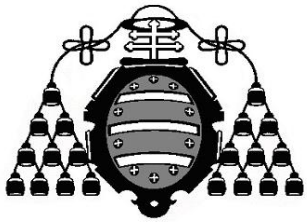

Universidad de Oviedo

Departamento de Biología de Organismos y Sistemas

TESIS DOCTORAL

Unravelling the polyploid complex *Ranunculus parnassiifolius* L. (Ranunculaceae)

A combined morphologic and molecular-genetic approach

Eduardo Cires Rodríguez

Oviedo, 2011

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Unravelling the polyploid complex Ranunculus parnassiifolius L.(Ranunculaceae). A combined morphologic and molecular-genetic approach

Eduardo Cires Rodríguez

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A mis padres y a mi hermano

A mi esposa

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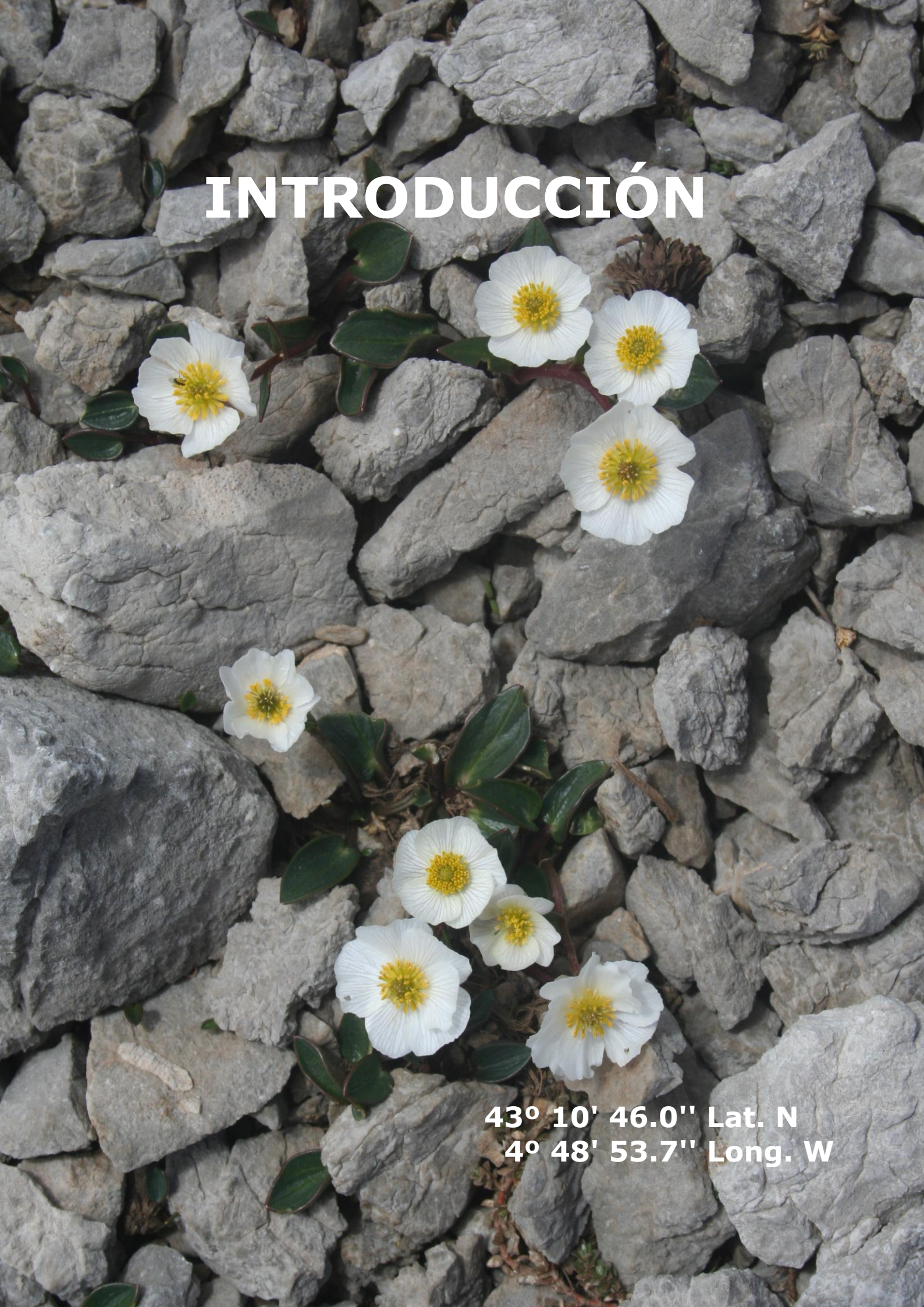
СЛАВА ТРУДУ
(pronúnciese: sláva trudú)

ÍNDICE

INTRODUCCIÓN



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4º 48' 53.7'' Long. W

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- Cires, E., Revilla Bahillo, M.A., Fernández Prieto, J.A. (2009). Atlas corológico y georreferenciación de *Ranunculus parnassifolius* en la Península Ibérica. En: Llamas, F. & Acedo, C. (eds). *Botánica Pirenaico-Cantábrica en el siglo XXI*. Universidad de León, pp. 375-386. ISBN 978-84-9773-471-4.

Marco taxonómico

La familia Ranunculaceae

Las Ranunculaceae Juss. (*nom. cons.*) es una familia de plantas con una distribución cosmopolita que comprende unos 59 géneros y cerca de 2500 especies (Tamura 1993). Ha sido considerada como una de las familias basales dentro de las Eudicotiledóneas (APG III 2009) y concretamente dentro del orden Ranunculales Juss. ex Bercht. & J.Presl (Figura 1).

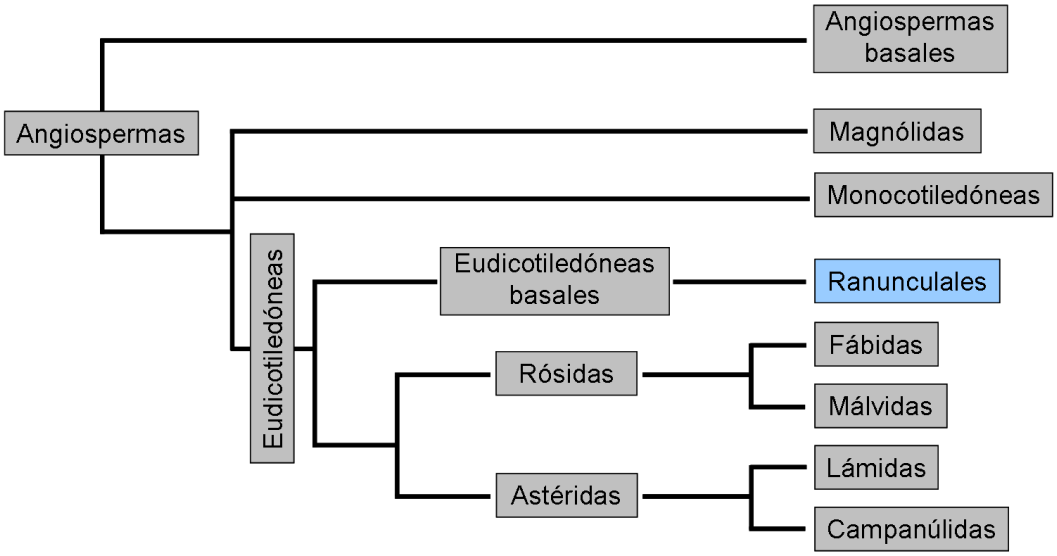

Figura 1. Diagrama simplificado que ilustra el árbol filogenético de las Angiospermas. Se muestran los grandes grupos de Angiospermas (Angiospermas basales, Magnólidas, Monocotiledóneas y Eudicotiledóneas). En color azul se indica el orden Ranunculales. Imagen modificada de APG III (The Angiosperm Phylogeny Group III 2009).

La familia Ranunculaceae muestra una gran variación morfológica, especialmente en lo que respecta al tipo de fruto y a su organización floral. Varias clasificaciones han sido propuestas en el seno de la familia basadas en criterios morfológicos (Hutchinson 1923; Janchen 1949; Tamura 1990, 1991, 1992a, 1992b, 1995), en datos moleculares (Jensen *et al.* 1995; Ro *et al.* 1997; Cai *et al.* 2009, 2010) o como resultado de una combinación de ambos tipos de criterios (Wang *et al.* 2009).

La familia ha sido subdividida en 3 subfamilias y 11 tribus (Tamura 1995) teniendo en cuenta tanto el número básico de cromosomas como el tipo de gineceo: Helleboroideae Hutch., Isopyroideae Tamura y Ranunculoideae Hutch. En esta última subfamilia se sistematiza la tribu Ranunculeae DC., que incluye 19 géneros (Emadzade *et al.* 2010) y cerca de 650 especies distribuidas por todos los continentes (Tamura 1995). Varias clasificaciones de la tribu Ranunculeae han sido propuestas por diversos autores (Candolle 1824; Prantl 1887; Ovczinnikov 1937; Tamura 1993, 1995; Whittemore 1997; Emadzade *et al.* 2010) (Tabla 1). Las

discrepancias entre dichas clasificaciones son debidas principalmente a la amplia variación de los caracteres florales que presentan.

El género Ranunculus

El género *Ranunculus* fue descrito por Carl Linnaeus (1707-1778) en *Species Plantarum* (1753). Linnaeus incluyó los géneros *Ranunculus* Tourn. y *Ficaria* Dill. ex Haller bajo el nombre genérico *Ranunculus* L.; y además describe 37 especies dentro del género, algunas de las cuales ya habían sido señaladas anteriormente por diversos autores pre-linneanos.

El nombre latino *ranunculus* = ranita, alude a la preferencia de estas plantas por vivir frecuentemente en terrenos húmedos, aunque algunas especies se han adaptado a vivir en condiciones de mayor xericidad e incluso en zonas semidesérticas o desérticas como *Ranunculus platyspermus* Fisch. ex DC., que habita en el desierto de Kirghisorum (República de Kazajstán) (Diosdado 1991; Diosdado & Pastor 1996).

Ranunculus L. es el género más amplio dentro de la familia Ranunculaceae, cuenta con aproximadamente unas 600 especies (Tamura 1993, 1995) y numerosas razas apomícticas (Hörandl *et al.* 2005), distribuidas desde las zonas templadas hasta las zonas árticas y subantárticas (Ziman & Keener 1989), siendo rara en los trópicos, donde está restringida a zonas de alta montaña (Tamura 1993, 1995). Por lo tanto, su distribución es cosmopolita encontrándose el mayor número de especies en zonas templadas de Norte y Sur América, Europa, Asia, Australia, Nueva Zelanda, y en las regiones alpinas de Nueva Guinea (Johansson 1998).

Las plantas del género *Ranunculus* se pueden encontrar en una gran variedad de hábitats, tales como bosques, prados secos y húmedos, lagos, ríos y sus riberas, así como en hábitats específicos de áreas de alta montaña. La mayoría de las especies parecen tener una gran amplitud ecológica; no obtante, no son infrecuentes las que medran en hábitats especializados.

Las especies del género *Ranunculus* reúnen plantas herbáceas perennes, aunque también incluyen otras anuales o bienales (Tamura 1993; Johansson 1998), con hojas enteras o divididas en muy diverso grado (Figura 2). Dentro del género prevalecen dos formas biológicas (biotipos): los geófitos (conjunto de formas vegetales en que la parte persistente del organismo queda completamente protegida bajo el nivel del suelo), y los hidrófitos (plantas con las yemas de recambio sumergidas en el agua, es decir, especies de ambientes acuáticos). Otros biotipos como los terófitos (plantas anuales, capaces de completar el ciclo de su existencia en la estación favorable) o hemicriptófitos (plantas cuya parte aérea muere anualmente y las yemas de reemplazo quedan apróximamente a la altura del suelo), son menos frecuentes entre las especies del género *Ranunculus*.

Tabla 1. Clasificaciones de la tribu Ranunculeae propuestas por distintos autores.

Emadzade *et al.* 2010 (a nivel mundial)	Whittemore 1997 (Norte América)	Tamura 1995 (a nivel mundial)	Ovczinnikov 1937 (U.S.S.R.)	Prantl 1887 (a nivel mundial)	Candolle 1824 (a nivel mundial)
Arcteranthis Greene	*R.* subg. *R.* sect. *Arcteranthis*	*Arcteranthis*	–	–	–
Beckwithia Jeps.	*R.* subg. *Crymodes*	*R.* subg. *Crymodes*	–	*R.*sect. *Hypolepium*	–
Callianthemoides Tamura	–	*Callianthemoides*	–	–	–
Ceratocephala Moench	*R.* subg. *Cerathocephalus*	*Ceratocephala*	*Ceratocephala*	*R.* sect. *Cerathocephalus*	*Ceratocephala*
Coptidium (Prantl) Beurl. ex Rydb.	*R.* subg. *Coptidium* & *Pallasiantha*	*R.* subg. *Coptidium* & *Pallasiantha*	*R.* subg. *Auricomus* sect. *Coptidium*	*R.* sect. *Marsypadenium*	*Ranunculus*
Cyrtorhyncha Torr. & A.Gray	*R.* subg. *R.* sect. *Cyrtorhyncha*	*Cyrtorhyncha*	–	*Oxygraphis* ?	–
Ficaria Guett.	*R.* subg. *Ficaria*	*R.* subg. *Ficaria*	*Ficaria*	*R.* sect. *Ficaria*	*Ficaria*
Halerpestes Greene	*R.* subg. *R.* sect. *Halodes*	*Halerpestes*	*Halerpestes*	*Oxygraphis*	*Ranunculus*
Hamadryas Comm. ex Juss.	–	*Hamadryas*	–	–	*Anemoneae*
Krapfia DC.	–	*Krapfia*	–	–	*Ranunculus*
Kumlienia Greene	*R.* subg. *R.* sect. *Pseudaphanostemma*	*Kumlienia*	–	*Oxygraphis* ?	–
Laccopetalum Ulbr.	–	*Laccopetalum*	–	–	–
Myosurus L.	*Myosurus*	*Myosurus*	*Myosurus*	*Myosurus*	*Myosurus*
Oxygraphis Bunge	*R.* subg. *Oxygraphis*	*Oxygraphis*	*Oxygraphis* subg. *Euoxygraphis*	*Oxygraphis*	–
Paroxygraphis W.W.Sm.	–	*Paroxygraphis*	–	–	–
Peltocalathos Tamura	–	*Peltocalathos*	–	–	–
Ranunculus L.	*Ranunculus*	*Ranunculus*	*Ranunculus*	*Ranunculus*	*Ranunculus*
–	–	*Aphanostemma*	–	*R.* sect. *Marsypadenium*	*Ranunculus*
–	*R.* subg. *Batrachium*	*R.* subg. *Batrachium*	*Batrachium*	*R.* sect. *Marsypadenium*	*R.* sect. *Batrachium*
Trautvetteria Fisch. & C.A.Mey.	*Trautvetteria*	*Trautvetteria*	*Trautvetteria*	*Trautvetteria*	–

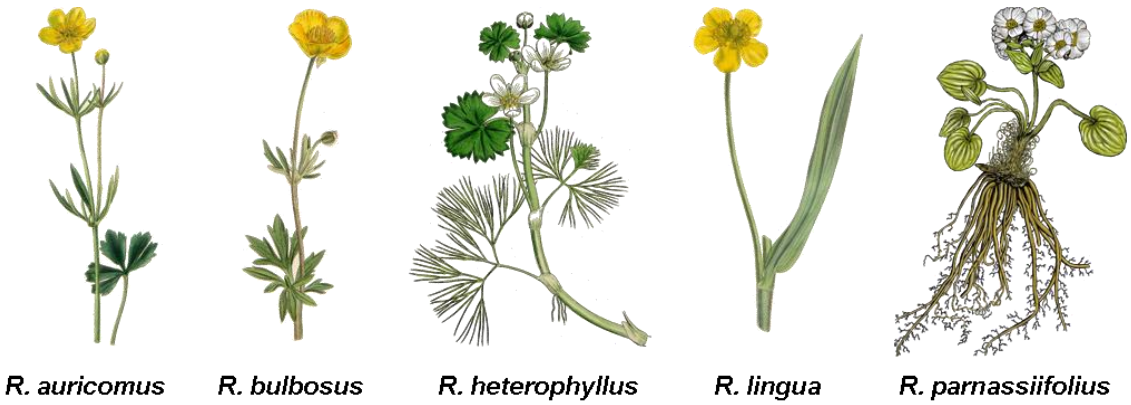

Figura 2. Aspecto morfológico de diferentes especies del género *Ranunculus*.

Las características de las plantas sistematizadas en el género *Ranunculus* son relativamente constantes, sobre todo en lo que refiere a las estructuras florales y a los frutos (Figura 3). Las flores son solitarias o más o menos numerosas, habitualmente reunidas en inflorescencias cimosas. Son flores actinomórficas, talamifloras, hemicíclicas, dialisépalas y hermafroditas, cuyo cáliz puede estar formado por (3)-5-(7) sépalos. La corola está formada por (0)-5-(12 o más) pétalos; éstos son generalmente amarillos, aunque en ocasiones son rojizos y, en particular en ciertos grupos, blancos; cada pétalo presenta cerca de su base una glándula nectarífera, que puede estar desnuda o cubierta por una escama. Los estambres son libres y numerosos, y se disponen helicoidalmente sobre la base del tálamo floral. El gineceo es pluricarpelar apocárpico originando frutos múltiples de tipo polinuez (es decir, un agregado de varias nueces en ocasiones denominadas aquenios), generalmente lisos, peludos o glabros, a veces alados o con espinas en forma de gancho.

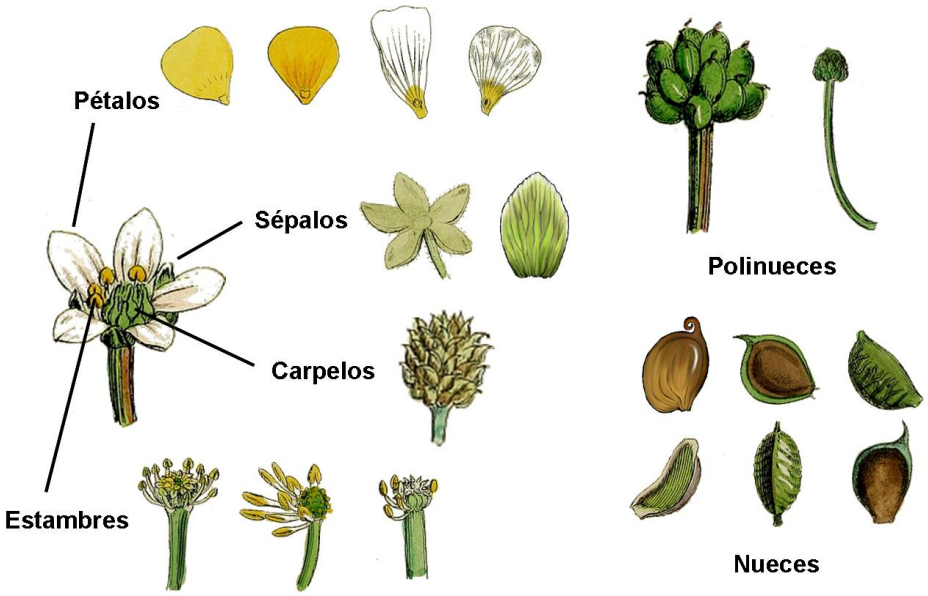

Figura 3. Estructuras florales y fructíferas típicas en plantas de diversas especies del género *Ranunculus*.

Tras la revisión a nivel global realizada por Tamura (1993, 1995), el género *Ranunculus sensu lato* (*s.l.*) puede ser dividido en 7 subgéneros: *Batrachium* (DC.) Peterm., *Coptidium* (Prantl) L.D.Benson, *Crymodes* A.Gray, *Ficaria* (Huds.) L.D.Benson, *Gampsoceras* (Steven) Tamura, *Pallasiantha* L.D.Benson y *Ranunculus*. El subgénero *Ranunculus* es el más extendido y numeroso en el género e incluye una gran diversidad de especies, algunas de ellas consideradas como ancestrales por Tamura (1995). Según el citado autor, este subgénero contiene al menos 20 secciones y más de 500 especies (Tabla 2).

