (L.) Vatke		Spain, Gran Canaria: Teror, *Aldasoro 9106* (MA *s. n.*)		**EF088777**
Craterocapsa congesta Hilliard & B. L. Burtt	Eddie *et al.* (2003)		AY322049, AY331462	
Diosphaera rumeliana (Hampe) Bornm.	Eddie *et al.* (2003)	Macedonia, Kavala: Mt. Pangeo, *Greuter 16056* (MA *540729*)	AY322051, AY331464	EF088778
Edraianthus graminifolius (L.) DC.	Eddie *et al.* (2003);	Italy, Sicily: Palermo, *Herrero 888* (MA *646860*)	AY322052, AY331465	EF088779
Edraianthus pumilio (Schultes) DC.	Eddie *et al.* (2003)		AY322053, AY331466	
Edraianthus tenuifolius DC.	Yugoslavia: Senj *Frost-Olsen s. n.* (MA *464019*)		**EF090556, EF090597**	
Feeria angustifolia (Schousb.) Buser	Eddie *et al.* (2003)	Morocco, Marrakech: High Atlas, Hir-n-Ifri, *Podlech 47779* (MA *472233*)	AY322054, AY331467	**EF088780**
Gadellia lactiflora (M. Bieb.) Schulkina	Eddie *et al.* (2003)		AY322055, AY331468	
Githopsis diffusa A. Gray	Eddie *et al.* (2003)		AY322056, AY331469	
Hanabusaya asiatica Nakai	Eddie *et al.* (2003)		AY322057, AY331470	
Heterocodon rariflorum Nutt.	Eddie *et al.* (2003)		AY322058, AY331471	
Jasione crispa (Pourr.) Samp.	Eddie *et al.* (2003)		AY322059, AY331472	
Jasione heldreichii Boiss. & Orph.	Bulgary, Rhodope Mts.: Koprivlen, *Navarro 5008* (MA *s. n.*)	Bulgary, Rhodope Mts.: Koprivlen, *Navarro 5008* (MA *s. n.*)	**EF090557, EF090598**	EF088781

Jasione laevis Lam.	Eddie *et al.* (2003)		AY322060, AY331473	
Jasione maritima (Duby) L. M. Dufour ex Merino	Eddie *et al.* (2003)		AY322061, AY331474	
Jasione montana L.	Eddie *et al.* (2003)	Spain, Barcelona: Saulons d'en Deu, *Sáez 6218* (BCB *s. n.*)	AY322062, AY331475	EF088782
Jasione sessiliflora Boiss. & Reut.	Eddie *et al.* (2003)		AY322063, AY331476	
Legousia falcata (Ten.) Fritsch	Eddie *et al.* (2003)		AY322064, AY331477	
Legousia hybrida (L.) Delarb.	Morocco: Atlas, Dayer Iffer, *Cirujano R10113 et al.* (BC *s. n.*)	Morocco: Atlas, Dayer Iffer, *Cirujano R10113 et al.* (BC *s. n.*)	**EF090558, EF090599**	EF088783
Legousia speculum-veneris (L.) Fisch.	Eddie *et al.* (2003)		AY322065, AY331478	
Michauxia tchihatchewii Fisch. & C. A. Mey.	Eddie *et al.* (2003)	Turkey: Ermenek, *Aldasoro 9138 et al.* (MA *s. n.*)	AT322068, AY331480	**EF088784**
Musschia aurea Dumort.	Eddie *et al.* (2003)	Portugal, Madeira: Encomiada, *Velayo 9727* (MA *655323*)	AY322067, AY331481	**EF088785**
Petromarula pinnata (L.) DC.	Eddie *et al.* (2003)	Greece: Kreta, *Shay 82-1059* (B *10 9010624*)	AY322069, AY331482	**EF088786**
Physoplexis comosa (L.) Schur	Eddie *et al.* (2003)		AY322070, AY331483	
Phyteuma orbiculare L.	Eddie *et al.* (2003)		AY322071, AY331484	
Phyteuma spicatum L.	Eddie *et al.* (2003)	Spain, Barcelona: Aiguafreda, *Roquet 8-V-05* (BC *s. n.*)	AY322072, AY331485	EF088787
Platycodon grandiflorum (Jacq.) DC.		Cultivated at Botanical Garden of Madrid (MA *573425*)		**EF088788**
Roella ciliata L. (1)	Eddie *et al.* (2003)		AY322074, AY331487	
Roella ciliata L. (2)	South-Africa: *Aldasoro 9014* (MA *s. n.*)	South-Africa, *Aldasoro 9014* (MA *s. n.*)	**EF090559, EF090600**	EF088789
Solenopsis laurentia (L.) C. Presl		Italy, Sardinia: Uroro Perdas de Fogu, *Garcia 3779* (MA *709006*)		EF088790
Trachelium caeruleum L.	Spain, Santander: Liencres, *Aldasoro 3503* (MA *s. n.*)	Spain, Santander: Liencres, *Aldasoro 3503* (MA *s. n.*)	**EF090560, EF090601**	EF088791
Triodanis leptocarpa (Nutt.) Nieuwl.	Eddie *et al.* (2003)		AY322079, AY331492	
Wahlenbergia hederacea L.	Eddie *et al.* (2003)	Spain, Oviedo: Cangas de Narcea, *Serra 6070* (MA *705618*)	AY322080, AY331493	EF088792
Wahlenbergia lobelioides Link		Portugal: Madeira, *Sequeira 4597* (MA *s. n.*)		**EF088793**

Phylogenetic analyses were performed for three data sets: ITS; *trn*L-F; and the combined ITS and *trn*L-F data of taxa for which both regions were available. Analyses were carried out using Maximum Parsimony (MP) and Bayesian Inference (BI). Parsimony analyses involved heuristic searches conducted with PAUP* 4.0b10 (Swofford 2002) with tree bisection-reconnection (TBR), MulTrees option in effect, branch swapping algorithm and character states specified as unordered and unweighted. The gaps were considered as missing data for all the analyses because of high ambiguity (ITS data) and complex patterns (*trn*L-F data). All most parsimonious trees (MPTs) were saved. Bootstrap (BS) analyses were performed (Felsenstein 1985). For the combined data set 100 replicates were performed. For independent data matrices, we used the approach by Lidén *et al.* (1997), BS analyses were performed using 1,000 replicates, random taxon addition with 10 replicates per replicate and no branch swapping. To explore the amount of phylogenetic signal for each data set, we calculated the Consistency Index (CI) (Kluge & Farris 1969) and the Retention Index (RI) (Swofford 2002). Furthermore, the data sets were explored with PAUP* executing the command files generated by PRAP (Parsimony Ratchet Analyses using PAUP, Müller 2004), which allow Parsimony ratchet searches (Nixon 1999), in order to explore the tree-space in more detail and attempt to find multiple islands of MP trees. Ten random addition cycles of 200 ratchet iterations each were applied, each with 25% up weighting of the characters in the PRAP iterations. Cycles were not extended beyond 200 iterations because each random addition cycle converged very soon on the same tree score.

The program MrModeltest 2.2 (Nylander 2004) was used to determine the best-fitting model of sequence evolution for each data set using Akaike Information Criteria (AIC). The best-fitting sequence evolution model required for ITS data was the symmetrical model (SYM + I + Γ, Zharkikh 1994), and for *trn*L-F the best was the General Time Reversible model (GTR + I + Γ, Rodríguez *et al.* 1990). The models and the resulting parameter estimates were then used in BI analyses conducted with MrBayes 3.1 (Huelsenbeck *et al.* 2001; Ronquist & Huelsenbeck 2003). In the BI analyses of the combined data, we set up a partitioned analysis to apply the parameters of the most appropiate model for each region, as MrBayes 3.1 allows heterogeneous models and data. Bayesian analyses were performed starting from the NJ tree, and four Markov chains during 10^6 generations were run in parallel to sample trees using the Markov Chain Monte Carlo (MCMC) principle. One sample of each 100 generations was saved, resulting in 10,000 sample trees. The first 1,000 trees were eliminated during the *burn-in* phase before computing the consensus tree because they did not reach a stationary posterior probability (PP). MacClade 4.06

(Maddison & Maddison 2003) was used to map the evolution of flower morphology in the context of molecular evolution.

2.3.4 Reproductive features and pollinators

We compiled data from the literature and data obtained during this work about reproductive features including pollen-ovule ratio and reproduction type, and other features such as the flower length, the corolla shape and the habit (see Table 3).

A large part of data about flower visitors was compiled from the literature and direct observation (see Table 4). Flower visitors for 23 species were recorded from the literature, and data for 17 species was obtained by field observations. Due to the difficulty of visiting each species, they were usually censused only three times (May, July and August) during the flowering seasons of 2004-2006. Four hours were spent each time, observing insect foraging. Insects were collected, mounted, identified, and their body length measured in the laboratory. The number of insect visits per hour was recorded.

Association between autogamy and habit was tested using Mann-Withney non-parametric test (Statistica 6.0). Autogamy rate was taken from references in all cases but *C. erinus* L., *C. fastigiata* Dufour ex A. DC. and *C. propinqua* Fisch. & C. A. Mey., which were tested for automatic self-pollination in bagged flowers at Madrid Botanic Garden. Mann-Withney tests were also used to test differences in taxa according to the three main corolla types. They were performed comparing the number of taxa visiting tubular-campanulate, broadly campanulate and rotate flowers (Table 4; we excluded from the analysis ants, Coleoptera and Lepidoptera because they are of low importance as *Campanula* pollinators).

2.4 RESULTS

The MP analyses for independent data collapsed at the first island explored, but further island exploration with the Parsimony ratchet did not yield trees with better scores. The topology of trees produced by BI is shown in Fig. 1 (consensus tree obtained by BI of ITS data), Fig. 2 (consensus tree obtained by BI of *trn*L-F data) and Fig. 3 (consensus tree-phylogram obtained by BI of combined data). Moreover, both kinds of analyses revealed the same topology in the strict consensus tree. Bootstrap support values greater than 70% obtained with PAUP* were added to BI trees, as the topologies of trees produced by MP and BI were virtually identical. Different data

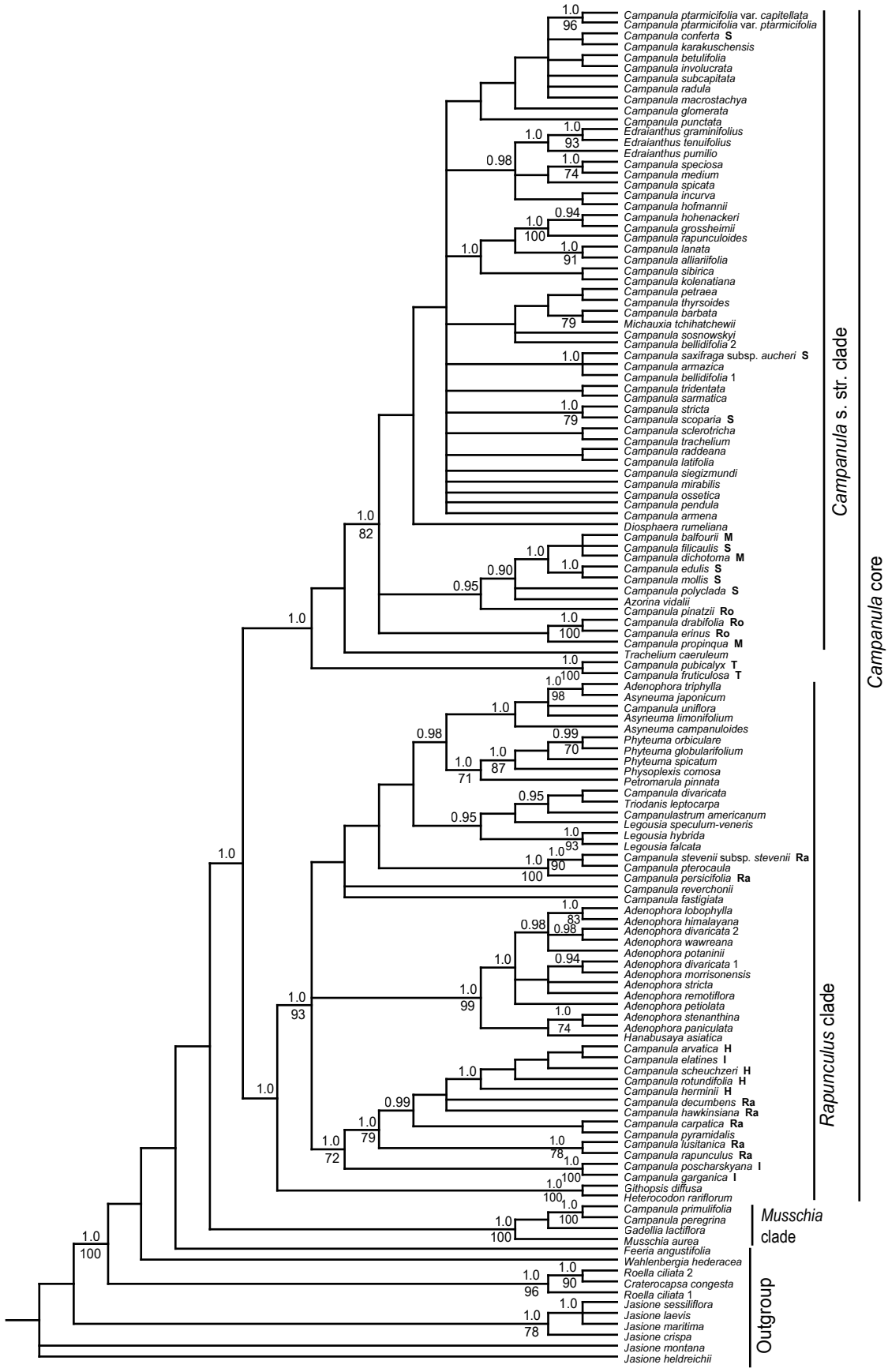

FIG. 1. Majority rule consensus tree obtained from BI of ITS data. Numbers above branches indicate Bayesian-credibility values (PP) > 0.90; numbers below branches indicate Parsimony BS > 70%. MP gave trees with identical topologies. Subgenera, sections and subsections discussed in the text have been mapped with the following abbreviations: H, section *Heterophylla* (Witas.) Fed.; I, subsection *Isophylla* Damboldt; M, subgenus *Megalocalyx* Damboldt; Ra, section *Rapunculus* (Fourr.) Boiss.; Ro, subgenus *Roucela* (Feer) Damboldt; S, section *Saxicolae* (Boiss.) Kharadze; T, section *Tracheliopsis* (Buser) Damboldt.

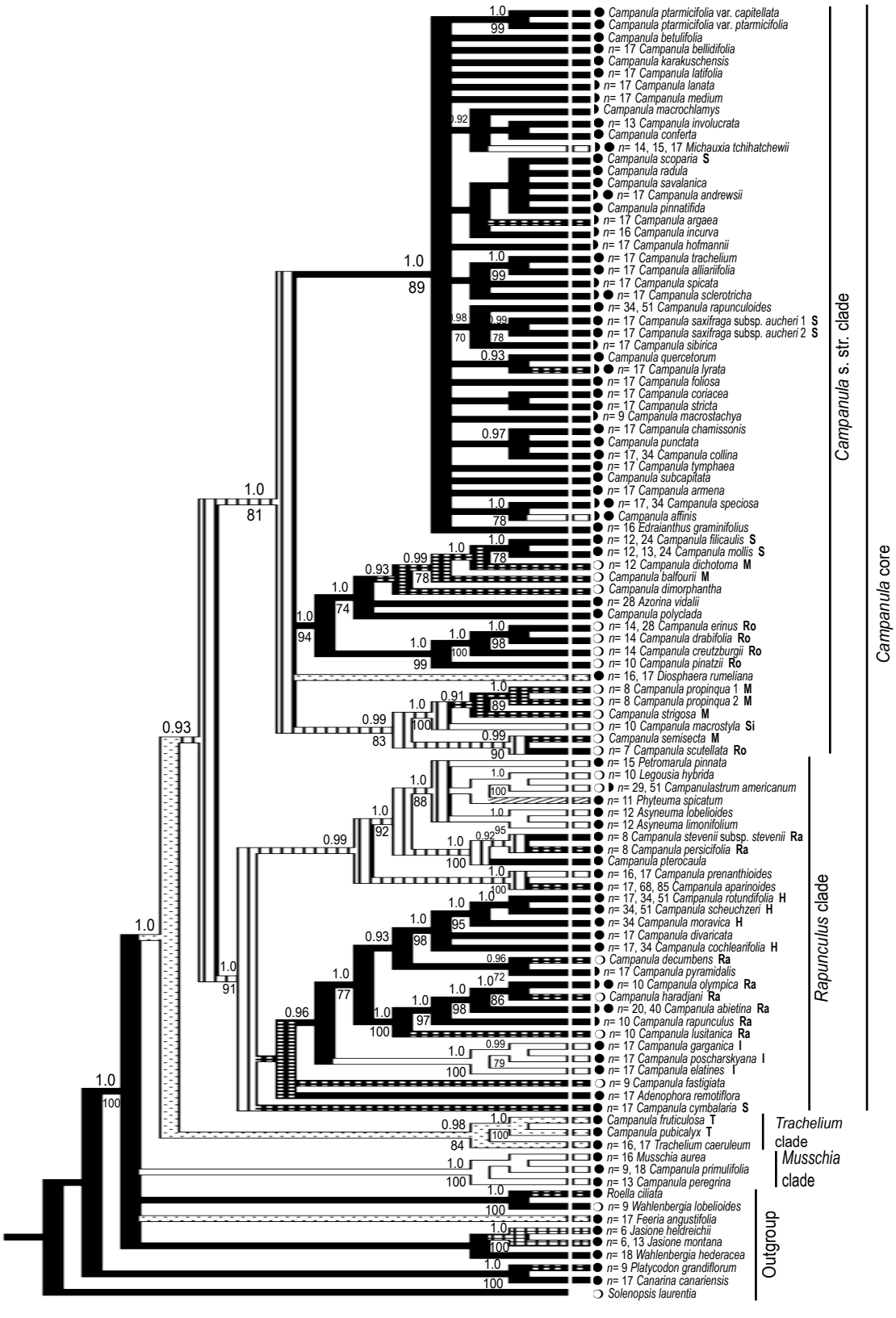

FIG. 2. Majority rule consensus tree obtained from BI of *trn*L-F data. Numbers above branches indicate Bayesian-credibility values (PP) > 0.90; numbers below branches indicate Parsimony BS > 70%. MP gave trees with identical topologies. The types of corolla (see Fig. 4) are mapped in the branches of the tree with the following patterns: white, rotate and sub-rotate; black with white points, broadly campanulate; black, tubular-campanulate; white with black points, narrowly tubular; white with stripes in diagonal, tubular with lateral dehiscence; and white with squared bottom, flowers in capitulum. Life cycle is indicated with the following symbols: white circle, annual; semi-circle, biennial; black circle, perennial. Haploid chromosome numbers are also indicated. Subgenera, sections and subsections discussed in the text have been mapped with the following abreviations: H, section *Heterophylla* (Witas.) Fed.; I, subsection *Isophylla* Damboldt; M, subgenus *Megalocalyx* Damboldt; Ra, section *Rapunculus* (Fourr.) Boiss.; Ro, subgenus *Roucela* (Feer) Damboldt; S, section *Saxicolae* (Boiss.) Kharadze; Si, subgenus *Sicyocodon* (Feer) Damboldt; T, section *Tracheliopsis* (Buser) Damboldt.

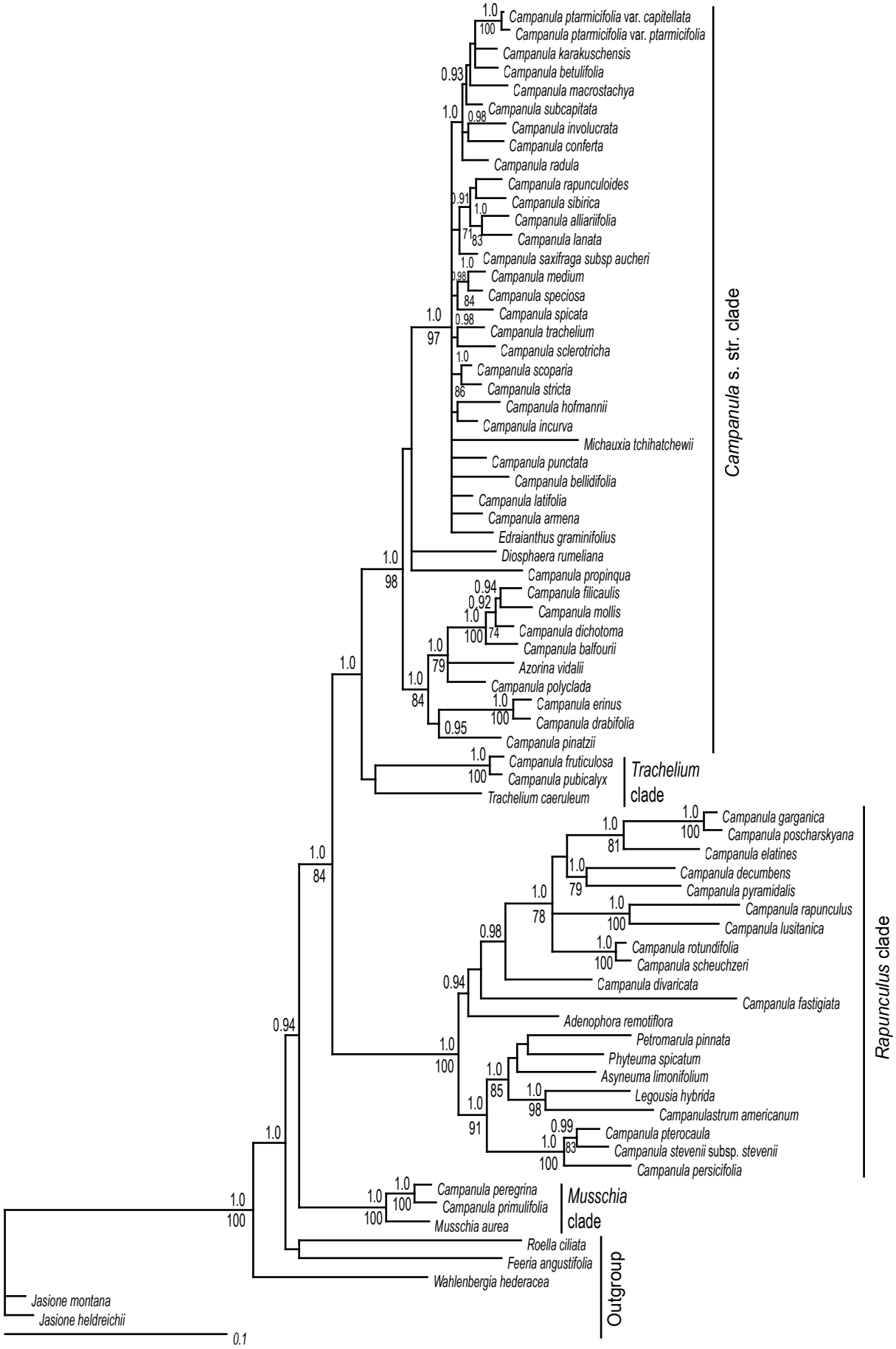

FIG. 3. Majority rule consensus tree-phylogram from BI of combined data of ITS and *trn*L-F. Numbers above branches indicate Bayesian-credibility values (PP) > 0.90; numbers below branches indicate Parsimony BS > 70%. MP gave trees with identical topologies.

TABLE 3. Compilation of results of literature and data obtained during this work about autogamy and other flower features in *Campanula* species and related species.

Corolla shape	Taxon	Reproduction features: P/O indexes and Autogamy rates. A, % seeds in self-crosses respect of control. In brackets, reproduction type: a, mainly autogamous; x, mainly xenogamous; f, facultative; -, unknown	Flower length (mm)	Habit: A: annual, B: biennial, P: perennial	Reference or sample data
Rotate or subrotate	*Campanulastrum americanum*	A= 88-100% (a)	20-30	A-B	Galloway *et al.* (2003)
	Campanula fastigiata	A= 100% (a)	1-2	A	Spain, Jaen, *Aldasoro & Alarcón s.n.*
	C. erinus	P/O= 145 (a)	1-6	A	Spain, Jaen, *Aldasoro & Alarcón s. n.*
	C. affinis	P/O= 1224 (x)	20-40	B-P	Simón *et al.* (2000)
	Triodanis perfoliata	P/O= 3; A= 100% (a)	5-10	A	Trent (1940); McVaugh (1948); Cruden (1977)
Broadly campanulate	*C. dichotoma*	A= 60-90% (a)	5-23	A	Nyman (1992a)
	C. propinqua	A= 78-95% (a)	5-25	A	Turkey, Gumushane, *Herrero 1287* (MA), cultivated in MA
	C. spatulata	A= 42% (f)	17-20	P	Blionis & Vokou (2002)
Tubular-campanulate	*Platycodon grandiflorum*	A= 54.6-62.7% (f/a)	15-23	P	Wei *et al.* (2006)
	C. uniflora	A= 100% (a)	5-9	P	Aegisdottir *et al.* (2006)
	C. microdonta	P/O= 580-2320; A= 30.9-84.3% (f/a)	30-45	P	Inoue (1990); Inoue *et al.* (1996)
	C. punctata	P/O= 3220; A= 26% (f)	50-57	P	Inoue & Amano (1986); Inoue (1990); Inoue *et al.* (1996); Kobayashi *et al.* (1997)
	C. rapunculoides	P/O= 166; A= 3-95% (f)	15-32	P	Vogler & Stephenson (2001); Good-Avila & Stephenson (2003)
	C. rapunculus	A= 2-14% (x)	12-23	B	Schlindwein *et al.* (2005)
	C. rotundifolia	A= 9-12.8 % (x)	10-18	P	Giblin (1998)
Tubular open laterally	*Phyteuma spicatum*	P/O= 630 (x)	8-15	P	Christ *et al.* (2000)
Capitula	*Jasione crispa*	P/O= 606 (x)	4-10	P	Pías & Guitián (2001)

TABLE 4. Pollinators and visitors of *Campanula*.

Corolla shape	Taxon	Reference or sample data	Pollinators and visitors. Asterisk signals the most frequent, if data is available.
Rotate or subrotate	*Asyneuma limonifolium*	Turkey, Ciflik to Altunishar, *Aldasoro et al. 9110* (MA)	**Hymenoptera**: Halictidae; Megachilidae: *Chelostoma*. **Diptera**: Syrphidae
	Campanula affinis	Simón *et al.* (2000)	**Hymenoptera**: Apidae: *Bombus sp., Apis mellifera;* Megachilidae*;* Sphecidae: *Stigmata* sp., *Ammophila* sp.; Ichneumonidae; Formicidae. **Diptera**: Syrphidae: *Eristalix* sp. **Coleoptera**: Oedemeridae: *Oedemera* sp.; Curculionidae: *Miarus* sp.; Cerambycidae: *Brachyleptura* sp.
	C. arvatica	Spain, Santander: Fuenté, *Aldasoro & Alarcón 9267* (MA)	**Hymenoptera**: Argidae; Apidae: *Apis mellifera*; Halictidae: *Halictus* sp., Andrenidae: *Andrena* sp.; Megachilidae: *Megachile* sp.; *Coelioxis* sp. **Diptera**: Syrphidae: *Syrphus ribesii*, *Scaeva* sp. *Rhyngia campestris*, *Sphaerophoria* sp.; Dolichopodidae: *Dolichopus*; Tachinidae: *Tachina fera*; Muscidae.
	C. isophylla	Spain, cultivated in Cuenca, *Aldasoro & Alarcón 9278* (MA)	**Hymenoptera**: Halictidae; Ichneumonidae; Megachilidae: *Megachile* sp.; Apidae: *Apis mellifera**. **Diptera**: Syrphidae: *Syrphus ribesii*
	C. macrostyla	Turkey, Ermenek, *Aldasoro et al. 9150* (MA)	**Hymenoptera**: Xylocopidae: *Xylocopa* sp. **Diptera**: Syrphidae. **Coleoptera**: Mordeliidae
	C. versicolor	Blionis & Vokou (2001)	**Hymenoptera**: Apidae: *Apis mellifera*; Collettidae: *Hylaeus* sp.; Megachilidae: *Chelostoma campanulorum*; Xylocopidae: *Xylocopa* sp.*. **Lepidoptera**. **Diptera**: Syrphidae*
	Campanulastrum americanum	Jonson *et al.* (1995)	**Hymenoptera**: Apidae: *Bombus* sp.*, *Apis mellifera*; Halictidae: *Halictus* sp.; Megachilidae: *Megachile* sp.
		Tooker *et al.* (2006)	**Diptera**: Syrphidae: *Trichopsomyia apisaon*
		Hilty (2006)	**Hymenoptera**: Apidae: *Bombus* sp.*, *Apis mellifera*; Halictidae: *Halictus* sp., *Lasioglossum* sp., *Agapostemon* sp., *Augochlorella* sp.*;* Anthophoridae: *Melissodes* sp.*;* Megachilidae: *Megachile* sp., *Coelioxis* sp.; Tiphiidae: *Myzinium* sp. **Diptera**: Muscidae: *Thricops* sp.
	Legousia speculum-veneris	Yeo (1993)	**Hymenoptera**: Halictidae: *Halictus* sp., *Lassioglosum* sp. **Often autogamous.**
	Michauxia campanuloides	Turkey, Ermenek, Akpinar, *Aldasoro et al. 9151*(MA)	**Hymenoptera:** Halictidae; Apidae: *Apis mellifera;* Megachilidae: *Anthidium*; Xylocopidae: *Xylocopa sp*.;* Formicidae. **Coleoptera**: Scarabeidae, Dasytidae, Cerambycidae
	M. tchihatchewii	Turkey, Ermenek, *Aldasoro et al. 9138* (MA)	**Hymenoptera**: Halictidae; Melittidae: *Macropis* sp.; Apidae: *Apis mellifera;* Xylocopidae: *Xylocopa* sp.**.* **Coleoptera**: Curculionidae; Dasytidae; Mordelidae
	Musschia wollastonii	Valido *et al.* (2004)	**Birds**: *Sylvia atricapilla*
	Triodanis perfoliata	Tooker *et al.* (2006)	**Diptera**: Syrphidae: *Toxomerus marginata.* **Often autogamous.**
Broadly campanulate	*Campanula decumbens*	Spain, Jaen: Mancha Real, *Aldasoro & Alarcón 9258* (MA)	**Hymenoptera**: Andrenidae; Halyctidae. **Diptera**: Bombylidae: *Anthrax* sp.; Muscidae: *Thricops* sp.*
	C. lusitanica	Spain, Salamanca, *Aldasoro & Alarcón 9247* (MA)	**Hymenoptera**: Andrenidae; Halyctidae. **Diptera**: Muscidae: *Thricops**
	C. patula	Spain, Santander: Fuenté, *Aldasoro & Alarcón 9268* (MA)	**Hymenoptera**: Andrenidae, Halictidae. **Diptera**: Syrphidae: *Rhyngia campestris*, *Sphaerophoria* sp.; Muscidae.
		Müller *et al.* (2006)	**Hymenoptera:** Halictidae: *Dialictus* sp., *Lasioglossum* sp.; Andrenidae: *Andrena* sp.; Apidae: *Bombus sp., Apis mellifera*; Megachilidae: *Coelioxis*

			Apis mellifera; Megachilidae: *Coelioxis* sp.
	C. persicifolia	Blionis & Vokou (2001)	**Hymenoptera**: Andrenidae: *Andrena bicolor*; Apidae: *Apis mellifera*, *Bombus lucorum*; Megachilidae: *Chelostoma campanulorum, C. Fuliginosum, Osmia mitis.*; **Diptera**: Muscidae: *Thricops* sp.
		Janzon (1983)	**Hymenoptera**: Andrenidae: *Andrena* sp.; Halictidae: *Lasioglossum calceatum*, *L. fratellum*., *Dufourea dentiventris, D. inermis*; Melittidae: *Melitta haemorrhoidalis.*; Collettidae: *Hylaeus communis, H. confusus, H. annularis*; Apidae: *Apis mellifera*, *Bombus* sp; Ichneumonidae; Braconidae; Formicidae. **Diptera**: Muscidae: *Thricops hirsutula*; Empididae: *Empis livida*, *Rhamphomya nigrescens*; Syrphidae: *Syrphus ribesii, Scaeva pyastri, Metasyrphus corollae, Episyrphus balteatus, Helophilus pendulus*; Anthomiidae: *Nupedia aestiva*. **Coleoptera**: Oedemeridae: *Oedemera virescens*; Melyridae: *Dasytes* sp.; Scarabeidae: *Trichius* sp.; Curculionidae: *Miurus campanulae;* Nitidulidae: *Meligethes* sp.
	C. semisecta	Spain, Cuenca, Valera, *Aldasoro & Alarcón 9256* (MA)	**Hymenoptera**: Halictidae: *Lassioglossum, Halictus*. **Diptera**: Syrphidae: *Episyrphus* sp.; Bombylidae: *Anthrax* sp*.
	C. spathulata	Blionis & Vokou (2001, 2002)	**Hymenoptera**: Andrenidae: *Andrena* sp.*, Halictidae; Melittidae. **Diptera**: Syrphidae: *Platycheirus scambus, Neocnemodon latitarsis. Metasyrphus corollae, Episyrphus balteatus*; Bombylidae: *Bombylius flavescens*. **Coleoptera**: Oedemeridae: *Oedemera mifofemorata*; Melyridae: *Dasytes*
Tubular-campanulate	*Adenophora grandiflora*	Chung & Epperson (1999)	**Hymenoptera**: *Bombus* sp.*, and several bee species
	A. triphylla	Nakano & Washitani (2003)	**Hymenoptera**: Apidae: *Bombus* sp.*. **Diptera**: Syrphidae
	Campanula glomerata	Blionis & Vokou (2001)	**Hymenoptera**: Andrenidae; Melittidae; Megachilidae: *Megachile* sp., *Chelostoma campanulorum**
	C. lingulata	Blionis & Vokou (2001)	**Hymenoptera**: Andrenidae*; Halictidae; Megachilidae: *Chelostoma campanulorum*
	C. microdonta	Inoue *et al.* (1996)	**Hymenoptera**: Apidae: *Bombus ardens**; Halictidae; Andrenidae; Megachilidae
	C. oreadum	Blionis & Vokou (2001)	**Hymenoptera**: Andrenidae; Melittidae*; Megachilidae: *Megachile* sp., *Hoplitis* sp.
	C. punctata	Inoue (1988, 1990); Inoue & Amano (1986); Inoue *et al.* (1996)	**Hymenoptera**: Apidae: *Bombus diversus**; Halictidae; Andrenidae; Megachilidae
	C. rapunculoides	Spain, Madrid, *Aldasoro et al.* (MA)	**Hymenoptera**: Apidae: *Apis mellifera;* Megachilidae: *Chelostoma campanulorum**; Xylocopidae: *Xylocopa* sp.
	C. rapunculus	Spain, Cuenca: Valera, *Aldasoro & Alarcón 9256* (MA)	**Hymenoptera**: Andrenidae: *Andrena* sp.; Halictidae. **Diptera**: Syrphidae: *Melanostoma* sp.; Bombylidae: *Anthrax* sp.; Muscidae: *Thrycops* sp.*
	C. rotundifolia	Bingham & Orthner (1998)	**Hymenoptera**: Apidae: *Bombus* sp*; Halictidae: *Dufourea* sp.; Andrenidae: *Andrena* sp.; Collettidae: *Collettes* sp.
		Hilty (2006)	**Hymenoptera**: Halictidae: *Augochlorella striata*; Collettidae: *Collettes brevicornis*; Megachilidae: *Megachile latimanus.* **Diptera**: Syrphidae: *Toxomerus marginatus*
		Hoffmann (2005)	**Hymenoptera**: Melittidae: *Melitta haemorrhoidalis*; Megachilidae: *Megachile* sp. **Diptera**: Tachinidae: *Syphona* sp.; Syrphidae: *Rhyngia campestris*
		Larson *et al.* (2006)	**Hymenoptera**: Halictidae: *Halictus* sp., *Dialictus* sp., *Lasioglossum* sp., *Agapostemon*; Apidae: *Apis mellifera*, *Bombus* sp.*. **Diptera**: Syrphidae

		Larson *et al.* (2006)	**Hymenoptera**: Halictidae: *Halictus* sp., *Dufourea* sp., *Lasioglossum* sp.; Melittidae: *Melitta hemorroidalis*; Megachilidae: *Chelostoma campanulorum, Hoplitis mitis*
	C. rotundifolia (as *C. gieseckiana*)	Lundgren & Olesen (2005)	**Diptera**: Fanniidae: *Delia* sp.; Dolichopodidae: *Dolichopus* sp.; Syrphidae: *Platychereus* sp., *Protophorma terranovae*
	C. scheuchzeri	Spain, Palencia: Alto Campoo, *Aldasoro & Alarcón 9199* (MA)	**Hymenoptera**: Apidae: *Bombus pratorum*, B. monticola, Bombus* sp.; Andrenidae; Halictidae
	C. sparsa	Blionis & Vokou (2001)	**Hymenoptera**: Andrenidae; Halictidae; Megachilidae: *Chelostoma campanulorum**
	Canarina canariensis	Valido *et al.* (2004)	**Hymenoptera**: Halictidae: *Lasioglossum* sp. **Birds**: *Parus caeruleus*, *Phylloscopus collybita, Sylvia conspicillata*
Narrowly tubular	*Diosphaera rumeliana*	Bulgaria, Rhodope Mts, *Aedo et al. 10256* (MA)	**Lepidoptera**: Hesperidae: *Thymelicus**
	Trachelium caeruleum	Yeo (1993)	**Hymenoptera**: Halictidae: *Halictus* sp. **Lepidoptera**: Pieridae: *Pieris* sp.*
Tubular open laterally	*Phyteuma hemisphaericum*	Spain, Palencia: Alto Campoo, *Aldasoro & Alarcón 9197* (MA)	**Hymenoptera**: Apidae: *Bombus monticola*, Apis mellifera*
	P. spicatum	Hoffmann (2005)	**Hymenoptera**: Apidae: *Bombus* sp.*
Capitula	*Jasione crispa*	Spain, Palencia: Alto Campoo, *Aldasoro & Alarcón 9196* (MA)	**Hymenoptera**: Apidae: *Bombus* sp.*
	J. montana	Spain, Salamanca: Ciudad Rodrigo, *Aldasoro & Alarcón 9200* (MA)	**Hymenoptera**: Halictidae: *Halictus* sp. **Diptera**: Syrphidae: *Spaerophoria* sp., *Eristalix* sp.; Conopidae; Empididae. **Lepidoptera**: Lycaenidae
		Yeo (1993)	**Hymenoptera**. **Diptera**: Syrphidae; Conopidae; Empididae; Muscidae. **Coleoptera**. **Lepidoptera**: Sphingidae

sets also yield similar topologies, and the few clades that are in conflict do no present high support. Thus, these data sets are suitable for combining them in a phylogenetic analysis (Seelanan *et al.* 1997). The consistency indices obtained for all analyses are low. A possible explanation for these results could be the high number of taxa included in the matrix (Archie 1989)**.** Numeric results of the analysis of each region and combined data are summarized in Table 5.

TABLE 5. Results from ITS and *trn*L-F regions and combined data. Consistency and retention indices and divergence were calculated excluding non-informative characters. * RAM limit computer was reached at this value.

Data set	ITS1 + ITS2	*trn*L-F	Combined
Total characters	513	1,084	1,597
Informative characters	324	340	504
Number of taxa	124	103	71
Number of MPTs found	891,000 (1 island)*	891,000 (1 island)*	6,177 (2 islands)
Number of steps	1835	1036	2119
Consistency index	0.3229	0.5425	0.425
Retention index	0.7506	0.7720	0.705

2.4.1 Data sets separately

Both the BI and the MP (not shown) consensus trees present two major clades (Fig. 1). They are: the clade containing the genera *Musschia, Gadellia*, *Campanula peregrina* L. and *C. primulifolia* Brot. (1.0 PP, 100% BS) and the large clade formed by the rest of taxa (core of *Campanula*); well supported in BI but not in MP analyses. This core of *Campanula* is formed by two main clades. The *Rapunculus* clade (1.0 PP) includes *Adenophora*, *Asyneuma, Campanulastrum, Githopsis, Hanabusaya, Heterocodon, Legousia, Petromarula*, *Physoplexis*, *Phyteuma* and *Triodanis.* The *Campanula* s. str. clade has good support (1.0 PP, 82% B.S.) and includes *Azorina*, *Michauxia, Edraianthus, Diosphaera* and many species of *Campanula*. The *Trachelium* branch and the species of *Campanula* section *Tracheliopsis* (represented here by the species *Campanula fruticulosa* (O. Schwarz & Davis) Damboldt and *C. pubicalyx* (Davis) Damboldt) are also included in the core of *Campanula*, and appear as sisters to *Campanula* s. str. clade.

Both the BI and the MP (not shown) consensus trees show similar results to ITS data, except for the position of *Trachelium* and *Campanula* section *Tracheliopsis* (Buser) Damboldt (see Fig. 2).

2.4.2 Combined nrDNA ITS and cpDNA *trn*L-F

The two large clades referred before as *Campanula* s. str. (it includes the type of the genus), and *Rapunculus* clade are congruent in all analyses. The positions of *Trachelium* and *Campanula* section *Tracheliopsis* with respect to the rest of *Campanula* do not agree in ITS and *trn*L-F topologies, and in the combined analysis they appear closer to *Campanula* s. str. However, the results derived from combined data should be considered with caution because of its limited taxon sampling due to incomplete overlap between the two data sets. Finally, the outgroup *Jasione* is sister to the well-supported clade (1.0 PP, 100% B.S.) formed by the other outgroups (*Feeria, Roella* and *Wahlenbergia*), the *Musschia* clade and the *Campanula* core.

2.4.3. Distribution of characters in the *trn*L-F tree

We mapped the distribution of the chromosome numbers compiled from the literature on the *trn*L-F tree (Fig. 2) but evolutionary patterns are not evident. Outgroups have different chromosome numbers: *Jasione* has as most frequent numbers *2n*= 12, 24 and 36 (the presumed basic number is *x*= 6), while *Platycodon* and *Wahlenbergia* have *2n*= 18, 36 and 72 (*x*= 9). In contrast, the most common number in *Campanula* is *2n*= 34 (see Fig. 2), but *Canarina* also has *2n*= 34. There are many successive and diverse numbers in *Campanula*, distributed in separate clades. There are also many cases of polyploid series in different clades such as *Campanula aparinoides* Pursh (*2n*= 34, 136 and 170), *C. cochlearifolia* Lam. (*2n*= 34 and 68), *C. glomerata* L. (*2n*= 30 and 60), *C. patula* L. (*2n*= 20, 40, 60 and 80), *C. rapunculoides* L. (*2n*= 68 and 102), *C. rotundifolia* L. (*2n*= 34, 68 and 102), *C. scheuchzeri* A. Gray (*2n*= 68 and 102), *C. speciosa* Pourr. (*2n*= 34 and 68) and *Campanulastrum americanum* (L.) Small (*2n*= 58 and 102).

Habit seems to have a high plasticity in this genus, annual or biennal forms (compiled from the literature) appear embedded among perennials in nearly all subclades. There are a large number of annuals and biennials in the subclades of section *Rapunculus* s. str. (Fourr.) Boiss. and *Legousia*. We have found a significant association between annual habit and increased autogamy (Mann-Whitney test: p= 0.021, z= 2.29, n= 17; Table 3). Furthermore, a significant negative correlation between petal size and autogamy rates has been found in the same species (r= 0.565, p= 0.018, n= 17).

Flower shape of *Campanula* and related genera has been classified with five main types and mapped on the tree terminals in the data set with the wider sampling (Fig. 2). The main forms are: rotate and sub-rotate, broadly campanulate, tubular-campanulate, tubular with lateral dehiscence of the corolla, and narrowly tubular (see Fig. 4). The more common type, the tubular-campanulate corolla, is distributed along many subclades. Rotate corollas are also embedded in various subclades suggesting repeated homoplasy. The tubular corollas with lateral dehiscence appear only in *Physoplexis* and *Phyteuma*, both in the *Rapunculus* clade. Finally, the species with long and narrow tubular corollas appear in three positions. One of these species falls in the core of *Campanula* s. str. [*Diosphaera rumeliana* (Hampe) Bornm.]. Others (*Campanula* section *Tracheliopsis* and *Trachelium*) are in an uncertain position: in a branch sister to *Campanula* s. str. clade (Figs. 1 and 3) or sister to both *Campanula* s. str. and *Rapunculus* clades (Fig. 2). Last, *Feeria angustifolia* (Schousb.) Buser is placed in a branch with the outgroups (Fig. 2).

2.4.4 Flower shape and pollinator preferences

Relationships between pollinator groups, reproductive success and flower shape have been widely studied in the bluebells. Mann-Whitney tests of data compiled from the literature and our own observations indicate that the total number of visitor taxa and the number of Hymenoptera are significantly higher in rotate flowers than in broadly campanulate flowers (total visitors: $p= 0.037$, $z= 2.08$; Hymenoptera: $p= 0.0054$, $z= 2.77$) and than in tubular-campanulate flowers (total visitors: $p= 0.012$, $z= 2.5$; Hymenoptera: $p= 0.03$, $z= 2.16$). In contrast, there was no significant difference in visitor type and frequency between broadly campanulate and tubular-campanulate flowers.

Other features that can be responsible for the restriction of the spectrum of visitors are: hanging corollas, which make landing difficult for all insects except for the bumblebees and large bees, or tubular corollas with lateral dehiscence, which need to be forced by the visitor to extract the nectar (Fig. 5). The narrow-tubular corollas, which are mainly visited by Lepidoptera, could create difficult access to short-tongued insects, but we do not have enough information to confirm it.

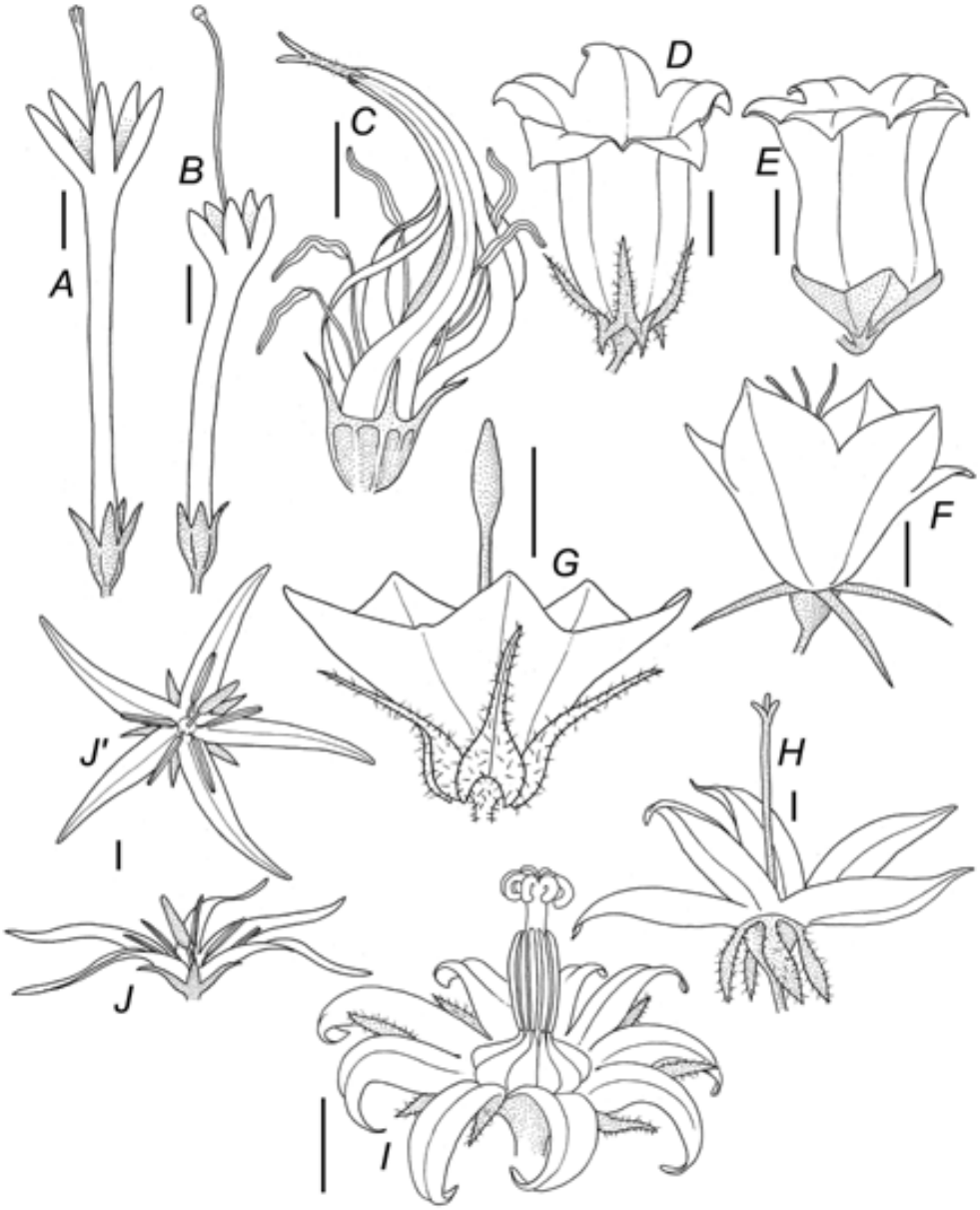

FIG. 4. Main types of corollas found in *Campanula* and related genera. A, narrowly tubular corolla of *Feeria angustifolia*; B, narrowly tubular corolla of *Trachelium caeruleum*; C, tubular corolla with lateral-dehiscence of *Phyteuma spicatum*; D, tubular-campanulate corolla of *Campanula speciosa*; E, tubular-campanulate corolla of *Azorina vidalii*; F, broadly campanulate corolla of *Campanula persicifolia*; G, nearly rotate corolla of *Campanula macrostyla*; H, rotate corolla of *Campanula elatines*; I, rotate corolla of *Michauxia tchihatchewii*; J and J' rotate corolla of *Asyneuma limonifolium*. Scale bars: A, B, D, E, F, H, J, J' = 1 mm; C, I = 5 mm; G = 1 cm.

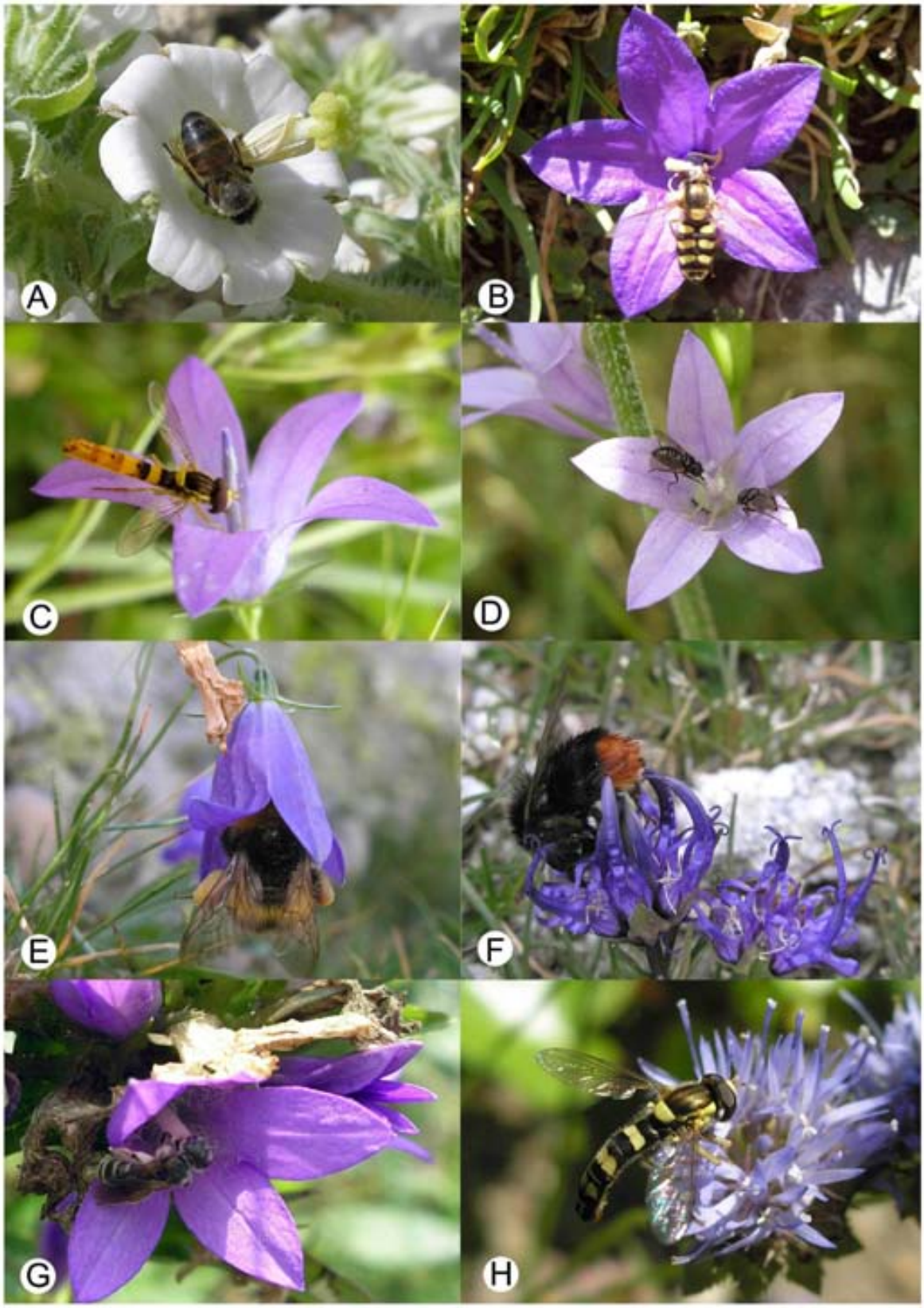

FIG. 5. Photographs of some Campanulaceae taxa and their visitors. A: *Michauxia tchihatchewii* with *Apis mellifera* and B: *Campanula arvatica* with *Syrphus ribesii*, both species have unspecialized rotate flowers; C: *Campanula patula* with *Sphaerophoria* sp. (Syrphidae) and D: *Campanula rapunculus* with *Thricops* sp. (Muscidae), both species showing small and unspecialized broadly-campanulate and campanulate flowers; E: *Campanula scheuchzeri* with *Bombus pratorum*, showing tubular-campanulate hanging flowers; F: *Phyteuma hemisphaericum* with *Bombus monticola*, with a tubular-closed corolla with lateral dehiscence, which can be opened by its visitor in the middle of the tube; G: *Campanula glomerata* with *Andrenida* sp.; and H: *Jasione montana*, which presents erect-patent lobed flowers in dense capitulum, with *Sphaerophoria* sp.

2.5 DISCUSSION

2.5.1 Phylogenetic relationships in *Campanula*

Campanula in its present circumscription has two groups: the large *Campanula* core and a small branch formed by *Musschia aurea* Dumort., two *Campanula* species: *C. peregrina* and *C. primulifolia,* and *Gadellia lactiflora* (M. Bieb.) Shulkina (Figs. 1-3). Kolakovsky (1986) included *Campanula peregrina* and *C. primulifolia* in a new genus: *Echinocodonia* Kolak., on the basis of several reproductive anatomical features, while Damboldt (1978) created for them the section *Pterophyllum*. Shulkina (1979) segregated *Gadellia lactiflora* (an endemism of the Caucasus) from *Campanula* on the basis of several anatomical and reproductive features such as its elongated and peculiar seedlings, the chromosome number (2*n*= 36), the biporate pollen and the capsule dehiscing by pores situated in the septum between locules (Kolakovsky 1986).

The ITS and *trn*L-F analyses coincide regarding the topology of the *Campanula* core in that two main groups were resolved: the *C. rapunculus* L. clade and the *Campanula* s. str. clade. Our results suggest that the *Musschia* clade is not transitional between these groups of *Campanula* (as it was suggested in Eddie *et al.* 2003) but sister to them. Even the ITS data set, the same marker that Eddie *et al.* (2003) used, indicates this. The difference between the two works might be due to the wider sampling done in this work.

2.5.2 The *Rapunculus* clade

Most of the species of this clade (Figs. 1-3) bear a trilocular capsule (except *Phyteuma* and *Physoplexis*, which are sometimes bilocular), tripartite stigma, no appendages in the calyx, and the pores or valves of the fruit situated medially or distally. However, two genera (*Adenophora* and *Hanabusaya*) and a few other *Campanula* species have basally dehiscing fruit [*C. pyramidalis* L. and all species of *Campanula* section *Heterophylla* (Witas.) Fed.] as signalled by Eddie *et al.* (2003). The clade is well supported in both ITS and *trn*L-F, but the group formed by *Githopsis* and *Heterocodon* (only included in the ITS analysis) resulted sister to the main part of the clade with very low support.

The *Rapunculus* clade includes many groups with low support. Only the terminal groups are well supported and are strongly suggested as monophyletic (Figs. 1-3). The main subclades supported are the group of section *Rapunculus* (*Campanula abietina* Griseb. & Schenk*, C. haradjanii*

Rech. f.*, C. lusitanica* Loefl.*, C. olympica* Boiss. and *C. rapunculus*, Fig. 2) and the heterogeneous group formed by the genera *Asyneuma, Campanulastrum* Small*, Legousia, Petromarula* and *Phyteuma*, and five *Campanula* species (*C. aparinoides*, *C. persicifolia* L., *C. prenanthoides* Durand, *C. pterocaula* Hausskn. and *C. stevenii* M. Bieb., Fig. 2). The *Rapunculus* clade has more autogamous or facultative taxa than *Campanula* s. str. clade, and also contains the largest spectrum of flower forms (Fig. 4). Its extraordinary diversity in plant form, flower shape, nectary types, plant habits, reproductive systems, chromosome number, distribution, etc., suggests a history of high diversification, under strong selective pressures on flower shape, size and structure. Climate changes may have played a role in the distribution of the *Rapunculus* clade, as suggested by its range centred in the Mediterranean, Caucasus, North East Asia and North America (all Northern Hemisphere). In the Late Tertiary the Northern Hemisphere suffered from sudden and great changes in climate (Robinson 1994). Pollinator assemblages were affected by these changes and probably changed repeatedly. The adaptation to a changing world in pollination agents can lead to an increase in autogamy or a more generalist strategy. However, while some species of the *Rapunculus* clade show a trend to autogamy, others show an increase in specialization. We will describe the clades to discriminate these trends.

Section *Heterophylla*, traditionally separated from *Rapunculus* (Fedorov 1957; Shulkina *et al.* 2003) by its basal capsule dehiscence, is included in *Rapunculus* clade, forming a highly supported subclade (Fig. 2). This subclade includes *C. divaricata* Michx. (Fig. 2), a North-American endemic with basal fruit dehiscence (characteristic of *Rapunculus*) and many intermediate features between sections *Heterophylla* and *Rapunculus*. Some species of section *Heterophylla* are extremely polymorphic (*e.g. C. rotundifolia*, which also presents a long polyploid series), leading to the description of many new species based on small morphological differences. Excepting *C. arvatica* Lag., most of the species of this clade show tubular-campanulate flowers (Fig. 5). It strongly attracts medium to large Hymenoptera (Table 4). In foothills of the Rocky Mountains, *C. rotundifolia* is visited by medium-sized solitary bees, while in high sites, bumblebees have higher pollination efficiency despite a lower rate of visits (Bingham & Orthner 1998). Related to this, it has been shown that corollas of *C. rotundifolia* are larger at higher altitudes (Shetler 1982; Maad & Armbruster 2005; Maad *et al.* 2006). The reason for this increase of size may be due to selective pressure of pollinators.

Section *Rapunculus* is also very polymorphic, formed mostly by annual or biennial species, with karyological heterogeneity and polyploid series. Nyman (1992) reported that some species of this section are self-incompatible (*C. lusitanica* and *C. persicifolia*). However, *C. rapunculus* can self-fertilize (Schlindwein *et al.* 2005). Their flowers vary in size and shape depending on species and habitats, and they are visited and pollinated by a large number of generalist taxa (Table 4). *Campanula pyramidalis* and *C. versicolor* Sibth. & Sm. are morphologically intermediate between sections *Rapunculus* and *Heterophylla*, both species with sub-rotate corollas which attract Syrphidae and small solitary bees (Blionis & Vokou 2001). Subsection *Isophylla* (starbells) clade is formed by several similar Mediterranean endemics; they are perennials, with characteristic star-like (rotate) flowers.

The genera *Adenophora* and *Hanabusaya* are monophyletic and belong to the *Rapunculus* group (Eddie *et al.* 2003), but in this work ITS data indicates that one species not included in Eddie *et al.* (2003), *A. triphylla* (Thunb.) DC., falls out of the group and forms a sister group to the genera *Petromarula* and *Phyteuma*. We cannot discard a misidentification of the sample *A. triphylla* because the sequence was obtained from GenBank, and we did not have material from that species to check this result. *Adenophora* is characterized by the tubular nectary at the base of the style. *Adenophora triphylla* has a nectar-tube at the base of the style but it is like a narrow membrane, seemingly different from those of the other *Adenophora*. The large flowers of this genus are tubular-campanulate, often with a long, exerted style, attracting mainly bumblebees.

Campanula fastigiata is a small selfing annual (Table 4) that has caused disagreement between many authors. De Candolle (1830), Fedorov (1957) and Damboldt (1976) indicated that *C. fastigiata* presents morphologic characteristics intermediate between *Campanula* and *Legousia*, a genus included here in the *Rapunculus* clade. In this work, *C. fastigiata* appears isolated within the *Rapunculus* clade, and shows a long branch length (Fig. 3).