En la Península Ibérica, según la propuesta sistemática realizada por Cook *et al.* (1986), están representados tres de los subgéneros citados, que reúnen 65 especies de las que una cuarta parte son endémicas: *Batrachium*[1] -ranúnculos acuáticos, provistos de flores blancas y aquenios con costillas transversales-, *Ficaria*[2] -ranúnculos de flores amarillas con 7-14 pétalos y 3 sépalos-, y por último, *Ranunculus* -ranúnculos no acuáticos de flores blancas, amarillas o rosadas, con aquenios sin costillas transversales regulares, provistos de 5 sépalos y generalmente 5 pétalos-.

Tabla 2. Subdivisión del género *Ranunculus s.l.* según Tamura (1993, 1995).

Género	Subgénero	Sección	Distribución	Nº Especies
Ranunculus	*Coptidium*	*Coptidium*	Asia, Europa, Norte América	1
	Pallasiantha	*Pallasiantha*	Asia, Europa, Norte América	1
	Ficaria	*Ficaria*	Asia, Europa, Norte África	5
	Crymodes	*Crymodes*	Europa, Norte América	4
	Bathachium	*Bathachium*	Cosmopolita	*ca.* 30
	Gampsoceras	*Gampsoceras*	Irán, Siria, Turquía	1
	Ranunculus	*Acetosellifolii*	Europa	1
		Aconitifolii	Europa	3
		Acris	Cosmopolita	*ca.* 150
		Casalea	Norte y Sur América	*ca.* 10
		Chloeranunculus	Europa, Norte África	1
		Echinella	África, Asia, Europa, Norte América	*ca.* 70
		Ficariifolius	Asia, Europa, Norte América	5
		Flammula	Asia, Europa, Norte América	*ca.* 22
		Hecatonia	Asia, Europa, Norte América	1
		Leptocaules	Australia, Nueva Zelanda, Sur América	*ca.* 13
		Leucoranunculus	Asia, Europa	4
		Micranthus	Asia, Europa, Norte África	2
		Physophyllum	Europa, Norte África	1
		Pseudoadonis	Australia, Nueva Zelanda, Tasmania	*ca.* 15
		Ranuncella	Europa, Norte África	6
		Ranunculastrum	Asia, Europa, Norte África	*ca.* 70
		Ranunculus	Asia, Europa, Norte y Sur América, Oceanía	*ca.* 160
		Thora	Europa	4
		Tuberifer	Asia	2
		Xanthobatrachium	Cosmopolita	*ca.* 8

La sistemática del género aún no está consolidada, y tras las últimas investigaciones realizadas por Hörandl *et al.* (2005), Emadzade *et al.* (2010) y Hörandl (en prensa), se considera *Ficaria* Guett. como un género independiente y no como un subgénero de *Ranunculus*.

[1]: *Ranunculus* subgen. *Batrachium* (DC.) A.Gray según Cook *et al.* (1986). Sin embargo, la autoría correcta debería ser: *Batrachium* (DC.) Gray (ver Index Nominum Genericorum, http://botany.si.edu/ing).
[2]: *Ranunculus* subgen. *Ficaria* (Guett.) L.Benson según Cook *et al.* (1986). Sin embargo, la autoría correcta debería ser: *Ficaria* (Schaeff.) L.D.Benson (ver Tropicos, http://www.tropicos.org/Home.aspx).

Además, tras la última revisión realizada por Hörandl (en prensa) desde una perspectiva geográfica, ecológica, morfológica y filogenética, se modifica sustancialmente la sistemática del subgénero *Ranunculus*, que puede ser dividido en 12 secciones:

- *Epirotes* (Prantl) L.D.Benson (sect. *Leucoranunculus* sensu Tamura p.p.)
- *Leucoranunculus* Boiss.
- *Ranuncella* (Spach) Freyn (incl. sect *Chloeranunculus* Janch. ex Tamura, incl. sect. *Acetosellifolii* Tutin)
- *Aconitifolii* Tutin (incl. sect. *Crymodes* (A.Gray) Tutin p.p.)
- *Thora* DC.
- *Hecatonia* (Lour.) DC. (incl. sect. *Xanthobatrachium* (Prantl) L.D.Benson)
- *Batrachium* DC. (*R.* subgen. *Batrachium* (DC.) Peterm.; *Batrachium* (DC.) Gray)
- *Auricomus* Schur (sect. *Ranunculus* sensu Tamura; sect. *Leucoranunculus* sensu L.D.Benson p.p.)
- *Flammula* (Webb ex Spach) L.D.Benson (incl. sect. *Micranthus* Ovcz. ex Tamura; incl. sect. *Casalea* (A.St.-Hil.) Tamura)
- *Echinella* DC.
- *Ranunculus* (sect. *Acris* Schur; sect. *Chrysanthe* (Spach) L.D.Benson)
- *Polyanthemos* (Luferov) Malacha

Finalmente, tres grupos no son asignados por la citada autora a un grupo sistemático concreto (*incertae sedis*): *Oreophili* Tschuradze (incl. sect. *Ampelophylli* (Ovcz.) Tschuradze); *Euromontani* Hörandl y *Ranunculastrum* DC.

En la Península Ibérica, de acuerdo con Cook *et al.* (1986), están representadas 12 secciones del subgénero *Ranunculus*: *Ranunculus*, *Echinella* DC., *Flammula* (Webb ex Spach) Freyn, *Ranuncella* (Spach) Freyn, *Physophyllum* Freyn, *Crymodes* (A.Gray) Tutin, *Thora* DC., *Leucoranunculus* Boiss., *Aconitifolii* Tutin, *Chrysanthe* (Spach) L.D.Benson, *Hecatonia* (Lour.) DC. y *Ranunculastrum* DC.

La sección Ranuncella

La sección *Ranuncella* (Spach) Freyn reúne plantas caracterizadas por tener raíces fibrosas o engrosadas y fusiformes junto con hojas enteras y dispuestas, la mayoría, en roseta basal.

De acuerdo con el tratamiento sistemático seguido por Cook *et al.* (1986), Tutin & Akeroyd (1993), Tamura (1995), con algunas variaciones (Maire 1964;

Hüber 1988; Bueno Sánchez *et al.* 1992; Sánchez Rodríguez *et al.* 2000), la sección *Ranuncella* del género *Ranunculus* incluye los siguientes taxa:

- ***R. abnormis*** Cutanda & Willk*., Linnaea* 30: 83 (1859).
- ***R. amplexicaulis*** L., *Sp. Pl*.: 549 (1753).
- ***R. angustifolius*** DC., *Nouv. Bull. Sci. Soc. Philom. Paris* 1: 118 (1808).
    - var. ***angustifolius***
    - var. ***uniflorus*** Boiss., *Voy. Bot. Espagne* 2: 6 (1839).
- ***R. bupleuroides*** Brot., *Fl. Lusit.* 2: 365 (1804).
    - subsp. ***bupleuroides***
    - subsp. ***cherubicus*** J.A.Sánchez Rodr., M.J.Elías & M.A.Martín, *Anales Jard. Bot. Madrid* 57(2): 402 (2000).
- ***R. calandrinioides*** Oliv., *Hooker's Icon. Pl.* 19: t. 1828 (1889).
- ***R. gramineus*** L*., Sp. Pl*.: 549 (1753).
- ***R. kuepferi*** Greuter & Burdet, *Willdenowia* 16: 452 (1987).
    - subsp. ***kuepferi***
    - subsp. ***orientalis*** W.Huber, *Veröff. Geobot. Inst. Rübel* 100: 59 (1988).
- ***R. parnassiifolius*** L*., Sp. Pl.*: 549 (1753).
    - subsp. ***parnassiifolius***
    - subsp. ***cabrerensis*** Rothm., *Bol. Soc. Esp. Hist. Nat.* 34: 148 (1934).
    - subsp. ***favargeri*** P.Küpfer, *Boissiera* 23: 191 (1975).
    - subsp. ***heterocarpus*** P.Küpfer, *Boissiera* 23: 192 (1975).
    - subsp. ***muniellensis*** Bueno Sánchez, Fern.Casado & Fern.Prieto, *Bot. J. Linn. Soc.* 109(3): 365 (1992).
- ***R. pyrenaeus*** L., *Mant. Pl*.: 248 (1771).
- ***R. wettsteinii*** Dörfler, *Anzeig. Akad. Wiss. (Wien)* 55: 282 (1918).

La sección *Ranuncella* reúne plantas distribuidas, fundamentalmente, por el suroeste y centro de Europa (Jalas & Suominen 1989; Tutin & Akeroyd 1993), aunque dos especies de la sección medran fuera de este ámbito geográfico: una en Europa oriental (*R. wettsteinii*) y otra en las montañas magrebíes (*R. calandrinioides*).

Está particularmente bien representada en la Península Ibérica y Pirineos, donde crecen siete de las diez especies: *R. abnormis*, *R. amplexicaulis*, *R. angustifolius*, *R. bupleuroides*, *R. gramineus, R. parnassiifolius* y *R. pyrenaeus*. La mayoría de estas plantas son estrictamente orófilas; únicamente *R. bupleuroides* y *R. gramineus* crecen fuera de la alta montaña.

Debido al elevado número de endemismos existentes en el norte de África y la Península Ibérica junto con la gran variabilidad morfológica que muestra en tales territorios, Diosdado (1991) postula que es probable que la sección *Ranuncella* tenga su centro de dispersión en los territorios mediterráneos occidentales.

El complejo poliploide Ranunculus parnassiifolius

Entre las plantas de la sección *Ranuncella*, *Ranunculus parnassiifolius s.l.* presenta un interés especial ya que, junto a *R. gramineus*, es la especie que presenta la más amplia distribución: desde las montañas del noroeste de la Península Ibérica hasta la mayor parte de los macizos alpinos.

Son geófitos de hasta 20 cm, con hojas basales arrosetadas, ovadas o anchamente elípticas, paralelinervias, de base cordada o redondeada, con pecíolo dilatado y membranoso en la base; las flores, reunidas en cimas corimbiformes, son actinomorfas, hermafroditas y pentámeras, de hasta 2,5 cm de diámetro, con pétalos rosados o blancos y estambres numerosos; carpelos numerosos, libres, dispuestos helicoidalmente, que originan fruto multiple de tipo polinuez, cada nuez de 2-4 mm con pico ganchudo (Cook *et al.* 1986; Bueno Sánchez *et al.* 1992).

La gran diversidad existente en su seno ha llevado a reconocer cinco subespecies (*R. parnassiifolius* subsp. *parnassiifolius*, *R. parnassiifolius* subsp. *cabrerensis* Rothm., *R. parnassiifolius* subsp. *favargeri* P.Küpfer, *R. parnassiifolius* subsp. *heterocarpus* P.Küpfer, y *R. parnassiifolius* subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto); se trata de razas orófilas disyuntas en unos casos y, en otros, vicariantes ecológicas (Tabla 3).

Tabla 3. Distribución, hábitat y número cromosomático (2n) de las distintas subespecies de *Ranunculus parnassiifolius s.l.*

Subespecie	Distribución	Hábitat	2n
parnassiifolius	Pirineos orientales	pedreras silíceas	2n = 16
cabrerensis	Montes de León; zonas central y oriental de la Cordillera Cantábrica (Macizos de Curavacas y Peña Prieta)	pedreras silíceas	2n = 16
favargeri	Picos de Europa; Pirineos occidentales	pedreras calcáreas	2n = 16
heterocarpus	Alpes; Pirineos centrales y orientales; Cordillera Cantábrica	pedreras calcáreas	2n = 32, 40
muniellensis	Reserva de la Biosfera de Muniellos (Asturias, España)	pedreras silíceas	2n = 16

Al igual que en otras especies orófilas del hemisferio norte, puede haber tenido en el pasado una distribución continua posteriormente fragmentada como consecuencia de cambios climáticos asociados a las glaciaciones. Por tanto, actualmente existe un considerable interés en reconstruir la historia glacial y post-glacial de estas plantas, con la intención de determinar las relaciones filogenéticas entre las diferentes razas y, en particular, de las poblaciones aisladas.

Antecedentes y objetivos

Antecedentes citogenéticos

Desde los primeros estudios citogenéticos realizados por Langlet (1927, 1932) y Gregory (1941), basados en el tamaño de los cromosomas y cambios en el número básico, la familia Ranunculaceae ha sufrido importantes reorganizaciones sistemáticas.

Tales caracteres cariológicos han permitido distinguir en la familia Ranunculaceae dos grandes grupos: el grupo *Ranunculus*, también llamado el grupo de los R-cromosomas, caracterizado por tener cromosomas grandes y largos y número básico igual a 8 y que incluye, entre otros, los géneros *Anemone*, *Ranunculus* y *Clematis*; y el grupo *Thalictrum*, o grupo de los T-cromosomas, con cromosomas pequeños y cortos, con números básicos que varían entre 7, 9 o 13, y que integra géneros tales como *Thalictrum*, *Hydrastis* y *Aquilegia*. Dichos caracteres resultan ser de los más congruentes cuando se han realizado filogenias de la familia Ranunculaceae basados en datos moleculares (Ro *et al.* 1997; Wang *et al.* 2009).

En cuanto a *Ranunculus parnassiifolius s.l.*, los primeros recuentos de números de cromosomas fueron realizados en plantas alpinas (Langlet 1936; Tischler 1950), resultando en ambos casos 2n = 32. Los siguientes resultados de conteos en *R. parnassiifolius s.l.* fueron realizados en plantas pirenaicas orientales, y resultaron ser n = 8 o 2n = 16 (Löve & Solbrig 1964; Küpfer & Favarger 1967; Favarger & Küpfer 1968). Posteriormente, Küpfer (1974) aportó abundantes datos de conteos de cromosomas, tanto n = 8 o 2n = 16 como n = 16 o 2n = 32, realizados en plantas procedentes de la mayor parte de su área de distribución. Esta información ha sido completada por otros autores (Löve 1987; Hüber 1988; Diosdado 1991; Diosdado & Pastor 1991; Baltisberger 1992; Bueno Sánchez *et al.* 1992).

Antecedentes filogenéticos moleculares

Un elevado número de estudios filogenéticos moleculares dentro de la familia Ranunculaceae sugieren que el género *Ranunculus* es monofilético (Hoot 1995; Johansson 1995, 1998; Ro *et al.* 1997; Hörandl *et al.* 2005; Paun *et al.* 2005; Lehnebach *et al.* 2007; Gehrke & Linder 2009; Emadzade *et al.* 2010; Hoffmann *et al.* 2010).

Además, dentro del género y mediante análisis de sitios de restricción del ADN del cloroplasto (Johansson 1998), secuencias de la región del Espaciador Interno Transcrito del ADN ribosomal del núcleo (conocido por sus siglas en inglés ITS: "Internal Transcribed Spacer") (Hörandl *et al.* 2005) y secuencias de ADN de plasto (Paun *et al.* 2005; Gehrke & Linder 2009; Hoffmann *et al.* 2010), han

demostrado la existencia de un gran clado denominado *Ranunculus* del que las secciones *Coptidium*, *Ficaria*, *Pallasiantha* y parte de la sección de *Crymodes* han sido excluidas. Este "clado básico" incluye al menos 19 subclados que corresponden a grupos ecológicos concretos (por ejemplo especies acuáticas y de humedales) o a determinados grupos geográficos (por ejemplo plantas de los sistemas montañosos europeos). Para una explicación más detallada de cada uno de los clados consultar Hörandl *et al.* (2005) y Paun *et al.* (2005).

Sin embargo, la sistemática supraespecífica y la delimitación de la sección *Ranuncella* del género *Ranunculus*, comúnmente aceptada y basada en caracteres morfológicos, no es refrendada por los resultados obtenidos al aplicar métodos moleculares (Hörandl *et al.* 2005; Paun *et al.* 2005).

Tal circunscripción de la sección *Ranuncella* se ha modificado como consecuencia de los resultados obtenidos por Hörandl *et al.* (2005) mediante el análisis de secuencias ITS, y confirmados por Paun *et al.* (2005) mediante el análisis de esas mismas secuencias junto a las del plasto *matK* y las regiones adyacentes *trnK*. Tales trabajos llevaron a la inclusión en un mismo clado a especies sistematizadas en la sección *Ranuncella -Ranunculus amplexicaulis*, *R. parnassiifolius* subsp. *parnassiifolius*, *R. calandrinioides*, *R. pyrenaeus* y *R. gramineus*-, esta última especie sistematizada por Tamura (1995) en la sección *Chloeranunculus*. En este clado se integra además *R. acetosellifolius,* especie diploide endémica de Sierra Nevada, sistematizada por unos autores (Tamura 1993) en una sección monotípica (*Acetosellifolii*) y por otros (Cook *et al.* 1986) en la sección *Aconitifolii.* Por otro lado, incluyen en un grupo bien diferenciado a *R. kuepferi* -tanto *R. kuepferi* subsp. *kuepferi* como *R. kuepferi* subsp. *orientalis*- junto con especies sistematizadas en la sección *Aconitifolii -R. seguieri, R. platanifolius* y *R. aconitifolius*-, en la sección *Crymoides -R. glacialis* y *R. chamissonis*- o en la sección *Leucoranunculus -R. alpestris*-.

Justificación y objetivos

Desde la primera clasificación del género *Ranunculus* a nivel mundial realizada por Candolle (1824), el género ha sido revisado en varias ocasiones pero aún está lejos de ser completada su clasificación y delimitación a nivel genérico e infragenérico. Los esfuerzos realizados en ocasiones se han visto frustrados debido al gran número de especies existentes en el seno del género, a la escasa representación en los herbarios de especímenes procedentes de floras regionales y a la gran variabilidad y/o homoplasia de ciertos caracteres morfológicos. Actualmente, el empleo de nuevas técnicas moleculares y la colaboración científica a nivel internacional están ayudando a desenmarañar la historia evolutiva y las relaciones filogenéticas del género *Ranunculus*.

Así, recientemente se han publicado una serie de trabajos donde se exponen los procesos biogeográficos que han dado forma a la distribución global del género *Ranunculus* (Emadzade 2010; Emadzade & Hörandl 2011; Emadzade *et al.* 2011) o colonizaciones en ciertas áreas como África y el Ártico (Gehrke & Linder 2009; Hoffmann *et al.* 2010). Sin embargo, aún estamos lejos de comprender todos los procesos biogeográficos y/o sistemáticos que engloban este enorme género de la familia Ranunculaceae.

Hay tres aspectos principales que justifican la presente tesis doctoral. En primer lugar, el enfoque de los estudios botánicos en el pasado solía tener una circunscripción más local, normalmente se estudiaba un grupo taxonómico en un área geográfica previamente delimitada, ello seguramente debido a la menor disponibilidad de recursos. En los trabajos de la presente memoria doctoral se ha intentado que el enfoque tenga un ámbito global con el objetivo de abarcar todas las subespecies descritas en *Ranunculus parnassiifolius*.

En segundo lugar, a pesar de la abundante y reciente bibliografía entorno al género *Ranunculus*, no se ha realizado ningún estudio en el complejo poliploide *R. parnassiifolius* desde el trabajo de Küpfer (1974). En este sentido, nos gustaría destacar que otros complejos apomícticos dentro del género *Ranunculus*, como por ejemplo *R. auricomus* y *R. kuepferi*, sí han sido profundamente investigados durante la última década (Hörandl 2008; Burnier *et al.* 2009; Hörandl & Temsch 2009; Hörandl *et al.* 2009; Cosendai & Hörandl 2010). En lo que respecta a *R. parnassiifolius s.l.*, solamente se ha empleado una única muestra (ver Hörandl *et al.* 2005; Paun *et al.* 2005; Emadzade *et al.* 2010) que corresponde a la subespecie típica *R. parnassiifolius* subsp. *parnassiifolius*. Los estudios que engloban esta única muestra han ido encaminados a obtener una visión global del género *Ranunculus* y no a profundizar en el mencionado complejo poliplode.

Por último y en tercer lugar, destacar que durante los últimos años ha habido un gran desarrollo de "herramientas" que cada día se hacen más imprescindibles cuando se aborda un trabajo de sistemática o de evolución en general, tales como las técnicas genéticas y la bioinformática.