The annual genera endemic to North America are included in the *Rapunculus* clade (*Campanulastrum, Githopsis, Heterocodon* and *Triodanis*, Fig.1). *Campanulastrum* and *Triodanis* appear as sister to *Asyneuma, Petromarula*, *Physoplexis* and *Phyteuma,* sister to *Campanula pterocaula* and *C. stevenii* (Fig. 1). The Eurasian genus *Legousia* also appears in the *Rapunculus* clade, close to the North-American endemics *Campanulastrum americanum*, *Triodanis*, *Campanula divaricata* and *C. reverchonii* A. Gray. *Legousia* and *Triodanis* have been considered as a single

genus by some authors because of high similarity (*cf.* McVaugh 1945, 1948). *Githopsis* and *Heterocodon* are closely related to each other and appear as sister to the rest of species of the *Rapunculus* clade. The position the genera endemic to North America in different subclades suggests that the ancestors of this alliance of *Campanula* have colonized North America at least twice. Eddie *et al.* (2003) found similar relationships for this heterogenous group and concluded that the ancestors of the *Rapunculus* clade might have radiated early in the Northern Hemisphere. All these genera except *Campanula* show high autogamy rates, and rotate flowers with some resemblance. *Physoplexis* and *Phyteuma* are monophyletic and similar morphologically. Both genera have narrow lateral incisions in the corolla tube (Fig. 4), which need to be forced by the visitor (*i.e. Bombus* and *Apis*; Fig. 5) in order to be able to extract the nectar (Richards 1997), a task that discourages the visits of Diptera and small Hymenoptera. *Asyneuma* and *Petromarula* show a vague similarity to *Phyteuma* and *Physoplexis*, due to the development of the flower as a tube that splits from the middle upwards and downwards, but that appear later as rotate. The genus *Petromarula* was established to separate *Phyteuma pinnatum* L., and it is distinguished by its pinnate leaves, absence of pollen collector hairs except in the upper part of the style as vestiges, and a showy stigma with a mace form. *Asyneuma* and *Phyteuma* are distinguished by their different floral shape (Fig. 4).

The North-American endemics *Campanula aparinoides* and *C. prenanthoides* are sister to the entire group with low support by the *trn*L-F. *Githopis* and *Heterocodon* are two North-American endemics closely related to each other and probably sister to all the *Rapunculus* clade (Fig. 1). The topology of the *Rapunculus* clade, which situates in different subclades the genera endemic to North America, suggests that the ancestors of this alliance of *Campanula* have colonized North America at least twice.

2.5.3 The *Campanula* s. str. clade

This clade presents four main groups: 1-the genus *Diosphaera*, 2-a heterogeneous group formed by *Azorina*, *Campanula* subg. *Roucela,* and species related to *C. mollis* L., 3-the core of *Campanula* subg. *Megalocalyx* plus the monotypic subg. *Sicyocodon* (Feer) Damboldt, and 4-a large but unresolved polytomy formed by the core of *Campanula* subg. *Campanula* (Figs. 1-3). In contrast to *Rapunculus* clade, all without appendages in the calyx, this clade can present or not appendages and a penta- or trilocular ovary. These characters have been used traditionally in its classification.

Megalocalyx, Roucela and *Sicyocodon* comprise annual plants with dichasial or subdichasial inflorescences. *Megalocalyx* and *Sicyocodon* share the presence of reflexed appendages between calyx lobes, while *Roucela* includes plants lacking these appendages. *Sicyocodon* differs from *Megalocalyx* in its long-exserted style (Sáez & Aldasoro 2003). Park *et al.* (2006) already detected a phylogenetic relationship between subgenera *Megalocalyx* and *Roucela*, but wider sampling in this study (see next paragraph) detects that both are not monophyletic (Fig. 2). In contrast to the *Rapunculus* clade, all without appendages in the calyx, *Campanula* s. s.tr may or may not have appendages, and can sometimes have a pentalocular ovary. These characters have been used traditionally in the sectional classification of *Campanula*.

The combination of annual plants with calyx appendages between lobes appears at least in two clades. One subclade includes 1-some typical *Megalocalyx* annuals, such as *Campanula balfourii* Wagner & Vierh. and *C. dichotoma* L.; 2-perennials traditionally included in sect. *Saxicolae* (Boiss.) Kharadze, such as *C. edulis* Forssk.*, C. mollis, C. polyclada* Rech. f. & Schiman-Czieka; 3-the atypical annual *C. dimorphantha* Schweinf. (with two different kinds of flowers: small autogamous and larger allogamous); and 4-the genus *Azorina,* which was separated from *Campanula* because of its shrub aspect and constricted corollas with a characteristic flat nectar disk (different from the nectary of *Adenophora*, which is tubular; Fig. 4). The other subclade traditionally included in subgenus *Megalocalyx* is formed by annuals: *C. propinqua, C. semisecta* Murb.*, C. strigosa* Banks & Sol. and *C. macrostyla* Boiss. & Heldreich (Damboldt 1976; Sáez & Aldasoro 2003). But this subclade has more open, somewhat different flowers. *Campanula macrostyla*, classified in the monotypic subgenus *Sicyocodon* by Damboldt (1976), has been considered closely related to subgenus *Megalocalyx* (Sáez & Aldasoro 2003).

The species of subgenus *Roucela* (*C. creutzburgii* Greuter, *C. drabifolia* Sibth. & Sm.*, C. erinus* and *C. pinatzii* Greuter & Phitos) included here constitute a well-supported group (Figs. 2-3) except for the ITS results, which do not include *C. pinatzii* in this subclade. Most of the *Roucela* species are autogamous or facultative. A few are endemic to Aegean islands (*C. creutzburgii, C. pinatzii*), while *C. erinus* has a wide distribution in the Mediterranean basin and the Macaronesian area.

The large polytomy formed by the remaining species of *Campanula* s. str. is highly supported as monophyletic (see Figs. 1-3) but shows no large well-supported subclades. There are many

informative characters (137 in the combined matrix, 94 in ITS and 43 in *trn*L-F), but many might be homoplastic, reporting incongruent information, due either to reticulate evolution or ancient hybridizations. Gadella (1964) obtained hybrids between some distantly related species in *Campanula*. We have also observed hybrids between the species *C. persicifolia* and *C. rapunculus* (*Rapunculus* clade).

The three species sampled for *Edraianthus* form a well-supported subclade also included in the polytomy (Fig. 1), thus its special capsule dehiscence (it opens from the top to the base, leading to a funnel form once it is opened) seems not sufficient to discriminate a genus. *Edraianthus* presents a restricted distribution in the SE Mediterranean and is morphologically similar to *Campanula* (Fedorov 1957; Hartvig 1991).

Some authors considered *Campanula armena* Stev., *C. betulifolia* K. Koch and *C. hofmannii* (Pant.) Greuter & Burdet as members of the genus *Symphyandra*, but later it has been considered as an artificial genus (Oganessian 1995) because it was defined only on the basis of its connate anthers, a character present in young flowers of all *Campanula* species. Our data supports this suggestion, as the species sampled fall in the polytomy.

Our results confirm previously known relationships between *Campanula* and *Michauxia* (Eddie *et al.* 2003). The position of *Michauxia* in the tree suggests derived flower morphology, where selective pressure of pollinators may have played an important role (Yeo 1993). The rest of the species of this large clade generally share a characteristic tubular campanulate shape and are mostly pollinated by *Bombus* or other large bees (Figs. 4, 5). The adaptation of *Michauxia* to dry, low to middle altitude habitats in the Caucasus and Anatolia might be the cause of its evolution to a more generalist strategy. These habitats are usually poor in *Bombus*, but rich in other Hymenoptera of different dimensions, such as Halictidae, Megachilidae, Melittidae, Xylocopidae, etc.

2.5.4 *Trachelium*

Trachelium is morphologically similar to *Campanula* section *Tracheliopsis,* and BI analysis of *trn*L-F region (Fig. 2) links them as sisters to the two main clades (*Campanula* s. str. and *Rapunculus*), but the ITS region and combined data set (Figs. 1, 3) do not support this relationship. *Diosphaera,* an Irano-Turanian and Mediterranean genus, has flowers similar to *Trachelium* and the same chromosome number *2n*= 34 (the most frequent in *Campanula*), but both taxa present

significative differences in inflorescence and vegetative aspects, and molecular data situates *Diosphaera* in *Campanula* s. str. The monotypic genus *Feeria* endemic to North Africa shares similar flowers with *Diosphaera* and *Trachelium* (Fig. 4), which present a partly coincident area of distribution, but our results do not support a close relationship among them. It seems that narrow tubular flowers have originated more than once (Fig. 2). This could be due to changes in pollinators. We suggest that adaptation to butterfly pollination could be the clue for evolution of deep-tubular flowers.

2.5.5 Implications for floral evolution

This study supports that rotate flowers have evolved independently in several subclades of *Campanula*. These species attract unspecialized pollinators (Blionis & Vokou 2001; Table 3; Fig. 5). In contrast, tubular-campanulate and widely campanulate flowers receive a greater number of specialist pollinators (mainly bumblebees and medium-large bees, like Andrenidae, Megachilidae and Melittidae) (Blionis & Vokou 2001; Hoffmann 2005; Fig. 5). Halictids and most Diptera are usually low-efficient pollinators in *Campanula* (Lau & Galloway 2004). Oligolectic species such as *Chelostoma campanulorum, C. rapunculi, Duforea dentiventris, D. inermis, Melitta hemorrhoidalis, Osmia mitis* (Westrich 1989), were recorded mainly in tubular-campanulate and widely campanulate species (Table 4).

A change to generalist strategies can lead to changes in corolla shape (more open) or size increase in *Campanula*. It also can lead to an increase in autocompatibility. Inoue (1988, 1990), Inoue & Amano (1986) and Kobayashi *et al.* (1997; 1999) demonstrated that *C. punctata* Lam. was mainly visited by bumblebees in Japan; while in the smaller Izu Islands, where bumblebees are absent, it was visited by small solitary bees. As a consequence of lower pollination rates, flowers of *C. punctata* in the Izu Islands are autocompatible, while their corolla is narrower and smaller. Other examples of reproductive or floral features variation related to differences in pollinators have been seen in *C. microdonta* Koidz. (Inoue *et al.* 1996), *C. persicifolia* (Hansen & Totland 2006) and *C. rotundifolia* (Maad & Armbruster 2005; Maad *et al.* 2006). Thus, species of Campanulaceae appear to be prone to considerable plasticity (Eddie 1997; Eddie & Ingrouille 1999). As previously mentioned this plasticity led to a considerable heterogeneity in systematic treatments. Besides, the phylogenetic results indicate the main morphological characters used in classifications such as flower shape and capsule dehiscence have arisen in parallel. We propose that strong selective pressures from

pollinators lead to floral convergence, and this might be one of the main reasons for morphological and molecular incongruence.

Deeper studies in phylogeny, reproductive and dispersive characters, mapping the morphologic characters on phylogenetic trees, dating the main evolutionary events and the study of could aid to improve the current classification.

2.5.6 Taxonomic Implications

According to its generic circumscription (Fedorov 1957; Damboldt 1976; Kovanda 1978), *Campanula* is paraphyletic. We propose two options and expose a third one, suggested by Park *et al.* (2006), to change the current classification of the genus and accomplish a monophyletic one. Another option not to be excluded is to keep *Campanula* as a paraphyletic genus. The matter of accepting or not paraphyletic taxa has been in the last decade one of the most important debates in taxonomy (Brummit 2006).

The first approach, more conservative, is to consider all genera that fall inside the three main clades as synonyms of *Campanula*: (1) *Campanula* s. str. (2) *Campanula* section *Rapunculus*, and (3) the small clade of *Musschia, Gadellia, Campanula peregrina* and *C. primulifolia*. In this way, *Campanula* would become monophyletic, including all the taxa of the tribe Campanuleae. They are easily distinguished from the plants included in the tribes Wahlenbergieae and Platycodoneae on the basis of ovarian characters (ovary inferior with lateral dehiscence). The last two tribes would include other genera (*e.g. Feeria, Jasione, Roella* and *Wahlenbergia;* Kovanda 1978; Yeo 1993). The only important change is to include in Campanuleae the genus *Edraianthus* (which presents ovary inferior, but irregular dehiscence), placed by Yeo (1993) in the tribe Wahlenbergiae.

The second approach is to limit the genus circumscription to the *Campanula* s. str. (that includes the type, *C. latifolia* L.) and change the generic nomenclature for all the species out of this clade. In both cases, laborious combinations of morphological characters are necessary to separate the taxa, making any easy taxonomic classification difficult. Interestingly, the subfamily Lobelioideae shows a similar problem for its main genus, *Lobelia*, which is paraphyletic (Knox & Muasya 2001).

The third approach is the one proposed by Park et al (2006): to split *Campanula* into numerous small genera taking as a starting point the taxonomic treatment of Kolakovsky (1994) based on fruit

characters. However, this treatment should be taken carefully as some of these genera are not supported by molecular results (Park *et al.* 2006).

We favor the first option in order to arrive at a generic delimitation that reflects the evolutionary history of *Campanula.* This approach is more consistent with previous taxonomic work, *Campanula* has always been very rich in number of species, and it does not seem to us reasonable to divide it ad nauseam. We can find numerous examples of this type of approach for big paraphyletic genera (*e.g. Asarum*, Kelly 1998; *Euphorbia,* Steinmann & Porter 2002; *Senecio,* Pelser *et al.* 2006). However, a comprehensive study of the currently recognized genera that fall within *Campanula* should be conducted before changing their taxonomic status.

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Capítol 2

Molecular Dating and Reconstruction of the Biogeographical History of *Campanula* L. and related genera

RESUM. Estudis filogenètics recents han mostrat que *Campanula* no és un gènere monofilètic, i que diversos gèneres propers s'hi troben inclosos. En aquest estudi hem intentat reconstruïr el passat en termes d'àrees ancestrals i episodis de divergència per millorar la comprensió de l'evolució d'aquest grup de plantes. Hem explorat l'evolució espaial i temporal de les campanulàcies en sentit estricte, i de *Campanula* en particular, mitjançant una aproximació bayesiana de la datació molecular i de l'ànalisi de dispersió-vicariança que té en compte la incertesa filogenètica. Per resoldre les relacions filogenètiques en els grups majors (Wahlenbergieae-Campanuleae) i la posició d'alguns gèneres com *Trachelium* respecte a *Campanula,* hem seqüenciat la regió conservada *rbc*L incloent tàxons dels principals llinatges de Platycodoneae i Wahlenbergieae. Les anàlisis de datació i biogeografia s'han aplicat a les noves dades del marcador *rbc*L i a les dades del marcador *trn*L-F obtingudes en un estudi previ. Les anàlisis filogenètiques mostren que Platicodoneae és el grup germà de les Wahlenbergiae-Campanulae, les quals apareixen combinades entre sí. Els resultats suggereixen que l'oest d'Àsia i l'est de la Mediterrània han jugat un paper important com a centres de migració i diversificació del grup *Campanula*. La història biogeogràfica d'aquest gènere sembla que ha estat molt complexe. Les taxes de diversificació de *Campanula* haurien incrementat durant el període Messinià. Suggerim que els canvis climàtics i l'expansió de regions muntanyoses durant aquest període actuaren com a fortes pressions selectives que expliquen el fet que moltes espècies de *Campanula* estan adaptades a ambients secs, freds o alterats.

ABSTRACT. Recent phylogenetic studies have shown that *Campanula* is not monophyletic, and that many satellite genera are nested within it. In this study, we attempted to reconstruct the past in terms of ancestral areas and divergence episodes of these genera, in order to increase the understanding of the evolution of this group of plants. We explored the spatial and temporal evolution of the Campanulaceae s. str., and of the *Campanula* alliance in particular, by applying a Bayesian approach to molecular dating and dispersal-vicariance analysis that takes into account phylogenetic uncertainty. To better resolve relationships among major groups (Wahlenbergieae-Campanuleae) and the position of some genera such as *Trachelium* L. with respect to *Campanula,* we have sequenced the *rbc*L-conserved region including taxa of some major lineages within Platycodoneae and Wahlenbergieae. Dating and biogeographic analyses were applied to the new *rbc*L data and to the *trn*L-F data obtained in a previous study. The phylogenetic analysis showed that Platicodoneae is the sister group of Wahlenbergiae-Campanulae, which appeared inter-graded. The results obtained suggest that Western Asia and Eastern Mediterranean seem to have played an important role as

centers of migration and diversification of the *Campanula* core. The biogeographical history of this genus seems to be highly complex. Rates of species diversification of *Campanula* seem to have increased during the Messinian period. Strong selective pressures from the climate changes and the expansion of mountainous regions during this period are suggested to explain the fact that many species of *Campanula* are adapted to drought, cold or disturbed environments.

3. MOLECULAR DATING AND RECONSTRUCTION OF THE BIOGEOGRAPHICAL HISTORY OF *CAMPANULA* L. AND RELATED GENERA

3.1 INTRODUCTION

Once seen as a valid aim in itself, phylogenetic reconstruction has come to be considered as an important tool for biologists to understand the processes governing organismal evolution. One aspect that has grown in importance is the reconstruction of past biogeographical ranges and divergence times. This is reflected in the plethora of new methods developed in the last five years to infer the spatial and temporal evolution of organisms (Sanderson 2002; Thorne & Kishino 2002; Ree *et al.* 2005). Dating methods have evolved from the strict-molecular clock (Zuckerkandl & Pauling 1965) to more realistic methods that use a "relaxed clock" approach, which models the rate variation among lineages (e. g. Penalized Likelihood, Sanderson 2002; Multidivtime, Thorne & Kishino 2002). Similarly, the last few years have witnessed the development of new biogeographical methods that incorporate to the analyses the error associated to phylogenetic and ancestral area estimates (e. g. Ree et al 2005; Sanmartin *et al.* in press) as well as new sources of fossil, geological or paleogeographical evidence (Ree *et al.* 2005; Moore *et al.* in press). For example, the widely popular dispersal-vicariance analysis method (DIVA, Ronquist 1996) has recently been updated with a Bayesian approach that allows incorporating phylogenetic error and nodal support to the biogeographic inference (Nylander *et al.* in press). Nevertheless, all of these methods still depend on a sound phylogenetic hypothesis for reliable and accurate reconstruction.

The "harebell" or "bell flower" family Campanulaceae s. str. is a derived angiosperm family comprising about 600-950 species and 35-55 genera (Cosner *et al.* 2004). They have a nearly cosmopolitan distribution, being present in all continents except Greenland and Antarctica. *Campanula* L. is the largest genus of the family, with *c.* 350-500 species inhabiting a wide range of habitats in the Northern Hemisphere (Fedorov 1957). The genus is mainly distributed in Eurasia, while poorly represented in North America and Africa. A large concentration of *Campanula* species is found in the Eastern Mediterranean region and the Caucasus.

Taxonomic treatments of *Campanula* and Campanulaceae have varied widely among authors (see Cosner *et al.* 2004 for a review) depending on each author's interpretation of the tribe morphological heterogeneity. Kovanda (1978) considered *Campanula* as part of the tribe

Campanuleae, differing from related tribes Wahlenbergieae and Platycodoneae in that the ovary is mostly inferior and the capsule dehisces at the sides (indehiscent in few cases). Thulin (1975) suggested that all taxa related to *Campanula* with porate pollen grains should be grouped within Wahlenbergieae and Campanuleae, whereas the tribe Platycodoneae would group those genera characterized by colporate or colpate grains (*Campanumoea* Blume, *Canarina* L., *Codonopsis* Wall., *Cyananthus* Wall. ex Benth., *Leptocodon* Lem. and *Platycodon* A. DC.). Recent phylogenetic analyses based on ITS-DNA sequence data (Eddie *et al.* 2003) and cpDNA rearrangements (Cosner *et al.* 2004) have helped to clarify phylogenetic relationships within the family. These studies show that the tribe Platycodoneae is the sister group of the tribes Wahlenbergieae and Campanuleae, which appear inter-graded into a well-supported clade (Cosner *et al.* 2004). In a recent analysis of phylogenetic relationships within the genus *Campanula* and allied genera, Roquet *et al.* (in press) showed that the tribe Campanuleae sensu Kovanda (1978) is monophyletic only if the genus *Edraianthus* DC. is included within it. *Edraianthus* was originally placed in tribe Wahlenbergieae (Yeo 1993) because of its irregular fruit dehiscence but inferior ovary.

There has been considerable debate regarding the geographic origin of Campanulaceae. In their classic paper of angiosperm biogeography, Raven & Axelrod (1974) hypothesized an Old World origin during the Paleogene. Hong (1995) suggested East Asia as the center of origin of the family, with the Mediterranean region and South-Africa as secondary centers of differentiation and diversification within the family starting no later than the Cretaceous. In contrast, Bremer & Gustafsson (1997) argued for a Gondwana origin for the family based on the current Southern Hemisphere distribution of many basal genera. Similarly, Eddie & Cupido (2001) suggested an African origin based on the present distribution of the subfamily Lobelioideae. Eddie *et al.* (2003) hypothesized that *Campanula* and related genera evolved in the Mediterranean region, from where it rapidly spread over other Northern Hemisphere landmasses during pre-glacial times; the origin of such morphologically distinct genera as *Phyteuma* L. or *Petromarula* Vent. ex R. Hedw. (included now within *Campanula*, Roquet *et al.* in press) would be associated to Alpine orogenic events and fluctuating Mediterranean sea levels during the Tertiary period (Favarger 1972; Greuter 1979). Regardless of the suggested origin, the great variety of distributions, ecological habitats, morphological characters, reproductive systems, and chromosome numbers exhibited by *Campanula* and allied genera (Roquet *et al.* in press) indicates a history of high and rapid diversification and a complex biogeographic pattern.

Attempts to date divergence times in Campanulaceae have been limited. In a large-scale study of angiosperms divergence times, Wikström *et al.* (2001) inferred a date of 41million years ago (mya) for the split of *Campanula* L. from *Codonopsis,* a basal genus within Campanulaceae (Cosner *et al.* 2004). A more specific study focused on the isophyllous species of *Campanula* (Park *et al.* 2006) dates the first diversification of the *Campanula garganica* Ten. complex within the Late Miocene. However, no attempt has been made to date the main diversification events within the genus *Campanula* and closely related genera.

3.2 MAIN OBJECTIVES

Here, we explore the spatial and temporal evolution of the Campanulaceae s. str., and of the *Campanula* alliance in particular, by applying a Bayesian approach to molecular dating and dispersal-vicariance that takes into account phylogenetic uncertainty: biogeographic and temporal reconstructions are averaged among a Bayesian distribution of trees weighted according to their posterior probability (Nylander *et al.* in press). Recent phylogenetic studies have shown *Campanula* is not monophyletic, and that many satellite genera are nested within it (Eddie *et al.* 2003; Park et al 2006; Roquet *et al.* in press). Morphological characters have proved of little utility in the natural characterization of the group. Reconstructing the past in terms of ancestral areas and divergence episodes of these genera could thus help to better understand the evolution of this group of plants.

Biogeographical and temporal analyses require a sound phylogenetic hypothesis to work with. Previous phylogenetic hypotheses based on ITS (Eddie *et al.* 2003; Park *et al.* 2006; Roquet *et al.* in press), *trn*L-F and combined data (Roquet *et al.* in press) provided a framework for the classification of the genus, but left the relationships among major groups (Wahlenbergieae-Campanuleae) unresolved, and disagreed in the position of some key genera, such as the relationships of *Trachelium* L. with respect to *Campanula* (Roquet *et al.* in press). To better resolve relationships within *Campanula,* we have added new data from the *rbc*L-conserved region to Roquet *et al.* study (in press) and increased the sampling of some major lineages within Platycodoneae and Wahlenbergieae.

3.3 MATERIALS AND METHODS

3.3.1 Plant material

The selection of taxa for the phylogenetic analysis based on the *rbc*L gene was done to represent the different groups appeared in previous molecular phylogenetic works with less conserved markers (Eddie *et al.* 2003; Roquet *et al.* in press), in order to confirm previous results and study unresolved phylogenetic relationships. With this purpose, we have done phylogenetic analyses of 54 *rbc*L sequences. A total of 32 new sequences were produced, and 22 were obtained from GenBank. Three sequences from Lobelioideae, another subfamily of Campanulaceae (Stevens 2006), were included as outgroups. Bayesian phylogenetic analyses from 105 sequences of *trn*L-F produced in Roquet *et al.* (in press) were also used for dating and biogeographic analyses. Sources of material and location of vouchers are in Table 1.

3.3.2 DNA extraction, PCR amplification and sequencing

DNA was extracted from herbarium material or, in few cases, from silica gel-dried plant tissue by the CTAB method (Doyle & Doyle 1987) modified following suggestions by Culling (1992). We also used the "Dneasy® Mini Kit" (Qiagen Inc., Valencia, CA) for difficult material, according to manufacturer's instructions.

The *rbc*L gene was amplified and sequenced in two overlapping fragments using the primers 1F/724 R (Olmstead *et al.* 1992) and 636F/1460R (Fay *et al.* 1997, 1998). PCR amplifications were performed with the thermocycler PTC-100™ Programmable Thermal Controller (MJ Research Inc.). The thermal cycling profile consisted of 1 minute at 95ºC; 5 minutes at 80ºC, while DNA-polymerase (Ecotaq, Ecogen S. R. L., Barcelona, Spain) was added, 29 cycles of 1 minute denaturing at 95ºC, 30 seconds annealing at 50ºC, and 1 minute of extension at 72ºC; with final extension of 7 minutes at 72ºC. PCR products were cleaned using the "QIAQuick® DNA cleanup system" (Qiagen Inc., Valencia, CA) according to manufacturer's instructions. DNA sequencing of PCR-purified templates was done using reactions based on chemistry of "Big Dye® Terminator v3.1" (Applied Biosystems, Foster City, CA) following the protocol recommended by the manufacturer. The products obtained were analyzed on an ABI Prism® 3730 PE Biosystems/Hitachi automated sequencer in the "Serveis Cientificotècnics de la Universitat de Barcelona", and the chromatograms obtained were edited with Chromas 2.0 (Technelysium Pty Ltd, Tewantin, Australia).

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

Species	**Voucher (*rbc*L)**	**Voucher (*trn*L-F)**
Adenophora confusa Nannf.	Cosner *et al.* (2004)	-
Adenophora remotiflora (Sich. & Zucc.) Miq.	Japan, Kawasaki: Honshu, *Estebánez 1511* (MA *s. n.*)	Japan, Kawasaki: Honshu, *Estebánez 1511* (MA *s. n.*)
Asyneuma limonifolium Bornm.	Turkey: Erzurum, *Nisa 1006* (MA *689405*)	Turkey: Erzurum, *Nisa 1006* (MA *689405*)
Asyneuma lobelioides Hand.-Mazz.	Turkey: Ermenek, *Aldasoro 9157 et al.* (MA *s. n.*)	Turkey: Ermenek, *Aldasoro 9157 et al.* (MA *s. n.*)
Asyneuma virgatum Bornm.	Cosner *et al.* (2004)	-
Azorina vidalii (Wats.) Feer	Portugal: Açores, *Sequeira 4493* (MA *s. n.*)	Portugal: Açores, *Sequeira 4493* (MA *s. n.*)
Campanula abietina Griseb. & Schenk		Turkey: Zongüldak, *Aedo 6469b* (MA *688196*)
Campanula affinis Roem. & Schult.		Spain, Barcelona: Montserrat, *Roquet V-2004* (BC *s. n.*)
Campanula alliarifolia Willd.		Cultivated at Botanical Garden of Madrid (MA *688448*)
Campanula andrewsii DC.		Greece, Peloponnese: Achaia, *Burri et al. 2-VII-1996*(LE *s. n.*)
Campanula aparinoides Pursh	Finland, Palkane: Lake Tykolanjawi, *Nunmi s. n.* (MA *451610*)	Finland, Palkane: Lake Tykolanjawi, *Nunmi s. n.* (MA *451610*)
Campanula argaea Boiss. & Bal.		Turkey, Kayseri: Erciyas Dag, *Alpinar et al. 2-VII-1994* (ISTE *s. n.*)
Campanula armena Stev.		Armenia, Ashtarak: Mt. Arailer, *Vasak 15-VII-1975* (MA *642322*)
Campanula balfourii Wagner & Vierh.		Yemen, Socotra: Qalansiyah, *Thulin 8712 et al.* (UPS *82575*)
Campanula bellidifolia Adams (2)		Cultivated at Botanical Garden of Madrid, *Alarcón 230* (MA *s. n.*)
Campanula betulifolia K. Koch		Turkey, Gümüshane: Tirebolu-Kürtün, *Herrero 1180* (MA *689193*)
Campanula canescens Wall.		Taiwan, Hualien: Hisiulin Hsiang, *Chih-Chia Wang 1353* (LE *s. n.*)
Campanula chamissonis Fed.	Japan: Honshu, *Estebánez 1478* (MA *s. n.*)	Japan: Honshu, *Estebánez 1478* (MA *s. n.*)
Campanula cochlearifolia Lam.		Spain, Huesca: Bielsa, *Roquet 12-X-2004* (BC *s. n.*)
Campanula collina M. Bieb.		Georgia, Javakhati: Mt. Taushan-Tagan, *Ketzkoveli 22-VII-80* (MA *575569*)
Campanula conferta DC.		Turkey, Sakaltutan Gecidi: Erzincan, *Aldasoro 2647* (MA *689787*)
Campanula coriacea Boiss. & Kotschy		Armenia, Arna: Ejevi Azor, *Oganessian 3-VIII-63* (MA *560762*)
Campanula creutzburgii Greuter	Greece, Kreta: Dia, *Alpinar* (ISTE *s. n.*)	Greece, Kreta: Dia, *Alpinar* (ISTE *s. n.*)
Campanula cymbalaria Sibth & Sm.		Turkey, Kayseri: Erciyas Dag, *Alpinar et al. 23-VII-94* (ISTE *62303*)
Campanula decumbens DC.		Spain, Cuenca: Barajas de Melo, *Arán et al. 30-V-98* (MA *623787*)
Campanula dichotoma L.		Italy: Sicily (MA *645874*)
Campanula divaricata Michx.	USA, Waterville: Pigeon River (MA *391570*)	USA, Waterville: Pigeon River (MA *391570*)
Campanula drabifolia Sibth. & Sm.		Greece, Peloponnese: Tolon, Argolida, *Buggenhourt 18481* (MA *625645*)
Campanula elatines Bout. ex Willk. & Lange	Cosner *et al.* (2004)	Bremer *et al.* (2002)
Campanula erinus L.	Spain, Mallorca: Cova Negra, *Sáez 6135* (BCB)	Spain, Mallorca: Cova Negra, *Sáez 6135* (BCB)
Campanula fastigiata Dufour ex Schult.	Spain: Albacete, *Aedo 3937* (MA *591308*)	Spain: Albacete, *Aedo 3937* (MA *591308*)
Campanula filicaulis Dur.		Morocco, Middle Atlas: Midelt, *Jury 17866* (MA *616923*)

Campanula foliosa Ten.		Italy: Mt. Vigula, *Snogerup 15903* (UPS *s. n.*)
Campanula fruticulosa (O. Schwarz & Davis) Damboldt	Turkey, Burdur, Dirmil: Masda Dagi, *Dumar 6279* (ISTE *s. n.*)	Turkey, Burdur, Dirmil: Masda Dagi, *Dumar 6279* (ISTE *s. n.*)
Campanula garganica Ten.		Italy: Foggia, *Aldobrandi et al. 12-VII-96* (MA *625685*)
Campanula haradjanii Rech. f.		Turkey, Gümüshane: Tirebolu-Kürtün, *Herrero 1234* (MA *688153*)
Campanula hofmannii (Pant.) Greuter & Burdet	Bosnia-Herzegovina: Torlokovac, *Frost-Olsen 4953* (MA *464670*)	Bosnia-Herzegovina: Torlokovac, *Frost-Olsen 4953* (MA *464670*)
Campanula incurva Aucher ex DC.		Cultivated at Botanic Institute of Barcelona, *Roquet s. n.* (BC *s. n.*)
Campanula involucrata Aucher ex DC.		Turkey, Gümüshane: Yagmürdere, *Herrero 1453* (MA *687604*)
Campanula karakuschensis Grossh.		Iran: Ghogeh Dag, *Rechinger 44029* (MA 417801)
Campanula lanata Friv.		Bulgary, Rila: Kostenec, *Frost-Olsen 484* (MA *463958*)
Campanula latifolia L.		Turkey, Trabzon: Sumelas, *Valcarcel 379* (MA *689767*)
Campanula lusitanica Loefl.	Spain, A Corunya: Carnota, *Louzan 1-VI-96* (MA *581374*)	Spain, A Corunya: Carnota, *Louzan 1-VI-96* (MA *581374*)
Campanula lyrata Lam.		Greece, Lesbos: Plomari, *Julin 22-IV-82* (UPS *s. n.*)
Campanula macrochlamys Boiss. & Huet		Turkey, Artvin: Lomasen Üstü, *Baytop 18-IV-82* (ISTE *48574*)
Campanula macrostachya Willd.		Turkey, Kirkareli: Pinarhisar arasi, *Baytop 17-VI-72* (ISTE *22508*)
Campanula macrostyla Boiss. & Heldreich	Turkey: Ermenek, *Aldasoro 9135 et al.* (MA *s. n.*)	Turkey: Ermenek, *Aldasoro 9135 et al.* (MA *s. n.*)
Campanula medium L.		Cultivated at Botanical Garden of Madrid, *MLA0183* (MA *s. n.*)
Campanula mollis L.	Spain, Almería: Gádor, Borja, *Navarro 1303* (MA *545932*)	Spain, Almería: Gádor, Borja, *Navarro 1303* (MA *545932*)
Campanula moravica (Spitzn.) Kovanda		Cultivated at Institut Botànic de Barcelona, *Roquet 5-V-2004* (BC *s. n.*)
Campanula olympica Boiss.		Turkey, Çamlik: Rize, *Nisa 772* (MA *s. n.*)
Campanula peregrina L.	Turkey, Alanya: Antalya, *Baytop 26-VII-57* (ISTE *5437*)	Turkey, Alanya: Antalya, *Baytop 26-VII-57* (ISTE *5437*)
Campanula persicifolia L.		Cultivated at Botanical Garden of Madrid, *MLA0179* (MA)
Campanula pinatzii Greuter & Phitos		Greece, Dhodhekanisos: Kastello, *Raus 9666* (MA *464542*)
Campanula pinnatifida Hub.-Mor.		Turkey: Gurun-Sivas, *Nydegger 16893* (MA *367632*)
Campanula polyclada Rech. f. & Schiman-Czieka		Afghanistan, Panjao: Waras, *Rechinger 36562* (MA *416822*)
Campanula poscharskyana Degen		Cultivated at Botanical Garden of Madrid, *Alarcón 178* (MA)
Campanula prenanthoides Durand		USA, California: Yosemite Park (MA *460216*)
Campanula primulifolia Brot.	Portugal, Algarve: Foia, *Julin 26-VI-1974* (UPS *s. n.*)	Portugal, Algarve: Foia, *Julin 26-VI-1974* (UPS *s. n.*)
Campanula propinqua Fisch. & C. A. Mey. (1)		Turkey, Gumushane: Kurtun-Torul, *Herrero 1287* (MA *688027*)
Campanula propinqua Fisch. & C. A. Mey. (2)		Armenia, Eghegnadsor: Egheg, *Oganessian 18-VI-04* (ERE *154863*)
Campanula ptarmicifolia Lam. (1)	Turkey: Tunceli, *Davis 31233 et al.* (ISTE *43633*)	Turkey: Tunceli, *Davis 31233 et al.* (ISTE *43633*)
Campanula ptarmicifolia Lam. (2)	-	Turkey: Erzincam, *Aedo 2593* (MA *690039*)
Campanula pterocaula Hausskn.		Turkey: Bolu, *Nydegger 19005* (MA *367633*)
Campanula pubicalyx (Davis) Damboldt	Turkey, Konya: Ermenek, *Davis 16244* (ISTE *43630*)	Turkey, Konya: Ermenek, *Davis 16244* (ISTE *43630*)

Campanula punctata Lam.	Japan: Honshu, *Estebánez 1508* (MA *s. n.*)	Japan: Honshu, *Estebánez 1508* (MA *s. n.*)
Campanula pyramidalis L.		Croatia: Rijeka, *Vitek 99440* (MA *641379*)
Campanula quercetorum Hub.-Mor. & C. Simon		Turkey, Evciler: Bayramiç, *Castroviejo 15236* (MA *644286*)
Campanula radula Fisch.		Turkey: Hakkari, *Archibald 8340* (ISTE *s. n.*)
Campanula rapunculoides L.		Turkey: Rize, *Nisa 763* (MA *689073*)
Campanula rapunculus L.		Spain, Barcelona: Viladrau, *Sáez 6121* (BCB *s. n.*)
Campanula ramosissima Sibth. & Sm.	Michaels *et al.* (1993)	-
Campanula rotundifolia L.		Andorra *Sáez 6134* (BCB *s. n.*)
Campanula savalanica Fed.		Iran: Shahbil *Herrero* s. n. (MA *s. n.*)
Campanula saxifraga M. Bieb. subsp. *aucheri* (DC.) Ogan. (1)		Armenia, Akhurian: Krashen, *Oganessian 26-VI-2004* (ERE *154864*)
Campanula saxifraga M. Bieb. subsp. *aucheri* (DC.) Ogan (2)		Turkey, Kars: Agri Dagh, Serdarbulah *Baytop et al. 14-VII-76* (ISTE *42896*)
Campanula scheuchzeri A. Gray		Spain, Huesca: Bielsa, *Roquet 12-X-2004*(BC *s. n.*)
Campanula sclerotricha Boiss.		Turkey, Van: Bahçesaray, *Baytop 19-IX-1978* (ISTE *30991*)
Campanula scoparia (Boiss. & Hausskn.) Damboldt		Turkey: Hakkari, *Duncan 71 et al.* (ISTE *s. n.*)
Campanula scutellata Griseb.		Macedonia: Veles (MA *555269*)
Campanula semisecta Murb.		Spain: Cazorla, *Muñoz-Garmendia et al. 16-VI-76* (MA *456218*)
Campanula sibirica L.		Russia, Altai: Artishtu-Karatsu, *Castroviejo 14132* (MA *613903*)
Campanula speciosa Pourr.		France: Ariège, Mijanes, *Montserrat et al. 8-VI-1983* (MA *256533*)
Campanula spicata L.		Italy, Teramo: Fondo de la Salsa, *Navarro 4323* (MA *699308*)
Campanula stevenii subsp. *stevenii* M. Bieb.		Armenia: Vayk, *Oganessian* (ERE *154865*)
Campanula stricta Labill.		Iran: Chadil Kuh, *Renz 48987* (MA *420241*)
Campanula strigosa Banks & Sol.		Turkey, Nemrut: Kahta, *Sorger 4-V-1980* (W *54340*)
Campanula subcapitata Popov		Turkey, Erzurum: Pasinler, *Herrero 1831* (MA *687545*)
Campanula thyrsoides L.	Switzerland, Berne: Geintrisch, *Hedberg 4037* (UPS *s. n.*)	-
Campanula trachelium L.		Spain, *Sáez 6133* (BCB *s. n.*)
Campanula tymphaea Hausskn.		Greece, Pindos: Kataras Pass, *Frost-Olsen 3685* (MA *544610*)
Campanulastrum americanum (L.) Small	USA, Nebraska: Seward Co., *Nieto-Feliner 2063* (MA *459958*)	USA, Nebraska: Seward Co., *Nieto-Feliner 2063* (MA *459958*)
Canarina canariensis (L.) Vatke	Spain, Gran Canaria: Teror, *Aldasoro 9106* (MA *s. n.*)	Spain, Gran Canaria: Teror, *Aldasoro 9106* (MA *s. n.*)
Codonopsis ovata Benth.	Cosner *et al.* (1994)	
Codonopsis viridis Wall.	Cosner *et al.* (2004)	
Cyananthus lobatus Wall. ex Benth.	Cosner *et al.* (2004)	
Diosphaera rumeliana (Hampe) Bornm.	Macedonia, Kavala: Mt. Pangeo, *Greuter 16056* (MA *540729*)	Macedonia, Kavala: Mt. Pangeo, *Greuter 16056* (MA *540729*)
Edraianthus graminifolius (L.) DC.	Cosner *et al.* (2004)	Italy, Sicily: Palermo, *Herrero 888* (MA *646860*)

Feeria angustifolia (Schousb.) Buser	Morocco, Marrakech: High Atlas, Hir-n-Ifri, *Podlech 47779* (MA *472233*)	Morocco, Marrakech: High Atlas, Hir-n-Ifri, *Podlech 47779* (MA *472233*)
Gadellia lactiflora (M. Bieb.) Schulkina	Turkey, Rize, *Nisa 732* (MA *688458*)	-
Jasione heldreichii Boiss. & Orph.	Cosner *et al.* (2004)	Bulgary, Rhodope Mts.: Koprivlen, *Navarro 5008* (MA *s. n.*)
Jasione montana L.	Spain, Barcelona: Saulons d'en Deu, *Sáez 6218* (BCB *s. n.*)	Spain, Barcelona: Saulons d'en Deu, *Sáez 6218* (BCB *s. n.*)
Legousia falcata (Ten.) Fritsch	Cosner *et al.* (2004)	-
Legousia hybrida (L.) Delarb.	Morocco: Atlas, Dayer Iffer, *Cirujano R10113 et al.*(BC *s. n.*)	Morocco: Atlas, Dayer Iffer, *Cirujano R10113 et al.*(BC *s. n.*)
Lobelia cardinallis L.	Kress & Erickson (2007)	
Lobelia erinus L.	Michaels *et al.* (1993)	
Lobelia nicotianifolia Heyne	Givnish *et al.* (unpublished)	
Michauxia tchihatchewii Fisch. & C. A. Mey.	Turkey: Ermenek, *Aldasoro 9138 et al.* (MA *s. n.*)	Turkey: Ermenek, *Aldasoro 9138 et al.* (MA *s. n.*)
Musschia aurea Dumort.	Cosner *et al.* (2004)	Portugal, Madeira: Encomiada, *Velayo 9727* (MA *655323*)
Merciera tenuifolia DC.	Cosner *et al.* (2004)	
Petromarula pinnata (L.) DC.	Cosner *et al.* (2004)	Greece: Kreta, *Shay 82-1059* (B *10 9010624*)
Phyteuma spicatum L.	Spain, Barcelona: Aiguafreda, *Roquet 8-V-05* (BC *s. n.*)	Spain, Barcelona: Aiguafreda, *Roquet 8-V-05* (BC *s. n.*)
Platycodon grandiflorum (Jacq.) DC.	Cosner *et al.* (2004)	Cultivated at Botanical Garden of Madrid (MA *573425*)
Prismatocarpus diffuses DC.	Cosner *et al.* (2004)	
Roella ciliata L.	Cosner *et al.* (2004)	South-Africa, *Aldasoro 9014* (MA *s. n.*)
Solenopsis laurentia (L.) C. Presl		Italy, Sardinia: Uroro Perdas de Fogu, *Garcia 3779* (MA *709006*)
Trachelium caeruleum L.	Cosner *et al.* (1994)	Spain, Santander: Liencres, *Aldasoro 3503* (MA *s. n.*)
Triodanis leptocarpa (Nutt.) Nieuwl.	Cosner *et al.* (2004)	-
Wahlenbergia gloriosa Lothian	Cosner *et al.* (2004)	
Wahlenbergia hederacea L.	Spain, Oviedo: Cangas de Narcea, *Serra 6070* (MA *705618*)	Spain, Oviedo: Cangas de Narcea, *Serra 6070* (MA *705618*)
Wahlenbergia lobelioides Link	Portugal: Madeira, *Sequeira 4597* (MA *s. n.*)	Portugal: Madeira, *Sequeira 4597* (MA *s. n.*)

3.3.3 Phylogenetic analyses

Sequences of *rbc*L gene were aligned using the text editor TextPad® 4.7.3 by eye, as there are no indels present, and bases 1-30 that formed the primer-binding region of the 1F primer used were excluded to reduce missing data. Phylogenetic analyses were performed for *rbc*L data using Maximum Parsimony (MP) and Bayesian Inference (BI).

Parsimony analyses involved heuristic searches conducted with PAUP* 4.0b10 (Swofford 2002) with tree bisection-reconnection (TBR), MulTrees option in effect, branch swapping algorithm, 10 replicates of random addition-sequence and character states specified as unordered and unweighted. Bootstrap (BS) analyses were performed (Felsenstein 1985). We used the approach by Lidén *et al.* (1997), performing BS analyses with 1,000 replicates, random taxon addition with 10 replicates per replicate and no branch swapping. To explore the amount of phylogenetic signal, we calculated the Consistency Index (CI) (Kluge & Farris 1969) and the Retention Index (RI) (Swofford 2002).

Previous to BI analyses, we used the program MrModeltest 2.2 (Nylander 2004) to determine the best-fitting model of evolution for the *rbc*L data with the Akaike Information Criteria (AIC). The best-fitting sequence evolution model selected was the General Time Reversible model (GTR + I + Γ) (Rodríguez *et al.* 1990). The model and parameter estimates were then used in BI analyses conducted with MrBayes 3.1 (Huelsenbeck *et al.* 2001; Ronquist & Huelsenbeck 2003). Two simultaneous and independent BI analyses were performed, for each analysis we run four Markov chains in parallel during 10^6 generations to sample trees using the Markov Chain Monte Carlo (MCMC) principle. One sample of each 100 generations was saved, yielding 20,000 sample trees. The first 2,000 trees were eliminated during the *burn-in* phase before computing the consensus tree in order to eliminate the trees that didn't reach a stationary posterior probability (PP).

3.3.4 Dating analyses

Two relaxed-clock methods based on different statistical basis were used to reconstruct divergence times for each marker: the Penalized Likelihood approach (PL, Sanderson 2002) and the Bayesian Relaxed Clock (BRC, Kishino *et al.* 2001; Thorne & Kishino 2002). These two methods seem to be the more successful at finding optimal levels of smoothing to correct for rate heterogeneity and less sensitive to undersampling (Linder *et al.* 2005).

Penalized Likelihood analyses were conducted with the r8s v.1.71 program (Sanderson 2002). This program assumes the tree topology and the branch lengths provided by the user, and it does not provide any confidence intervals on the parameters. To obtain these intervals and account for both branch length and topological uncertainties, we applied an abbreviation of the Bayesian approach suggested by Lopez-Vaamonde *et al.* (2006): we selected randomly one hundred trees from the 9000 trees with higher posterior probability (PP) and we run the r8s program for each of these hundred trees, each with its specific smoothing value (calculated by cross-validation). Each estimate was perturbed and restarted up to three times to avoid local stability. To summarize the values obtained for each node of interest, we report their modes (the most likely value for each dated node) and 90% highest posterior density (HPD) limits to provide a confidence interval for each estimate (Table 2; see Appendix 1 for all the nodes of *rbc*L data and Appendix 2 for all the nodes of *trn*L-F data). The modes and 90% HPD were obtained by local density estimation using the program LOCFIT (Loader 1999), implemented in the "R" statistical package (Ihaka & Gentlemen 1996; see script in Lopez-Vaamonde *et al.* 2006).

Bayesian dating was done with the packages of programs PAML (Yang 1997) and Multidivtime (Thorne & Kishino 2002). We used thee programs: Baseml (PAML, Yang 1997), to estimate model parameters; Estbranches, to estimate the maximum likelihood of the branch lengths and a variance-covariance matrix; and Multidivtime (Kishino *et al.* 2001; Thorne & Kishino 2002), to perform a Bayesian analysis with the Markov Chain Monte Carlo (MCMC), to approximate the posterior distributions of substitution rates and divergence times. Multidivtime provides direct confidence intervals for all dated nodes. The 50% majority rule consensus trees obtained from the BI analyses of each marker were used for the Bayesian dating analyses.

For each marker, we run two separate analyses with each method to obtain divergence times. In the first analysis, we set arbitrarily the root to 100 mya, and then converted the relative times obtained to absolute by means of fossil data (see next section). In the second analysis we used simultaneously multiple constraints (fossil, geological and independent molecular dating data) such as minimum age (fossil) or maximum age constraints (geological events and independent molecular results).

The ages estimated from *trn*L-F and *rbc*L data with PL and BRC yielded similar results with very few exceptions, and credibility intervals overlap in great part. All calibrations produced almost

TABLE 2. Estimated ages and standard deviation using Penalized Likelihood (r8s) and Bayesian Relaxed Molecular Clock (Multidivtime) with multiple calibrations for *rbc*L and *trn*L-F data. Node letters correspond to those given on the chronograms. (s. d.)= standard deviation; LHPD= 90 % lower highest posterior density limit; UHPD= 90 % upper highest posterior density limit. * indicates age constrained in this node.

Nodes	***rbc*L r8s mode (LHPD-UHPD)**	***rbc*L Multidivtime (s. d.)**	***trn*L-F r8s mode (LHPD-UHPD)**	***trn*L-F Multidivtime (s. d.)**
A - Split between Platycodoneae and the ancestor of Campanuleae and Wahlenbergieae	34.8 (30.6-41)	19.3 (2.3)*	-	-
B - Divergence of Campanuleae and Wahlenbergieae	16*	17 (1)*	16*	17 (1)*
C - Divergence of *Musschia* clade	6.6 (2.8-9.8)	5.9 (2.1)	4.8 (3.2-6.2)	5.5 (1.4)
D - Beginning of diversification of the *Campanula* core	13.9 (12.3-15.4)	14.3 (1.5)	14 (13.1-15.2)	14.7 (1.5)
E - Separation of the *Campanula* s. str. from *Rapunculus* clade	-	-	13.5 (12.4-14.8)	13.5 (1.5)
F - Split of *Campanula* s. str. clade in three lineages plus the *Diosphaera* branch	-	-	11.7 (9.4-13.4)	10.2 (1.5)
G - Split of *Rapunculus* clade in two main lineages (*Rapunculus* I and II)	-	-	10.5 (9.5-12.6)	10.9 (1.8)
H - Separation of *Adenophora*	9.8 (6.6-11.6)	9.1 (2.1)	10 (8.6-11.5)	10.3 (1.8)
I - Separation of *C. divaricata* from the ancestor of the *C. rotundifolia* complex	-	-	2.3 (1.6-4.2)	3.3 (1.2)
J - Separation of *C. rotundifolia*	-	-	0.2 (0-0.7)	0.5 (0.5)
K - Diversification of Section *Rapunculus*	-	-	3.4 (2.4-4.1)	4.4 (1.5)
L - Separation of the ancestor of *C. aparinoides* and *C. prenanthoides*	-	-	5 (4.1-6.9)	6 (1.7)
M - Separation of *Asyneuma, Petromarula* and the ancestor of *Campanulastrum, Legousia* and *Triodanis.*	6.9 (6.1-7.5)	6.7 (2.0)	5.4 (4.5-7.7)	6.4 (1.5)
N - Separation of *Campanulastrum* and *Legousia*	3.7 (2.4-5.3)	4.2 (1.8)	3.5 (2.3-4.7)	3.9 (1.3)
O - Separation of *Campanulastrum* and *Triodanis*	2 (1-4.2)	2.4 (1.5)	-	-
P – Start of diversification of the ancestor of *Campanula* s. str. clade 3	-	-	10.1 (8.3-11.2)	8.9 (1.6)
Q - Start of diversification of the ancestor of *Campanula* s. str. clade 2	-	-	8.3 (6.2-10.3)	7.5 (1.7)
R - Diversification of *Campanula* s. str. clade 1	-	-	9.3 (4.6-10.9)	5.1 (1.4)
S - Separation of *Azorina* from *Campanula*	7.9 (4.6-8)	5.5 (1.7)*	6.5 (3.5-8)	5.2 (1.3)*
T - Separation of *Michauxia* from *Campanula*	7.4 (4.7-9.6)	7.1 (1.9)	4.9 (2.4-8.5)	4.1 (1.3)
U - Separation of the ancestor of *Platycodon-Canarina* from the ancestor of *Codonopsis-Cyananthus*	26 (17-32)	12.7 (3.2)	-	-

coincident results except for for the basal clade of the outgroups *Canarina*, *Codonopsis, Cyananthus* and *Platycodon* in *rbc*L data (Appendix 1). However, the credibility intervals tend to overlap, except for the root node. Optimal smoothing levels (λ) obtained with the PL approach for 100 different *rbc*L Bayesian trees varied widely, from 0.0032 to 3200, but most of them (73%) were intermediate (between 0.32 and 3.2), suggesting a moderate rate heterogeneity among lineages (Sanderson 2002). Smoothing levels for *trn*L-F trees also indicated moderate rate heterogeneity among lineages (between 0.1 and 32). Mean age estimates and their credibility intervals for each node and dating/calibration method are given in Appendixes 1 and 2. The modes of divergence times obtained by PL and the age estimates with highest probability obtained by BRC with multiple calibrations are also indicated for each node in the Bayesian consensus phylogenetic trees obtained with the *rbc*L (Fig. 1) and *trn*L-F (Fig. 2) data.

3.3.5 Calibrations

We used four different calibration points to place minimal age constraints on internal nodes in the phylogeny: (1) a fossil seed found by Lancucka-Srodoniowa (1979) from the Early-Middle Miocene (*c*. 16 mya; see Appendix 3 for a geologic timescale table), described by the author as *Campanula* sp., and whose structure resembles those of *Campanula* and related genera such as *Adenophora* Fisch., *Jasione* L.*, Phyteuma* and *Wahlenbergia* Schrad. ex Roth., this fossil was used as fixed calibration date for the node of the most recent common ancestor (mrca) to all the above-cited genera; (2) the sub-aerial stage of the oldest island of the Azores, Punta Delgada, dated as 8 mya (Abdel-Monem *et al.* 1975), which was used as an upper age constraint ("maximum age constraint") for the appearance of the endemic species *Azorina vidalii* (Wats.) Feer; (3) the age of the emerged part of Madeira (dated as being maximum 5.2 mya, Ferreira *et al.* 1988) as the upper limit for the appearance of *Musschia aurea* Dumort., endemic to Madeira; and (4) a maximum age of 41 mya for the root node, obtained from the dating analyses of Wikström *et al.* (2001).

3.3.6 Biogeographic analyses

The areas of endemism were defined by the presence of one or more endemic taxons. Thirteen areas are proposed for the biogeographic analyses: A, Western Asia (from Anatolia to Iran, including the Caucasus); B, Eastern Mediterranean Basin; C, Western Mediterranean Basin; D, North Africa; E, Macaronesia; F, North and Central Europe; G, Eastern Asia; H, Central Asia; I, North Ame-

rica; J, East Africa; K, South Africa; L, Himalayan range; M, India (except the higher mountains area) and Indonesia; and N, Australia and New Zealand (Fig. 3).

Dispersal-vicariance analysis (Ronquist 1997) as implemented in DIVA (Ronquist 1996) was used to reconstruct ancestral distributions on the phylogeny of Campanulaceae. DIVA optimizes distributions for each node of the tree, assuming allopatric speciation by vicariance as the null model and minimizing the number of assumed dispersals and extinctions under a parsimony criterion (Ronquist 1997). The maximum number of ancestral areas was first unconstrained and then constrained to five (Ronquist 1996), which is the maximum geographic range of the most widespread species. The second type of analysis proved more effective to reduce uncertainty in the biogeographical reconstructions and it is the only one presented here.

Current methods of biogeographic inference, including DIVA, reconstruct biogeographic patterns on a fixed, fully resolved tree, thus ignoring the error usually associated with phylogenetic reconstruction (Ronquist 2004). This can be problematic since usually phylogenetic hypotheses contain some level of ambiguity or different support for different nodes. To account for phylogenetic uncertainty in our biogeographic reconstructions, we used a new method here that averages DIVA biogeographical reconstructions over a Bayesian sample of trees reflecting confidence (credibitlity values) on the different clades (Nylander *et al.* in press). DIVA analyses were run on each individual tree from the posterior probability distribution of a Bayesian analysis, using scripts graciously provided by Johan Nylander (Nylander *et al.* in press). The scripts summarize/average ancestral area reconstructions for a given node across all the trees in the Bayesian sample. When there were several equally parsimonious reconstructions at a given node (e. g. A / B / AB), these were downweighted by 1/*n*, where *n* was the total number of alternative reconstructions at the node. Thus, pie charts in Figs. 1 and 2 represent the uncertainty in ancestral reconstruction at a given node, while controlling for the uncertainty in phylogenetic relationships in the rest of the tree, conditional in that this node exists. This means that uncertainty in the node existence is not included in the analysis because only those trees where the node is present are used in the summary reconstructions (*i.e.* “node-by-node reconstruction”).

3.4 RESULTS

3.4.1 Phylogenetic results of *rbc*L and *trn*L-F data.

Figure 1 shows the topology of the majority rule consensus tree from the Bayesian analysis of the conserved region of the *rbc*L marker. Bayesian and Maximum Parsimony analyses yielded the same topology in the sense that relationships that were strongly supported (>70% BS; >0.90 PP) in one analysis were also found in the other. Numeric results of the analyses of the *rbc*L and *trn*L-F markers are summarized in Table 3. Figure 2 shows the Bayesian consensus tree for the *trn*L-F analysis, which is virtually identical to the one presented by Roquet *et al.* (in press).

TABLE 3. Numeric results from *rbc*L and *trn*L-F data. Consistency and retention indices and divergence were calculated excluding non-informative characters. * RAM limit computer was reached at this value.

Data set	*rbc*L	*trn*L-F
Total characters	1,358	1,084
Informative characters	192	340
Number of taxa	54	103
Number of MPTs found	30,856	891,000 (1 island)*
Number of steps	497	1036
Consistency index	0.5311	0.5425
Retention index	0.7465	0.7720

Our results confirm previous known relationships within Campanulaceae based on the cpDNA rearrangements (Cosner *et al.* 2004), ITS (Eddie *et al.* 2003; Park *et al.* 2006; Roquet *et al.* in press), *trn*L-F and ITS plus *trn*L-F combined data (Roquet *et al.* in press). Campanulaceae s. str. (sensu Kovanda 1978; Cosner *et al.* 2004) is confirmed as a natural, monophyletic group, formed by all genera traditionally ascribed to Platycodoneae, Campanuleae and Wahlenbergieae.

The tribe Platycodoneae, which includes *Canarina, Codonopsis, Cyananthus* and *Platycodon*, appears as the sister-clade to the remaining Campanulaceae (1.0 PP, 100% BS, Fig. 1), in agreement with the cpDNA rearrangement data (Cosner *et al.* 2004). Platycodoneae have sepals alternating with carpels and colpate or colporate pollen, while Wahlenbergieae and Campanuleae show opposite sepals and porate pollen (Dunbar 1975; Thulin 1975; Yeo 1993; Cosner *et al.* 2004). Campanuleae (including *Campanula, Gadellia* Shulkina and *Musschia* Dumort.) and Wahlenbergieae (including *Feeria* Buser*, Jasione, Merciera* A. DC.*, Prismatocarpus* L'Hér.*, Roella* L. and *Wahlenbergia*) also form a well-supported clade (1.0 PP, 100% BS, Figs. 1-2). The analyses also

recognize a monophyletic group within Campanuleae, the *Campanula* core (sensu Roquet *et al.* in press), which is divided into a rapunculoid and campanuloid groups (1.0 PP, Figs. 1-2). However, the analyses failed to recognize the monophyly of the Campanuleae or the Wahlenbergieae, which appear inter-graded. The Wahlenbergieae appear divided into two main branches, which form a polytomy with the *Campanula* core and the *Musschia* clade: the branch of *Jasione* plus *Feeria* and the clade formed by *Wahlenbergia, Roella, Prismatocarpus* and *Merciera* (Figs. 1-2). Thus, the monophyly of Wahlenbergieae and Campanuleae requires taxonomic revision.

Apart from the *Musschia* group, the *Campanula* core appears divided into the *Campanula* s. str. clade (1.0 PP, 81% BS, Fig. 2) and the *Rapunculus* clade (1.0 PP, Fig. 1; 1.0 PP, 91% BS, Fig. 2). Unfortunately, *rbc*L could not resolve the position of the *Trachelium* clade with respect to *Campanula:* there is no bootstrap support or very low Bayesian support for its placement in the phylogeny (Fig. 1). In fact, the three molecular markers, ITS, *rbc*L and *trn*L-F gave quite different results, with *Trachelium* appearing alternatively as the sister group to the *Rapunculus* clade (Fig. 1), to the clade formed by *Campanula* s. str. and *Rapunculus* (Fig. 2), or to the core of the *Campanula* s. str. clade (ITS, Roquet *et al.* in press).

The *Rapunculus* clade (1.0 PP, Fig. 1; 1.0 PP, 91% BS, Fig. 2) includes two main subclades. The first one, the *Rapunculus* clade 1 (R1) shows a heterogeneous core (1.0 PP, 92% BS, Fig. 2) including taxa distinct in morphology, distribution and habitat: (1) the endemic Cretan *Petromarula*; (2) *Asyneuma* Griseb. & Schenk, spread in the Caucasus, the Irano-Turanian region, and the Eastern Mediterranean; (3) the alpine or subalpine *Phyteuma*; and (4) the widespread annual and drought-adapted genera *Legousia* Durand (circum-Mediterranean) and *Triodanis* Raf. and *Campanulastrum* Small (North-American endemics). Basal to these taxa, there are the groups of *Campanula prenanthoides* Durand and *C. stevenii* M. Bieb. (Fig. 2). The *Rapunculus* clade 2 (R2) includes a central core of species and two basal branches (0.99 PP, Fig. 2). The two branches correspond to: one species belonging to the genus *Adenophora*, which includes *c.* 60 species centered in Asia; and *Campanula fastigiata* Dufour ex Schult., present in North Africa, Western Mediterranean and the Irano-Turanian region. The core is formed by the assembly of the subsection *Isophylla* Damboldt and the sections *Heterophylla* (Witas.) Fed. and *Rapunculus* (Fourr.) Boissier (1.0 PP, 77% BS, Fig. 2).