Atendiendo a las anteriores consideraciones, los objetivos de la presente memoria son los siguientes:

1) Estimar la cantidad de ADN nuclear mediante citometría de flujo en plantas de *Ranunculus parnassiifolius s.l.* procedentes de toda su área de distribución.

2) Relacionar dicho nivel de ploidía (basado en cantidad de ADN nuclear) con los caracteres morfológicos y los recuentos de cromosomas previamente realizados por otros autores.

3) Abordar el primer estudio sistemático y de relaciones filogenéticas en el complejo poliploide *Ranunculus parnassiifolius s.l.* Se evaluarán las clasificaciones

tradicionales del complejo y se investigarán los distintos procesos responsables del origen y especiación de los distintos linajes.

4) Una vez establecido el marco sistemático básico de *Ranunculus parnassiifolius s.l.*, establecer estrategias de conservación en aquellos taxones en los que sea necesario, bien por su carácter endémico o por formar parte de catálogos de protección.

5) Se propondrán las reorganizaciones taxonómicas que se consideren necesarias para proponer la clasificación más acorde posible con la historia evolutiva.

Para la consecución de los objetivos propuestos se ha estructurado la memoria doctoral en seis capítulos.

El Capítulo 1 supone el punto de partida de la tesis; en el mismo se exponen los antecedentes taxonómicos y citogenéticos de *R. parnassiifolius s.l.* y sirve como puesta a punto de la técnica de citometría de flujo en poblaciones de la Cordillera Cantábrica. La citometría de flujo es una técnica que permite el análisis de las propiedades ópticas (dispersión de la luz y fluorescencia) de partículas que fluyen en una suspensión líquida. Es un método rápido y directo para conocer la ploidía de una planta a partir de la cantidad de ADN existente en sus células (Figura 4).

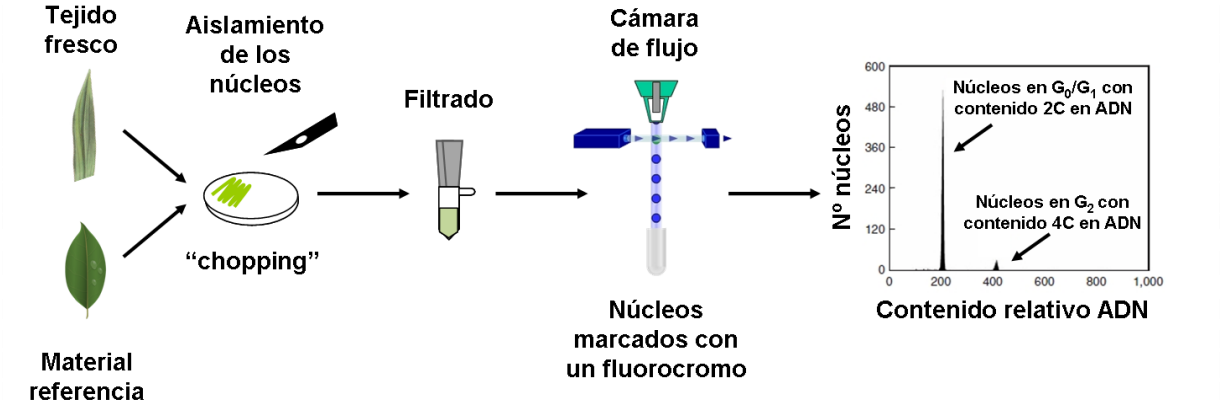

Figura 4. Esquema explicativo para la preparación y determinación del contenido en ADN mediante citometría de flujo en *Ranunculus parnassiifolius*.

En el Capítulo 2, ampliamos el rango de estudio de la técnica de citometría de flujo a las poblaciones pirenaicas y alpinas. Estudios enfocados en la determinación del tamaño del genoma han sido particularmente importantes en las áreas de la taxonomía y la sistemática. Por ejemplo, la citometría de flujo es una herramienta muy útil en la identificación de especies próximas con un número de cromosomas similar pero con contenidos en ADN distintos o en la identificación de variaciones intraespecíficas en el tamaño del genoma. Además, en este capítulo también caracterizamos morfológicamente las poblaciones estudiadas.

En el Capítulo 3, se describe la estructura poblacional de *R. parnassiifolius s.l.* a través de una red de haplotipos de plasto. Los marcadores moleculares de ADN son un fragmento (secuencia) que por sí solo, o combinado en alineación con otros, puede ser físicamente localizado dentro del genoma de un organismo (Figura 5). Tales marcadores moleculares son específicos para cada individuo, especie o grupos sistemáticos supraespecíficos, lo cual los convierte en herramientas útiles para el análisis tanto de individuos como de poblaciones.

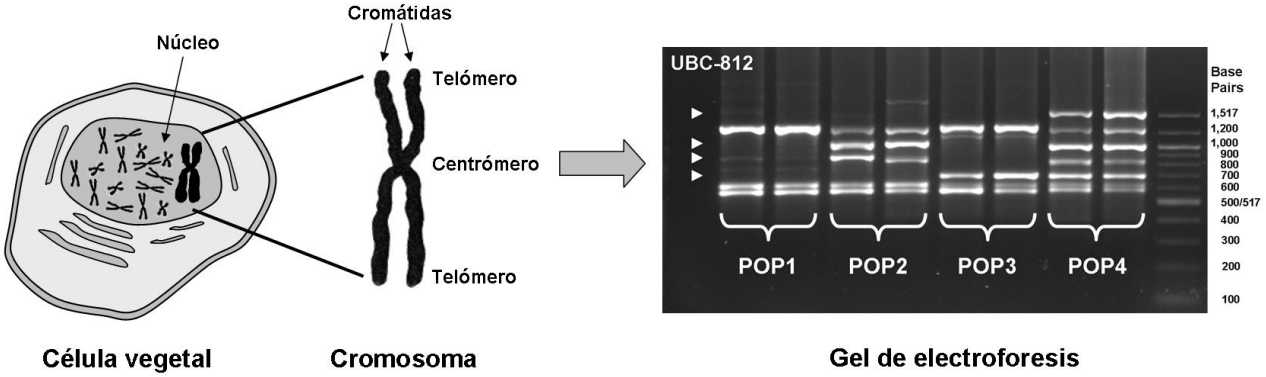

Figura 5. Representación esquemática de la estructura de un cromosoma vegetal y patrón de bandas obtenido con técnicas de marcadores moleculares.

Los resultados moleculares, morfológicos y citométricos obtenidos en el presente capítulo, nos llevan a sistematizar *R. parnassiifolius* subsp. *cabrerensis* como una especie independiente, así como considerar que *R. parnassiifolius* subsp. *muniellensis* debe ser considerado una raza de *R. cabrerensis*: *R. cabrerensis* subsp. *muniellensis*.

A la vista de los resultados obtenidos, surgen los Capítulos 4 y 5 enfocados en estudios de conservación genética en *R. cabrerensis s.l.* mediante el empleo de Amplificación de Fragmentos de Longitud Variable (AFLP: Amplified Fragment Length Polymorphism), Microsatélites Anclados (ISSR: Inter Simple Sequence Repeat) y ADN Polimórfico Amplificado al Azar (RAPD: Random Amplified Polymorphic DNA).

El Capítulo 6 aporta la primera filogenia molecular de *Ranunculus* grex *parnassiifolius* incluyendo también algunas especies de las sección *Ranuncella*. Dicha filogenia está basada en la región ITS del ADN nuclear y dos regiones del ADN plastidial (*rpl32-trnL*, *rps16-trnQ*). Se extraen conclusiones sistemáticas y biogeográficas a la luz de los resultados obtenidos y se somete a prueba la sistemática propuesta por Küpfer (1974).

Finalmente se enumeran las conclusiones más relevantes de la tesis y se proponen futuras líneas de investigación.

Summary

The genus *Ranunculus* was first described by Carl Linnaeus in 1753. The Latin name means "little frog" and makes allusion to the wet habitats in which some species grow. *Ranunculus* L. represents the largest genus within Ranunculaceae and comprises nearly 600 species, distributed from temperate to artic/subantartic zones, and rarely in the tropics where it is restricted to high mountain areas. In the Iberian Peninsula around 65 species are recognized, a quarter of which are endemic.

Since the first worldwide classification of *Ranunculus* by Candolle in 1824, the genus has been revised on several occasions but delimitation and classification at the generic and infrageneric level is still far from complete. Nowadays, the use of molecular techniques and the establishment of international links of scientific cooperation are helping to untangle the phylogenetic affinities and complex evolutionary history of this genus. The aim of this PhD Thesis was to study the phylogenetic relationships within the polyploid complex *Ranunculus parnassiifolius*.

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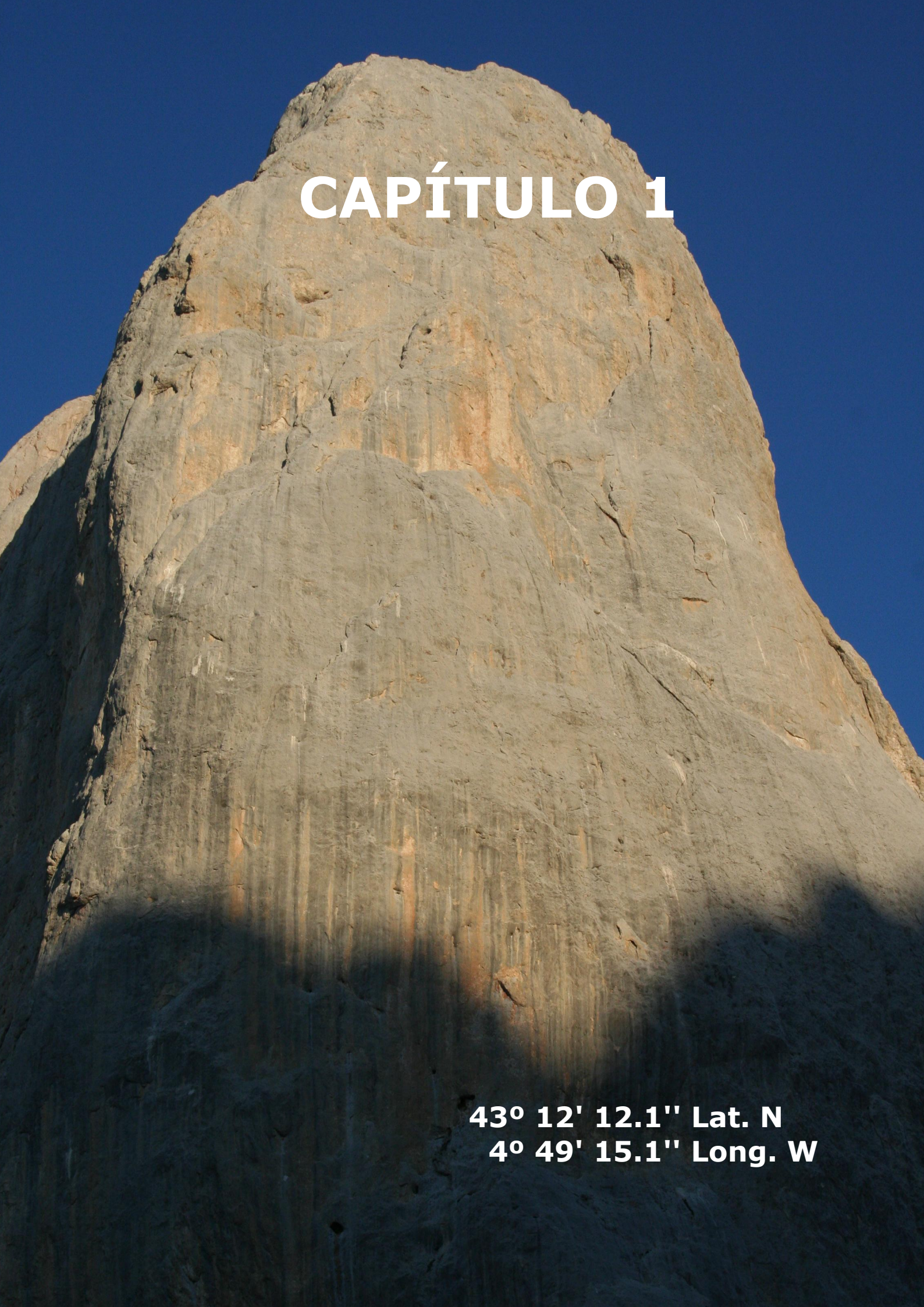

CAPÍTULO 1

43º 12' 12.1'' Lat. N
4º 49' 15.1'' Long. W

Genome size variation and morphological differentiation within *Ranunculus parnassiifolius* group (Ranunculaceae) from calcareous screes in the Northwest of Spain

Summary

Ranunculus parnassiifolius is an orophilous plant distributed throughout Central and Southwestern Europe (Alps, Pyrenees and Cantabrian Mountains). Its evolutionary history and taxonomy are often complicated, having been little studied before now. The purpose of this article is to present flow cytometry measurements and multivariate morphometric analyses to ascertain cytotype distribution patterns and the morphological differentiation of *R. parnassiifolius s.l.* from calcareous screes in the Northwest of Spain. DNA ploidy level and morphometric analysis were determined for plants of *R. parnassiifolius s.l.* using flow cytometry (112 individuals) and multivariate analysis (152 individuals). Specimens were collected in eight localities in the Northwest of the Iberian Peninsula. Different sample preservation methods (fresh, frozen, and herbarium specimens) were employed as well as the use of various buffers and internal standards, in order to test the reproducibility of DNA flow cytometry.

Three ploidy levels were detected in the study area (diploid, tetraploid, and pentaploid), and mixed-cytotype populations were also found. The mean nuclear DNA content of the *R. parnassiifolius* group ranged from 7.43 ± 0.185 to 7.63 ± 0.339 pg/2C in diploids and from 15.09 ± 0.161 to 15.85 ± 0.587 pg/2C in tetraploids. The analysis of the monoploid genome sizes (1Cx) did not reveal a clear difference among cytotypes. These results suggest low intraspecific variation, at least among the populations studied. In addition, a comparison of different DNA reference standards was conducted. A new value for the chicken genome size was used as internal reference standard (2C = 3.14 ± 0.155 pg), with similar results found using both animal and plant standards (*Pisum sativum* and *Solanum lycopersicum*). Finally, herbarium vouchers and frozen tissue were proved to be suitable for DNA ploidy level measurements.

This study provided a first assessment of C values in the *R. parnassiifolius* group using flow cytometry. The weak morphological distinction of the cytotypes and the existence of mixed-cytotype populations in the Northwest of Spain are reported here for the first time. The different distribution pattern of the two cytotypes is discussed.

Este capítulo se corresponde con la publicación:

- Cires, E., Cuesta, C., Peredo, E.L., Revilla, M.A., Fernández Prieto, J.A. (2009). Genome size variation and morphological differentiation within *Ranunculus parnassifolius* group (Ranunculaceae) from calcareous screes in the Northwest of Spain. *Plant Systematics and Evolution* 281: 193-208.

Introduction

Ranunculus L. represents the largest genus within Ranunculaceae and comprises nearly 600 species (Tamura 1993), distributed from temperate to artic/subantarctic zones (Ziman & Keener 1989), being rare in the tropics where it is restricted to high mountain areas (Hörandl *et al.* 2005). According to the systematics proposed by Cook *et al.* (1986), in the Iberian Peninsula around 65 species are recognized, a quarter of which are endemic. They are divided into three subgenera: *Ranunculus* subgen. *Batrachium* (DC.) Gray (8 species), *Ranunculus* subgen. *Ficaria* (Schaeff.) L.D.Benson (1 species), and *Ranunculus* subgen. *Ranunculus* L. This latter subgenus, *Ranunculus*, includes at least 56 species classified into 13 sections. One of them, the section *Ranuncella* (Spach) Freyn, comprises a group of plants spread throughout Central and Southwestern Europe and with a local presence in the North African Mountains (*Ranunculus calandrinioides* Oliv.) and in the Korab Mountains in the Republic of Macedonia (*R. wettsteinii* Dörfl.) (Jalas & Suominen 1989; Tutin & Akeroyd 1993). In the Iberian Peninsula, seven species from this section *Ranuncella* are represented (*Ranunculus abnormis* Cutanda & Willk., *R. amplexicaulis* L., *R. angustifolius* DC., *R. bupleuroides* Brot., *R. gramineus* L., *R. parnassiifolius* L., and *R. pyrenaeus* L.), most of them being endemic to small areas.

Traditionally, *R. parnassiifolius* has been considered an orophilous plant distributed through the Alps, the Pyrenees, and the Cantabrian Mountains (Tutin 1964); nevertheless, some authors studying distribution in different locations have attributed different taxonomic ranks (from varietal to species level) to the taxa *R. parnassiifolius* (Candolle 1817; Rothmaler 1934; Guinea López 1953). After obtaining data from across the range of *R. parnassiifolius s.l.*, Küpfer (1974) proposed the following systematics, which has been accepted by most authors (Cook *et al.* 1986; Jalas & Suominen 1989; Tutin & Akeroyd 1993): *R. parnassiifolius* subsp. *parnassiifolius* diploid (2n = 16), silicicole, endemic in the Eastern Pyrenees; *R. parnassiifolius* subsp. *cabrerensis* Rothm. diploid (2n = 16), silicicole, endemic to the mountains of Northwestern Spain; *R. parnassiifolius* subsp. *favargeri* P.Küpfer diploid (2n = 16), calcicole, endemic in the Picos de Europa (Cantabrian Mountains) and Western Pyrenees, and finally, *R. parnassiifolius* subsp. *heterocarpus* P.Küpfer tetraploid (2n = 32, 40), calcicole, spread throughout the Cantabrian Mountains, the Central Pyrenees and the Alps. Later, Bueno Sánchez *et al.* (1992) described another subspecies, *R. parnassiifolius* subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto as diploid (2n = 16), silicicole, with a single population in the Biological Reserve of Muniellos (Western Cantabrian Mountains). Despite the relatively small area covered by the Cantabrian Mountains, it may be considered to be the area with the highest global diversity of *R. parnassiifolius*, with four out of five of the recognized subspecies being found within it (Küpfer 1974; Cook *et al.* 1986; Bueno Sánchez *et al.* 1992).

Although many authors have reported the presence of *R. parnassiifolius* subsp. *favargeri* and *R. parnassiifolius* subsp. *heterocarpus* in the calcareous screes

of the Cantabrian Mountains (e.g. Küpfer 1974; Laínz 1976; Fernández Prieto 1983; Rivas Martínez *et al.* 1984; Nava 1988), chromosome counts from these natural populations have only been reported by Küpfer (1969, 1974). In the cited studies, a chromosome count was undertaken on a total of three plants (two tetraploid and one diploid) in very close locations in the Central Massif of the Picos de Europa, this being the first and only record in the Cantabrian Mountains. In all other published work, identification has been solely based on morphological characters provided by Küpfer (1974), such as the relationship between the number of stamens and carpels, the presence of microspores and carpels aborted, and the regularity of the corolla. However, although ploidy level is considered a differentiating feature among these taxa, there is little information about the within- and among-population variation in ploidy levels in natural populations of limestone-dwelling *R. parnassiifolius*.

Polyploidy is recognized as an important evolutionary phenomenon and a significant source of angiosperm diversity (Soltis *et al.* 2007), thus the geographical distributions of cytotypes, within species or between closely related species, provide useful insights into the evolutionary history of polyploid complexes (Kron *et al.* 2007; Perný *et al.* 2008; Ståhlberg & Hedrén 2008). Therefore, one aim of polyploidy research is to obtain better characterization of the distribution of cytotypes at various spatial and temporal scales. In recent years, a growing body of data has become available concerning the geographical distribution of cytotypes (e.g. Mandáková & Münzbergová 2008; Perný *et al.* 2008; Španiel *et al.* 2008). Nevertheless, even if the cytotypes represent distinct biological entities, present taxonomic treatments largely rely on a morphology-based concept, and assessment of their morphological differentiation is therefore still highly relevant (Soltis *et al.* 2007).