The *Campanula* s. str. clade has good support (1.0 PP, 81% BS, Fig. 2) and includes *Azorina* Feer*, Diosphaera* Feer*, Edraianthus, Michauxia* L'Hér. and many species of *Campanula.* It is formed

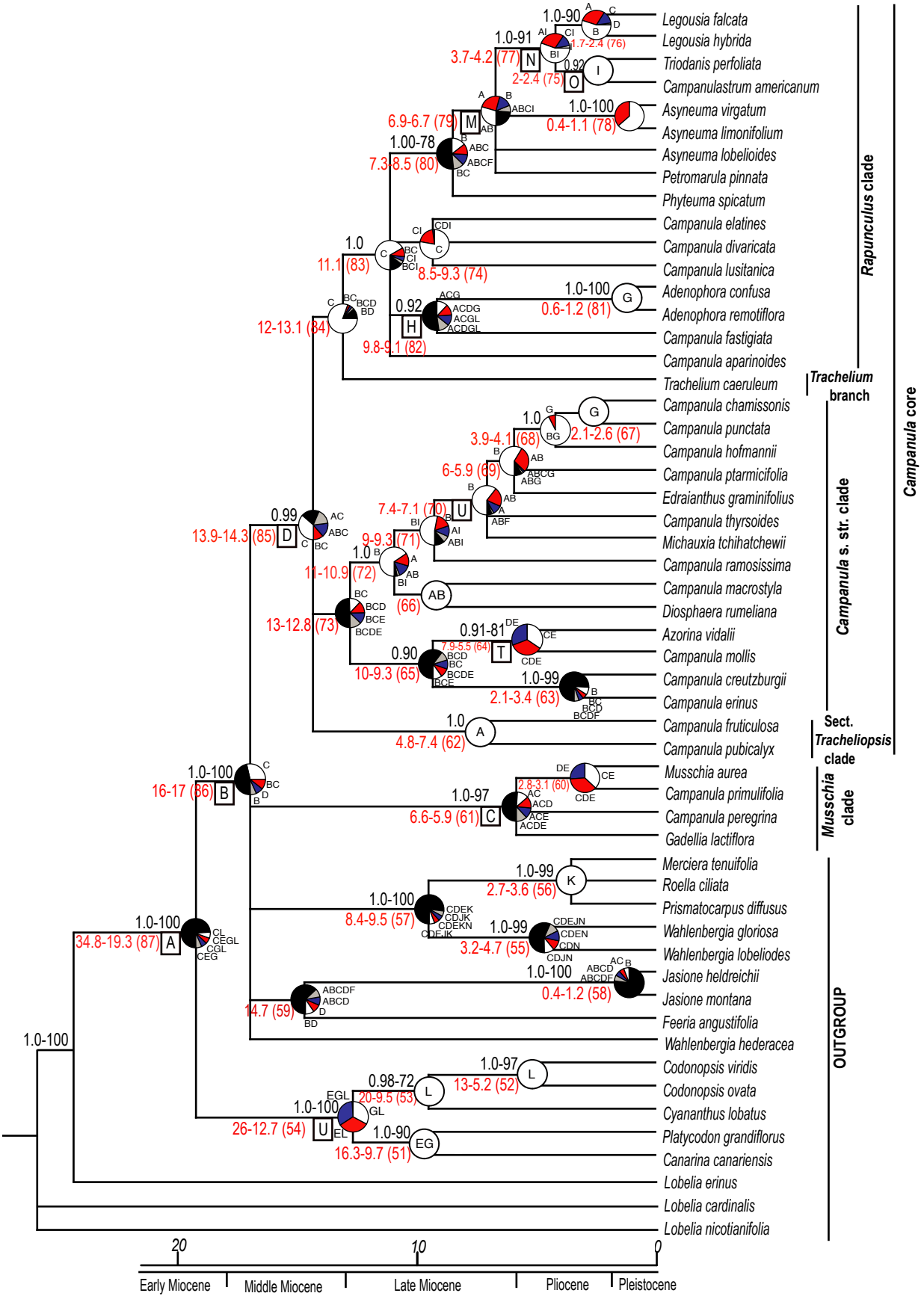

Figure 1. Chronogram obtained with BRC analysis of *rbc*L data. Numbers in black above branches indicate Bayesian-credibility values (PP) > 0.90 and Parsimony BS > 70%. MP gave trees with identical topologies. Numbers in red below branches indicate the mode of age estimates obtained with PL and the mode of age estimates obtained with BRC, and the numbers in brackets in red indicate the node number. The pie charts represent the reconstructions of ancestral areas for each node. The white portion of each piechart corresponds to the most probable ancestral area reconstruction, the red to the second most probable, the blue to the third, the grey to the fourth, and the black portion correspond to re-constructions with a probability < 0.10. Letters next to pie charts correspond to the reconstruction of ancestral areas: A, Western Asia (from Anatolia to Iran, including the Caucasus); B, Eastern Mediterranean Bassin; C, Western Mediterranean Bassin; D, North Africa; E, Macaronesia; F, North and Central Europe; G, Eastern Asia; H, Central Asia; I, North America; J, East Africa; K, South Africa; L, Himalayan range; M, India (except the higher mountains area) and Indonesia; and N, Australia and New Zealand. Letters inside squares indicate the nodes referred in Table 2.

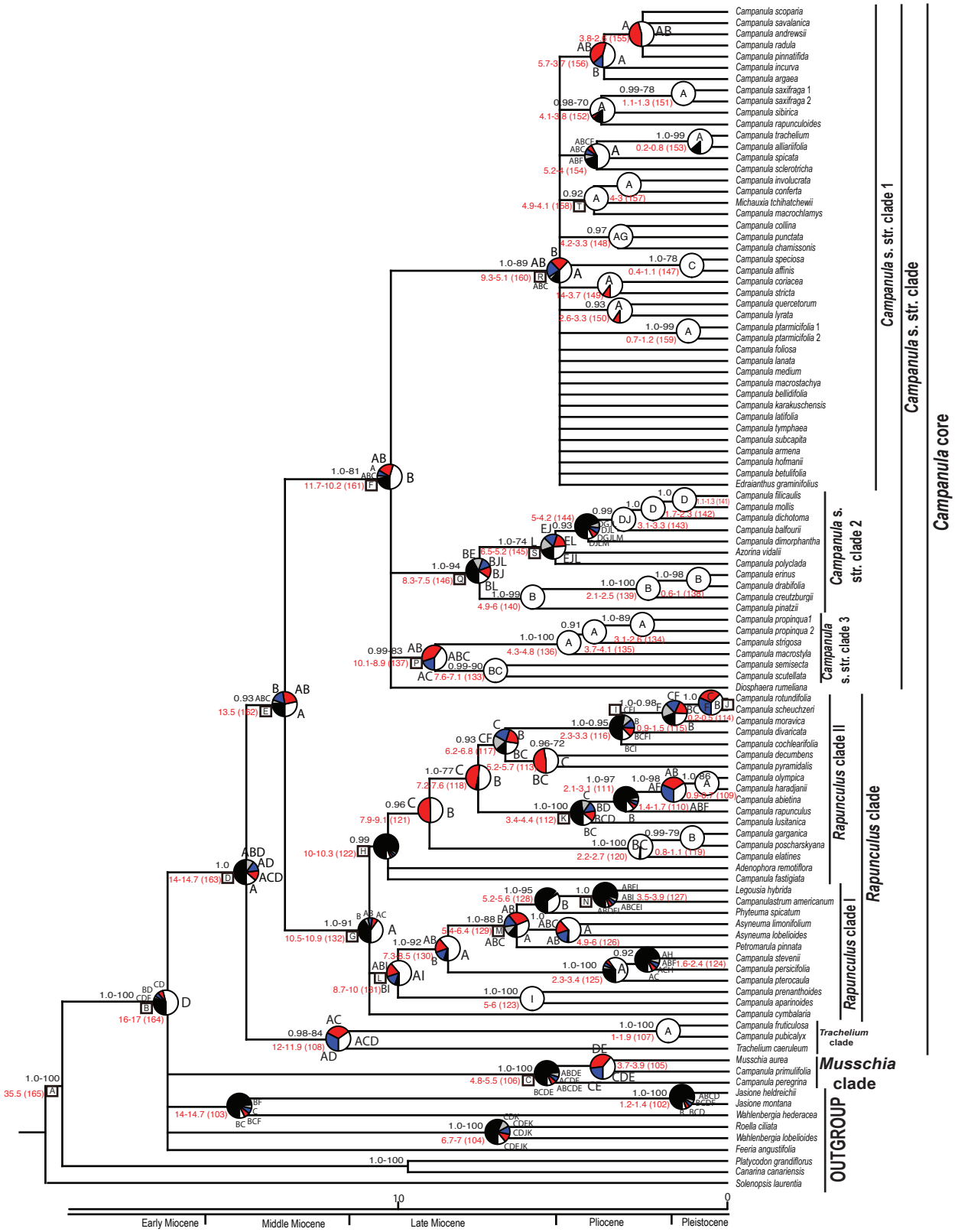

Figure 2. Chronogram obtained with BRC analysis of *trn*L-F data. Numbers in black above branches indicate Bayesian-credibility values (PP) > 0.90 and Parsimony BS > 70%. MP gave trees with identical topologies. Numbers in red below branches indicate the mode of age estimates obtained with PL and the mode of age estimates obtained with BRC, and the numbers in brackets in red indicate the node number. The pie charts represent the reconstructions of ancestral areas for each node. The white portion of each piechart corresponds to the most probable ancestral area reconstruction, the red to the second most probable, the blue to the third, the grey to the fourth, and the black portion correspond to re-constructions with a probability < 0.10. Letters next to pie charts correspond to the reconstruction of ancestral areas: A, Western Asia (from Anatolia to Iran, including the Caucasus); B, Eastern Mediterranean Bassin; C, Western Mediterranean Bassin; D, North Africa; E, Macaronesia; F, North and Central Europe; G, Eastern Asia; H, Central Asia; I, North America; J, East Africa; K, South Africa; L, Himalayan range; M, India (except the higher mountains area) and Indonesia; and N, Australia and New Zealand. Letters inside squares indicate the nodes referred in Table 2.

by three main subclades: the large *Campanula* s. str. clade 1 (C1) including the core of *Campanula* s. str. and the genera *Edraianthus* and *Michauxia* (1.0 PP, 89% BS, Fig. 2); a second subclade (*Campanula* s. str. clade 2, C2) formed by *Azorina,* a part of *Campanula* subgenus *Megalocalyx* Damboldt, the subgenus *Roucela* (Feer) Damboldt, the group of *C. mollis* L. plus *C. dimorphanta* Schweinf. and *C. polyclada* Rech. f. & Schiman-Czieka (1.0 PP, 94% BS, Fig. 2); and a third subclade (*Campanula* s. str. clade 3, C3) formed by the remaining species of the subgenus *Megalocalyx* (0.99 PP, 83% BS., Fig. 2). *Diosphaera rumeliana* (Hampe) Bornm. appears as an independent branch in the polytomy (Fig. 2).

3.4.2 Biogeographical and temporal analyses

The Bayes-DIVA analyses suggest a complex biogeographical history involving several events of intercontinental dispersal and a history of rapid diversification in the Mediterranean Basin. Bayes-DIVA analysis based on *rbc*L data (Fig. 1) indicates considerable ambiguity regarding the origin of the Campanulaceae, probably as a result of the low taxon sampling. Basal diversification within the subfamily (the split between Platycodoneae and Campanuleae-Wahlenbergieae) is dated around the Late Oligocene-Early Miocene (35.5-19.3 mya, node A in Table 2). The ancestral area of the Platycodoneae is reconstructed as Asia or Africa plus Asia, with the African-Macaronesian genera *Canarina* and the Eastern Asian *Platycodon* as the sister group of the Central Asian *Cyananthus* and *Codonopsis*. Diversification within this tribe is dated by the *rbc*L marker as starting *c.* 26-12.7 mya (node U in Table 2; Fig. 1).

According to our reconstruction, the ancestor of Campanuleae and Wahlenbergieae could have originated around the Middle Miocene (16-17 mya; Node B in Table 2), either in the Western Mediterranean region (Fig. 1) or in North Africa (Fig. 2). These differences are likely attributed to differences in taxa composition between the two analyses. Given that taxon sampling within the Campanulae was considerable higher in the *trn*L-F analysis than in the more general *rbc*L analysis, we follow the *trn*L-F reconstruction here.

Although ancestral reconstructions for the *Musschia* clade are ambiguous, they point to a circum-Mediterranean ancestor with first diversification during the Pliocene (Fig. 2). Ancestral area reconstructions for the *Campanula* core are ambiguous but suggest also a Mediterranean ancestor, with only Western Asia or Western Asia combined with Western Mediterranean, North Africa and/or

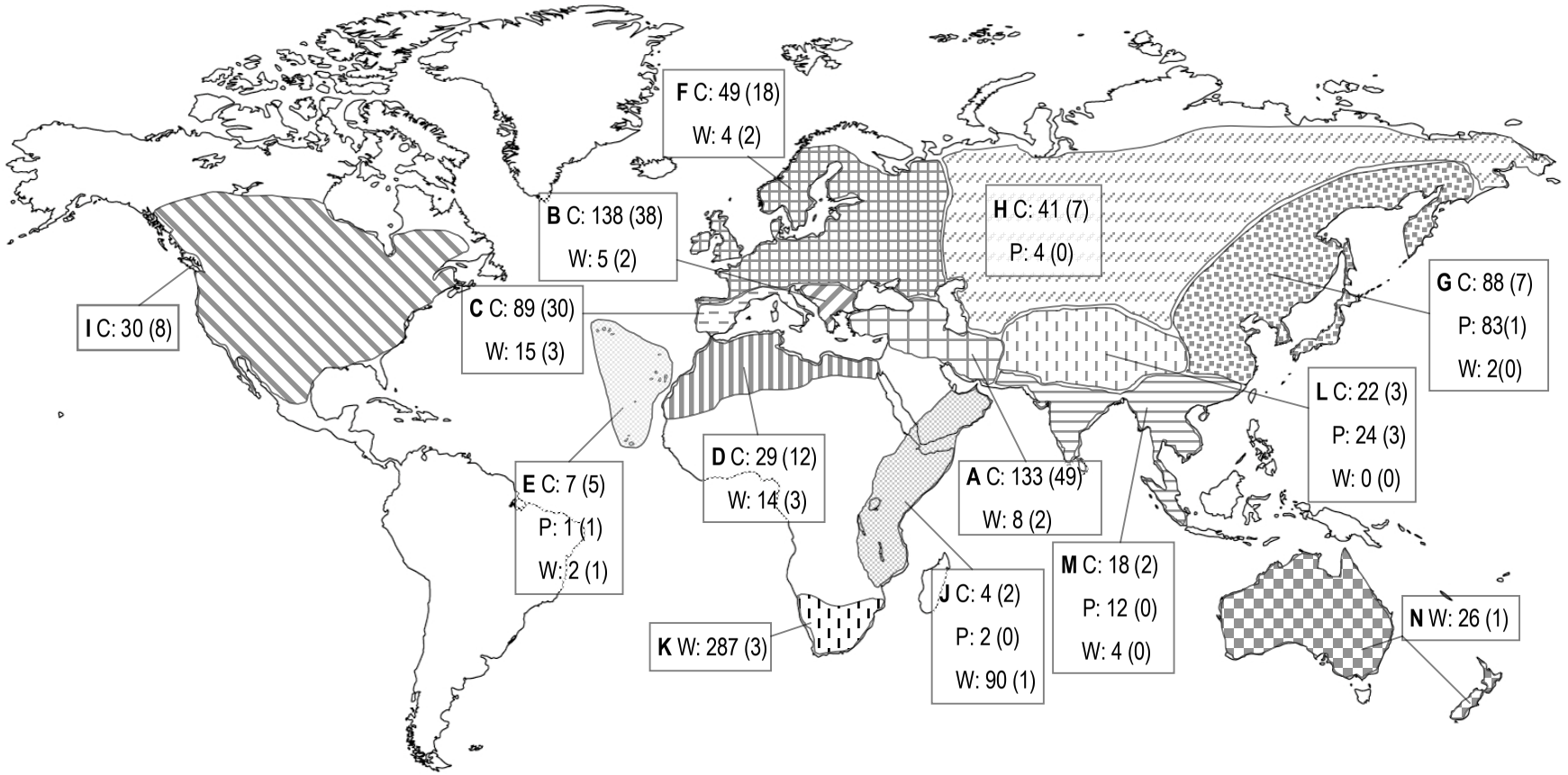

Figure 3. Distribution map of Campanulaceae s. str. showing the areas used for DIVA analysis. Bold letters A to N are the defined geographic areas for the analysis: A, Western Asia (from Anatolia to Iran, including the Caucasus); B, Eastern Mediterranean Bassin; C, Western Mediterranean Bassin; D, North Africa; E, Macaronesia; F, North and Central Europe; G, Eastern sia; H, Central Asia; I, North America; J, East Africa; K, South Africa; L, Himalayan range; M, India (except the higher mountains area) and Indonesia; and N, Australia and New Zealand. Numbers next to letters C, P and W in each area box refer to the number of species belonging to the tribes Campanuleae, Wahlenbergiae and Platycodoneae. The number between brackets indicates the number of species present in the area that have been sampled in this study.

Eastern Mediterranean as the most likely ancestral areas (Fig. 2). Divergence times based on *trn*L-F place the start of the diversification within the crown group of Campanuleae during the Middle Miocene (14.7-13.9 mya, node D in Table 2, separation of *Trachelium*, *Campanula* s. str. and *Rapunculus* clades).

The ancestor of the *Trachelium* clade is also reconstructed as circum-Mediterranean, with first diversification in the Middle Miocene (Fig. 2). This genus is found in the Western Mediterranean and North Africa. Biogeographic analyses suggest an origin in Western Asia combined with Western Mediterranean and/or North Africa. However, depending on the molecular marker used, *Trachelium* is situated in very different positions: basal to the *Rapunculus* clade (*rbc*L), sister to the *Campanula* s. str. clade (ITS, Roquet *et al.* in press); or as the sister group of both clades *Rapunculus* and *Campanula* s. str. (*trn*L-F). The incongruent phylogenetic signal between the three markers could be attributed to past hybridization events between species belonging to the two main *Campanula* clades.

The ancestral area reconstruction with the highest probability suggests a Western Asian origin for the ancestor of *Rapunculus* and *Campanula* s. str. clades, whose split is also dated as Middle Miocene (13.5 mya, node E in Table 2). The Eastern Mediterranean is the most probable ancestral area for the *Campanula* s. str. clade, whereas the most favored reconstruction for the rapunculoid group is Western Asia. Main diversification within these subclades started in the Late Miocene (11.7-10.2 mya for the *Campanula* s. str. clade, node F in Table 2; 10.5-10.9 mya for the *Rapunculus* clade, node G in Table 2).

The largest clade of *Campanula* s. str. (clade C1) is assigned to Western Asia as the most probable area, followed by Eastern Mediterranean, and Eastern Mediterranean plus Western Asia as other possible areas (Fig. 2). Divergence times place the start of this radiation in the Late Miocene-Pliocene (9.3-5.1 mya, node R in Table 2). The other two smaller clades of *Campanula* s. str. are assigned to a large combination of areas: clade 3 is reconstructed as having a Mediterranean origin, whereas clade 2 shows a possible vicariance between Eastern Mediterranean and Eastern Asia and/or Eastern Africa. Within *Rapunculus*, the clade 1 is assigned to a Western Asian origin, whereas ancestral area reconstructions are ambiguous for the clade 2, comprising subection *Isophylla* and sections *Heterophylla* and *Rapunculus.*

It is interesting to note that though several taxa within the *Rapunculus* clade 1 have been described as different genera – they exhibit large differences in flower morphology (*e.g. Asyneuma, Campanulastrum*, *Legousia* and *Phyteuma*) – our dating results suggest a relatively recent origin: the separation of *Asyneuma, Petromarula* and the ancestor of *Campanulastrum, Legousia* and *Phyteuma* is dated as only 5.4-6.5 mya (node M in Table 2), whereas the split between *Phyteuma* and the ancestor of *Campanulastrum* and *Legousia* is dated as 5.2-5.6 mya, and the split of *Campanulastrum* and *Legousia* as recent as 3.5-3.9 mya.

3.5 Discussion

3.5.1 Origin of Campanuleae

Recent phylogenetic studies of the Campanulales (Cosner *et al.* 1994; Gustafsson & Bremer 1995; Bremer & Gustafsson 1997) indicate that this order is composed of several families/subfamilies including Campanulaceae, Stylidiaceae and Lobelioideae, many of which have a Southern Hemisphere distribution. The apparently African origin (Eddie *et al.* 2003) of the Lobelioideae (included within Campanulaceae sensu lato), as well as the mainly Southern Hemisphere distribution of the tribe Wahlenbergieae, have led some authors to hypothesize that the Southern Hemisphere is also the ancestral area for the Campanulaceae (Bremer & Gustafsson 1997; Cosner *et al.* 2004).

Our phylogenetic hypothesis confirms that the Platycodoneae are sister to the rest of Campanulaceae in agreement with cpDNA rearrangements (Cosner *et al.* 2004) and morphological data (Thulin 1975). This tribe is distributed in two main areas: (1) Eastern Africa and Macaronesia (*Canarina*), and (2) Central and Eastern Asia (*Codonopsis, Cyananthus* and *Platycodon*). Cosner *et al.* (2004) proposed an Asian origin for the Platycodoneae based on the basal position of the genus *Platycodon* within the tribe. Despite some ambiguity, our results suggest also an Asian origin for the tribe, with possible dispersal to the African region of *Canarina*. The *rbc*L and *trn*L-F data show the Wahlenbergieae and Campanuleae inter-graded (see also Cosner *et al.* 2004). According to Cosner *et al.* (2004), Campanuleae should include genera of the Northern Hemisphere, while Wahlenbergieae should contain mainly Southern Hemisphere taxa. Thulin's definition (1975) of Wahlenbergieae includes the genera *Merciera, Microcodon* A. DC., *Prismatocarpus, Rhigiophyllum* Hochst., *Roella* and *Treichelia* Vatke (all endemic to South Africa), *Heterochaenia* A. DC. (Mascarene

Islands), *Gunillaea* Thulin (tropical Africa and Madagascar), *Craterocapsa* Hilliard & B. L. Burtt (SE tropical Africa) and *Wahlenbergia* (81% of species in Africa, 13% in Australasia, the rest in Southern China, Japan, India, South America and a few Pacific islands, Lammers 1992). Other taxa sometimes included in Wahlebergieae are: *Jasione* (circum-Mediterranean), *Feeria* (North-Western Africa) and *Wahlenbergia hederacea* L. (phylogenetically not a true *Wahlenbergia*) (Kovanda 1978; Yeo 1993). Our *rbc*L phylogeny shows Wahlebergieae divided into two main clades: the branch formed by *Wahlenbergia*, *Roella*, *Merciera* and *Prismatocarpus*, mainly distributed in the Southern Hemisphere, and a clade comprising *Jasione, Feeria* and *Wahlenbergia hederacea*, which is mainly distributed in the Mediterranean Basin. Bayes-DIVA reconstructions for these two clades are ambiguous, but in general the ancestral area reconstructions for the *Jasione* clade favor North Africa as either the sole ancestral area or part of it, whereas all possible reconstructions for the *Wahlenbergia* clade include South Africa as part of the ancestral area – the genus *Wahlenbergia* is specially rich in this area (150 species). The ancestor of Wahlenbergieae and Campanuleae is most likely reconstructed as being of North African or Western Mediterranean origin, depending on the marker considered. Both markers, however, coincide in placing the first split between Wahlenbergieae and Campanuleae in the Early-Middle Miocene (Table 2).

Considering the distribution of Platycodoneae and Wahlenbergieae plus Campanuleae, the ancestor of the family Campanulaceae could have originated in Africa and migrated to Asia (*e.g.* via the mountains of Syria and Lebanon and the Saharo-Sindian deserts, Van Zinderen Bakker 1969), or diverged first in the Asian continent, dispersing later to Africa and the Mediterranean region. Similar movements from Asia to Africa, generally involving the Middle East and Levante regions, have been found in many other groups of organisms, including mammals (Vrba 1993; Cox & Moore 2005) and passerine birds (Voelker 1999; Nylander *et al.* in press). These dispersal events have been attributed to the cooling and drying trends of the climate during the Neogene that led to the development of open, grassy habitats in South-Western Asia and Eastern Africa (Vrba 1993; Fernandes *et al.* 2006).

The split of the ancestor of Platycodoneae and Wahlenbergieae plus Campanuleae is dated as Oligocene or Early Miocene (35-20 mya) in the *rbc*L analysis. Final collision between the Arabian Plate and the Eurasian Plate in the Mid-Late Miocene (16-10 mya) and subsequent rising of mountain chains in Arabia, Turkey and the Middle East, closed the Tethys seaway and interrupted the circum-equatorial world oceanic circulation, leading to climatic deterioration in Eurasia and Africa (Cox &

Moore 2005). African aridification increased in the early Miocene as a result of the uplift of the continent and the formation of the East African Rift Valley. Fossil evidence indicates that the East African rain forest was replaced by open woodland habitats around 25-17 mya (Axelrod & Raven 1972, 1978; Retallack *et al.* 1990; Jacobs 1999), with grassy savannas becoming prominent at the end of the Miocene (Fernandes *et al.* 2006). Furthermore, the Neogene collision of the Arabian Plate with Eurasia provided a new dispersal route between Eurasia and Africa via the Anatolian and Levante regions, and closed the opening of the Red Sea to Mediterranean (Pliocene), allowing dispersal between these regions and North Africa (Sanmartin 2003). It is possible that early diversification within Campanulaceae was correlated to geographic expansion (movement into new areas) favored by the formation of new biomes and the availability of new dispersal routes between Africa and Eurasia during the Neogene.

3.5.2 Diversification in the *Campanula* core

The split of the three main clades in the *Campanula* core (*Campanula* s. str., *Rapunculus* and *Trachelium*) is dated as Middle Miocene (14.7-12.3 mya), coincident with the gradual cooling of the climate that began 15 mya and lasted until 13 mya (Flower & Kennett 1994). These cooling trends led to an important sea level fall 14.8-11.2 mya associated to the rapid expansion of the Antarctic Ice sheet (Rögl & Steininger 1984). This fall closed the Tethyan seaway, connecting the Mediterranean and the Indian Ocean, increasing the land in the Eastern Mediterranean and connecting areas previously separated in the Mediterranean, the Caucasus and the Western and Central Asia. The rising of mountains in these new continental sheets may have provided an adequate scenario for the diversification and expansion of the ancestors of *Campanula.* Increasingly cooler climates from the Middle Miocene onwards may also have favored diversification within *Campanula*, a cold-adapted genus whose highest species richness occurs in high steppes and mountain ranges.

3.5.3 Western Asia and Eastern Mediterranean as a cradle of diversification in *Campanula*

The two main clades of *Campanula*, *Rapunculus* and *Campanula* s. str., seem to have originated and evolved in the Eastern part of the Mediterranean Basin – the Balkan Peninsula in the case of *Campanula* s. str. and the Anatolian region in the case of *Rapunculus* – around the same period (10-12 mya). These two areas have also the highest richness of Campanuleae species (Fig.

3). The main subclade in *Campanula* s. str. (clade C1), a large non-resolved polytomy of perennial and monocarpic species, also originated in Western Asia and/or the Balkan Peninsula during the Late Miocene (9.5-5.1 mya), from where it dispersed to other regions. Three reasons may have favored diversification in this area: first, the strategic position of Anatolia between Europe and Asia made this area a connecting route from which species could migrate to North Africa, Central-Northern Europe and Asia. Secondly, the intense orogenic activity that took place in Western Asia during the Late Miocene onwards. The collision of the Arabian Plate with Eurasia resulted in the formation of several mountain belts surrounding the Iranian Plateau, including the Zagros Mountains (10 mya) and the Kopet-Dagh and Lesser Caucasus (5 mya). These mountains are still been uplifted as the Arabian Plate continues its indentation into Eurasia (Dercourt *et al.* 1986). Similarly, the squeezing of the Turkish Plate along the Anatolian Fault Zone by the Arabian Plate is responsible for the intense orogenic activity in the Anatolian region. Finally, the fact that Pleistocene glaciations affected Western Asia to a limited extent could have favored Anatolia as a refugial area for many temperate organisms (Davis 1965).

The low phylogenetic resolution of clade C1 in *Campanula* s. str. can be explained by a rapid radiation in Western Asia, from 5-10 mya, a time with intense orogenic activity, due to the movements of the Arabian plate (Quennell 1984; Steininger & Rögl 1984). The rising of mountains in these new continental sheets may have provided an adequate scenario for the diversification and expansion of the ancestors of *Campanula.* Climate changing trends might have affected insect communities, which seem to have exercised a strong selective pressure in floral aspects of *Campanula* (Roquet *et al.* in press). The pollinators could have selected different flower types in the three main clades: the tubular corollas in *Trachelium* clade; the hanging-campanulate corollas that prevail in *Campanula* s. str.; and the heterogeneous shaped-corollas found in the *Rapunculus* clade.

The diversification of the clade C2 dates back to the end of Miocene. Most species included in the first subclade (which corresponds to subgenus *Roucela*: *C. creutzburgii* Greuter*, C. drabifolia* Sibth. & Sm., *C. erinus* L. and *C. pinatzii* Greuter & Phitos) are endemic to the Aegean islands and the Greek and Turkish coasts. The second subclade (part of subgenus *Megalocalyx* and part of sect. *Saxicolae* (Boiss.) Kharadze) is formed by dry-tolerant annuals and short-lived perennials. This group includes two pairs of East-West disjunct taxa such as *Campanula polyclada* (Iran-Pakistan), sister to *Azorina vidalii* (Azores), and *C. balfourii* Wagner & Vierh. (Socotra) sister to the clade of *C. mollis, C.*

filicaulis Dur. and *C. dichotoma* L. (North-Western Africa and South-Western Europe). These patterns can be explained by a vicariant differentiation due to climatic fluctuations in North Africa. There are at least 15 genera or sections of angiosperms that exhibit in their phylogenies geographical disjunctions between Macaronesia/Mediterranean Basin and East/South Africa/Southern Arabia (Andrus *et al.* 2004). The increase of precipitation during the periods of the Pliocene wiped out a great part of the desert and permitted the exchange of species along areas now isolated by the Sahara (Quézel 1978).

3.5.4 Dispersal to North America

At least four dispersal events to North America are inferred within the *Rapunculus* clade. One is the ancestor of *Campanula aparinoides* Pursh. and *C. prenanthoides*: the first is native to slopes near coniferous forests of Western North America and the second is found in wetlands of the Northern Great Plains. These two sister-species are placed basal within the *Rapunculus* clade 1, whose ancestor is reconstructed as already widespread in Western Asia and North America 8.7-10 mya (Fig. 2). Migration between Asia and North America could have been possible via the Beringian Land Bridge (Wolfe 1975; Tiffney 1985; Sanmartín *et al.* 2001), which began to cool down significantly from the Middle Miocene (15 mya) onwards (Milne 2006). Many *Campanula* taxa are adapted to cool and dry conditions. Increasing cooling of the BLB in the Late Miocene led to vicariance speciation of Old and New World populations of the *Rapunculus* clade 1 around 9-10 mya, coinciding with the most prominent temperature decrease of the Miocene (Mosbrugger *et al.* 2005).

A recent example of dispersal to North America is *Campanula rotundifolia,* a very polymorphic species with numerous infraspecific taxa and characterized by polyploidy superimposed on segmental chromosome rearrangements (Bocher 1960). This species probably originated during the Middle Pleistocene (Fig. 2), when the Beringian Land Bridge was dominated by tundra, steppe-like vegetation. Dispersal across the bridge was then limited to arctic, tundra species (Tiffney 1993; Sanmartín *et al.* 2001). As in other arctic species, intraspecific variation in *C. rotundifolia* may have been promoted by glacial-interglacial cycles, when population underwent episodes of isolation in small enclaves during periods of extremely adverse conditions (Murray 1995).

At least one long-distance dispersal event from the Mediterranean area to North America seems to have occurred in the *Rapunculus* clade 1, by the ancestor of the Mediterranean-Asian

Legousia and the North-American *Triodanis* and *Campanulastrum. Legousia* is a drought-tolerant annual, with rotate autogamous flowers which presents a large distribution in Eurasia and Africa. The separation of *Legousia* from the ancestor of *Campanulastrum* and *Triodanis* is dated here as 3.7-4.2 mya, some time after the Messinian salinity crisis (Hsü *et al.* 1973) and after the opening of the Bering Strait that finally broke terrestrial connections between Asia and North America (Milne 2006). Similarly, the Eastern North America distribution of *Campanula divaricata* Michx. and its recent age of origin (2.3-3.3 mya) suggests another long-distance dispersal event within the *Rapuculus* clade.

Only plant species bearing seeds with very low falling velocity (*e.g.* plumed seeds and dust-like seeds) are dispersed over long distances in appreciable numbers (Tackenberg *et al.* 2003). The seeds of bellflowers (Campanulaceae) seem to be streamlined to facilitate wind dispersal (Emig & Leins 1996; Maier *et al.* 1999; Kuss *et al.* 2007). They are dust-like seeds, egg or spindle-shaped, somewhat compressed and in some taxa with a narrow wing. They are released from capsules by wind under dry conditions, and a part of them fall on leaves, bracts or sepals if the wind speed and turbulence are weak.

Although seeds are very similar in all *Campanula* species, only members of the *Rapunculus* clade are present nowadays in North-America. This clade presents much more heterogeneity in chromosome numbers, basic numbers and ploidy levels than *Campanula* s. str. clade (Roquet *et al.* in press). Stebbins (1966) pointed out that aneuploid reduction in chromosome number and polyploidy are characteristic of many species groups which occupy pioneer habitats. These characteristics might be one of the factors involved in the success of this group to settle several times in the New World.

3.6 CONCLUSIONS

Western Asia and Eastern Mediterranean seem to have played an important role as centers of migration and diversification for the different clades of the *Campanula* core. The biogeographical history of this genus seems to be highly complex: repeated diversification events in Western Asia, spreading to adjacent areas and posterior isolation that constitute new endemisms; at least two independent dispersal events to Macaronesia; and occurrence of both vicariant and long-distance dispersal events to North America.

Rates of species diversification of at least four of the five *Campanula* clades (*Campanula* s. str. 1 and 2, and *Rapunculus* clade 1 and 2) seem to have increased during the Messinian salinity crisis. During this period, drought and erosion were more intense and may have promoted diversification in annual, xeromophic and other pioneer lineages (Bocquet *et al.* 1978; Kellogg 2001). These climate changes and the expansion of mountainous regions probably lead the ancestors of some *Campanula* species to adapt to disturbed, dry or cold environments.

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Capítol 3

Molecular phylogeny and historic biogeographic reconstruction of *Campanula* L. subgenus *Roucela* (Dumort.) Damboldt

RESUM. *Campanula* subgènere *Roucela* comprèn espècies que conformen unitats evolutives difícilment delimitables morfològicament degut a la manca de caràcters estables. Per aquesta raó, el tractament taxonòmic del subgènere *Roucela* és molt controvertit. Les seqüències nuclears i cloroplàstiques (ITS, *trn*G i *trn*L-*trn*F) obtingudes han estat analitzades independentment i de forma combinada mitjançant els mètodes de parsimònia i inferència Bayesiana, per millorar els coneixements de les relacions filogenètiques d'aquest grup. S'han mapat en els arbres filogenètics els nombres cromosòmics i els principals caràcters morfològics per tal d'esbrinar possibles patrons evolutius. Les anàlisis filogenètiques de *Roucela* han revelat que la circumscripció actual no és monofilètica. *Campanula scutellata* presenta tendències morfològiques i moleculars que suggereixen la seva pertanyença a una unitat evolutiva diferent. La resta d'espècies de *Roucela* conformen un grup monofilètic, tot constituint tres clades que no es corresponen amb els principals trets morfològics. Es suggereix una proposta taxonòmica per tal d'obtenir una circumscripció que es correspongui amb l'evolució del subgènere *Roucela.*

ABSTRACT. *Campanula* subgenus *Roucela* includes species that constitute evolutionary units very difficult to delimit morphologically due to the lack of stable characters. Because of this, the taxonomic treatment of *Roucela* is highly controversial. Independent and combined data from nuclear and chloroplast sequences (ITS, *trn*G and *trn*L–*trn*F) were analyzed with Bayesian inference and parsimony methods to elucidate the phylogenetic relationships of *Roucela,* in order to ameliorate the understanding of its phylogenetic relationships. Chromosome numbers, and morphological characters were mapped on the phylogenetic trees searching for evolutionary patterns. The phylogenetic analyses revealed that *Roucela*, as currently circumscribed, is not monophyletic. *Campanula scutellata* shows morphological and molecular trends that suggest that it belongs to a different evolutionary unit. The rest of the *Roucela* species constitute a monophyletic group with three different lineages, which are not in consonance with the main morphological features. We suggest a taxonomic proposal in order to obtain a more natural circumscription for the subgenus *Roucela.*

4. MOLECULAR PHYLOGENY AND HISTORIC BIOGEOGRAPHIC RECONSTRUCTION OF *CAMPANULA* L. SUBGENUS *ROUCELA* (DUMORT.) DAMBOLDT

4.1 INTRODUCTION

Campanula subgenus *Roucela* (Dumort.) Damboldt includes dichotomously branched annual plants, without appendages between the calyx lobes, and capsules dehiscing by 3 basal valves (Damboldt 1978; Carlström 1986). These plants are mainly distributed in the Eastern Mediterranean. However, the species with the widest distribution, *Campanula erinus* L., is found throughout the Mediterranean basin and Western Asia, reaching also the Azores and the Canary Islands in the West and Iran and Oman in the East.

The taxonomic complexity of *Roucela* led to different and somewhat confusing treatments in different floras and other geographically restricted studies (Fedorov 1976; Damboldt 1978; Greuter *et al.* 1984; Carlström 1986). Several authors presented studies for some species or groups of species of this subgenus, and they also commented about taxonomic difficulties and problematic patterns of character variation. For some species, taxonomic information is deficient. In other cases, local catalogues, floras or checklists provide additional interesting information (Rechinger & Schimann-Czeika 1965; Greuter *et al.* 1984). In addition, a great number of taxa have been described at various taxonomic ranks, and most of these have also been combined at other ranks.

Carlström (1986) studied the group of *Campanula drabifolia* Sibth. & Sm., recognizing *C. creutzburgii* Greuter, an endemism of Crete; *C. drabifolia*, distributed in Southern Greece and Ionian islands; *C. kastellorizana* Carlström, endemism of the islands Kastellorizo and Ro; *C. pinatzii* Greuter & Phitos, restricted to the islands of Karpathos and Kasos; *C. rhodensis* A. DC., endemism of Rhodes; *C. simulans* Carlström, located in South-Western Anatolia; and *C. veneris* Carlström, endemic to Cyprus. Other species of the subgenus are *C. delicatula* Boiss., distributed in Cyprus, Eastern Aegean islands, Karpathos and South and Western Anatolia; *C. raveyi* Boiss., endemic to Western Anatolia; and *C. scutellata* Griseb., distributed in Central and Northern Greece, reaching Bulgaria and Macedonia; all of them recognized but not studied by Carlström (1986). Tan & Sorger (1987) described a new species of the subgenus *Roucela* (*C. lycica* Tan & Sorger*)* restricted to Southern Anatolia.

The molecular work done in Chapter 1 included 5 species that belong to subgenus *Roucela.*

The results show that *Roucela* is not a monophyletic group because *C. scutellata* appears in a different clade, separated from the other *Roucela* species and closely related to some species belonging to subgenus *Megalocalyx* Damboldt such as *C. propinqua* Fisch. & C. A. Mey. However, these results should be considered cautiously because of the limited sampling. Polo (2007), in its taxonomic study of subgenus *Roucela*, indicated that *C. scutellata* is the more morphologically distinct species in respect to the other species recognized in the subgenus. Moreover, its chromosome number (*2n*= 14) is not shared with any other *Roucela* species.

4.2 Main Objectives

Campanula subgenus *Roucela* includes species very closely related, and its evolutionary units are very difficult to delimit morphologically due to the lack of stable characters. The complexity of this subgenus, and the lack of definite results about its phylogenetic relationships, led us to explore it by means of a combined analysis of nuclear and plastid sequences: the internal transcribed spacer (ITS) of nuclear ribosomal DNA plus the chloroplast DNA regions *trn*L-*trn*F (*trn*L-F) and *trn*G. Our goals in the present work are: (1) to identify the evolutionary entities within this group in order to ameliorate the understanding of the phylogenetic relationships of *Campanula* subgenus *Roucela*; (2) to search for evolutionary patterns in morphological and cytological aspects; (3) to test and compare the phylogenetic utility of ITS, *trn*G and trnL-*F* at the intrasubgeneric level; and (4) to infer the biogeographic events that occurred during the evolution of this subgenus by means of the dispersal-vicariance analysis DIVA (Ronquist 1997).

4.3 Material and Methods

4.3.1 Plant material

We have included in this work 10 species of *Campanula* subgenus *Roucela*, plus three outgroup species (*C. balfourii* Wagner & Vierh., *C. dichotoma* L. and *C. propinqua*), which belong to the subgenus *Megalocalyx*. We have chosen these species as outgroups because *Megalocalyx* is the subgenus morphologically closer to *Roucela,* and phylogenetic results of Chapter 1 also relate them closely. Moreover, recent phylogenetic work on the genus *Campanula* showed that *Megalocalyx* is

not monophyletic, and that the two clades that include *Megalocalyx* species are related to *Roucela* (Roquet *et al.* in press). For this reason, we have included species of both *Megalocalyx* clades.

In this work, we have produced a total of 10 new sequences of ITS region, 15 of *trn*G region, and 9 of *trn*L-F region of *Campanula* species. Five sequences of ITS and 7 sequences of *trn*L-F were obtained in the previous work done in Chapter 1. Sources of material and location of vouchers are in Table 1. The *Roucela* species not included here because of lack of material were *C. kastellorizana*, *C. lycica* and *C. raveyi*. It was not possible to obtain ITS sequences of *C. scutellata*, neither *trn*G sequence of two outgroup species, *C. balfourii* and *C. propinqua*.

4.3.2 DNA extraction, amplification and sequencing

Total DNA was extracted from herbarium material or, in some cases, from silica gel-dried plant tissue following the CTAB method (Doyle & Doyle 1987) with the modifications suggested by Culling (1992). For difficult material we used the kit "DNeasy® Mini Kit" (Qiagen Inc., Valencia, CA), according to manufacturer's instructions.

PCR amplifications were performed with the thermocycler PTC-200™ Programmable Thermal Controller (MJ Research, Inc.). The complete ITS region was amplified with primers ITS1 and ITS4 (White *et al.* 1990). In some cases we substituted ITS1 by 1406F (Nickrent *et al.* 1994). The PCR profile for ITS included 2 minutes at 94ºC; 5 minutes at 80ºC, while DNA-polymerase (Ecotaq, Ecogen S. R. L., Barcelona, Spain) was added; 30 cycles of 1 minute denaturing at 94°C, 2 minutes annealing at 55°C, and 3 minutes of extension at 72°C; with final extension of 15 minutes at 72°C. The *trn*G region was amplified with the primers 3'*trn*G and 5'*trn*G2G (Shaw *et al.* 2005). The PCR profile of the *trn*G region consisted of 4 minutes at 95ºC; 34 cycles of 1 minute denaturing at 95ºC, 1 minute and 30 seconds annealing at 52ºC, 2 minutes extension at 72ºC; and final extension of 10 minutes at 72ºC. The *trn*L-F region was amplified using external primers "c" and "f" and internal primers "d" and "e" (Taberlet *et al.* 1991), amplifying the *trn*L (UAA) intron and the intergenic spacer between the *trn*L (UAA) 3' exon and the *trn*F (GAA) 5' exon. The PCR profile consisted of 1 minute and 35 seconds at 95ºC; 5 minutes at 80ºC, while DNA-polymerase (Ecotaq, Ecogen S. R. L.) was added; 34 cycles of 1 minute denaturing at 93ºC, 1 minute annealing at 50ºC, 2 minutes of extension at 72ºC; and final extension of 10 minutes at 72ºC.

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

Species	Voucher ITS	Voucher *trn*G	Voucher *trn*L-F
Campanula balfourii Wagner & Vierh.	Yemen, Socotra: Qalansiyah, *Thulin 8712 et al.* (UPS *82575*)	-	Yemen, Socotra: Qalansiyah, *Thulin 8712 et al.* (UPS *82575*)
Campanula creutzburgii Greuter	Greece, Kreta: Dia, *Alpınar* (ISTE *s. n.*)	Greece, Kreta: Dia, *Alpınar* (ISTE *s. n.*)	Greece, Kreta: Dia, *Alpınar* (ISTE *s. n.*)
Campanula delicatula Boiss.	Turkey, Mugla: Marmaris, Hisarönii to Türgut (ISTE *s. n.*)	Turkey, Mugla: Marmaris, Hisarönii to Türgut (ISTE *s. n.*)	Turkey, Mugla: Marmaris, Hisarönii to Türgut (ISTE *s. n.*)
Campanula dichotoma L.	Italy: Sicily (MA *645874*)	Italy: Sicily (MA *645874*)	Italy: Sicily (MA *645874*)
Campanula drabifolia Sibth. & Sm. (1)	Greece, Peloponnese: Tolon, Argolida, *Buggenhourt 18481* (MA *625645*)	Greece, Peloponnese: Tolon, Argolida, *Buggenhourt 18481* (MA *625645*)	Greece, Peloponnese: Tolon, Argolida, *Buggenhourt 18481* (MA *625645*)
Campanula drabifolia Sibth. & Sm. (2)	Greece, Kefallínia: Kardakála (W *04020*)	Greece, Kefallínia: Kardakála (W *04020*)	Greece, Kefallínia: Kardakála (W *04020*)
Campanula drabifolia Sibth. & Sm. (3)	Greece, Laconia: Mistras (W *01065*)	Greece, Laconia: Mistras (W *01065*)	Greece, Laconia: Mistras (W *01065*)
Campanula erinus L.	Eddie *et al.* (2003)	Spain, Mallorca: Cova Negra, *Sáez 6135* (BCB)	Spain, Mallorca: Cova Negra, *Sáez 6135* (BCB)
Campanula pinatzii Greuter & Phitos	Greece, Dhodhekanisos: Kastello, *Raus 9666* (MA *464542*)	Greece, Dhodhekanisos: Kastello, *Raus 9666* (MA *464542*)	Greece, Dhodhekanisos: Kastello, *Raus 9666* (MA *464542*)
Campanula podocarpa Boiss.	Turkey, Burdur: Kizilhisar-Burdur (ISTE *s. n.*)	Turkey, Burdur: Kizilhisar-Burdur (ISTE *s. n.*)	Turkey, Burdur: Kizilhisar-Burdur (ISTE *s. n.*)
Campanula propinqua Fisch. & C. A. Mey	Armenia, Eghegnadsor: Egheg, *Oganessian 18-VI-04* (ERE *154863*)	-	Armenia, Eghegnadsor: Egheg, *Oganessian 18-VI-04* (ERE *154863*)
Campanula rhodensis A. DC. (1)	Greece, Nomos Dodekanisos: Kattavia (P *s. n.*)	Greece, Nomos Dodekanisos: Kattavia (P *s. n.*)	Greece, Nomos Dodekanisos: Kattavia (P *s. n.*)
Campanula rhodensis A. DC. (2)	Greece, Nomos Dodekanisos: Stegna (P *s. n.*)	Greece, Nomos Dodekanisos: Stegna (P *s. n.*)	Greece, Nomos Dodekanisos: Stegna (P *s. n.*)
Campanula scutellata Griseb.	-	Macedonia: Veles (MA *555269*)	Macedonia: Veles (MA *555269*)
Campanula simulans Carlström (1)	Turkey, Mugla: on southern promontory, West of Marmaris (MA *s. n.*)	Turkey, Mugla: on southern promontory, West of Marmaris (MA *s. n.*)	Turkey, Mugla: on southern promontory, West of Marmaris (MA *s. n.*)
Campanula simulans Carlström (2)	Turkey, Mugla: Kaunos (W *24013*)	Turkey, Mugla: Kaunos (W *24013*)	Turkey, Mugla: Kaunos (W *24013*)
Campanula veneris Carlström	Cyprus: Roudhkio's Valley (P *s. n.*)	Cyprus: Roudhkio's Valley (P *s. n.*)	Cyprus: Roudhkio's Valley (P *s. n.*)

PCR products were cleaned using the "QIAQuick® DNA cleanup system" (Qiagen Inc., Valencia, CA) according to manufacturer's instructions and sequenced with ITS4 and 1406F primers for ITS region, with *trn*G3' and *trn*G2G for the *trn*G region, and with the *trn*L-F c and *trn*L-F f primersprimers for the *trn*L-F region. DNA sequencing of PCR-purified templates was done using reactions based on chemistry of "Big Dye® Terminator v3.1" (Applied Biosystems, Foster City, CA) following the protocol recommended by the manufacturer. The products obtained were analyzed on an ABI Prism® 3730 Applied Biosystems/Hitachi automated sequencer in the "Serveis Cientificotècnics de la Universitat de Barcelona", and the resulting chromatograms were edited with Chromas 2.0 (Technelysium Pty Ltd, Tewantin, Australia).

4.3.3 Phylogenetic analyses

Sequences were aligned independently and manually using the text editor TextPad® 4.7.3. For the ITS sequence alignment, the highly conserved 5.8 subunit was not included in phylogenetic analyses to reduce missing data, because it was not available for all the species. For the *trn*L-F alignment, bases 1-36 that formed a primer-binding region were also excluded to reduce missing data.

Phylogenetic analyses were performed for four data sets: ITS; *trn*G; *trn*L-F; and the combined ITS, *trn*G and *trn*L-F data. We included in the combined data all the taxa, even if one region was missing. Analyses were carried out using Maximum Parsimony (MP) and Bayesian Inference (BI). Parsimony analyses involved heuristic searches conducted with PAUP* 4.0b10 (Swofford 2002) with tree bisection-reconnection (TBR), MulTrees option in effect, branch swapping algorithm and character states specified as unordered and unweighted. Bootstrap (BS) analyses were performed with 1000 replicates, no swapping and simple addition sequence (Felsenstein 1985). To explore the amount of phylogenetic signal for each data set, we calculated the Consistency Index (CI) (Kluge & Farris 1969) and the Retention Index (RI) (Swofford 2002).

The program MrModeltest 2.2 (Nylander 2004) was used to determine the best-fitting model of sequence evolution for each data set using the Akaike Information Criteria (AIC). The models and the resulting parameter estimates were then used in BI analyses conducted with MrBayes 3.1 (Huelsenbeck *et al.* 2001; Ronquist & Huelsenbeck 2003). In the BI analyses of the combined data, we set up a partitioned analysis to apply the parameters of the most appropriate model for each

region, as MrBayes 3.1 allows heterogeneous models and data. Bayesian analyses were performed starting from the NJ tree, and four Markov chains during 2 millions of generations were run in parallel to sample trees using the Markov Chain Monte Carlo (MCMC) principle. One sample of each 100 generations was saved, resulting in 20,000 sample trees. The first 5,000 trees were eliminated during the *burn-in* phase before computing the consensus tree to eliminate the trees that still did not reach a stationary posterior probability (PP).

4.3.4 Mapping of characters and chromosome numbers

We have mapped the main morphological characters used in the different treatments of *Roucela* and also the chromosome numbers available in the literature to search for evolutionary patterns within the subgenus. The characters mapped are: calyx lobes erect or convergent in fruit *vs*. calyx lobes stellate-patent in fruit; flexuous stems *vs*. rigid; sinus of the calyx lobes rounded *vs.* acute; and flower shape, which has been classified in three types to map it on the tree (sub-rotate, broadly campanulate, and tubular-campanulate). Figures 1 and 2 illustrate some of these morphological characters.

4.3.5 Biogeographic analyses

The ancestral biogeographic reconstruction was inferred with the dispersal-vicariance analysis implemented in DIVA (Ronquist 1996, 1997), using as input data the tree obtained with the combined matrix. The areas of endemism were defined by the presence of one or more endemic taxa. Nine areas of endemism are proposed: (a) Southern-Western Anatolia; (b) Cyprus; (c) Southern Greece; (d) Rhodes; (e) Karpathos; (f) Crete; (g) Central and Northern Greece, Bulgaria and Macedonia; (h) Macaronesia, Northern Africa, Western and Central Mediterranean; and (i) Western Asia. The areas of endemism are shown in Fig. 3. The area of distribution of each species is shown in Figs. 4 and 5. The dichotomic tree needed as input file for the DIVA was extracted from the Bayesian trees with higher posterior probability. We run the analysis without constraining the number of areas for each node.

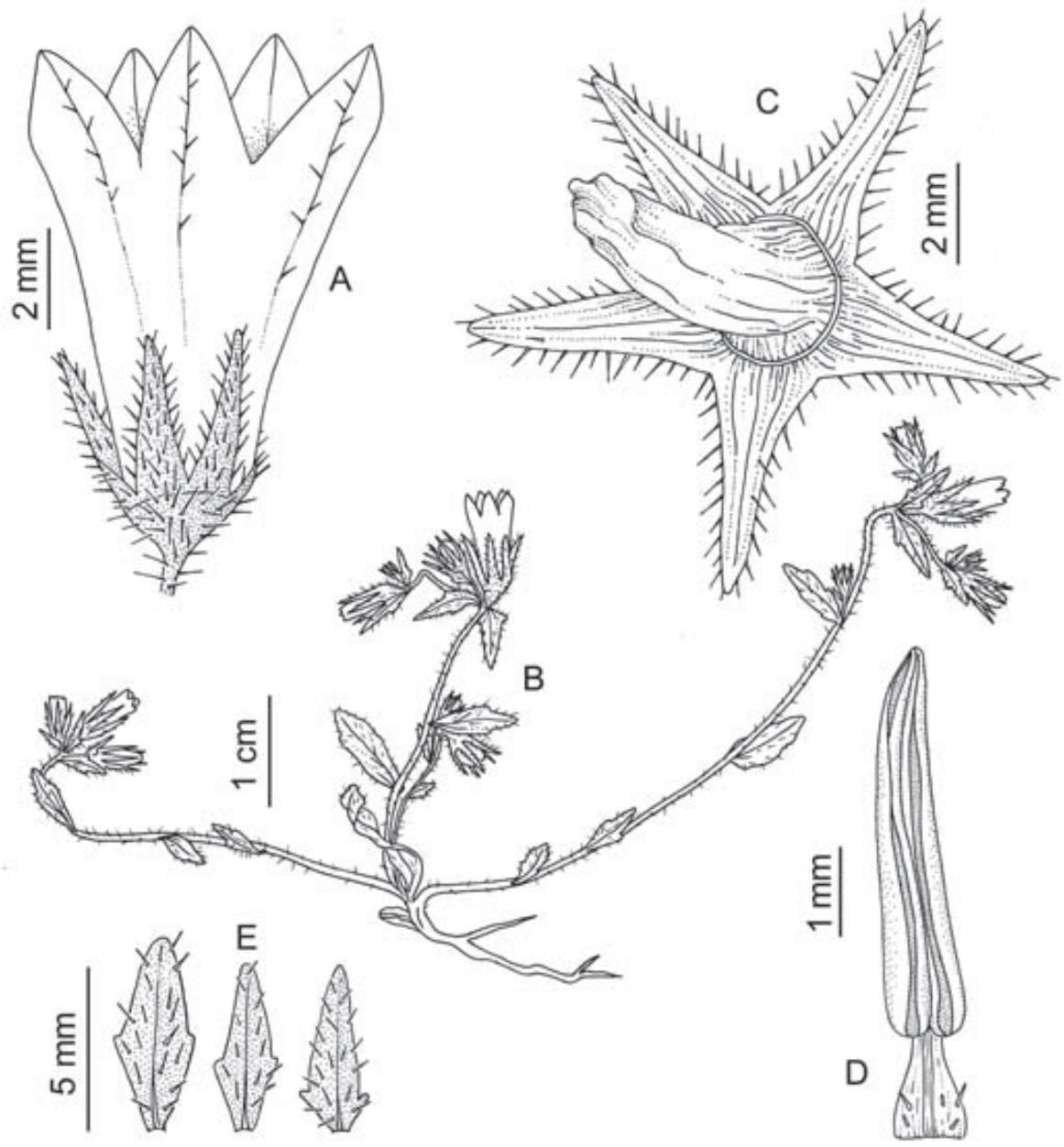

FIG 1. *Campanula rhodensis*. A: corolla and calyx with acute sinus of the lobes, B: flexuous stem, C: fruit with stellate-patent calyx lobes, D: stamen, E: bracts.

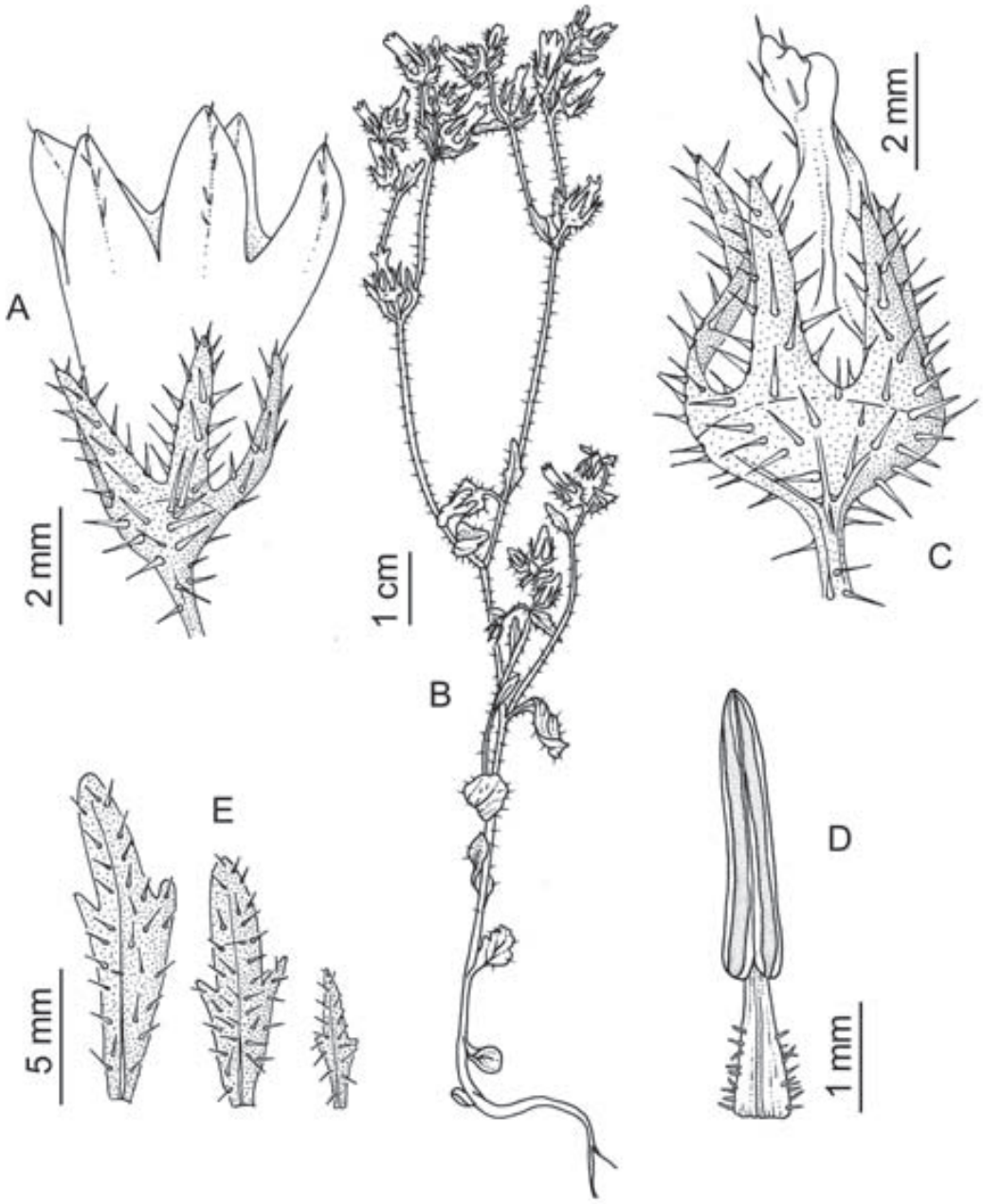

FIG 2. *Campanula podocarpa*. A: corolla and calyx with rounded sinus of the lobes, B: erect stem, C: fruit with convergent calyx lobes, D: stamen, E: bracts.

4.4 RESULTS

4.4.1 Phylogenetic results

Bayesian and MP analyses yield highly coincident topologies for all the data. The topology of trees produced by BI is shown in Fig. 6 (consensus tree obtained by BI of ITS data), Fig. 7 (consensus tree obtained by BI of *trn*G data), Fig. 8 (consensus tree obtained by BI of *trn*L-F data) and Fig. 9 (consensus tree obtained by BI of combined data). Bootstrap support values greater than 70% obtained with PAUP were added to Bayesian trees, as the topologies of trees produced by MP and BI were coincident. The consistency indices obtained for the analyses is high, and CI and RI indicate low homoplasy. Numeric results of the analysis of each region and combined data are summarized in Table 2.

4.4.2 Nuclear ITS data

The best-fitting sequence evolution model required for BI for ITS data was the General Time Reversible (GTR) model, with equal base frequencies and variable sites assumed to follow a gamma distribution (GTR + Γ) (Rodríguez *et al.* 1990). This data shows three clades well-supported by BS and/or BI: the first one (clade 1) formed by *C. creutzburgii, C. drabifolia* and *C. erinus*; the clade 2 formed by *C. pinatzii* and *C. simulans*; and the clade 3 by *C. delicatula*, *C. podocarpa* Boiss., *C. rhodensis* and *C. veneris*. The outgroup species *C. propinqua* appears basal to clade 1 (Fig. 6).