Flow cytometry (FCM) has become a frequently used method in the estimation of DNA ploidy and genome size in plants. The basic process of plant FCM is as follows: isolation of nuclei from plant material, staining of their DNA with fluorochrome, followed by analysis of their fluorescence emission. It is a convenient method that can provide useful information for phylogenetic and taxonomic purposes and is proving to be a useful tool in delimitation between some species that are sometimes not so easily distinguished using morphoanatomic analyses (e.g. Loureiro *et al.* 2007; Perný *et al.* 2008). However, it is widely accepted that FCM has some drawbacks. A common problem is related to the difficulty of the estimation of DNA content for various reasons, such as the isolation of insufficient intact nuclei for analysis, the aggregation of fluorescent debris particles to the surfaces of isolated nuclei, or the decreasing resolution of DNA content histograms. Considering that the estimation of DNA content in absolute units requires internal standardization and that it is necessary to have a close but non-overlapping genome size in relation to the target species, the use of different plant and animal standards in different studies has to be taken into account when comparing results. In short, some striking discrepancies between FCM estimations of genome sizes in different laboratories may be explained by the use of different reference standards, sample preparation

and staining protocols, and flow cytometers (Doležel *et al.* 1998). In fact, these differences in peak ratios obtained in different laboratories play an important role in the correct estimation of plant genome size. Additionally, the buffer composition is crucial for obtaining precise, reliable, and high-resolution results; hence a first step in studying FCM is to test different buffers, selecting the most suitable. According to Loureiro *et al.* (2006b), the most appropriate buffer depends on the species, the tissue type, and the presence of cytosolic compounds that interfere with DNA staining.

Based on the current knowledge of the *R. parnassiifolius* group in the Cantabrian Mountains, we addressed the following questions: (1) what is the frequency of the different cytotypes? (2) are diploids and tetraploids morphologically differentiated? If so, which morphological characters contribute to their differentiation? (3) are there any mixed-cytotype populations in the study area? and (4) what is the spatial distribution pattern of both cytotypes in this geographical area? In addition, it is hoped that this work will contribute to a more thorough knowledge of FCM in plant cells of the *R. parnassiifolius* group of the Cantabrian Mountains, through the evaluation of different sample preservation methods (fresh, frozen, and herbarium vouchers), as well as the use of various buffers and internal standards for the estimation of nuclear DNA content in the studied populations and for testing the reproducibility of DNA flow cytometry for genome size estimation.

Materials and methods

Study area and plant material

The studied populations of *R. parnassiifolius* are situated in the Cantabrian Mountains, located in the Northwest of Spain and running from east to west, parallel to the Atlantic coast (at a distance from the coast never exceeding 80 km) (Figure 1A-B). The climate of the area is temperate and humid, and at higher levels, especially in the oro-template belt (subalpine zone), snow cover extends from the middle of autumn until at least early spring. In the northeastern area of these Cantabrian Mountains, dominated by paleozoic limestones, are the Picos de Europa, an area divided into three independent massifs: the Western Massif (El Cornión), the Central Massif (Los Urrieles), and the Eastern Massif (Ándara). All of them are almost totally calcareous, with peaks of around, or even over, 2500 m. It should be noted that, although separated geographically from the Picos de Europa, Peña Ubiña and Picos Albos also form part of the Cantabrian Mountains. In all three areas, the rock type and the extreme climatic variations cause strong freeze-thaw processes, with resulting gravels and pebbles abundant in a wide extension along the mountainside.

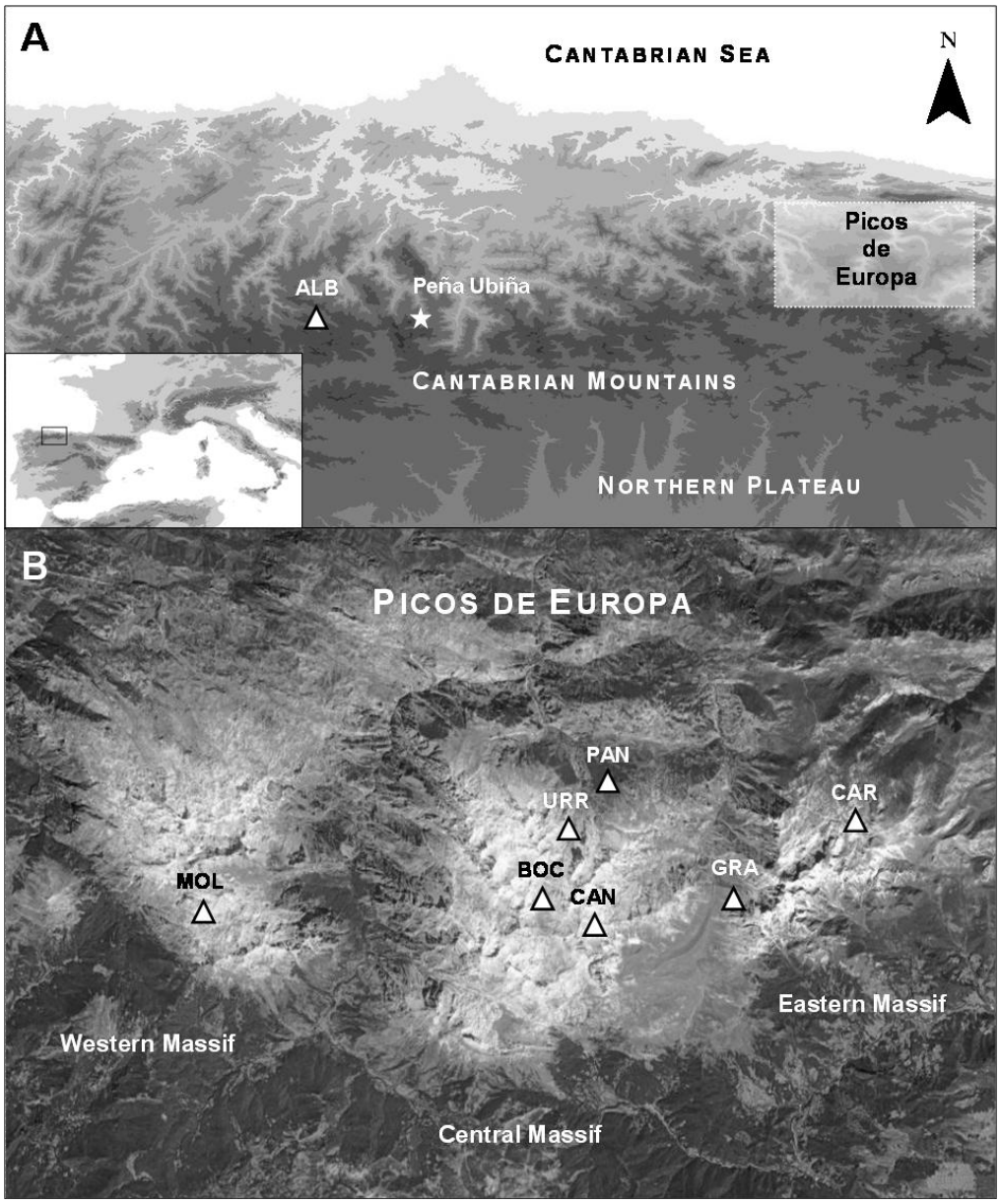

Figure 1. Map of the study area. A) Geographical distribution of *R. parnassiifolius s.l.* in the Cantabrian Mountains, including the sampled area of Picos Albos (ALB). B) Sampled territories in the Picos de Europa. Triangles (△) refer to localities of sample collection (BOC, CAN, CAR, GRA, MOL, PAN, URR). Image modified from Google Earth 4.0.

Plant samples of eight populations of *R. parnassiifolius s.l.* were collected; one population from Picos Albos (Somiedo Natural Park, Biosphere Reserve) and the other seven populations from the three massifs of the Picos de Europa (Picos de Europa National Park, Biosphere Reserve) (Table 1). Sampling was carried out during the summers of 2007 and 2008, between the months of May and July. Geographical coordinates were recorded in the field (Garmin-Etrex GPS instrument), and topographic information was derived from the geographic information system (GIS), the resulting data being processed with GIS software (ArcGis 9.2, ESRI, Inc. 2006). The identification of each plant was verified by consulting the original description of the species and other relevant literature. As a rule, 15-20 plants from

each population were sampled. Efforts were made to avoid collecting samples originating from one clone (usually plants growing very close together in a very small area of several square centimeters). Field-collected specimens were potted and kept in a greenhouse at room temperature and are currently maintained as a collection of living plants at the Botany Area of the University of Oviedo. Leaves taken from plants at the site were wrapped in moistened paper enclosed in a plastic bag and transported in a cool box to avoid high temperatures during transportation; then frozen plant tissues were stored at -80 ºC. Additionally, voucher specimens were collected, dried by pressing in absorbent paper, stored at room temperature, and kept in the Herbarium of the University of Oviedo (FCO).

Table 1. Collection locations of the *R. parnassiifolius s.l.* samples.

Acronym	Locality and collector	Zone	Coordinates	Altitude (m a.s.l.)	Date	Voucher specimens
ALB	Massif of Picos Albos, above Lago Cerveriz (Lagos de Saliencia, Somiedo; Asturias), *EC & JAFP*	29T	x = 734311 y = 4770131	1946	2007-06-18 2008-07-01	31103 31104
BOC	Central Massif of the Picos de Europa, Jou de los Boches (Cabrales; Asturias), *EC*	30T	x = 351469 y = 4783099	2136	2007-07-13 2008-07-10	31105 31106
CAN	Central Massif of the Picos de Europa, Collado de la Canalona (Camaleño; Cantabria), *EC*	30T	x = 352498 y = 4782342	2455	2007-07-11 2008-07-07	31107 31108 31109 31110
CAR	Eastern Massif of the Picos de Europa, Collado de San Carlos (Camaleño; Cantabria), *EC*	30T	x = 362152 y = 4785130	2050	2008-07-30	31111 31112
GRA	Eastern Massif of the Picos de Europa, Canal de las Grajas (Camaleño; Cantabria), *EC*	30T	x = 357333 y = 4782264	1688	2008-07-30	31113
MOL	Western Massif of the Picos de Europa, Los Moledizos (Posada de Valdeón; León), *EC*	30T	x = 340430 y = 4782283	2043	2008-07-30	31114
PAN	Central Massif of the Picos de Europa, pathway from Pandébano to Vega de Urriellu. Opposite Jou Lluengu (Cabrales; Asturias), *EC*	30T	x = 352622 y = 4786134	1632	2007-07-12 2008-05-20	31115 31116
URR	Central Massif of the Picos de Europa, Vega de Urriellu just past the refuge J.D. Ubeda in the direction of Horcados Rojos (Cabrales; Asturias), *EC*	30T	x = 352074 y = 4785006	1967	2007-07-12 2008-07-10	31117 31118 31119

Geographical coordinates and altitudes are in accordance with European 1950 and Universal Transverse Mercator datums. Collectors: *EC* - E. Cires; *JAFP* - J.A. Fernández Prieto.

Genome size estimations using flow cytometry

An assessment of nuclear isolation buffers was previously carried out, comparing the most common buffers of different chemical compositions: LB01 [15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 15 mM of *ß*-mercaptoethanol, 0.1% (v/v) Triton X-100, pH 7.5] (Doležel *et al.* 1989), Otto's [Otto I: 100 mM citric acid monohydrate, 0.5% (v/v) Tween 20 (pH approx. 2-3); Otto II: 400 mM $Na_2HPO_4.12H_2O$ (pH approx. 8-9)] (Otto 1990; Doležel & Göhde 1995) and Tris.$MgCl_2$ [200 mM Tris, 4 mM $MgCl_2.6H_2O$, 0.5% (v/v) Triton X-100, pH 7.5] (Pfosser *et al.* 1995).

In order to compare the performance of these nuclear isolation buffers, a set of parameters was selected to evaluate sample quality. Among the parameters chosen, the coefficient of variation of DNA peaks (CV = standard deviation/peak mean × 100%) was of major importance. In analyzing each plant, the CV of the standard and the sample were calculated, considering 5.0% as the maximum acceptable CV value in plant DNA flow cytometry (Greilhuber *et al.* 2007). In order to check the presence of substances that interfere with the intercalating fluorescent dye, all the plant species used for the DNA content determination were tested by FCM; an inhibitor test was conducted (Greilhuber 2008), putting the samples one on top of another in a sandwich-like fashion and chopping into sections of both materials (standard and sample).

For FCM analyses, nuclear suspensions were prepared according to the protocol of Galbraith *et al.* (1983). A new razor blade was used to simultaneously chop 150 mg of leaf tissue of *R. parnassiifolius s.l.* and 50 mg of internal standard leaves in a glass Petri dish containing 1 mL of ice-cold LB01 nuclear isolation buffer (the buffer having been stored frozen at -20 °C in aliquots for convenient use). The samples were maintained at ice-cold temperature following isolation of nuclei in order to decrease nuclease activity. Then, the nuclear suspension was filtered through a 42 µm nylon filter to remove large debris, and 50 µg mL^{-1} of propidium iodide (PI, Sigma) was added to the samples to stain the DNA. As propidium iodide is an intercalating fluorescent dye that binds to DNA and double-stranded RNA, samples were treated with RNase (50 µg mL^{-1}, Sigma) to avoid the staining of double stranded RNA. After mixing well, the samples were put on ice and kept in darkness for a 30-min period and then analyzed. Experiments were carried out using a Cytomics FC 500 (Beckman Coulter) with 488-nm excitation from an argon ion laser. Data analysis was carried out using Cytomics RXP Analysis (Beckman Coulter, Inc. 2006).

At least three individuals from each population were analyzed on three different days to avoid errors due to instrumental drift, and at least 5000 nuclei were analyzed per sample. The holoploid genome size (2C; sensu Greilhuber *et al.* 2005) of *R*. *parnassiifolius* subspecies was estimated according to the following formula:

2C nuclear DNA content (pg)

$$= \frac{\textit{Ranunculus}\ \text{sp.G0/G1 peak mean}}{\text{reference standard G0/G1 peak mean}}$$ x nuclear DNA content of reference standard

The monoploid genome size (1Cx; sensu Greilhuber *et al.* 2005) of all plants was also calculated in mass values (pg) and Mbp (1 pg = 978 Mbp, Doležel *et al.* 2003). Due to very low mitotic activity in *R. parnassiifolius* leaf tissues (as indicated by the absence or low incidence of peaks corresponding to nuclei in G2 phase) and the lack of endopolyploidy, potential bias in DNA ploidy level estimation can be excluded.

DNA reference standards

Plant and animal standards were used for the present work. *Pisum sativum* cv. 'Ctirad' (2C = 9.09 pg of DNA, Doležel *et al.* 1998) and *Solanum lycopersicum* cv. 'Stupicke' (2C = 1.96 pg of DNA, Doležel *et al.* 1992), both of which have a genome size close to that of *R. parnassiifolius s.l.* samples, were used as internal reference standards for all the studied species to minimize potential instrument non-linearity. Seeds from both cultivars were supplied by the Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany (Olomouc, Czech Republic), and germinated in a greenhouse at 22 ± 2 ºC.

Additionally, nuclei from chicken red blood cells (CRBC; 2C = 2.33 pg of DNA, Galbraith *et al.* 1983), kept at the Flow Cytometry Area (Scientific-Technical Services, the University of Oviedo), were used for plant DNA flow cytometry analysis. Fixed CRBC were self-prepared and stored at low temperatures (J. Doležel, unpublished results; http://lmcc.ieb.cz/research/protocols.php?protocols=particles). Only one male domestic chicken (*Gallus gallus domesticus*) was used throughout the analysis, and the nuclei from the animal reference standard were added after preparing plant nuclei suspension.

Morphometric measurements

Materials from all localities (20 plants per population) were subjected to multivariate morphometric study. Characters measured or scored, always made on fresh specimens, are listed in Table 2. The selection of the morphological characters to be scored followed a literature review of previous taxonomic studies on this species and a preliminary examination of herbarium material. They included characters traditionally used for differentiation of the two recognized taxa/cytotypes according to accepted determination keys and floras. For logistical reasons, it was not possible to collect samples of each population in exactly the same flowering and/or fructification period. In addition to this, in some specimens, it was not possible to measure all the characters because the specimen either did not contain enough flowering individuals or had been damaged by grazing. These facts should be taken into consideration in the interpretation of the morphometric analysis results.

Statistical analysis

Genome size data obtained by FCM and differences among standards were analyzed using Kruskal-Wallis one-way ANOVA on ranks, and Dunn's method for pair-wise comparison. For each species, within-population differences in nuclear DNA content and differences among sample preservations were analyzed (when applicable) and compared using a one-way ANOVA procedure, and a Holm-Sidak multiple comparison test was used for pair-wise comparison. Basic statistical measures for the morphometric analysis were evaluated using Mann-Whitney rank

sum test. All statistical studies were performed using Statistica 7 (StatSoft, Inc. 2004).

Table 2. List of characters measured and used in morphometric analyses of *R. parnassiifolius s.l.* samples.

Continuous quantitative characters:	
LL	lamina length of the basal stem leaf
LW	lamina width of the basal stem leaf
LS	length of sepals
WS	width of sepals
LP	length of petals
WP	width of petals
LA	length of achene
WA	width of achene
Discrete quantitative characters:	
NF	number of flowers
NS	number of sepals
NP	number of petals
Ratio characters:	
LLLW	lamina length of the basal stem leaf / lamina width of the basal stem leaf
LSWS	length of sepals / width of sepals
LPWP	length of petals / width of petals
LAWA	length of achene / width of achene
Binary characters:	
SL	base shape of the basal leaves (0: ovate-subcordate; 1: broadly cordate)
IAD	Indumentum of adaxial surface of basal leaves (0: basal leaves uniformly hairy above; 1: basal leaves more densely hairy on the veins than elsewhere above or occasionally only at base)
IAB	Indumentum of abaxial surface of basal leaves (0: basal leaves hairy beneath; 1: basal leaves glabrous beneath, except sometimes at base)
TI	Type of inflorescence (0: dense cyme; 1: open cyme)
IS	Indumentum of sepals (0: sepals densely villous; 1: sepals glabrous or sparsely hairy)
FS	Floral regularity (0: corolla usually irregular, with unequal or absent petals; 1: corolla more or less regular)
CL	Color of petals (0: petals white; 1: petals usually pinkish white)
SA	Surface of achene (0: achenes strongly veined; 1: achenes smooth or with inconspicuous veins)
NSTC	number of stamens / number of carpels (0: ≤1, 1: >1)

In terms of morphometric measurement, exploratory data analysis was used for each DNA ploidy level to obtain basic statistics related to quantitative and ratio characters (mean, standard deviation, minimum and maximum values, 5th and 95th percentiles). The morphometric information based on quantitative, binary, and ratio characters was analyzed using Principal Coordinates Analysis (PCoA) and Principal Components Analysis (PCA), techniques commonly used in numerical classifications. These methods reduce the dimensions of the original data and allow visual interpretation of the relationships. The principal components of PCA are linear combinations of the original descriptors. In contrast, principal coordinates in PCoA are complex functions of the original descriptors mediated through a dissimilarity measure. Therefore, the relative position of the points is an indication of their taxonomic relationship. For the PCoA, a similarity matrix (*S*) was first built, using the Gower similarity coefficient (Gower 1971) that permits simultaneous work on qualitative and quantitative characters. Then, a dissimilarity (or distance) matrix (*D*) was built. Distance and similarity are intimately connected through a set of

mathematical formulas, where *D* = 1-*S* (*D*: distance; *S*: similarity) is the simplest (Escudero *et al.* 1994). Based on this new matrix, a PCoA was carried out in order to display the "natural groups" that might appear in the sample and identify the factors that may be responsible for such clusters. Different PCoA and PCA were run, including all vegetative (LL, LW, LLLW, SL, IAD), floral (LS, WS, LP, WP, NF, NS, NP, LSWS, LPWP, TI, IS, FS, CL, NSTC) and/or fructification characters (LA, WA, LAWA, SA) (see Table 2). In addition, multivariate analysis methods were performed using Ginkgo version 1.5.8 software (Bouxin 2005), the statistical module from the VegAna package (http://biodiver.bio.ub.es/vegana), that provides several tools for editing and analyzing flora and vegetation.

Results

Nuclear DNA content estimations

The 2C nuclear DNA content of 112 specimens of *R. parnassiifolius s.l.* was determined using FCM, providing histograms with well-defined peaks of both sample and internal reference standards (CRBC) (Table 3).

Table 3. Nuclear DNA content estimations of the *R. parnassiifolius* group studied in this work.

		Nuclear DNA Content							
			2C range						
Population	Ploidy Level	2C (pg)	Min.	Max.	Dif. Pop.	1Cx (pg)	1Cx (Mbp)[1]	CV (%)	*n*
ALB	4x	15.85 ± 0.587	14.76	16.81	a	3.96 ± 0.147	3877	4.37	13
	5x	20.45 ± 0.975	19.32	21.04	b	4.09 ± 0.195	4000	5.33	3
MOL	4x	15.30 ± 0.432	14.74	15.94	a	3.82 ± 0.108	3743	4.52	10
PAN	2x	7.54 ± 0.145	7.24	7.74	c	3.77 ± 0.072	3689	3.34	16
URR	2x	7.43 ± 0.185	7.19	7.75	c	3.71 ± 0.092	3636	3.98	14
	4x	13.94	-	-	a	3.48	3409	4.57	1
BOC	2x	7.63 ± 0.339	7.13	8.32	c	3.81 ± 0.170	3734	4.64	15
CAN	2x	7.57 ± 0.471	7.08	8.53	c	3.78 ± 0.235	3705	3.79	14
GRA	4x	15.25 ± 0.463	14.73	16.18	a	3.81 ± 0.116	3729	3.27	10
CAR	4x	15.09 ± 0.161	14.79	15.31	a	3.77 ± 0.040	3689	3.82	16

The values are given as mean and standard deviation of the holoploid nuclear DNA content (2C in pg) of individuals of each population. The 2C range is defined by the minimum (Min.) and maximum (Max.) value obtained for each population. The monoploid nuclear DNA content (1Cx) in mass values (pg) and Mbp, the mean sample coefficient of variation of G0/G1 DNA peak (CV, %) and the number of analyzed individuals (*n*) are also provided for each population. Differences among populations (Dif. Pop.) were analyzed using Kruskal-Wallis one-way ANOVA on ranks and Dunn's method. Differences within populations were analyzed using a one-way ANOVA procedure and a Holm-Sidak multiple comparison test. Populations identified by the same letter are not statistically different ($P < 0.001$).

Internal reference standard: CRBC 2C = 3.14 pg of DNA (this study).

[1] pg = 978 Mbp (Doležel *et al.* 2003).

Three different DNA ploidy levels were revealed in the study area: diploid (2n ~ 2x, 52.67%), tetraploid (2n ~ 4x, 44.64%) and pentaploid (2n ~ 5x, 2.67%). Mean holoploid genome size of diploid plants (2C) ranged from 7.43 pg/2C in URR to

7.63 pg/2C in BOC, whilst in tetraploid plants the holoploid genome size ranged from 15.09 to 15.85 pg/2C in CAR and ALB, respectively. Statistical analyses revealed significant differences between DNA ploidy level in all tested populations ($P < 0.001$). A high homogeneity of the values within each ploidy was also observed. The occurrence of pentaploid plants deserves particular interest as it represents the second recorded incidence in the species.

Fluorescence histograms (Figure 2A-B) of relative nuclear DNA content showed diploid and tetraploid plants with CVs usually below 4.0% (3.97 ± 0.778, mean ± SD; n = 109). Indeed, 88.07% of the estimations presented a CV value below 5.0%, and in only 11.93% of cases were these values above 5.0% (CV maximum value 5.50%). Intraspecific variation in genome size within each cytotype was detected (2.71% in diploid and 5.12% tetraploid), but on overall examination, the analysis of the monoploid genome sizes (1Cx) did not reveal a clear difference between diploid and tetraploid level (Table 3), the low variability thus presumably being due to instrumental or methodological errors.

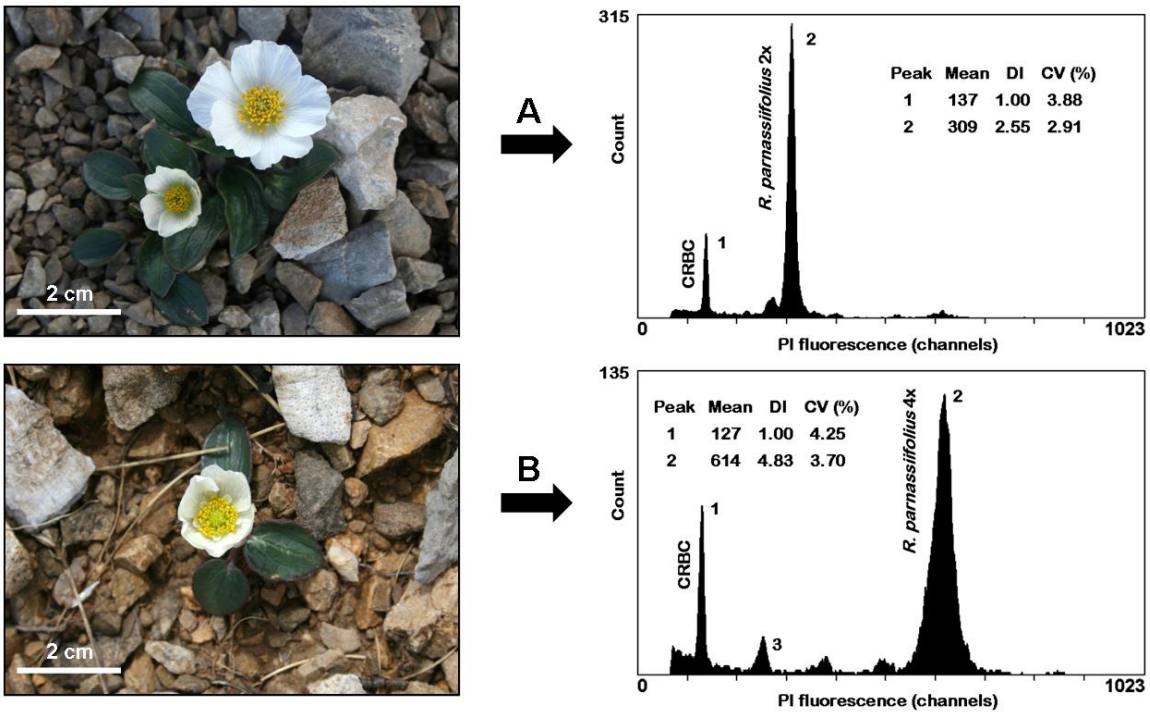

Figure 2. Morphological aspect and histograms of relative fluorescence intensity obtained after analysis of propidium-iodide-stained nuclei of *R. parnassiifolius s.l.* A) Diploid plant from CAN, Central Massif of the Picos de Europa. B) Tetraploid plant from ALB, Somiedo. The peaks marked with 1 and 2 indicate nuclei at the G0/G1 phase of the internal standard and the G0/G1 phase of the sample, respectively. The mean channel number (PI fluorescence), DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given. In the histogram in B, the number 3 corresponds to G2/M peaks.

Comparison of different DNA reference standards

For the estimation of the genome size in absolute units, an internal standard of "known" genome size was co-processed with the specimen. In this way, internal standardization eliminates the risk of error due to variations in sample preparation and instrument instability. In the resulting flow histogram, the ratio of specimen/standard peak means was calculated and was then multiplied by the absolute genome size of the reference standard to give the absolute genome size of the specimen. Therefore, as accurate a genome size of internal standard as possible should be used in order to compare the results with other works. The nuclear DNA content of *R. parnassiifolius s.l.* samples was initially determined by assuming the value of CRBC nuclei as 2C = 2.33 pg of DNA (Galbraith *et al.* 1983). However, taking into account the different values presented in the literature for chicken genome size (Greilhuber *et al.* 2007), we decided to calculate the size of the chicken blood used by our research group. For this purchase, *Pisum sativum* cv. 'Ctirad' (2C = 9.09 pg of DNA, Doležel *et al.* 1998; *n* = 10) and *Solanum lycopersicum* cv. 'Stupicke' (2C = 1.96 pg of DNA Doležel *et al.* 1992; *n* = 10) were used as internal standards. The new value for the CRBC used in our laboratories was 2C = 3.14 ± 0.155 pg with 2.93 and 3.43 pg as maximum and minimum values, respectively, (CV = 3.73%; *n* = 20). After that, plant and animal standards were compared with each other to calculate the nuclear DNA content in populations of *R. parnassiifolius s.l.* and to test the reproducibility of FCM in the DNA genome size estimation.

Table 4 shows the mean and standard deviation of the holoploid (2C) and monoploid (1Cx) nuclear DNA content in mass values (pg) of ploidy level with different standards, and also the CV and minimum and maximum values obtained for each ploidy. Clear differences were observed when using different standards in the estimation of DNA content in absolute units, for example, comparing the Galbraith's value and the new value for the CRBC (Table 4).

Table 4. Comparison of plant and animal DNA reference standards in cytotypes of *R. parnassiifolius s.l.*

Cytotype	Standard	2C (pg)	Min.	Max.	Dif. Standards	1Cx (pg)	CV (%)	*n*
Diploid	CRBC[1]	5.60 ± 0.227	5.25	6.33	a	2.80 ± 0.113	3.93	59
	PS	7.68 ± 0.372	6.99	7.97	b	3.84 ± 0.186	3.50	10
	SL	7.21 ± 0.391	6.77	8.01	b	3.60 ± 0.195	3.24	10
	CRBC[2]	7.55 ± 0.306	7.09	8.54	b	3.78 ± 0.153	3.93	59
Tetraploid	CRBC[1]	11.41 ± 0.382	10.93	12.48	a'	2.85 ± 0.095	4.03	49
	PS	14.99 ± 0.178	14.68	15.42	b'c'	3.74 ± 0.044	3.31	17
	SL	13.82 ± 0.305	13.08	14.46	c'	3.42 ± 0.200	3.40	22
	CRBC[2]	15.37 ± 0.514	14.75	16.84	b'	3.84 ± 0.129	4.03	49

Differences among standards (Dif. Standards) within cytotypes were analyzed using a Kruskal-Wallis one-way ANOVA on ranks, and a Dunn's method. Standards identified by the same letter are not statistically different ($P < 0.001$).

CRBC[1]: chicken red blood cells, 2C = 2.33 pg of DNA (Galbraith *et al.* 1983); PS: *Pisum sativum* cv. 'Ctirad', 2C = 9.09 pg of DNA (Doležel *et al.* 1998); SL: *Solanum lycopersicum* cv. 'Stupicke', 2C = 1.96 pg of DNA (Doležel *et al.* 1992); CRBC[2]: chicken red blood cells, 2C = 3.14 pg of DNA (this study).

Although plant standards are preferable for determination of plant nuclear DNA content in absolute units (J Doležel, Institute of Experimental Botany, Olomouc, Czech Republic, personal communication), the new value for CRBC used in our laboratory is an adequate standard for ploidy determinations and is convenient because a single blood sample can be used for all runs.

Sample preservation

The feasibility and quality of FCM measurements largely depend on the species/tissue analyzed, the age of the material, and the storage conditions. Although fresh material is normally used, the potential use of frozen leaves and desiccated tissues may be an attractive alternative for many species and functions. It is also important to consider that, sometimes, the analysis of the tissue collection is quite difficult, especially when the samples are taken in distant areas, so the use of dehydrated and frozen plant tissues can be very useful for sample preservation in field botany. As a result, the relative nuclear DNA content of fresh plant material versus frozen and herbarium specimens from *R. parnassiifolius s.l.* was analyzed (Table 5). As expected, peak attributes gradually changed with the type of material; fresh material provided smaller CVs and higher yields of intact nuclei while background fluorescence became more prominent with frozen and herbarium material. Analysis of fresh leaves typically yielded histogram results with a single prominent DNA peak, while histograms from desiccated tissues and frozen leaves presented peaks with poor resolution. The mean CV of fresh and non-fresh material ranged from 3.24 to 5.97% in diploid and from 3.31 to 5.24% in tetraploid.

Table 5. Comparison of relative nuclear DNA contents from different sample preservation types of *R. parnassiifolius s.l.*

Cytotype	Sample Preservation	Standard	2C (pg)	Dif. Sample	CV (%)	*n*
Diploid	Fresh material	SL	7.21 ± 0.391	a	3.24	10
	-80 ºC material (9 months old)	SL	7.25 ± 0.132	a	4.22	10
	Herbarium (6 months old)	SL	8.16 ± 0.519	b	5.97	5
	Herbarium (12 months old)	SL	No signal	-	-	5
Tetraploid	Fresh material	PS	14.99 ± 0.178	a’	3.31	17
	-80 ºC material (9 months old)	PS	16.44 ± 0.234	b’	4.93	10
	Herbarium (6 months old)	PS	16.27 ± 0.479	b’	5.24	5
	Herbarium (12 months old)	PS	No signal	-	-	5

Pisum sativum cv. ‘Ctirad’ was used as the standard tetraploid cytotype due to its genome size being close to that of *Ranunculus* samples. In the diploid cytotype, *Solanum lycopersicum* cv. ‘Stupicke’ was employed to avoid the peaks of the standard overlapping with the peaks of the unknown sample. Differences among sample preservation types (Dif. Sample) within cytotypes were analyzed using a Holm-Sidak multiple comparison test. Standards identified by the same letter are not statistically different ($P < 0.001$).

PS: *P. sativum* cv. ‘Ctirad’, 2C = 9.09 pg of DNA (Doležel *et al.* 1998); SL: *S. lycopersicum* cv. ‘Stupicke’, 2C = 1.96 pg of DNA (Doležel *et al.* 1992).

Fresh material showed less debris than that from the frozen/dehydrated samples, and the nuclei count was higher (Figure 3A-D). The low nuclear count, the presence of debris, and the reported high CV for non-fresh material, are presumably

due to mechanical damage caused to the cells by storage at -80 ºC and possible interference by cytosolic compounds. However, the existence of debris did not affect the determination of DNA ploidy levels of *R. parnassiifolius s.l.* Herbarium vouchers of up to 6 months old seemed to be suitable for cytometric determination. However, in older individuals, more than 12 months old, or poorly preserved specimens, measurement result were highly degraded (high side scatter) or even lacked a DNA signal. Although desiccated tissues and frozen leaves of *R. parnassiifolius s.l.* often experience a certain decrease in fluorescence intensity after several months of storage, the reliability of DNA ploidy value is not compromised and fluorescence intensity of isolated nuclei was highly comparable with that of fresh material, allowing reliable DNA ploidy estimation (Figure 3A-D).

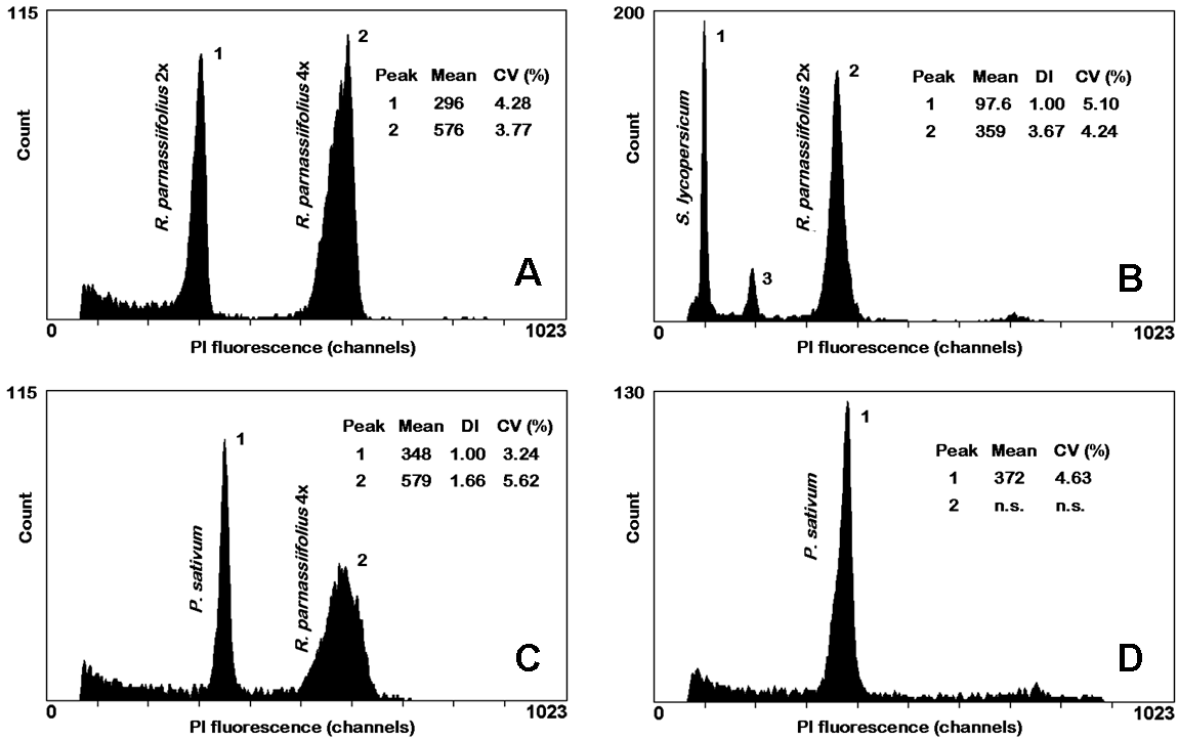

Figure 3. Comparison of flow cytometric histograms obtained from relative nuclear DNA content of fresh, frozen, and herbarium-specimen materials from *R. parnassiifolius s.l.* Changes in the quality of DNA signals and the reproducibility of the results are shown. Flow histograms show decreasing quality of signal and standard/sample ratio (A > B > C > D). A) Simultaneous multi-ploidy analyses of fresh green leaves from CAN and CAR. B) Frozen plant tissues (9 months old) from CAN. C) Young herbarium specimens (6 months old) from GRA. D) Damaged signal of old herbarium specimens (12 months old) from ALB. In the above histograms, the peaks are marked as follows: 1 - nuclei of internal standard at G0/G1 phase (except in histogram A where it corresponds to a multi-ploidy analysis); 2 - nuclei of sample at G0/G1 phase. The mean channel number (PI fluorescence), DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given. In histogram B, the number 3 corresponds to G2/M peaks, and in histogram D; n.s. means "no signal".

Morphometric measurements

Results of the exploratory data analysis of quantitative characters for diploid and tetraploid populations of *R. parnassiifolius s.l.* from the Cantabrian Mountains

are given in Table 6. The ranges of the measured characters of both DNA ploidy levels broadly overlapped, although showing significant differences between diploid and tetraploid cytotypes. These differences could be influenced by the date of sample collection. After using a one-way ANOVA procedure on all the morphological characters of each population (data not shown), the significant differences were attributable to ALB (Somiedo). These differences were detected between ALB and the rest of the populations, and no differences were recorded within the Picos de Europa, regardless of their ploidy level.