4.4.3 Chloroplast *trn*G data

The best model for *trn*G was also the GTR, but in this case with some sites assumed to be invariant (GTR + I) (Rodríguez *et al.* 1990). The topology obtained with *trn*G is the less resolved. The clades 1 and 2 are highly supported. The rest of species appear in a polytomy, except *C. scutellata* which appears in a basal polytomy with the outgroup species *C. dichotoma* (Fig. 7).

4.4.4 Chloroplast *trn*L-F data

The best model for *trn*L-F was the GTR, with equal base frequencies and variable sites assuming gamma distribution (GTR + Γ) (Rodríguez *et al.* 1990). This dataset is the one with the best resolution and highest support. The *trn*L-F data shows the same three clades obtained with the ITS data plus the basal branch of *C. scutellata* (Fig. 8).

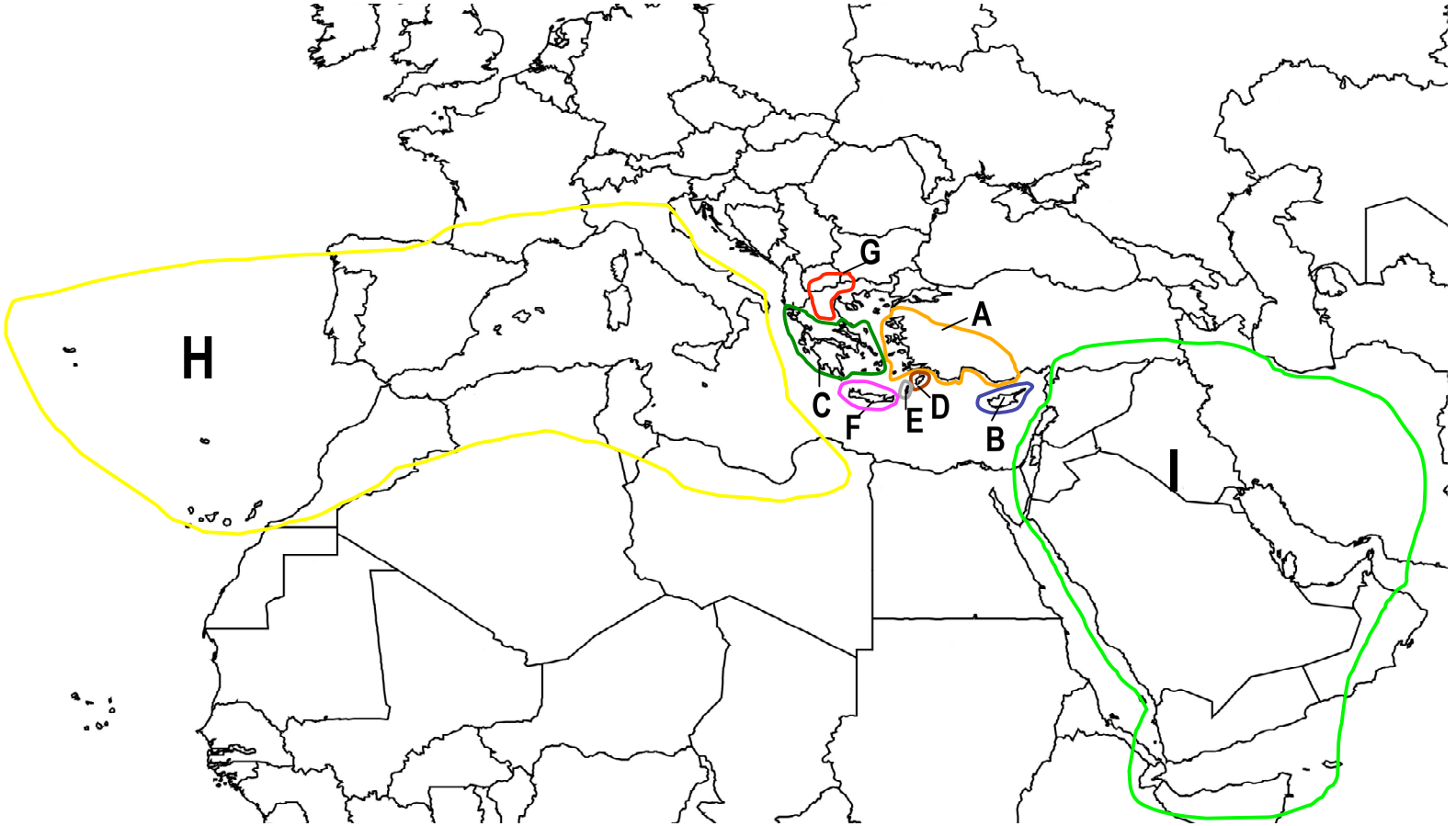

FIG. 3. Distribution map of *Campanula* subgenus *Roucela* showing the areas used for DIVA analysis. Letters A to H are the defined geographic areas for the analysis: A, Southern-Western Anatolia; B, Cyprus; C, Southern Greece; D, Rhodes; E, Karpathos; F, Crete; G, Central and Northern Greece, Bulgaria and Macedonia; H, Macaronesia, Northern Africa, Western and Central Mediterranean; and I, Western Asia.

TABLE 2. Results from ITS, *trn*G and *trn*L-F regions and combined data. Consistency and retention indices and divergence were calculated excluding non-informative characters.

Data set	ITS1 + ITS2	*trn*G	*trn*L-F	Combined
Total characters	482	629	922	2033
Informative characters	71	25	48	144
Number of taxa	16	15	17	17
Number of MPTs found	4	4	11	1
Number of steps	124	28	64	217
Consistency index	0.6503	0.8966	0.8214	0.7094
Retention index	0.7872	0.9500	0.8881	0.8205

4.4.5 Combined data

The combination of all the data has produced a very well resolved and highly supported topology, with a basal branch formed by *C. propinqua* and *C. scutellata* plus the three main clades already specified above (Fig. 9). The analyses of the combined data matrix were done specifying each model for the respective data set, as MrBayes 3.1 allows dealing with heterogeneous models and data.

4.4.6 Distribution of characters in the combined tree

We have mapped the distribution of the main morphological characters used in different treatments of *Campanula* subgenus *Roucela* in the combined tree (Fig. 9), but evolutionary patters are not evident. We have mapped also the chromosome numbers. The species with the lowest chromosome number is *C. scutellata*, which appears as basal to the rest of the ingroup species. Clade 1 presents tetraploid (*C. drabifolia*, *C. erinus*) and octoploid species (*C. creutzburgii*) with the basic number *x*= 7. Clade 2 includes one tetraploid species with basic number *x*= 7 (*C. simulans*) plus one diploid species with *x*= 10 (*C. pinatzii*). Clade 3 presents diploid species with *x*= 8.

4.4.7 Biogeographic analyses

The reconstruction of the ancestral biogeographic areas is shown in Fig. 9. The result of dispersal-vicariance analysis indicates a widespread ancestor of the subgenus *Roucela* in the areas of endemism of the Eastern Mediterranean (Greece, Anatolia, plus the islands between these lands) as the most parsimonious result. The results also indicate that several vicariant events and few dispersals occurred along the evolution of the subgenus.

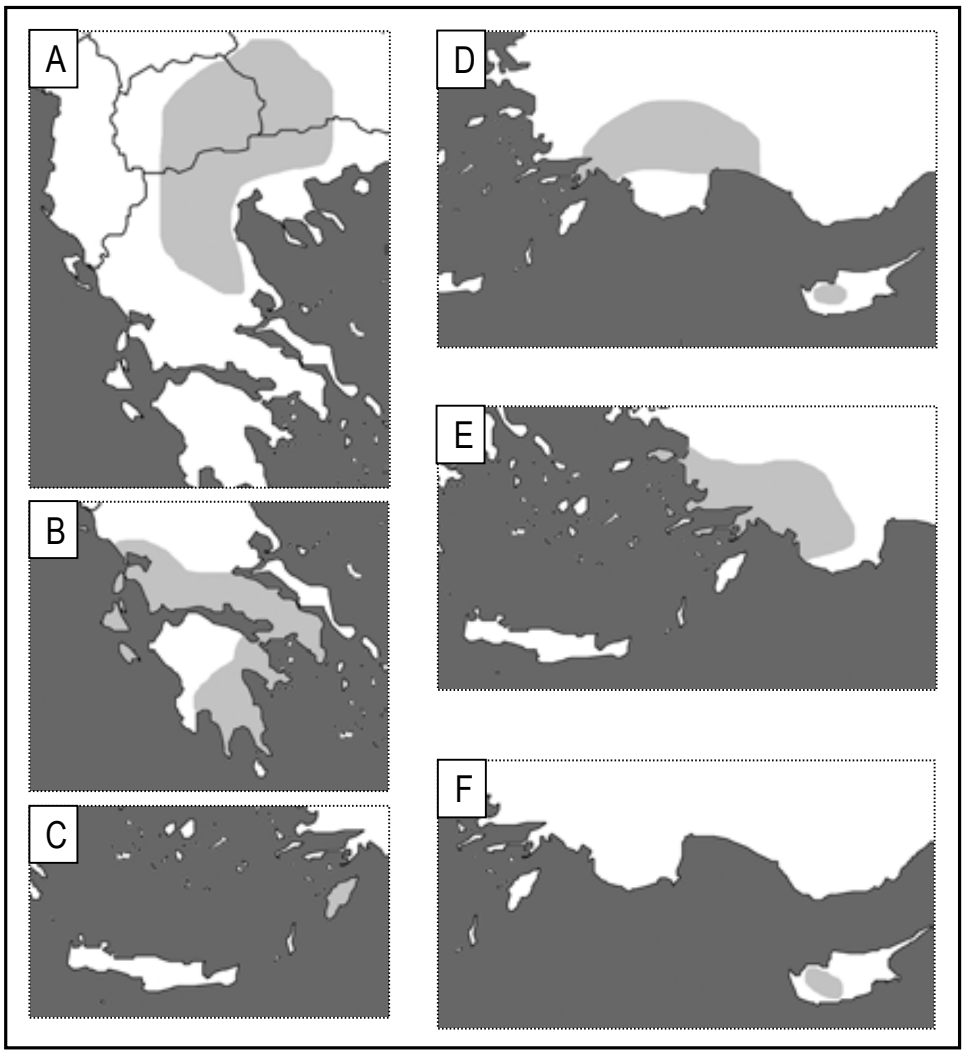

FIG. 4. Area of distribution of the following species belonging to *Campanula* subgenus *Roucela*: A, *C. scutellata*; B, *C. drabifolia;* C, *C. rhodensis;* D, *C. podocarpa*; E, *C. simulans;* and F, *C. veneris.*

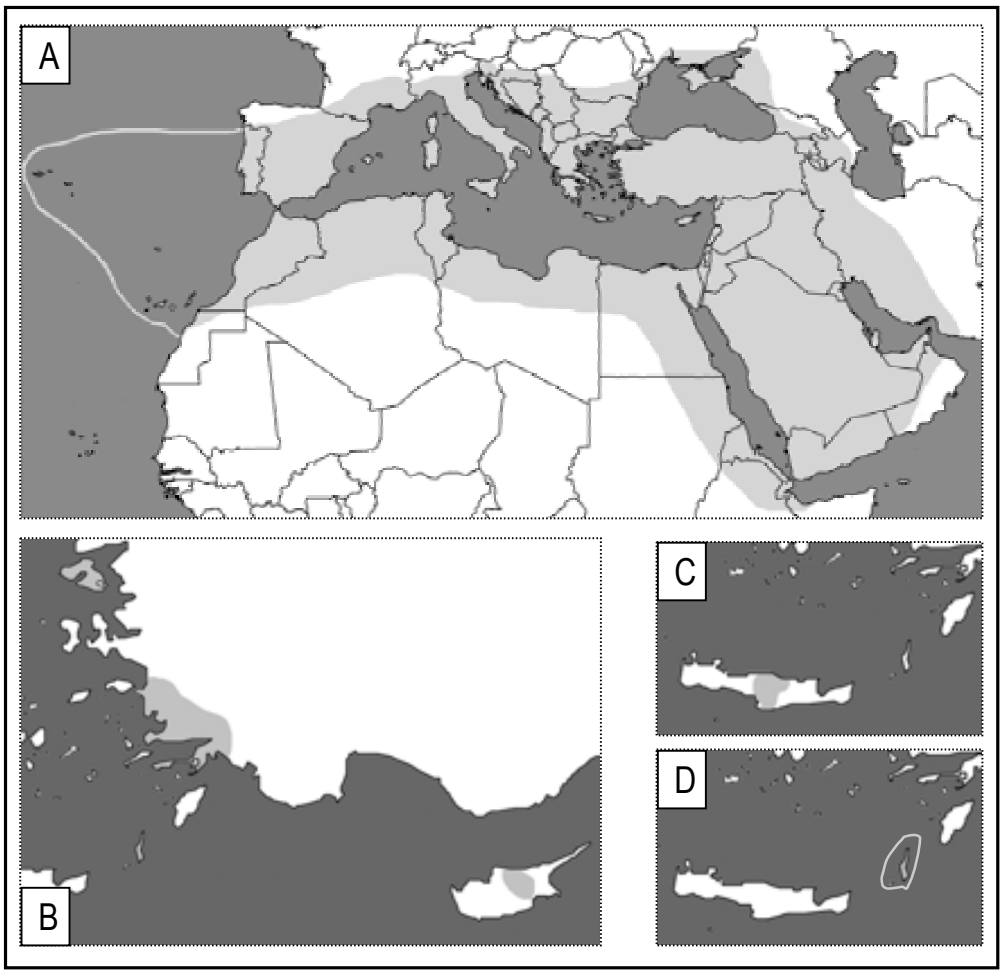

FIG. 5. Areas of distribution of some *Campanula* subgenus *Roucela* species. A, *Campanula erinus;* B, *C. delicatula*; C, *C. creutzburgii*; and D, *C. pinatzii.*

4.5 DISCUSSION

4.5.1 The circumscription of *Campanula* subgenus *Roucela*

The diagnostic characters of *Campanula* subgenus *Roucela* are: dichotomously branched annual plants, without appendages between lobes of calyx, and capsules dehiscing by 3 basal valves. All the species here included (except the 3 outgroups *C. balfourii*, *C. dichotoma* and *C. propinqua*) fit in with this definition, but as already indicated by Carlström (1986) and Polo (2007), there is one species that shows morphological trends that suggest a more distant relationship with the other species included in the subgenus *Roucela*. *Campanula scutellata* is the only *Roucela* species that presents the corolla divided until the half of its length or more; it presents the highest size and the higher longitudes of leaves; it is the only species with two different types of trichomes (short and long); it bears the largest corolla, which is nearly sub-rotate, while the other *Roucela* species present tubular-campanulate or broadly-campanulate corollas; and it presents exerted styles, while all the other species present included or sub-exerted styles.

The phylogenetic results also support that *C. scutellata* is the species less related to the other species of the subgenus. The combined dataset indicates with high support (Fig. 9) that *C. scutellata* is a lineage apart within *Roucela*, basal to the rest of species and closer to the outgroup *Campanula propinqua* than to the other *Roucela* species. However, the close phylogenetic relationship of *C. scutellata* and *C. propinqua* obtained here should be considered with caution, because wider sampling of *Campanula* species (Chapter 1) indicates that *C. scutellata* forms part of a wider lineage of annual species including *C. propinqua* but also other taxa of subgenus *Megalocalyx* even closer to *C. scutellata*, and also the subgenus *Sicyocodon* (Feer) Damboldt (*C. macrostyla* Boiss. & Heldr.).

4.5.2 Cryptic species within *Roucela*

Carlström (1986) described the new species *C. simulans* for the eastern populations of *Campanula drabifolia*, mainly located in Turkey. The taxonomic work of Polo (2007) showed that the diagnostic characters proposed by Carlström (1986) are not consistent and appear also in plants from populations of *C. drabifolia* in Greece. However, the molecular data supports these two species as different evolutionary entities, as they appear in different clades (Figs. 6-9), despite accurate morphological revision has shown no differences between them (Polo 2007). They present neither different ecological requirements. The only clear difference existing between these two species is its

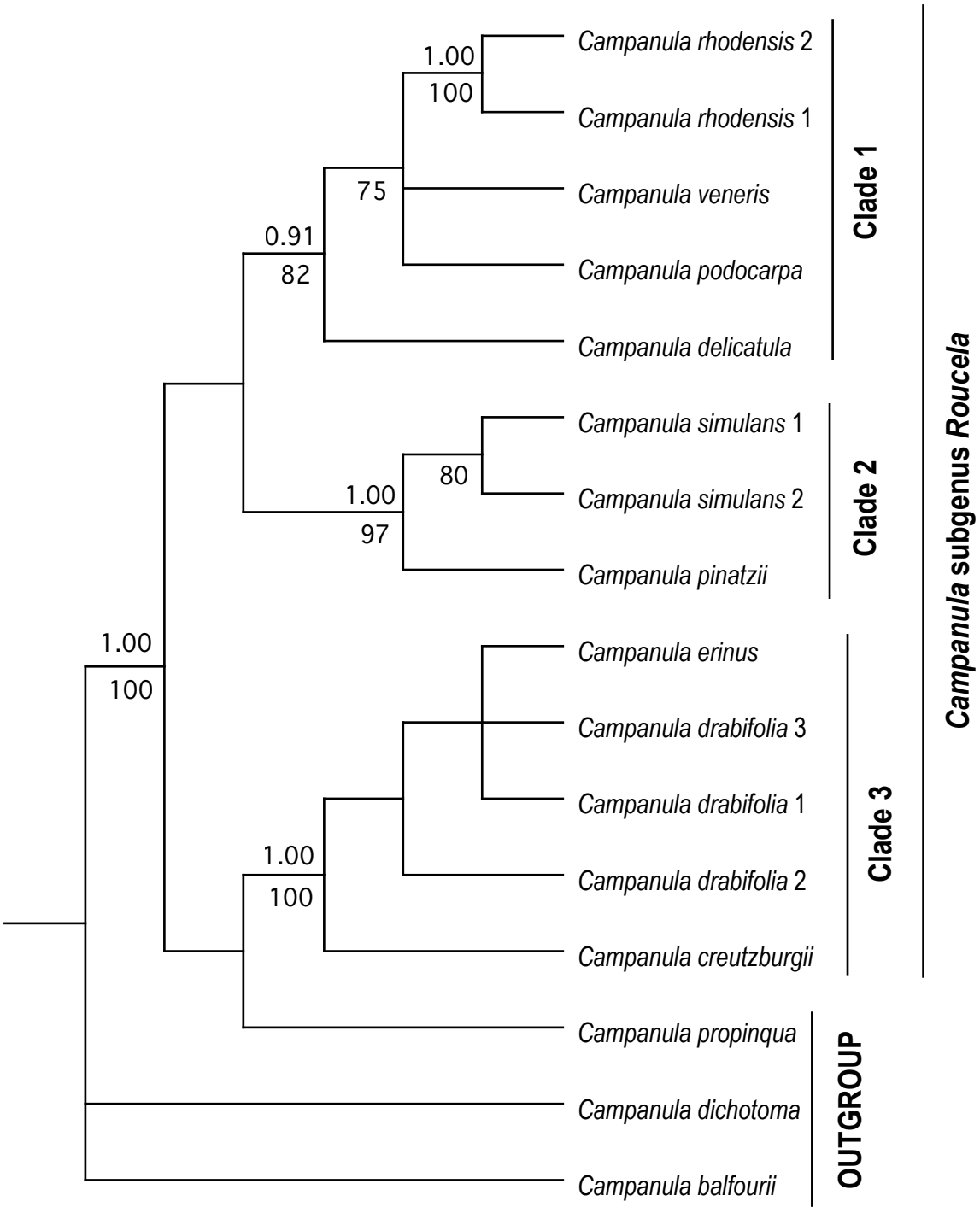

FIG. 6. Majority rule consensus tree obtained from BI of ITS data. Numbers above branches indicate Bayesian-credibility values (PP) > 0.90; numbers below branches indicate Parsimony BS > 70%. MP gave trees with identical topologies.

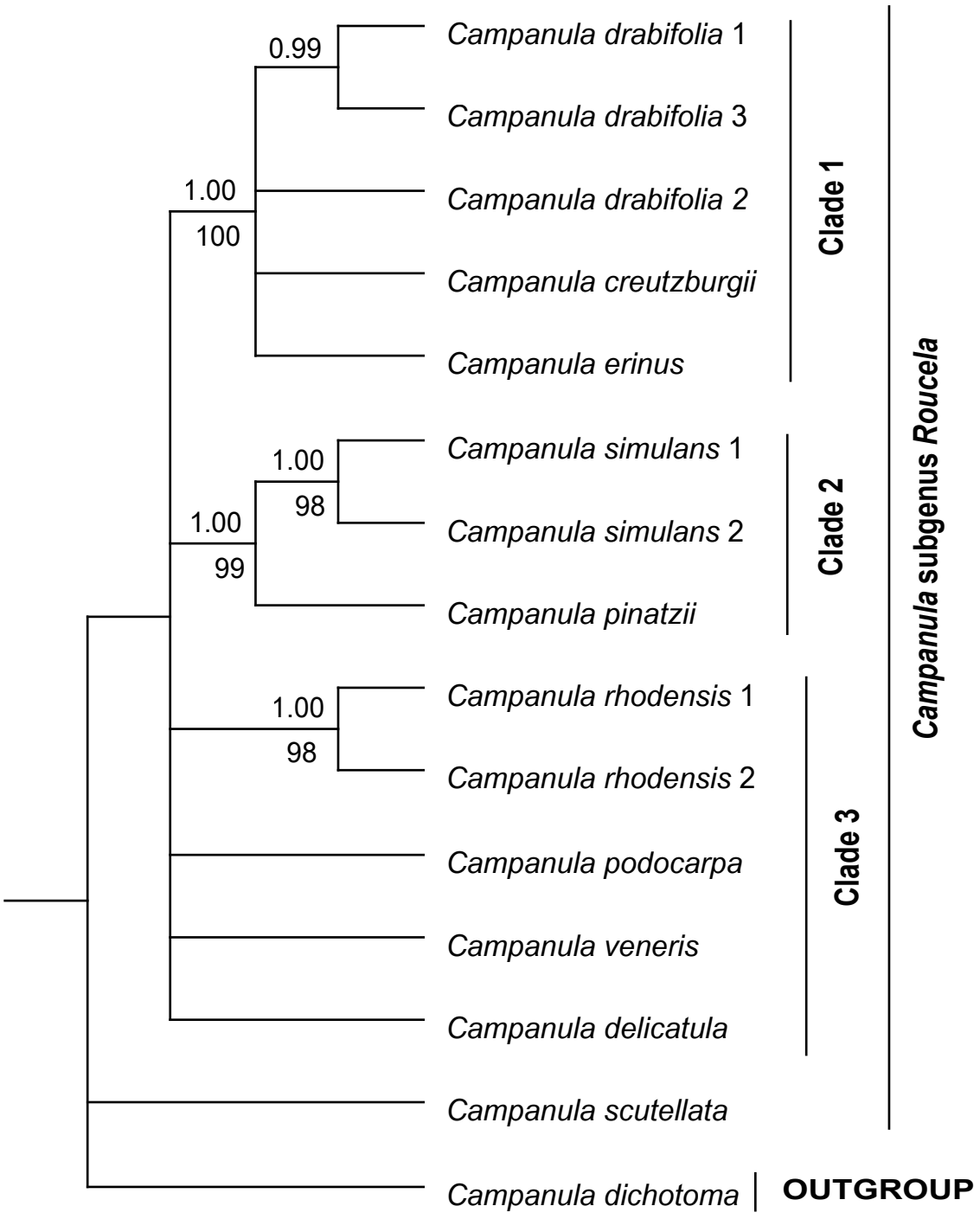

FIG. 7. Majority rule consensus tree obtained from BI of *trn*G data. Numbers above branches indicate Bayesian credibility values (PP) > 0.90; numbers below branches indicate Parsimony BS > 70%. MP gave trees with identical topologies.

geographical range.

Reproductive isolation and genetic differentiation can occur without much associated morphological change, leading to the formation of cryptic species (Gornall 1997). This poses an evident problem for the taxonomic treatment of these species. In these cases it is difficult to build a classification that reflects evolutionary units providing a morphological character for species recognition. Cryptic species are not rare in aquatic plants (Waycott *et al.* 2002; Whittall *et al.* 2004) and ferns (*e.g.* Paris & Windham 1988; Haufler & Windham 1991; Adjie *et al.* 2007), but studies of cryptic species in flowering plants are scarce. However, cryptic species whitin flowering plants of the arctic flora have been reported (Grundt *et al.* 2006). Cryptic species are particularly common in lineages that diversify in habitat that impose substantial physiological constraints (*e.g.* the aquatic environment) and also in lineages with morphological constraints (which is the case of ferns), resulting in low or non-existent morphological divergence between lineages, particularly in adaptive traits (Whittall *et al.* 2004). In other cases, morphological boundaries between species can be obscured by hybridization, but it does not seem to be the case of *C. drabifolia* and *C. simulans*, as maternally-inherited chloroplast and biparental nuclear sequences yield the same phylogenetic relationship between those two species.

Molecular methods have great potential to resolve the nature of species boundaries due to the large number of unanmbiguous characters they provide (Avise 1994). The application of molecular tools is highly relevant in these cases. The results here obtained suggest that one of the three individuals sequenced of *C. drabifolia* (the one proceeding from Kephallinia, near the limits of distribution) could represent another cryptic species, as its position in the combined analysis (Fig. 9) is basal to the other two sequences of *C. drabifolia* and *C. erinus*. Additional molecular work at populations level should be done in the *C. drabifolia* complex and *C. erinus* to confirm possible additional cryptic species.

4.5.3 Phylogenetic relationships within *Roucela*

Molecular data suggests that, apart from the *C. scutellata* branch, there are three main lineages within the subgenus *Roucela*. Clade 1 includes 3 species (*C. creutzburgii*, *C. drabifolia* and *C. erinus*) with a very different extent on its distribution (Figs. 4-5). *Campanula creutzburgii* is a rare species endemic to Crete, while *C. drabifolia* is distributed in the south of Greece and in the adjacent

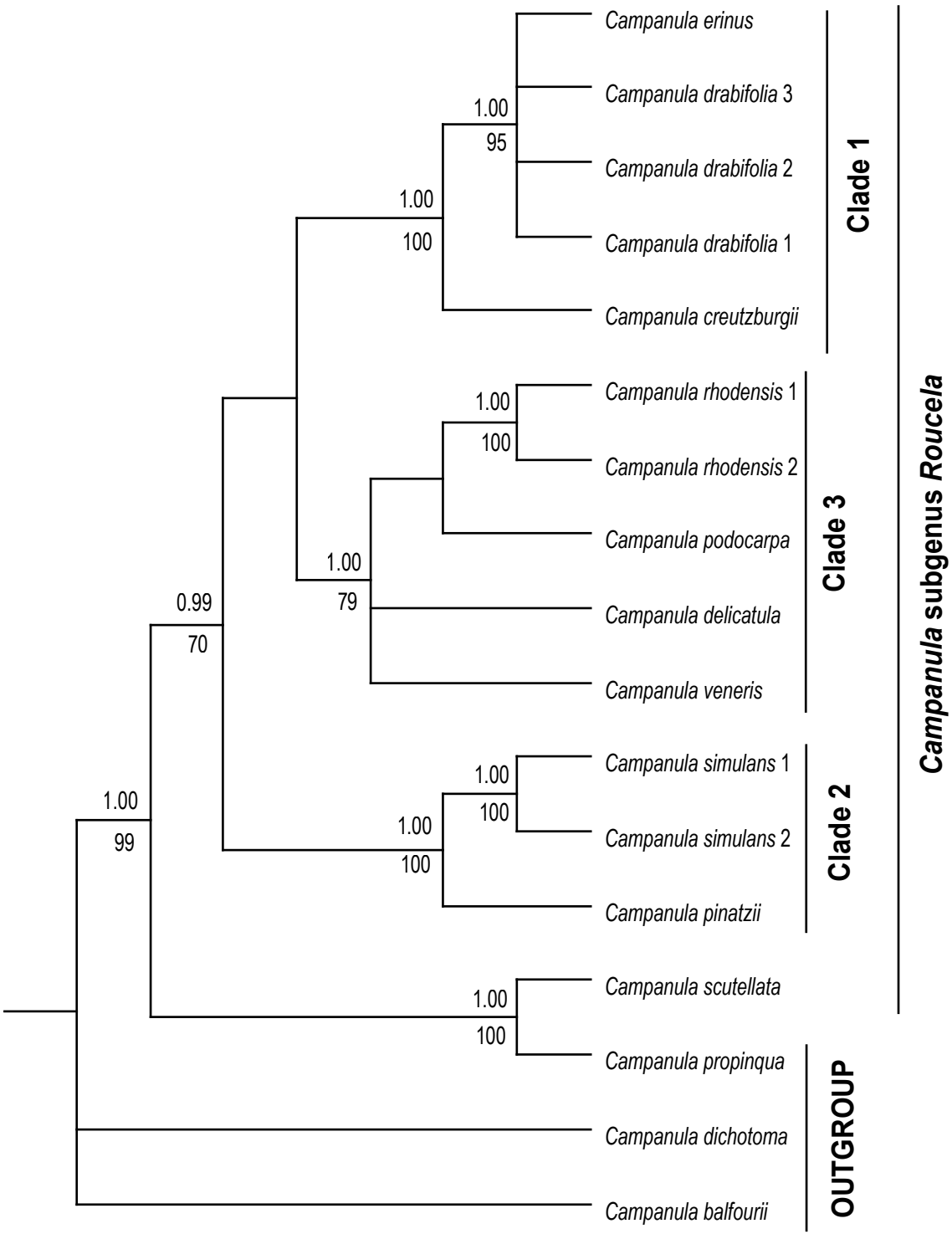

FIG. 8. Majority rule consensus tree obtained from BI of *trn*L-F data. Numbers above branches indicate Bayesian-credibility values (PP) > 0.90; numbers below branches indicate Parsimony BS > 70%. MP gave trees with identical topologies.