Table 6. Results of exploratory data analysis of DNA ploidy level in *R. parnassiifolius s.l.*

Character	Ploidy Level	Mean	SD	Min.	5%	95%	Max.	*n*
LL	2x	1.57	0.29	1.10	1.12	2.06	2.47	86
	4x	1.75	0.58	0.80	0.90	2.88	3.43	66
LW*	2x	0.94	0.22	0.57	0.63	1.28	1.70	86
	4x	1.15	0.43	0.50	0.60	1.94	2.23	66
LS*	2x	0.48	0.09	0.31	0.36	0.71	0.71	40
	4x	0.56	0.13	0.36	0.37	0.76	0.77	17
WS*	2x	0.26	0.07	0.14	0.17	0.41	0.43	40
	4x	0.37	0.09	0.25	0.25	0.53	0.53	17
LP*	2x	0.78	0.11	0.55	0.64	1.00	1.10	39
	4x	1.12	0.29	0.56	0.57	1.58	1.60	13
WP*	2x	0.69	0.13	0.47	0.50	0.92	1.11	39
	4x	0.90	0.23	0.45	0.45	1.17	1.17	13
LA*	2x	0.24	0.04	0.15	0.17	0.30	0.32	49
	4x	0.27	0.04	0.20	0.20	0.30	0.30	21
WA*	2x	0.15	0.03	0.09	0.10	0.20	0.21	49
	4x	0.18	0.03	0.10	0.13	0.20	0.20	21
NF*	2x	2.97	1.70	1.00	1.00	6.00	7.00	76
	4x	2.17	1.40	1.00	1.00	5.00	7.00	46
NS	2x	5.00	0.00	5.00	5.00	5.00	5.00	40
	4x	5.00	0.00	5.00	5.00	5.00	5.00	17
NP*	2x	5.30	0.52	5.00	5.00	6.00	7.00	40
	4x	4.77	0.44	4.00	4.00	5.00	5.00	13
LLLW*	2x	1.73	0.35	0.90	1.18	2.37	2.59	86
	4x	1.57	0.28	1.06	1.15	2.03	2.25	66
LSWS*	2x	1.89	0.44	1.10	1.14	2.72	2.84	40
	4x	1.59	0.38	1.08	1.12	2.34	2.52	17
LPWP	2x	1.15	0.22	0.74	0.80	1.60	1.81	39
	4x	1.27	0.26	0.96	0.97	1.85	1.88	13
LAWA	2x	1.69	0.39	1.00	1.17	2.37	2.78	49
	4x	1.54	0.30	1.00	1.00	2.00	2.00	21

For character abbreviations, see Table 2. Differences between cytotypes were analyzed using a Mann-Whitney rank sum test. Asterisks indicate statistical differences ($P < 0.001$).

Using an ordination diagram of PCoA with qualitative and quantitative characters, based on Gower's similarity coefficient, diploids from the Picos de Europa and tetraploids from ALB samples showed distinct groupings (Figure 4A). The first three axes accounted for 52.79% of the total variance (29.16, 13.27 and 10.35%,

respectively). However, analyzing the ratio characters in the previously-cited populations by PCA, the samples did not show distinct groupings and appeared to overlap (Figure 4B) with 40.09% of the total variability explained by the first characteristic vector. In addition, the plots of the centroids of the *R. parnassiifolius s.l.* samples, according to the two first dimensions of PCoA and including all vegetative and fructification characters, did not allow discrimination of the two cytotypes (Figure 4C). The first three axes accounted for 64.06% of the total variance (32.31, 17.99 and 13.75%, respectively). Furthermore, similar values were obtained with PCoA based on all vegetative, fructification, and floral characters from populations of higher and lower altitudinal distribution sampled (CAN and PAN), of which 75.65% of the variance (Figure 4D) was explained by three factors (37.82, 33.65 and 4.17%, respectively).

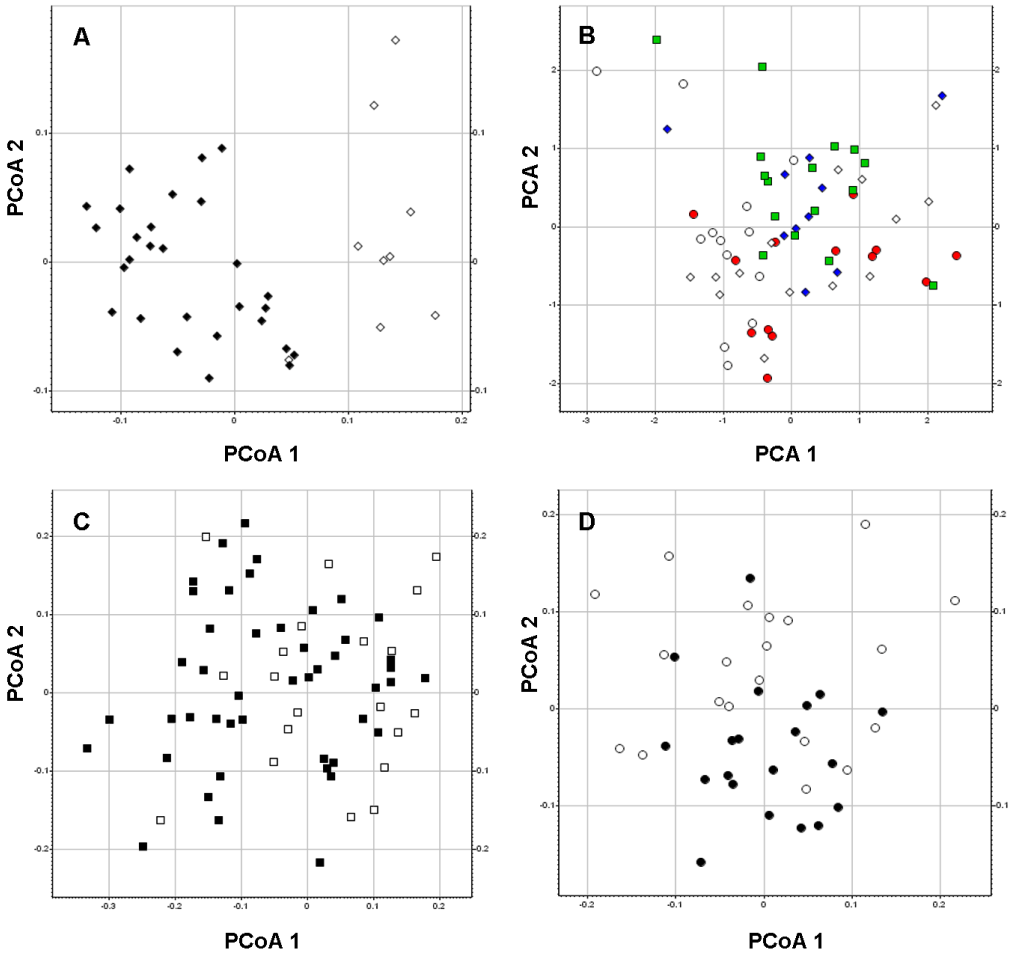

Figure 4. Principal Coordinates and Components Analyses of *R. parnassiifolius s.l.* cytotypes based on morphological characters (quantitative, ratio, and binary). The diploid and tetraploid samples were found to be intermingled. A) Floral and fructification characters from diploids (Picos de Europa, represented as filled diamonds:◆) and tetraploids (Somiedo, represented as open diamonds:◇). B) Ratio characters in the previously cited populations by PCA. The symbols correspond to BOC (filled circles:●), ALB (open circles:○), CAN (filled diamonds:◆), PAN (open diamonds:◇) and URR (filled squares:■). C) Vegetative and fructification characters from diploid (filled squares:■) and tetraploid (open squares:□). D) Vegetative characters between the populations of higher and lower altitudinal distribution sampled (CAN represented as filled circles:●, and PAN represented as open circles:○).

Hence, both PCoA and PCA based on vegetative, floral, and fructification characters were performed on all the Cantabrian material analyzed, giving similar results. No morphological character or combination of characters could distinguish the plants as belonging to different ploidy levels. Similar results were obtained when analyzing the significantly different characters alone.

The *R. parnassiifolius* group in the Cantabrian Mountains has similar morphotypes, with basal leaves that are subcordate-ovate and glabrous (except for the base and/or on the veins), glabrous or sparsely hairy sepals, smooth achenes, and in 96% of cases, the petals were white instead of pinkish white. Nevertheless some grouping tendencies can only be seen in the frequency of the occurrence of the number of stamens, e.g. tetraploid plants had a smaller number of stamens than diploid (see Figure 2A-B). However, there were no events of aborted carpels, and the corolla was similarly regular in all the samples tested. We would also like to point out that the phenotypic uniformity in the field was consistent with the low variation reported between cytotype populations.

Discussion

Within the framework of broader taxonomic and evolutionary investigation of the *R. parnassiifolius* group, we focused on unravelling the geographical distribution of ploidy levels both within and among populations in the Cantabrian Mountains, usually considered the western boundary of the European alpine chain. This area represents the southwestern boundary of the Atlantic biogeographical region (Ozenda & Borel 2000) and it has also been defined as a unique phytogeographical territory and is within the Eurosiberian region (Atlantic European province, Orocantabrian subprovince) (Rivas Martínez 2007). Prevailing climatic conditions, in both the past and present, have had a decisive effect on the flora and vegetation of the Cantabrian area (Fernández Prieto 1983). The existence of sympatric cytotypes of *R. parnassiifolius s.l.* on the limestone territory of the Massif Central of the Picos de Europa (Küpfer 1974) raises some questions worthy of analysis.

For the first time, nuclear DNA content for the *R. parnassiifolius* group has been analyzed by using FCM. Our results are consistent with previous cytological data and with those provided by Goepfert (1974), who calculated the relative nuclear DNA content of one tetraploid sample of *R. parnassiifolius* by cytophotometry with a value of 1C = 7.25 pg (Bennett & Leitch 2004). According to the basic chromosome number x = 8, it seems evident that the two *R. parnassiifolius* groups are characterized by 2n = 16 and 2n = 32 chromosomes. Over the years there have been numerous studies which attempted to correlate genome size with various ecological parameters (e.g. latitude, altitude, elevation), but after comparing the results, their findings seem to be contradictory (Knight *et al.* 2005). An altitudinal distribution model has been proposed for this species by Küpfer (1974), as

R. parnassiifolius subsp. *heterocarpus* is distributed in an altitudinal range between 1600 and 2200 m, being replaced by the diploid *R. parnassiifolius* subsp. *favargeri* at higher altitudes (i.e. 2450 m, such as CAN). What it is more, Nava (1988), in the study of some orophyte taxa from the Picos de Europa, stated that the tetraploid taxa are the most frequent, the diploid cytotype being much rarer. However, and as mentioned by Laínz (1976), we have found there to be no clear altitudinal distribution model, with diploid samples being found in the less elevated populations studied (such as PAN) and the frequency of both cytotypes seems to be quite similar. On the other hand, we have in fact found a pattern of spatial distribution within the Picos de Europa with the diploid cytotype only being located in the Massif Central, while the tetraploid was present in the other massifs (Western and Eastern).

It is worthy of note that this is the first time that mixed populations have been reported, demonstrating the co-existence of two different ploidies (2x + 4x in the Picos de Europa-URR; 4x + 5x in Somiedo-ALB) in the Cantabrian Mountains. Until now, the existence of the pentaploid cytotype (2n = 5x = 40) had only been confirmed in two locations in the Central Pyrenees (Küpfer 1974; Vuille & Küpfer 1985). Previous studies by Küpfer (1974) revealed the existence of tetraploid plants (two counts) in Peña Vieja and its surroundings. In addition, Rivas Martínez *et al.* (1984) indicated the presence of *R. parnassiifolius* subsp. *favargeri* (diploid) in the three massifs of the Picos de Europa. Based on this information, and considering the possible influence of plant phenology in the flowering of the two cytotypes, more comprehensive future samplings should be considered in such massifs, to determine a clearer distribution model.

The increased number of reports concerning mixed-ploidy populations highlights the interest generated in the evolutionary dynamics of polyploidy, as these populations provide conditions similar to the early stages of polyploid evolution (Suda *et al.* 2007a). The presence and extent of intraspecific variation in genome size has been a controversial topic over the last two decades (Doležel *et al.* 2007b), with intraspecific variation for some species having been verified (e.g. Creber *et al.* 1994; Sugiyama *et al.* 2002). However, the variability of nuclear DNA content within species has been dismissed by several reports applying better practice methodologies when conducting further investigations (Greilhuber 2005). Our data did not reveal a clear difference in monoploid genome sizes (1Cx) between diploid and tetraploid plants, supporting the findings of Smith & Bennett (1975), who suggested that the consequent doubling of the nuclear DNA content in tetraploid species resulted in minimal variation in Cx value.

For quite some time, there has been debate about the use of animal reference standards. In many studies, CRBC are used as the reference standard in FCM of nuclear DNA content in higher plants (Emshwiller 2002; Lim *et al.* 2006). However, their use has been called into question, mainly because there is no general agreement regarding the size and stability of the chicken genome and The Plant Genome Size Meeting in 1997 recommended not using it

(http://data.kew.org/cvalues/homepage.html). Even at a suitable genome size ratio to the unknown sample, CRBC are not considered to be an ideal standard due to their strong DNA compaction (Hardie *et al.* 2002) and, more importantly, because of differences in their fixation and storage history compared to that of the sample under investigation. In addition, an instability in genome size has been reflected by the results of several studies, with 2C values ranging from 1.88 (Chen *et al.* 2002) to 3.01 pg (Johnston *et al.* 1999), with intraspecific variation and sex-related differences also being found. For instance, male and female chicken genomes differ by 2.7% because of sex chromosomes (Nakamura *et al.* 1990), which may introduce additional, though minor, experimental errors. However, animal reference standards, in the form of a nuclear suspension, are easy to use, they can be bought commercially or self-prepared, and can be fixed and stored, often for years, at low temperatures. Therefore, the use of CRBC as an internal standard is not totally reliable unless the genome size of chicken used in each individual study has been previously validated, and its use should be strongly discouraged in works where precise estimations of genome size in absolute units are required.

Although the use of animal standards for plant samples may cast some doubt upon these estimates as absolute values, the use of a consistent methodology for all samples and the reproducibility of measurements of the same accessions, assuming the exact chicken genome size used is known, allow for comparisons of similarities and differences among species and populations. As such, we were able to obtain similar results using both animal and plant standards in the study of different populations of *R. parnassiifolius s.l.*

Although it was originally developed for fresh plant tissues, Galbraith's protocol (Galbraith *et al.* 1983) would seem to be suitable for the estimation of relative nuclear DNA content in herbarium (Suda & Trávníček 2006a, b), or fixed specimens (e.g. Lucretti *et al.* 1999) and seeds (e.g. Sliwinska 2006). There has also been significant progress in the use of frozen plant tissues (e.g. Hopping 1993; Nsabimana & van Staden 2006), though some difficulties have been detected using these types of sample preservation. Firstly, analysis of samples from frozen and dehydrated tissues usually results in DNA content histograms of lower resolution and with excessive background from debris (Doležel *et al.* 2007a). Secondly, in measuring reliable nuclear DNA amounts, CVs of the peaks should not exceed 5.0% (Greilhuber *et al.* 2007), the problem being that these preservation techniques often result in samples that have resultant CVs over this limit. Other problems are due to the chemical composition of the cytosol, as plant cells produce a vast array of secondary metabolites that may interfere with a particular assay, for example with the staining of nuclear DNA. In addition, autofluorescence of some cellular components (chloroplasts and cell walls) may mask the weak fluorescence of stained targets (Loureiro *et al.* 2008).

The use of dry samples and frozen tissue in investigations would open up many new possibilities for plant research and significantly increase the power of

FCM. The results of nuclear DNA content in herbarium samples of *R. parnassiifolius s.l.* are consistent with those presented by Suda & Trávníček (2006b), where it was found that samples of *R. repens* analyzed with FCM gave no signal at 20 months old. While the preparation of herbarium specimens of *R. parnassiifolius s.l.* up to 6 months of age showed a good signal, herbarium vouchers of more than 1 year exhibited unsatisfactory histogram quality. Furthermore, according to the same authors, the family Ranunculaceae is not FCM-friendly when estimating relative nuclear DNA content in dehydrated plant tissues (Suda & Trávníček 2006a). In recent years, the success of FCM studies utilizing desiccated plant material has increased considerably, but the use of frozen tissue has received less attention. In this study the feasibility of using *Ranunculus* leaves stored at -80 ºC for the analysis of ploidy using FCM with CV below 5.0% has been demonstrated. This fact is very important as the lifetime of deep freeze-stored samples is substantially longer than with other preservation methods, although the age limit of sample viability still needs to be determined. That there is a slight variation in the genome size of frozen tetraploid material with respect to its fresh counterpart (which is not seen with diploid material) should also be mentioned. This could be due to a lower hardiness or tolerance to cold of tetraploid individuals compared with diploid plants as has been seen in other perennial species (e.g. Sugiyama 1998). The decrease in cold tolerance in tetraploids seems to result from changes in plant cells such as membrane fluidity, protein and nucleic acid conformation, and/or metabolite concentration (Chinnusamy *et al.* 2007). Moreover, the aggregation of water molecules to form a stable ice nucleus, sometimes associated with coatings of debris, could interfere with staining and/or the fluorescence of the fluorochromes. The aggregation of minor particles with nuclei can play a key role in this interference and can even lead to an apparent increase in nuclear fluorescence (Loureiro *et al.* 2006a). Although a small decrease in DNA signal in herbarium specimens (under 6 months old) and frozen material compared to fresh material is documented, both stored specimen types have proved satisfactory in the determination of ploidy level. The use of both sample preservation methods opens up new perspectives for analyzing field-collected material, and this advance greatly expands the potential application of FCM in botany, where samples frequently cannot be immediately analyzed.

The co-occurrence, frequency distribution, and origins of infraspecific cytotypes are challenging topics that have not been thoroughly explored. Recent studies have indicated that co-distribution of cytotypes may be more widespread than had been previously expected (e.g. Rosenbaumová *et al.* 2004; Suda *et al.* 2007b; Španiel *et al.* 2008; Kolář *et al.* 2009). Although only very limited information concerning potential sympatric growth of plants with different ploidy levels and their distribution pattern at fine spatial scales is available, the current work depicts the landscape patterns of the calcareous screes in the Cantabrian Mountains. Our recordings of *R. parnassiifolius* subsp. *favargeri* and *R. parnassiifolius* subsp. *heterocarpus* from apparently identical habitats within a geographically limited area, like the Picos de Europa, indicate that their niches could overlap. Whilst DNA ploidy differences were found, it is important to acknowledge

that at the morphological level, the data were not reliable enough to enable the differentiation of the two cytotypes of *R. parnassiifolius* in the Cantabrian Mountains. Some recent studies have identified similar cases, where, despite slight morphological tendencies expressed at the population level, the cytotypes cannot be reliably distinguished morphologically, thus concluding that the coexistence of different cytotypes within a single species, without recognition of infraspecific taxa, is rather frequent (e.g. Perný *et al.* 2008; Španiel *et al.* 2008; Zonneveld 2008).

This work represents the first study dealing with FCM in *R. parnassiifolius* and has found the use of FCM in ploidy estimation to be reliable and efficient, suggesting it may prove to be a significant tool for the delimitation of those cytotypes that are sometimes not readily separated by morphoanatomic studies. In light of the results obtained in this work, new issues are raised, such as the distribution of both taxa in other regions such as the Pyrenees and the Alps, or whether the distribution of polyploid phenomena is associated with apomixis as in other species within the same genus (Paun *et al.* 2006). Moreover, genome size data are helpful in generating or supporting hypotheses about parental taxa and, consequently, about allo- versus auto-polyploid origins. We conclude that the existence of mixed-cytotype populations along the study area is not common and the frequency of diploid and tetraploid cytotypes seems to be quite similar. Furthermore, we have not found evidence supporting a model of altitudinal distribution between the two cytotypes. These data are therefore important for establishing strategic priorities for conservation programmes throughout the Cantabrian Mountains in general, and more specifically, for intensifying the strategies implemented in the Picos de Europa.