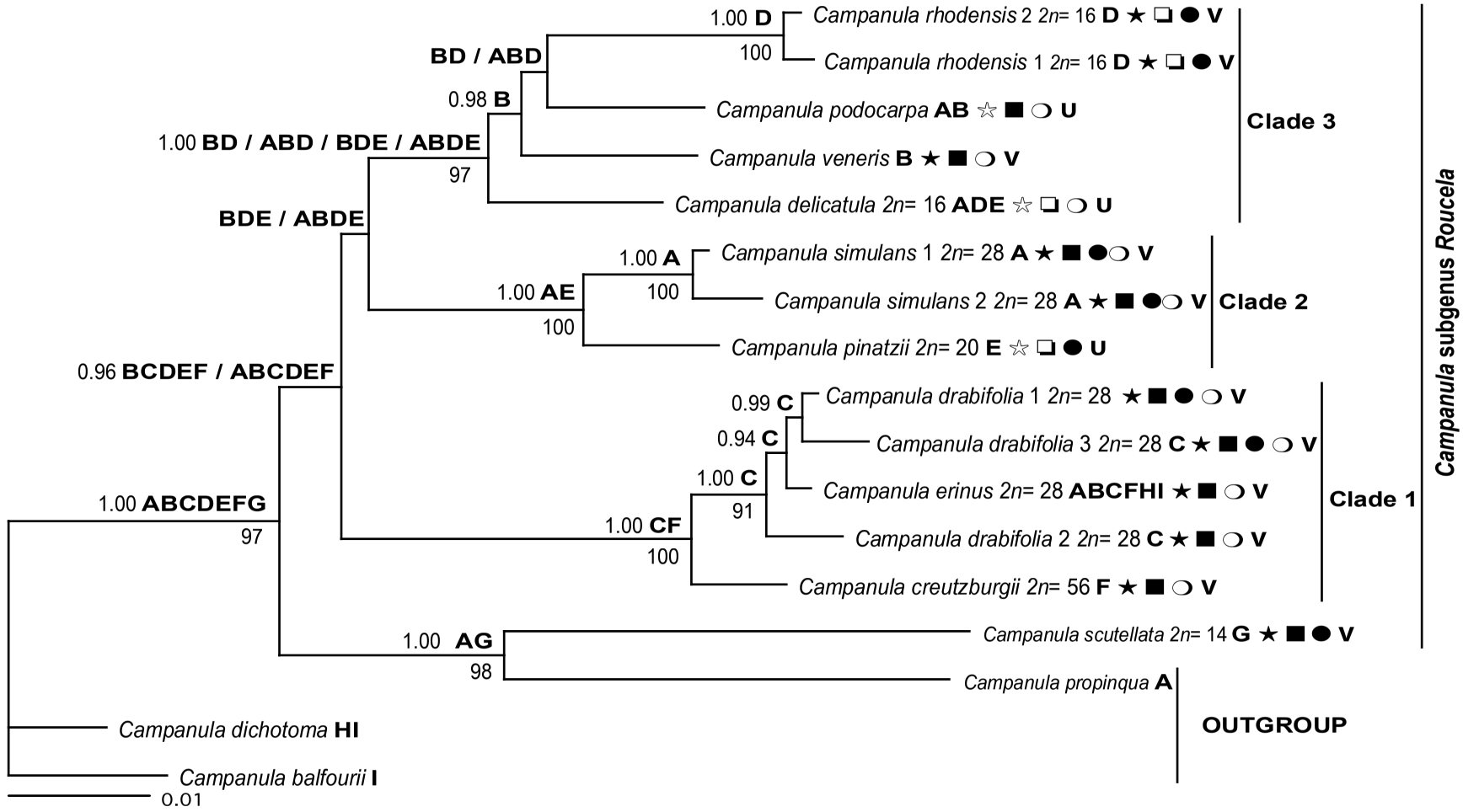

FIG. 9. Majority rule consensus tree-phylogram from BI of combined data of ITS, *trn*G and *trn*L-F. Numbers above branches indicate Bayesian-credibility values (PP) > 0.90; numbers below branches indicate Parsimony BS > 70%. MP gave trees with identical topologies. Chromosome numbers obtained from the literature are indicated for each species. Morphological features are mapped in the branches of the tree with the following patterns: black star, calyx lobes stellate-patent in fruit; white star, calyx lobes erect or convergent in fruit ; black circle, corolla broadly campanulate; white circle, corola tubular-campanulate; black square, rigid stems; white square, flexuose stems; V, sinus of the calyx lobes acute; U, sinus of the calyx lobes rounded. Letters A to H are the defined geographic areas for DIVA analysis: A, Southern-Western Anatolia; B, Cyprus; C, Southern Greece; D, Rhodes; E, Karpathos; F, Crete; G, Central and Northern Greece, Bulgaria and Macedonia; H, Macaronesia, Northern Africa, Western and Central Mediterranean; and I, Western Asia. The letters next to each species indicate the distribution of each taxon. The let- ters in each node are the ancestral areas inferred by DIVA analysis.

islands, reaching its northern limits in the island of Kephallinia; and the circum-Mediterranean *C. erinus* is the more widespread, reaching Macaronesia and Oman.

Campanula erinus ressembles *C. drabifolia* and it is mainly distinguished by its reduced flowers, with the corolla shorter than the calyx or slightly larger. Both species show considerable morphological variability. Moreover, these two species are the only ones that can present tetraporate pollen (Polo 2007) in addition to triporate pollen. *Campanula creutzburgii* ressembles greatly *C. drabifolia*, *C. erinus*, but also *C. rhodensis* (Greuter & Rechinger 1967). Absence of conspicuous teeth in the bracts of *C. creutzburgii* is the main difference with *C. drabifolia* and *C. erinus*. *Campanula rhodensis* presents longer anthers than *C. creutzburgii*. Molecular data indicates that all of them but *C. rhodensis* form part of the same lineage.

Clade 2 is constituted by *C. pinatzii* and *C. simulans*. It is surprising that these two species appear in the same clade since they are rather different morphologically: *Campanula pinatzii* presents calyx lobes erect or convergent in fruit, sinus of the calyx lobes rounded, and flexuose stems, while *C. simulans* presents calyx lobes stellate-patent in fruit, sinus of the calyx lobes acute and rigid stems. They are also different in cytological aspects: *Campanula pinatzii* presents $2n = 20$, while *C. simulans* has $2n = 28$.

Clade 3 also constitutes a morphological heterogeneous group of species (Fig. 9). However, the common chromosome number $2n = 16$ in *C. delicatula* and *C. rhodensis* supports the indication of a close relationship. Unfortunately the chromosome numbers of *C. podocarpa* and *C. veneris* are not known.

4.5.4 Character evolution

The distribution of characters in Fig. 9 shows that the main morphological characters used in the taxonomic treatments of *Roucela* do not define natural groups. Characters such as the form of calyx lobes in fruit, the sinus of the calyx lobes, flexuose or rigid stems and the form of the corolla have arisen more than once in parallel evolution. The only clade uniform in characters is clade 1, which is the one with shorter branch lengths (Fig. 9). This heterogeneity is in consonance with the plasticity shown by the genus *Campanula* (see Chapter 1).

4.5.5 Cytological evolution

Plants included in subgenus *Roucela* present at least three different basic chromosome numbers, *x*= 7, 8 and 10. The complex cytological pattern of the subgenus *Roucela* contrasts with the fact that there are few morphological characters that are useful to distinguish these species.

There are three ploidy levels for the *x*= 7: the basal diploid *C. scutellata*, the tetraploids *C. drabifolia, C. erinus* and *C. simulans*, located in two different clades; and the octoploid *C. creutzburgii*. Two hypothesis could explain the position of this octoploid appears in the base of the clade 1, formed also by the tetraploids *C. drabifolia* and *C. erinus*. First of all, *C. creutzburgii* could have originated by autopoliploidy of a tetraploid ancestor, probably widespread if we consider the ancestral biogeographic reconstruction. Then, *C. creutzburgii* could be considered as an apoendemism of the ancestor of clade 1. On the other side, *C. creutzburgii* could be an allopolyploid, hybrid of *C. drabifolia* and *C. erinus*, resulting in a less adaptative species, restricted to a smaller area. However, *C. creutzburgii* is endemic to Crete, and *C. erinus* is also found there, but *C. drabifolia* is not present in this island, thus the hybridization hypothesis seems less probable.

4.5.6 Biogeographic reconstruction

The dispersal-vicariance optimization suggests a widespread ancestor in all the areas of endemism of the Eastern Mediterranean for the ancestor of the three main lineages within *Roucela.* This concordes with the palaeogeography of this area during the Quaternary: at the beginning of the Pliocene, alpine orogeny caused the uplift and folding of Tertiary materials, creating several recent mountain ranges. One of these new mountain arcs constituted a former South Aegean mountain range connecting the Peloponnese peninsula to Crete, Karpathos, Rhodes and South-Western Turkey (Strid 1997; Thompson 2005). Cyprus is also related to this arch, as its geological foundation is an extension of the Taurus/Amanus folding system (Thompson 2005). Again, the only species of *Roucela* that presents a differentiated pattern is *C. scutellata*, which is distributed in Central and Northern Greece, Macedonia and Bulgaria, which were connected during the Pleistocene to the North and central Aegean islands by land. These areas were separated by sea from the South Aegean arch because of the deep basin south of the Cyclades.

The biogeographic analyses point that vicariance has played a greater role in the evolution of the subgenus than the dispersal events. Besides the repeated disappearance of land connections

between these areas, the gradual cooling and drying of the climate from the late Pliocene to early Pleistocene (Thompson 2005) could have accentuated the isolation of the ancestral populations in coastal refugia, leading to its differentiation. However, sympatric speciation may have played also an important role in some cases via cytological changes.

The clade 1 involves a ancestor distributed in Southern Greece and Crete, implying a geographic vicariance between Southern Greece and Crete that lead to the posterior differentiation of the ancestral populations. This historical biogeographic relationships between the Peloponnese and Crete has been noted before in other genera (e. g. *Cyclamen* L. in Gielly *et al.* 2001; *Silene* L. in Oxelman 1996). The clades 2 and 3 are distributed in the Eastern islands of the Southern Aegean arch (Karphatos and Rhodes), plus South-Western Anatolia and Cyprus, both areas in the other side of the "Rechinger's line" (Strid 1997), that stands out the floristic differences between Europe and Asia.

The reconstructions of the ancestral areas of the clades 2 and 3 advocate for a widespread ancestor in the areas mentioned and vicariance as the main factor to explain the present biogeographic distribution, with few dispersal events. The clade 2 would have been widespread in South-Western Anatolia and Karphatos. Perhaps it was also distributed (and later extinct) in Rhodes, which connected these two lands until the late Pliocene/early Pleistocene, when the strait of Marmaris was established between Rhodes and Anatolia (Meulenkamp *et al.* 1972). The ancestor of clade 3 was widespread and suffered a vicariant event that separated the populations of Cyprus from those located in Anatolia, Rhodes and Karphatos, which lead to the differentiation of *C. delicatula*. The ancestral population restricted to Cyprus would have originated there *C. veneris*, and it would also have dispersed to Anatolia and Rhodes, leading to the formation of new species (*C. podocarpa* and *C. rhodensis*).

Another dispersal event in the subgenus is linked to the widespread *C. erinus*, whose ancestor seems to have been restricted to Southern Greece. *Campanula erinus* is mainly autogamous (see Chapter 1). This reproductive feature and its terophitic habit have undoubtely helped it to colonize new habitats.

4.5.7 Taxonomic implications

According to its present circumscription (Carlström 1986), the subgenus *Roucela* is not monophyletic. We suggest that a new monotypic subgenus should be described for *Campanula scutellata*, after an exhaustive revision of herbarium specimens of this species, in order to confirm the different morphological trends observed by Polo (2007). This species presents cytological, morphological and molecular differences from all the other species included in the subgenus *Roucela*. The morphological traits that distinguish it from the plants of subgenus *Roucela* are the following: corolla divided until the half of its longitude or more; presence of two different types of trichome (short and long); size and shape (subrotate) of the corolla; and exerted styles. Excluding *C. scutellata* from *Roucela*, this subgenus would represent a natural unit.

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Conclusions

5. CONCLUSIONS

1. Sobre la base de les anàlisis filogenètiques moleculars de màxima parsimònia i inferència Bayesiana dels marcadors moleculars ITS i *trn*L-F, podem concloure que *Campanula*, en la seva circumscripció actual, no és un gènere monofilètic. Aquest gènere es divideix en dos clades principals: en primer lloc, un ampli clade format per la majoria d'espècies de *Campanula* incloent gèneres propers (*Adenophora, Asyneuma, Azorina, Campanulastrum, Diosphaera, Edraianthus, Githopsis, Hanabusaya, Heterocodon, Legousia, Michauxia, Petromarula, Physoplexis, Phyteuma*, *Trachelium* i *Triodanis*), i en segon lloc, un clade constituït per *Musschia* més dos espècies de *Campanula*. L'ampli clade de *Campanula* es compòn de dos grups principals, un tipus *Rapunculus* i un tipus *Campanula*.

2. Basant-nos en la filogènia molecular, podem concloure que els principals caràcters morfològics utilitzats en les classificacions tals com la forma de la flor i la dehiscència de la càpsula han aparegut paral.lelament en l'evolució de les espècies de *Campanula*. S'hipotetitza que la convergència floral pot ser deguda a fortes pressions selectives per part dels pol.linitzadors.

3. Sobre la base de la filogènia obtinguda, fem una proposta per tal d'obtenir una classificació de *Campanula* que reflecteixi més acuradament l'evolució d'aquest gènere: considerar tots els gèneres que s'inclouen en els clades amb espècies de *Campanula* com a sinònims d'aquest gènere. D'aquesta manera, *Campanula* inclouria tots els tàxons de la tribu Campanuleae, que són fàcilment distingibles de les tribus Wahlenbergiae i Platycodoneae perquè tots els gèneres de Campanuleae presenten ovari inferior i dehiscència capsular lateral. L'únic canvi important que caldria fer és incloure el gènere *Edraianthus*, fins ara considerat com a membre de Wahlebergiae perquè presenta dehiscència irregular, malgrat l'ovari és inferior.

4. Sobre la base de l'anàlisi filogenètic realitzat amb el marcador molecular conservat *rbc*L, podem confirmar que la tribu Platycodoneae és el grup germà de les Campanuleae i les Wahlenbergiae.

5. Donada la incongruència del senyal filogenètic entre els marcadors ITS, *rbc*L i trnL-F sobre la posició del gènere *Trachelium* respecte a *Campanula*, s'hipotetitza que s'han produit hibridacions durant la història evolutiva d'aquest gènere.

6. Basant-nos en les anàlisis de reconstrucció biogeogràfica, podem concloure que l'oest d'Àsia així com la regió est de la Mediterrània han jugat un paper important com a centres de migració i diversificació dels principals llinatges de *Campanula.*

7. Les anàlisis de reconstrucció biogeogràfica aplicades a les dades dels marcadors *rbc*L i *trn*L-F ens permeten concloure que la història biogeogràfica de *Campanula* ha estat molt complexa: s'han produït repetidament esdeveniments de diversificació des de l'oest d'Àsia (Anatòlia i la regió Irano-Turaniana), des d'on les espècies s'han extès a àrees adjacents, on el posterior aïllament ha donat peu a la formació de nous endemismes; almenys han tingut lloc dos esdeveniments de dispersió independents a Macaronèsia; i s'han produït esdeveniments de vicariança i dispersió a llarga distància cap a Nord-Amèrica des de la regió Mediterrània i des d'Àsia.

8. Les filogènies obtingudes amb els diferents marcadors mostren que només els tàxons que apareixen en el clade *Rapunculus* han arribat a Nord-Amèrica, malgrat tenir llavors de característiques molt similars als altres llinatges de *Campanula.* A més, l'arribada a Nord-Amèrica de *Rapunculus* s'ha produït repetides vegades, en esdeveniments independents. El clade *Rapunculus* presenta una major heterogeneitat en nombres cromosòmics, nombres bàsics i nivells de ploïdia. S'hipotetitza que aquestes característiques poden haver estat un dels factors clau implicats en l'èxit de colonització d'aquests tàxons.

9. Basant-nos en les anàlisis de datació molecular dutes a terme amb les dades de les regions *rbc*L i *trn*L-F, es conclou que la diversificació dels dos llinatges principals de *Campanula* (*Campanula* s. str i *Rapunculus*) es va iniciar al Miocè Mitjà. En aquest període es va iniciar un refredament del clima seguit de l'establiment d'una nova connexió entre el Mediterrani, la regió caucàsica i l'oest d'Àsia, que es trobaven aïllats pel mar de Tetis. Concloem que aquests esdeveniments paleoclimàtics i paleogeològics han estat de gran importància en la diversificació de *Campanula*, un gènere adaptat a regions fredes que té una major diversitat d'espècies en regions muntanyoses i d'estepes.

10. Sobre la base de les anàlisis de datació molecular, deduïm que les taxes de diversificació de diversos llinatges de *Campanula* van augmentar durant la crisi del Messinià. En aquest període l'erosió i la sequera foren més intenses, per tant, deduïm que aquests fenòmens poden haver

promogut la diversificació de plantes anuals, xeromòrfiques i altres llinatges pioners.

11. Les regions ITS, *trn*G i *trn*L-F són bons marcadors per a estudiar les relacions filogenètiques de *Campanula* subgenère *Roucela.*

12. Els resultats filogenètics moleculars revelen que la circumscripció actual de *Roucela* no constitueix un grup monofilètic. *Campanula scutellata* presenta tendències morfològiques i moleculars que suggereixen la seva pertinença a una unitat evolutiva diferent. La resta d'espècies de *Roucela* conformen un grup monofilètic, tot constituint tres clades que no es corresponen amb els prinicipals trets morfològics, però que sí presenten coherència biogeogràfica. Suggerim la conveniència de descriure un nou subgènere per a *Campanula scutellata,* prèvia revisió exhaustiva d'exemplars d'herbari d'aquesta espècie.

Apèndixs

APPENDIX 1. Estimated ages and standard deviation using Penalized Likelihood (r8s) and Bayesian Relaxed Molecular Clock (Multidivtime) for *rbc*L data. Node numbers correspond to those given on the chronograms. * = fixed node; **= constrained node; (s. d.)= standard deviation; LHPD= 90 % lower highest posterior density limit; UHPD= 90 % upper highest posterior density limit.

Nodes	r8s Mode (LHPD-UHPD)		Multidivtime (s. d.)	
	Fossil calibration	Multiple calibrations	Fossil calibration	Multiple calibrations
51	12.8 (8.2-14.9)	16.3 (12.6-25.6)	8 (2.3)	9.7 (3)
52	8.2 (3.8-11.4)	13 (4.4-18.2)	4.2 (1.9)	5.2 (2.5)
53	13.7 (9.3-15.5)	20 (11.9-25.6)	7.8 (2.3)	9.5 (3.0)
54	16.3 (12.8-19.1)	26 (17-32)	10.4 (2.3)	12.7 (3.2)
55	2.9 (1.8-5.8)	3.2 (2.1-5.6)	4.5 (2.1)	4.7 (2.2)
56	3.8 (1.8-5.8)	2.7 (1.8-5.4)	3.4 (1.9)	3.6 (1.9)
57	9 (5.4-11)	8.4 (5.7-10.8)	9.1 (2.4)	9.5 (2.6)
58	0.4 (0.1-2.5)	0.4 (0-2.1)	1.2 (1.1)	1.2 (1.1)
59	14.8 (14.3-15)	14.7 (14.2-15)	13.8 (1. 3)	14.7 (1.6)
60	5 (1.5-7.4)	2.8 (2.3-5.2)**	4.4 (2.3)	3.1 (1.4)**
61	6.3 (2.2-11.5)	6.6 (2.8-9.8)	6.6 (2.3)	5.9 (2.1)
62	8.8 (2.5-12.9)	4.8 (2-11.7)	7.1 (2. 6)	7.4 (2.7)
63	2.4 (1-4.4)	2.1 (1-4.3)	3.4 (2.4)	3.4 (2.3)
64	6.5 (4.2-10.5)	7.9 (4.6-8)**	6.4 (2.4)	5.5 (1.7)**
65	10 (7.4-12.9)	10 (7.2-12.6)	9.5 (2.2)	9.3 (2.2)
66	8.8 (8.1-9)	9 (8.5-10)	8.8 (2)	9.2 (2.2)
67	1.3 (0.4-3.2)	2.1 (0.6-5)	2.5 (1.5)	2.6 (1.6)
68	4.4 (1.6-6)	3.9 (1.9-6.3)	4.1 (1.6)	4.1 (1.8)
68	5.9 (5-6.7)	6 (5-6.5)	5.7 (1.8)	5.9 (1.9)
70	7.5 (4.6-9.8)	7.4 (4.7-9.6)	6.9 (1.8)	7.1 (1.9)
71	8.8 (7.8-9)	9 (8.2-9.5)	8.9 (1.9)	9.3 (2.1)
72	9.5 (7-12.5)	11 (8-12.2)	10.6 (1.8)	10.9 (2.0)
73	12 (9.3-14.5)	13 (10.1-14.3)	12.4 (1.5)	12.8 (1.8)
74	8 (7.4-8.8)	8.5 (7.6-9)	8.9 (1.9)	9.3 (2.1)
75	1.7 (1.1-4)	2 (1-4.2)	2.3 (1.4)	2.4 (1.5)
76	2.1 (0.9-3.1)	1.7 (1.1-3.2)	2.3 (1.4)	2.4 (1.4)
77	3.5 (2.2-5.3)	3.7 (2.4-5.3)	4 (1.7)	4.2 (1.8)
78	0.4 (0.1-1.2)	0.4 (0.2-1.2)	1 (1)	1.1 (1.0)
79	6.6 (6-7)	6.9 (6.1-7.5)	6.4 (1.9)	6.7 (2.0)
80	7.8 (6-9.4)	7.3 (6.1-10.4)	8.1 (29	8.5 (2.1)
81	0.6 (0-1.7)	0.6 (0-1.5)	1.2 (1)	1.2 (1.1)
82	9.1 (6.3-11.1)	9.8 (6.6-11.6)	8.7 (1.9)	9.1 (2.1)
83	10.8 (8.3-12.6)	11.1 (9-13.7)	10.6 (1.8)	11.1 (2.0)
84	11.8 (11-12.6)	12 (11.2-12.7)	12.5 (1.5)	13.1 (1.7)
85	12.7 (11.4-15.5)	13.9 (12.3-15.4)	13.7 (1.2)	14.3 (1.5)
86	16 (12.6-16)*	16**	16*	17.0 (1.0)**
87	23.5	34.8 (30.6-41)	18.4 (2.2)	19.3 (2.3)**

APPENDIX 2. Estimated ages and standard deviation using Penalized Likelihood (r8s) and Bayesian Relaxed Molecular Clock (Multidivtime) for *trn*L-F data. Node numbers correspond to those given on the chronograms. * = fixed node; **= constrained node; (s. d.)= standard deviation; LHPD= 90 % lower highest posterior density limit; UHPD= 90 % upper highest posterior density limit.

Nodes	r8s Mode (LHPD-UHPD)		Multidivtime (s. d.)	
	Fossil calibration	Multiple calibrations	Fossil calibration	Multiple calibrations
102	1.2 (0.7-2.3)	1.2 (0.7-2.3)	1.3 (0.7)	1.4 (0.8)
103	14 (11.8-15.8)	14 (11.8-15.8)	13.9 (1.2)	14.7 (1.6)
104	6.7 (5.2-8.7)	6.7 (5.2-8.7)	6.5 (1.7)	7.0 (1.9)
105	3.7 (2.5-4.9)	3.7 (2.5-4.9)	5.1 (1.8)	3.9 (1.0)**
106	4.8 (3.2-6.2)	4.8 (3.2-6.2)	6.4 (1.8)	5.5 (1.4)
107	1 (0.4-3.1)	1 (0.4-3.1)	1.8 (1.3)	1.9 (1.4)
108	12 (7.5-14)	12 (7.5-14)	11.2 (1.4)	11.9 (1.7)
109	0.9 (0.3-1.3)	0.9 (0.3-1.3)	0.7 (0.5)	0.7 (0.5)
110	1.4 (0.8-1.9)	1.4 (0.8-1.9)	1.6 (0.8)	1.7 (0.8)
111	2.1 (1.6-3)	2.1 (1.6-3)	3.0 (1.1)	3.1 (1.2)
112	3.4 (2.4-4.1)	3.4 (2.4-4.1)	4.1 (1.4)	4.4 (1.5)
113	5.2 (4-6.7)	5.2 (4.1-6.7)	5.4 (1.4)	5.7 (1.5)
114	0.2 (0-0.7)	0.2 (0-0.7)	0.5 (0.4)	0.5 (0.5)
115	0.9 (0.4-1.7)	0.9 (0.4-1.7)	1.4 (0.8)	1.5 (0.8)
116	2.4 (1.6-4.2)	2.3 (1.6-4.2)	3.1 (1.1)	3.3 (1.2)
117	6.2 (5.1-7.6)	6.2 (5.1-7.6)	6.4 (1.4)	6.8 (1.6)
118	7.2 (5.7-8.2)	7.2 (5.7-8.2)	7.2 (1.5)	7.6 (1.6)
119	0.8 (0.4-1.6)	0.8 (0.4-1.6)	1.1 (0.8)	1.1 (0.8)
120	2.2 (1.4-3.4)	2.2 (1.4-3.4)	2.5 (1.1)	2.7 (1.2)
121	7.9 (7.1-9.8)	7.9 (7.1-9.8)	8.6 (1.5)	9.1 (1.7)
122	10 (8.6-11.5)	10 (8.6-11.5)	9.7 (1.6)	10.3 (1.8)
123	5 (4.1-6.9)	5 (4.1-6.9)	5.7 (1.5)	6 (1.7)
124	1.6 (1-2.8)	1.6 (1-2.8)	2.3 (1)	2.4 (1.1)
125	2.4 (1.6-3.8)	2.3 (1.6-3.8)	3.2 (1.2)	3.4 (1.3)
126	3.3 (2.3-5.4)	3.3 (2.3-5.4)	4.6 (1.3)	4.9 (1.4)
127	3.5 (2.3-4.7)	3.5 (2.3-4.7)	3.6 (1.2)	3.9 (1.3)
128	5.2 (3.4-6.8)	5.2 (3.4-6.7)	5.2 (1.3)	5.6 (1.4)
129	5.5 (4.5-7.7)	5.4 (4.5-7.7)	6 (1.4)	6.4 (1.5)
130	7.4 (6.3-9.4)	7.3 (6.3-9.4)	8 (1.5)	8.5 (1.7)
131	8.7 (7.7-10.5)	8.7 (7.7-10.5)	9.4 (1.6)	10 (1.8)
132	11.4 (9.5-12.6)	10.5 (9.5-12.6)	10.3 (1.6)	10.9 (1.8)
133	7.6 (6.1-9.7)	7.6 (6.1-9.5)	6.7 (1.5)	7.1 (1.7)
134	3.1 (1.7-4.5)	3.1 (1.7-4.5)	2.4 (1.2)	2.6 (1.3)
135	3.7 (3-5.4)	3.7 (3-5.4)	3.8 (1.4)	4.1 (1.5)
136	4.3 (3.3-6)	4.3 (3.3-6)	4.5 (1.5)	4.8 (1.6)
137	10.2 (8.2-11.2)	10.1 (8.3-11.2)	8.4 (1.5)	8.9 (1.6)
138	0.6 (0.2-1.1)	0.6 (0.2-1.1)	0.9 (0.7)	1.0 (0.8)
139	2.1 (1.2-3)	2.1 (1.2-3)	2.4 (1.2)	2.5 (1.2)
140	4.9 (3.3-6.1)	4.9 (3.3-6.1)	5.8 (1.6)	6.0 (1.7)

141	1.1 (0.5-2.4)	1.1 (0.5-2.4)	1.3 (0.9)	1.3 (0.9)
142	1.7 (1.2-3.7)	1.7 (1.2-3.7)	2.3 (1)	2.3 (1)
143	3 (1.8-5.1)	3.1 (1.8-5.1)	3.3 (1.2)	3.3 (1.2)
144	5.1 (2.7-6.6)	5 (2.7-6.6)	4.2 (1.4)	4.2 (1.3)
145	5.5 (3.4-8.3)	6.5 (3.5-8)	5.2 (1.5)	5.2 (1.3)**
146	8.4 (6.2-10.3)	8.3 (6.2-10.3)	7.3 (1.7)	7.5 (1.7)
147	0.4 (0-3)	0.4 (0-3)	1 (0.8)	1.1 (9.9)
148	4.2 (2.6-9)	4.2 (2.6-9)	3.2 (1.3)	3.3 (1.4)
149	4 (2-9.2)	4 (2-9.2)	3.5 (1.3)	3.7 (1.4)
150	2.6 (1.4-6)	2.6 (1.4-6)	3.1 (1.3)	3.3 (1.4)
151	1.1 (0.3-3.7)	1.1 (0.3-4)	1.3 (0.9)	1.3 (1)
152	4.1 (1.8-7.3)	4.1 (1.8-7.3)	3.7 (1.3)	3.8 (1.3)
153	0.3 (0-1.5)	0.2 (0-1.5)	0.8 (0.7)	0.8 (0.7)
154	5.9 (2.6-8.6)	5.2 (2.6-8.6)	3.8 (1.3)	4.0 (1.3)
155	3.8 (1.7-5.8)	3.8 (1.7-5.8)	2.5 (1.1)	2.6 (1.1)
156	5.7 (2.2-8.7)	5.7 (2.2-8.7)	3.6 (1.3)	3.7 (1.3)
157	4 (1.6-6.2)	4 (1.6-6.2)	2.9 (1.3)	3.0 (1.3)
158	4.9 (2.4-8.5)	4.9 (2.4-8.5)	3.9 (1.3)	4.1 (1.3)
159	0.7 (0.3-2.2)	0.7 (0.3-2.2)	1.2 (0.9)	1.2 (1)
160	9.3 (4.6-10.9)	9.3 (4.6-10.9)	4.9 (1.3)	5.1 (1.4)
161	11.7 (9.4-13.3)	11.7 (9.4-13.4)	9.7 (1.4)	10.2 (1.5)
162	13.5 (12.4-14.8)	13.5 (12.4-14.8)	12.7 (1.2)	13.5 (1.5)
163	14 (13.1-15.2)	14 (13.1-15.2)	13.8 (1.1)	14.7 (1.5)
164	16 (0)*	16 (0)*	16 (0)*	17 (1)**
165	35.2 (29-42.7)	35.5 (30.3-41)	-	-

APPENDIX 3. Table of geologic time from the beginning of the Upper Cretaceous until present based on the dates and nomenclature proposed by the International Comission on Stratigraphy.

Eonothem Eon	Erathem Era	Sub-Era	System Period	Series Epoch	Stage Age	Age mya
Phanerozoic	Cenozoic	Quaternary	Neogene	Holocene		0.0118
				Pleistocene	Upper	0.126
					Middle	0.781
					Lower	1.806
		Tertiary		Pliocene	Gelasian	2.588
					Piacenzian	3.6
					Zanclean	5.332
				Miocene	Messinian	7.246
					Tortonian	11.608
					Serravallian	13.82
					Langhian	15.97
					Burdigalian	20.43
					Aquitanian	32.03
			Paleogene	Oligocene	Chattian	28.4
					Rupelian	33.9
				Eocene	Priabonian	37.2
					Bartonian	40.4
					Lutetian	48.6
					Ypresian	55.8
				Paleocene	Thanetian	58.7
					Selandian	61.7
					Danian	65.5
	Mesozoic		Cretaceous	Upper	Maastrichtian	70.6
					Campanian	83.5
					Santonian	85.8
					Coniacian	89.3
					Turonian	93.5
					Cenomanian	99.6