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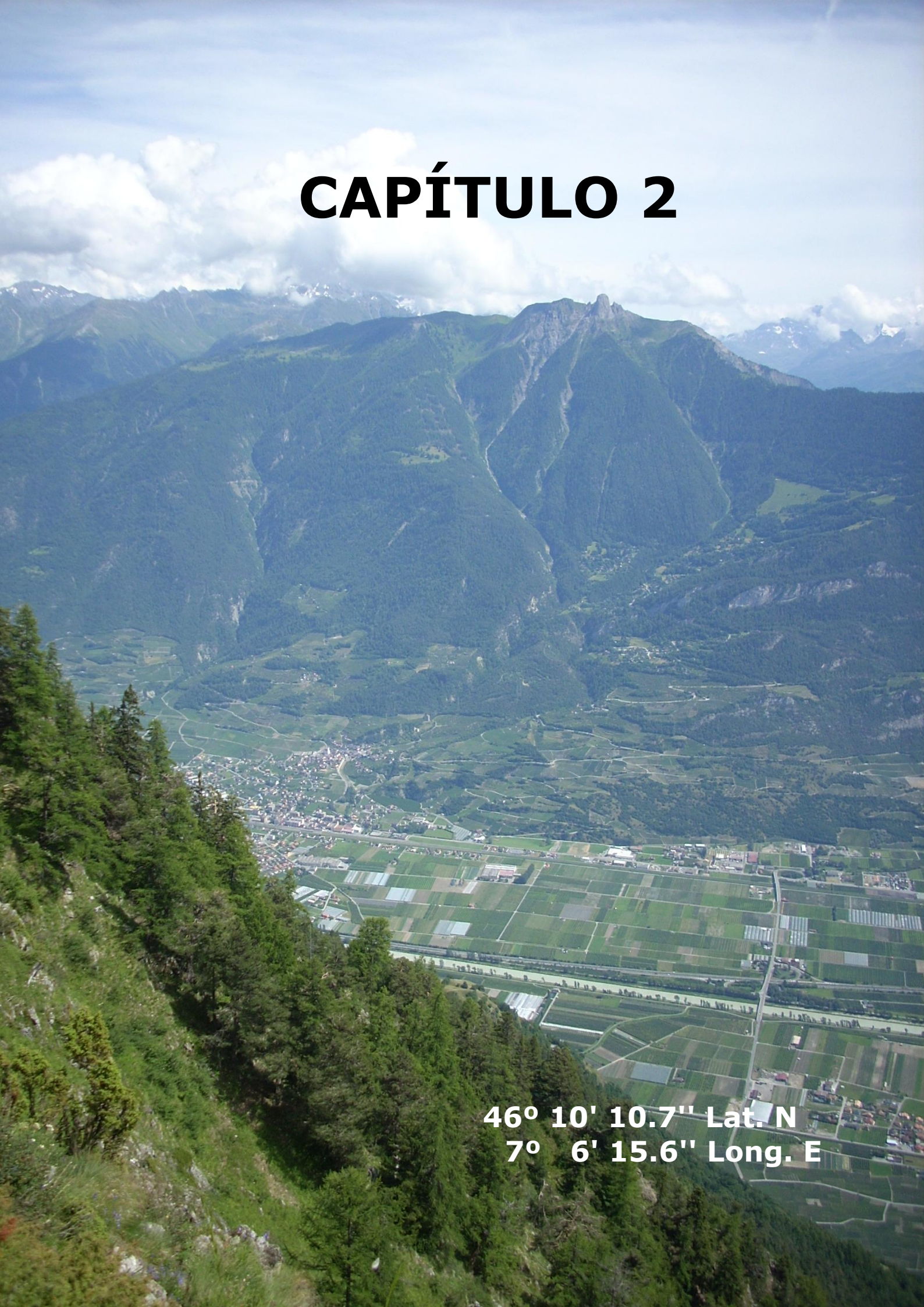
CAPÍTULO 2



Intraspecific genome size variation and morphological differentiation of *Ranunculus parnassiifolius* (Ranunculaceae), an Alpine-Pyrenean-Cantabrian polyploid group

Summary

The aim of this study is to assess genome size variation and multivariate morphometric analyses to ascertain cytotype distribution patterns and the morphological differentiation within the *Ranunculus parnassiifolius* group in the Pyrenees and the Alps. Although divergences in nuclear DNA content among different species within a genus are widely acknowledged, intraspecific variation is still a somewhat controversial issue. Holoploid and monoploid genome sizes (C- and Cx-values) were determined using propidium iodide flow cytometry in 125 plants of *R. parnassiifolius s.l.* distributed across four European countries.

Three different DNA ploidy levels were revealed in the study area: diploid (2n ~ 2x, 57.14%), triploid (2n ~ 3x, 1.19%) and tetraploid (2n ~ 4x, 41.67%). The mean population 2C-values ranged from 8.15 pg in diploids to 14.80 pg in tetraploids, representing a ratio of 1:1.8. Marked intra-specific/inter-population differences in nuclear DNA content were found. Diploid populations prevail in the Pyrenees, although tetraploid cytotypes were reported throughout the distribution area. In general, mixed-cytotype populations were not found. The Spearman correlation coefficient did not reveal significant correlations between genome size and altitude, longitude or latitude. Morphometric analyses and cluster analyses based on genome size variation revealed the presence of three major groups, which exhibited a particular biogeographical pattern. A new cytotype, DNA triploid, was found for the first time. Tetraploid populations showed constant nuclear DNA levels, whereas diploid populations from the Pyrenees, in which introgressive hybridization is suggested as a presumable trigger for genome size variation, did not. Scenarios for the evolution of geographical parthenogenesis in *R. parnassiifolius s.l.* are discussed. Finally, the different levels of effectiveness between plant and animal reference standards are analysed.

Este capítulo se corresponde con las publicaciones:

- Cires, E. (2010). Citotipos en *Ranunculus parnassiifolius* determinados mediante citometría de flujo. *Boletín de Ciencias del Real Instituto de Estudios Asturianos* 51: 379-380.
- Cires, E., Cuesta, C., Revilla, M.A., Fernández Prieto, J.A. (2010). Intraspecific genome size variation and morphological differentiation of *Ranunculus parnassifolius* (Ranunculaceae), an Alpine-Pyrenean-Cantabrian polyploid group. *Biological Journal of the Linnean Society* 101: 251-271.
- Cires, E., Cuesta, C., Fernández Prieto, J.A. (2011). *Flow cytometric analysis in* Ranunculus parnassiifolius *(Ranunculaceae)*. Monografies del CENMA (Centre d'Estudis de la Neu i la Muntanya d'Andorra) (en prensa).

Introduction

Since the introduction of flow cytometry (FCM) to plant sciences in the 1980s, estimation of nuclear DNA content has been the major application of FCM in research (Doležel & Bartoš 2005) with more than 800 publications to date (Loureiro *et al.* 2008). Despite this fact, recent reports indicate that genome size has only been estimated for roughly 1.8% of angiosperms (Leitch & Bennett 2007). FCM has had a profound influence on population and evolutionary biology, as it combines the ability to collect qualitative information about biological particles with the capacity to quantify these measures for large numbers of particles (Kron *et al.* 2007). Perhaps the most obvious application of FCM is in resolving taxonomic complexities in groups with variation in ploidy.

Polyploidization is a common evolutionary process that plays a key role in the adaptation and speciation of plants. Up to 80% of angiosperms and 95% of pteridophytes have experienced one or more episodes of polyploidization in their evolutionary history (Leitch & Bennett 1997). Recently, Wood *et al.* (2009) established that 15% of angiosperm and 31% of fern speciation events are accompanied by ploidy increase. Consequently, ploidy is often an important criterion guiding taxonomic delineation of plants. FCM offers a rapid and precise method for identifying taxa of different ploidy number, enabling cytotype surveys over large spatial scales and involving large population samples. It is of particular value in polyploid complexes that show considerable phenotypic plasticity or lack distinct morphological characters. Lately, FCM has been used to examine the systematics and taxonomy of a number of plant groups, and more specifically has frequently been used in ploidy analysis (e.g. Mráz *et al.* 2008; Šmarda 2008; Kolář *et al.* 2009). Consequently, the use of the nuclear DNA content as a taxonomically informative marker, and its value in resolving complex groups has been acknowledged repeatedly (e.g. Mishiba *et al.* 2000; Suda *et al.* 2007a).

In the genus *Ranunculus s.l.*, the use of Internal Transcribed Spacer region (ITS) of nuclear ribosomal DNA and plastid sequences (*matK-trnK*) has provided evidence for at least three independent origins of apomixis, each of them showing a pattern of geographical parthenogenesis (Hörandl 2009). One example is the complex polyploid *R. parnassiifolius* L.: an orophyte plant distributed throughout the Alps, the Pyrenees and the Cantabrian Mountains (Cires *et al.* 2009a, b). At least five taxa have been recognized within *R. parnassiifolius*, treated either at the species or subspecies level (Rothmaler 1934; Guinea López 1953; Küpfer 1974; Bueno Sánchez *et al.* 1992), and referred to as *R. parnassiifolius* subsp. *parnassiifolius*: endemic in the Eastern Pyrenees; *R. parnassiifolius* subsp. *cabrerensis* Rothm.: endemic in the Northwestern mountains of Spain; *R. parnassiifolius* subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto: endemic in the Western Cantabrian Mountains (Muniellos Biosphere Reserve) with a single population; *R. parnassiifolius* subsp. *favargeri* P.Küpfer: endemic in the Cantabrian Mountains and the Western

Pyrenees; and finally, *R. parnassiifolius* subsp. *heterocarpus* P.Küpfer: spread throughout the Cantabrian Mountains, the Central Pyrenees and the Alps. The first three subspecies live on slope deposits of gravel and siliceous pebbles, while the two latter are considered to be from calcareous environments. In addition to the type of substrate, several characters have been reported for the differentiation within subspecies, such as the regularity of the corolla and the presence of aborted carpels (Küpfer 1974). A total of 22 chromosome counts in 18 localities of the Iberian Peninsula (including the Pyrenees) have been reported by Löve & Solbrig (1964), Küpfer & Favarger (1967), Favarger & Küpfer (1968), Küpfer (1969, 1974), Vuille & Küpfer (1985), Diosdado (1991) and Diosdado & Pastor (1991, 1996). It is worth noting that no ploidy levels other than diploids (2n = 2x = 16) and tetraploids (2n = 4x = 32) are known within the calcareous/silicicolous populations, with the exception of a pentaploid cytotype (2n = 5x = 40) in two locations in the Central Pyrenees (Küpfer 1974; Vuille & Küpfer 1985; Vuille 1987) and another locality in the Cantabrian Mountains (Cires *et al.* 2009a). A number of other investigations undertaken in the rest of Europe (Alps) by Favarger & Küpfer (1968), Küpfer (1974), Vuille & Küpfer (1985), Baltisberger & Huber (1987) and Hüber (1988), have reported chromosome number for *R. parnassiifolius s.l.* in Central Europe from Italy, Switzerland and Austria (eight records of six localities altogether), finding that all specimens were tetraploids. Beside the above mentioned reports, the cytogeography of this species has been poorly investigated throughout its range.

To extend the boundaries of our knowledge on genome size differentiation in the *R. parnassiifolius* group and to assess the usefulness of such information for taxonomic purposes, nuclear DNA contents were estimated in a representative set of populations in the Pyrenees, the Alps and adjacent territories, including material from the type localities. The aim of this article is therefore: (1) to verify the geographical distribution of DNA ploidy levels of the *R. parnassiifolius* complex, (2) to look for intraspecific variation in genome size across the range of the cytotypes, (3) to examine the morphological differentiation within calcicole and silicicole subspecies, and finally (4) to establish a possible evolutionary pattern of geographical parthenogenesis in *R. parnassiifolius s.l.* based on nuclear DNA variation.

Materials and methods

Plant material

Plants of *R. parnassiifolius s.l.* were collected in 2007 and 2008 in their natural habitats throughout the Pyrenees and the Alps (Figure 1; Table 1). Field-collected specimens were potted and kept in a greenhouse at room temperature (20-22 ºC) and are currently maintained as a collection of living plants at the Botany Area of the University of Oviedo.

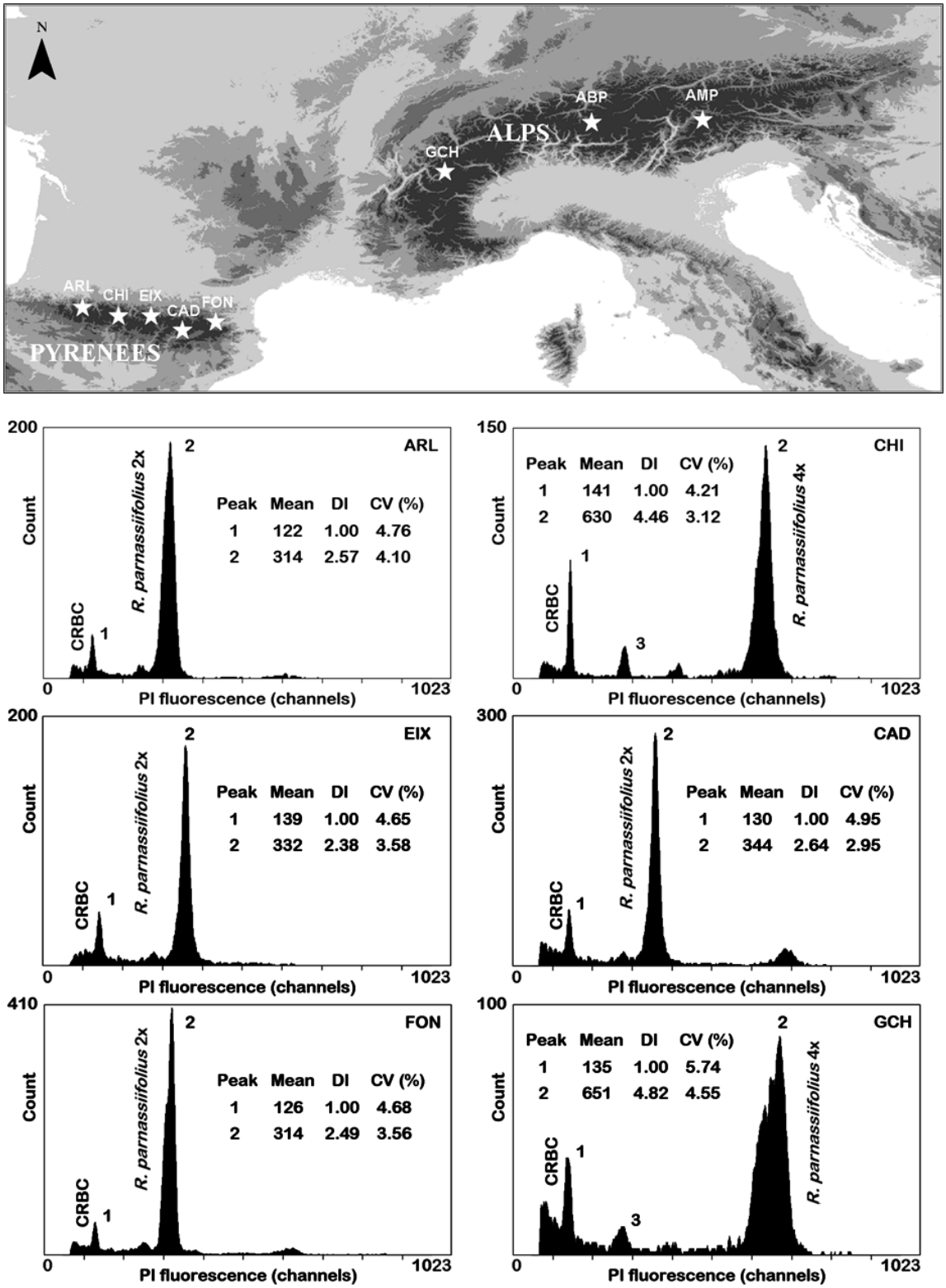


Figure 1. Geographical distribution and FCM histograms of the populations of *R. parnassiifolius s.l.* studied. The peaks marked with 1 and 2 indicate nuclei at the G0/G1 phase of the internal standard and the G0/G1 phase of the sample, respectively. The mean channel number (PI fluorescence), DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given. The peaks numbered 3 correspond to G2/M peaks.

As a rule, 15–20 plants from each population were sampled. Geographical coordinates were recorded in the field (Garmin-Etrex GPS instrument), and topographic information was derived from the geographic information system (GIS), the resulting data being processed with GIS software ArcMap 9.2 (ESRI, 2006). Additionally, voucher specimens were collected and kept in the Herbarium of the University of Oviedo (FCO).

Table 1. Localities of sample collection of *R. parnassiifolius s.l.* studied in this work. Voucher specimens are kept in the Herbarium of the University of Oviedo (FCO).

Acronym	Locality/collector/date	Zone	Coordinates	Bedrock	Altitude (m a.s.l.)	Subsp. Syst.†	Voucher specimens
ABP	Albulapass, Blais Cuorta (Graubünden, Switzerland); *CC, EC, JAFP & MC*; 2008/07/19	32T	x = 564448 y = 5159889	C	2507	RPH	31353
AMP	Behind the Rifugio of Auronzo, Tre Cime di Lavaredo (Véneto, Belluno, Auronzo di Cadore, Italy); *CC, EC, JAFP & MC*; 2008/07/18	33T	x = 293014 y = 5165900	C	2353	RPH	31354
ARL	Col d'Arlas (Aquitaine, France); *EC & JAFP*; 2007/07/27, 2008/06/10	30T	x = 683386 y = 4759904	C	1971	RPF	31355 31356 31357
CAD	Top of Puigllançada, Parc Natural Cadí-Moixeró (Bagà, Barcelona, Spain); *EC & JAFP*; 2007/06/28, 2008/06/08	31T	x = 411552 y = 4684235	C	2165	RPH	31358 31359
CHI	End of the track that begins in Chisagües, south of Sierra de Liena (Bielsa, Huesca, Spain); *EC & JAFP*; 2007/07/25, 2008/06/27	31T	x = 267122 y = 4730103	C	2346	RPH	31360
EIX	Coll de la Creu de l'Eixol (Espot, Lérida, Spain); *EC & JAFP*; 2007/06/29, 2008/06/09	31T	x = 342739 y = 4711539	S	2246	RPH	31361 31362
FON	Collada de Fontalba (Queralbs, Gerona, Spain); *EC & JAFP*; 2007/06/27, 2008/06/08	31T	x = 429305 y = 4690908	S	2223	RPP	31363 31365 31364
GCH	Grand Chavalard, versant ouest (Valais, Switzerland); *CC, EC, JAFP & MC*; 2008/07/21	32T	x = 353665 y = 5114642	C	2071	RPH	31366 31367

Geographical coordinates and altitudes are in accordance with European 1950 and Universal Transverse Mercator datums.

Collectors: *CC* = C. Cuesta; *EC* = E. Cires; *JAFP* = J.A. Fernández Prieto; *MC* = M. Ceballos.

Bedrock type: C = calcareous; S= siliceous

† Subspecific systematization of samples according to previous studies [RPH: *Ranunculus parnassiifolius* subsp. *heterocarpus*; RPF: *R. parnassiifolius* subsp. *favargeri*; and RPP: *R. parnassiifolius* subsp. *parnassiifolius*] (see Küpfer 1974; Vigo i Bonada 1983; Cook *et al.* 1986; Jalas & Suominen 1989; Carrillo i Ortuño & Ninot i Sugranyes 1992) and herbarium material (ABH, AH, ALME, ARAN, B, BC, BCN, BHUPM, BIO, BOLO, BOZ, CCEC, CLF, COA, DR, EMMA, FCO, FR, GDA, GDAC, GJO, HEID, HJBS, HSS, JACA, JAEN, JBAG, KL, LEB, LYJB, MA, MACB, MAF, MSTR, OSN, P, PAD, PAMP, REG, RO, ROST, ROV, SALA, SANT, SEV, STR, TFC, UPNA, VAL, VIT, WFBVA, WU, Z, ZT and GBIF (2008), http://www.gbif.org/).

Genome size estimation

Nuclear DNA content was analysed for 125 plants of *R. parnassiifolius s.l.*, using FCM with plant and animal standards. Nuclei from chicken red blood cells (CRBC; 2C = 3.14 pg, Cires *et al.* 2009a) and leaves of *Pisum sativum* cv. 'Ctirad' (2C = 9.09 pg of DNA, Doležel *et al.* 1998), were the standards used for the present work. CRBC were self-prepared and stored at -20 ºC (see Cires *et al.* 2009a for more

details) and then added to the suspension of released nuclei as an internal reference standard. Nuclear suspensions were obtained following the protocol of Galbraith *et al.* (1983). Fresh intact leaf tissue from the sample for analysis (150 mg) and internal reference standard (50 mg) were placed together in a sandwich-like fashion and chopped with a new razor blade in a glass Petri dish containing 1 mL of ice-cold LB01 nuclear isolation buffer [15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 15 mM ß-mercaptoethanol, 0.1% (v/v) Triton X-100, pH 7.5] (Doležel *et al.* 1989) which is kept stored frozen at -20 °C in aliquots for convenient use.

The nuclear suspension was then filtered through a 42 µm nylon filter to remove large debris and 50 µg mL^{-1} of propidium iodide (PI, Sigma) was added to the samples to stain the DNA. Additionally, 50 µg mL^{-1} of RNase (Sigma) was added to avoid staining of double-stranded RNA. Samples were kept on ice throughout the process and analyzed after a 15-min incubation period using a Cytomics FC 500 (Beckman Coulter) with 488-nm excitation from an argon ion laser. Data analysis was carried out using Cytomics RXP Analysis (Beckman Coulter, Inc. 2006). At least three individuals from each population were analyzed on three different days to avoid errors due to instrumental drift, and at least 5000 nuclei were analyzed per sample. The position of nuclei G0/G1 peaks in histograms of relative fluorescence intensity was used to estimate nuclear DNA content. The 2C DNA content of *R. parnassiifolius s.l.* (2C = holoploid genome size; sensu Greilhuber *et al.* 2005) was calculated as:

$$\text{2C value (pg)} = \frac{\text{sample 2C mean peak position}}{\text{standard 2C mean peak position}} \text{ x standard 2C value}$$

The monoploid genome size (1Cx; sensu Greilhuber *et al.* 2005) of all plants was also calculated in mass values (pg) and Mbp (1 pg = 978 Mbp, Doležel *et al.* 2003). Throughout the process, we incorporated recommendations proposed by Galbraith *et al.* (1997), Suda *et al.* (2006), Doležel *et al.* (2007a, b), Greilhuber & Doležel (2009), and the conclusions of the first and second Plant Genome Size Meetings in 1997 and 2003 respectively (see "the nine key recommendations" at http://data.kew.org/cvalues/homepage.html).

In addition, flow cytometric seed screen (FCSS), a method developed by Matzk *et al.* (2000) and recently reviewed (Matzk 2007) based on DNA content measurement and ploidy estimation in embryos versus endosperm in mature seeds, was used to discriminate between sexual and apomictic plants. We therefore used achenes of *R. parnassiifolius* (embryo and testa; and exclusively embryo) to test if the mode of reproduction is apomictic as suggested by Vuille & Küpfer (1985) and Hüber (1988). FCSS analyses followed the same protocol as for FCM.

Genome size data obtained by FCM was analysed by means of a Kruskal–Wallis one-way ANOVA on ranks and Dunn's method for pair-wise comparison. The

Spearman rank correlation coefficient (a non-parametric measure of correlation, which quantifies the strength of the association between the variables) was used to test whether mean genome sizes were related to the geographical location/position of populations (altitude, latitude and longitude).

The values were represented by cluster analyses [using Euclidean distance as the similarity coefficient and UPGMA (unweighted pair group method with arithmetic mean) as the clustering algorithm]. The data were analyzed using the following software packages: Geotrans 2.4.2 (Geographic Translator, 2008), Past 1.79 (Hammer *et al.* 2001), SigmaStat 3.11 (Systat, Inc. 2004) and STATISTICA 7.0 (StatSoft, Inc. 2004).

Morphometric analyses

Twenty-three characters successfully employed in the past were measured in plants collected in the field (20 plants per population) (Cires *et al.* 2009a). They included quantitative and qualitative characters, as well as ratios. The character set was chosen on the basis of published determination keys, flora handbooks, and our own observations.

Principal Coordinates Analysis (PCoA) together with a Gower similarity coefficient test (Gower 1971) were conducted. PCoA is a fundamental tool in multivariate data analysis, used to reduce the dimensionality of multivariate data-sets by transforming the original variable set to a smaller number of composite variables, the first few of which usually represent most of the sample variance. The method was used to display an overall variation pattern across the first three components, extracting most of the original multidimensional character space. Different PCoA were performed, considering all vegetative (LL, LW, LLLW, SL, IAD), floral (LS, WS, LP, WP, NF, NS, NP, LSWS, LPWP, TI, IS, FS, CL, NSTC) and/or fructification characters (LA, WA, LAWA, SA) (see Table 2 in Chapter 1 for abbreviations, p. 32).

The matrices thus generated (datasets) were analyzed taking into account different levels of ploidy, the subspecies described, the region of origin and the geological substrates in which the plants live, in order to find possible correlations. Furthermore, within-matrix analysis was performed considering not only the vegetative, floral and fructification characters separately, but also all their possible combinations (vegetative-floral; vegetative-fructification; floral-fructification). In terms of morphometric measurement, exploratory data analysis, including mean, standard deviation, minimum and maximum values as well as 5th and 95th percentiles were computed for each DNA ploidy level and each subspecies. Multivariate analysis methods were performed using Ginkgo 1.5.8 (Bouxin 2005) and Past 1.79 (Hammer *et al.* 2001).

Finally, to provide a more detailed study of the polyploid *R. parnassiifolius* complex, previously published information (211 samples) from the Northwest Iberian

Peninsula (Cires *et al.* 2009a) was included. Consequently, the biogeographical territories studied were: Atlantic European province (Orocantabrian subprovince), Cévenno-Pyrenean province (Central Pyrenean and Eastern Pyrenean subprovinces) and Alpine province (Western Alpine, Central Alpine and Eastern Alpine subprovinces) (Rivas Martínez *et al.* 2004). For convenience these biogeographical provinces are henceforth treated as Orocantabrian, Pyrenean and Alpine populations respectively.

Results

Intraspecific genome size variation

FCM analyses of *Ranunculus* samples for genome size resulted in high resolution histograms (Figure 1) with mean population coefficients of variation of G0/G1 peaks ranging from 2.82 to 5.38%. Indeed, 81.92% of the estimations presented a coefficient of variation value below 5.0%. Due to the very low mitotic activity detected in *R. parnassiifolius* leaf tissues (with only a negligible peak corresponding to nuclei in G2 phase being recorded) and the absence of endopolyploidy, potential bias in DNA ploidy level estimation was discounted.

Three different DNA ploidy levels were revealed in the study area: diploid (2n ~ 2x, 57.14%), triploid (2n ~ 3x, 1.19%) and tetraploid (2n ~ 4x, 41.67%). The occurrence of triploid plants deserves particular mention as it represents the first recorded incidence in the species. Averaged absolute genome size values (2C) for each population, as well as ranges for 2C and 1Cx (monoploid genome size) are given in Table 2.

The mean population 2C-values ranged from 8.15 pg in diploid to 14.80 pg in tetraploid, representing a ratio of 1:1.8 rather than the expected 1:2. Intraspecific variation in genome size within each cytotype was detected (8.58% in diploid and 1.29% tetraploid). In the tetraploid cytotype this low level of variability can easily be attributed to instrumental or methodological errors. However, in diploid populations of the Pyrenees considerable intraspecific variation was detected. Subsequent repeated measurements of the same sample resulted in highly similar nuclear DNA content values with low coefficients of variation (3.58% on average). This fact, taken together with the symmetrical G0/G1 peaks observed, implies that the initial data cannot account for the presence of disturbing secondary metabolites (Loureiro *et al*. 2006).

Table 2. Nuclear DNA content of *R. parnassiifolius s.l.* populations analysed in this study using FCM. The values are given as means with standard deviation of the mean (SD) of the nuclear DNA content (pg/2C). The 2C range of values (min.- minimum, max.- maximum) obtained for each population (referred by its acronym), the mean coefficient of variation (CV, %) of the 2C peak, the number of individuals analyzed (*n*) and the monoploid nuclear DNA content (1Cx) in mass values (pg) and Mbp are also given.

			Nuclear DNA Content						
				2C range					
	Population	Ploidy Level	2C (pg) ± SD	Min.	Max.	1Cx (pg)	1Cx (Mbp)[1]	CV (%)	*n*
Alps	ABP	4x	14.84 ± 0.336 a	14.36	15.12	3.71 ± 0.084 ac	3628 ± 82.176	5.19	5
	AMP	4x	14.73 ± 0.789 a	14.22	15.95	3.68 ± 0.197 ac	3602 ± 192.892	5.38	10
	GCH	4x	14.91 ± 0.194 a	14.58	15.22	3.72 ± 0.048 ac	3645 ± 47.375	3.91	10
Pyrenees	ARL	2x	7.93 ± 0.332 b	7.40	8.32	3.96 ± 0.166 ab	3879 ± 162.392	4.85	14
	CAD	2x	8.37 ± 0.237 c	7.94	8.76	4.19 ± 0.119 b	4097 ± 116.061	3.95	14
	CHI	4x	14.72 ± 0.482 a	14.02	15.72	3.68 ± 0.120 c	3601 ± 117.769	3.61	14
	EIX	2x	8.47 ± 0.528 c	7.50	8.90	4.23 ± 0.264 b	4144 ± 258.192	3.60	13
	EIX	3x*	13.12	-	-	4.37	4277	2.82	1
	FON	2x	7.80 ± 0.229 b	7.42	8.35	3.90 ± 0.114 abc	3817 ± 111.947	3.55	14

Differences between populations were analyzed using a one-way ANOVA procedure and Dunn's method for pair-wise comparison. Means followed by different letters are statistically different ($P < 0.001$). * Data expressed without standard deviation refer to a single individual.

Internal reference standard: CRBC 2C = 3.14 pg of DNA (Cires *et al.* 2009a).

[1] 1 pg = 978 Mbp (Doležel *et al.* 2003).

Consequently, we are strongly of the opinion that the observed genome size divergence in diploid was not caused by methodological shortcomings. More importantly, simultaneous measurements of individuals with divergent genome sizes always gave histograms with two or more distinct peaks (see Figure 2), which is regarded as robust proof of genuine genome size variation (Greilhuber *et al*. 2007).

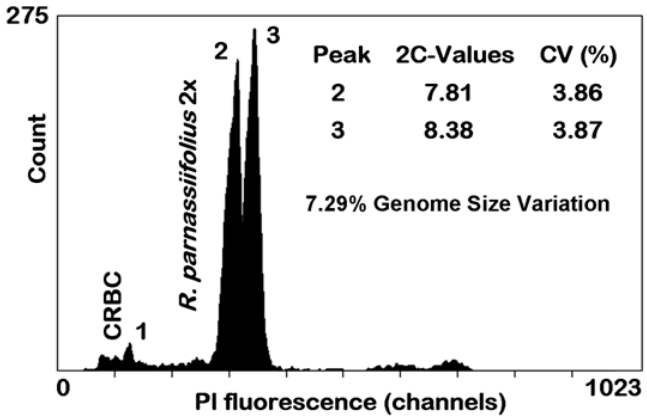


Figure 2. Differences in genome size in diploid cytotype of *R. parnassiifolius s.l.* from two different populations (ARL and CAD). The nuclei from all individuals were isolated, stained with propidium iodide and analysed simultaneously. The peak marked with 1 indicates nuclei at the G0/G1 phase of the internal standard. The peaks marked with 2 and 3 indicate nuclei at the G0/G1 phase of different samples. Nuclear DNA content (pg/2C) and coefficient of variation value (CV, %) of each peak are also given.

Statistical analyses revealed significant differences ($P < 0.001$) between DNA ploidy level in diploid populations tested, and also between diploid and tetraploid cytotypes (Table 2). In our study, 6.98% populational variation in 2C-values was found among the most dispersed Pyrenean diploid populations (lowest 2C-value: ARL and FON) and the most concentrated ones (highest 2C-value: CAD and EIX). The maximum difference between the 2C-values of diploid cytotype ranged from 7.40 pg in ARL to 8.90 pg in EIX (a 1.20-fold difference). In fact tetraploid populations showed constant nuclear DNA amounts, in contrast to the diploid populations from the Pyrenees, in which introgressive hybridization may be suggested as a presumable trigger for genome size variation. For example, EIX recorded the highest maximum intra-populational variation (18.66%).

Finally, comparisons were made between the use of plant and animal standards, in order to confirm the nuclear DNA content results, and also to test the reproducibility of DNA genome size estimation using FCM. The populations of *R. parnassiifolius s.l.* from the Alps were chosen as they were potentially the most damaged samples as a result of their transportation. Fluorescence histograms of nuclear DNA content, with plant internal reference standard (Figure 3), from different localities of the Alps also exhibited coefficients of variation that were usually below 5.0%.

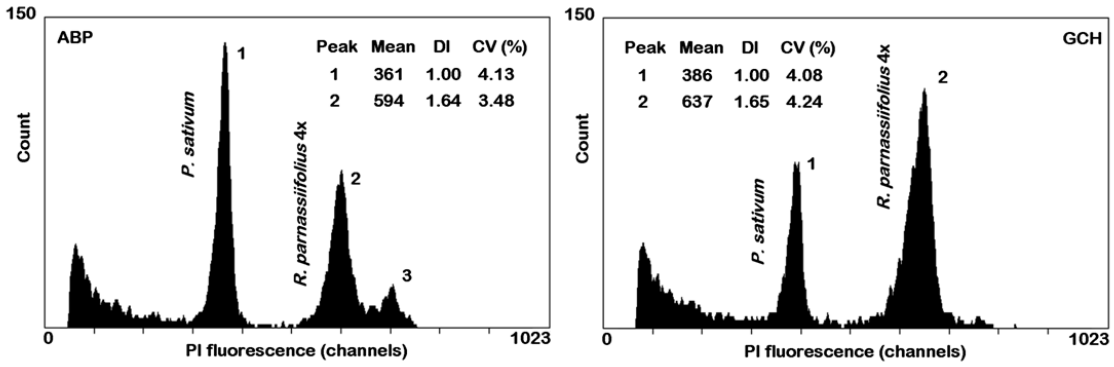


Figure 3. Flow cytometric histograms obtained with plant internal reference standard in *R. parnassiifolius s.l.* from different localities of the Alps. The peaks marked with 1 and 2 indicate nuclei at the G0/G1 phase of the plant internal standard (*Pisum sativum* cv. 'Ctirad', 2C = 9.09 pg of DNA; Doležel *et al.* 1998), and the G0/G1 phase of the sample, respectively. The mean channel number (PI fluorescence), DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given. The peaks numbered 3 correspond to G2/M peaks.

Table 3 shows the mean and standard deviation of the holoploid (2C) and monoploid (1Cx) nuclear DNA content in mass values (pg) of ploidy level with different standards, and also the coefficient of variation and minimum and maximum values obtained for each population. Similar values were observed when using different standards (plant/animal) in the estimation of DNA content in absolute units, applying a previously determined chicken genome size (Cires *et al*. 2009a).

Table 3. Comparison of plant and animal DNA reference standards in populations of *R. parnassiifolius s.l.* from the Alps.

Population	Standard	2C (pg)	Min.	Max.	1Cx (pg)	CV (%)	*n*
ABP	CRBC	14.84 ± 0.336	14.36	15.12	3.71 ± 0.084	5.19	5
	PS	14.81 ± 0.183	14.50	14.95	3.70 ± 0.045	4.00	10
AMP	CRBC	14.73 ± 0.789	14.22	15.95	3.68 ± 0.197	5.38	10
	PS	14.93 ± 0.058	14.87	14.99	3.73 ± 0.045	4.60	10
GCH	CRBC	14.91 ± 0.194	14.58	15.22	3.72 ± 0.048	3.91	10
	PS	14.96 ± 0.043	14.91	15.01	3.74 ± 0.010	3.47	10
TOTAL	CRBC	14.85 ± 0.497 a	14.22	15.95	3.71 ± 0.124	4.66	25
	PS	14.89 ± 0.138 a	14.50	15.01	3.72 ± 0.034	3.97	30

Differences between standards were analyzed using a Kruskal-Wallis one-way ANOVA on ranks. Means and standard deviations followed by different letters are statistically different ($P < 0.001$). CRBC: chicken red blood cells, 2C = 3.14 pg of DNA (Cires *et al.* 2009a); PS: *Pisum sativum* cv. 'Ctirad', 2C = 9.09 pg of DNA (Doležel *et al.* 1998).

FCSS histograms of ten seeds of the tetraploid populations of *R. parnassiifolius s.l.* showed unclear peaks for the endosperm (low fluorescence of nuclei was recorded) (data not shown). Although the expected ratios of Cx-values of embryo versus endosperm seemed to correspond to a pseudogamous apomict processing, we could not discriminate whether this was the result of pseudogamous I (one pollen nucleus fertilises the endosperm nuclei, expected ratio 4:10) or pseudogamous II (both pollen nuclei fertilise the endosperm nuclei, expected ratio 4:12) (Hörandl *et al.* 2008). Moreover, studies conducted to assess geographical variation in pollen production in diploid and tetraploid plants in *R. parnassiifolius* have also allowed us to reveal clear evidence of apomixis (unpublished data), showing a low viability of pollen in tetraploid populations (85.27% non-viable pollen) compared to diploid populations (17.72%).

Morphometric analyses

Results from the exploratory data analysis of quantitative characters for diploid and tetraploid populations of *R. parnassiifolius s.l.* from the Pyrenees and the Alps are given in Table 4.

The analysis was conducted taking into account, firstly, the cytotype distribution in the different biogeographical areas studied, and secondly, the subspecific systematization adopted by several authors in previous works. The morphometric study of quantitative characters between diploid and tetraploid populations revealed that the main differences ($P < 0.001$) of cytotypes were attributable to the geographical distribution and not to the previously classified different subspecies.

Using an ordination diagram of PCoA with qualitative and quantitative characters, based on Gower's similarity coefficient, and taking into account either bedrock type, subspecies or cytotype (Figure 4A-G); clear models of clustering were not detected.

Table 4. Exploratory data analysis of morphometric characters in *R. parnassiifolius s.l.* according to ploidy level (P.Lv.) from different biogeographical territories and previous subspecific systematization (Subsp. Syst.) of the samples. Differences between cytotypes (2x-Pyrenees, 4x-Pyrenees, 4x-Alps) and subspecies (RPF: *R. parnassiifolius* subsp. *favargeri*, RPH: *R. parnassiifolius* subsp. *heterocarpus*, RPP: *R. parnassiifolius* subsp. *parnassiifolius*) were analyzed individually, using a Kruskal-Wallis one-way ANOVA on ranks. The different values are separated by a slash. Letters indicate statistical differences ($P < 0.001$). For character abbreviations see Table 2 in Chapter 1 (p. 32).

Character	P.Lv. / Subsp. Syst.	Mean	s.d.	Min.	5%	95%	Max.	*n*
LL	2x - Pyrenees / RPF	1.61 a / 1.42 a	0.42 / 0.28	0.81 / 0.90	0.98 / 1.00	2.36 / 1.90	3.06 / 1.94	138 / 34
	4x - Pyrenees / RPH	1.25 b / 1.69 b	0.16 / 0.49	0.90 / 0.90	0.95 / 1.00	1.46 / 2.79	1.47 / 3.10	20 / 111
	4x - Alps / RPP	2.19 c / 1.72 b	0.50 / 0.49	1.40 / 0.81	1.40 / 1.00	2.98 / 2.77	3.10 / 3.06	22 / 35
LW	2x - Pyrenees / RPF	1.19 a / 1.06 a	0.31 / 0.18	0.50 / 0.77	0.78 / 0.80	1.81 / 1.40	2.50 / 1.40	138 / 34
	4x - Pyrenees / RPH	0.84 b / 1.21 a	0.16 / 0.39	0.61 / 0.50	0.62 / 0.67	1.15 / 2.00	1.20 / 2.50	20 / 111
	4x - Alps / RPP	1.71 c / 1.39 b	0.35 / 0.38	1.10 / 0.70	1.10 / 0.85	2.32 / 2.32	2.50 / 2.50	22 / 35
LS	2x - Pyrenees / RPF	0.73 a / 0.74 a	0.11 / 0.10	0.50 / 0.50	0.50 / 0.51	0.90 / 0.89	0.90 / 0.90	50 / 11
	4x - Pyrenees / RPH	0.09 b / 0.57 a	0.01 / 0.27	0.08 / 0.08	0.08 / 0.09	0.11 / 0.90	0.11 / 0.90	5 / 41
	4x - Alps / RPP	0.31 b / 0.68 a	0.13 / 0.13	0.20 / 0.50	0.20 / 0.50	0.60 / 0.88	0.60 / 0.90	10 / 13
WS	2x - Pyrenees / RPF	0.28 a / 0.30 a	0.04 / 0.07	0.18 / 0.20	0.20 / 0.20	0.30 / 0.49	0.50 / 0.50	50 / 11
	4x - Pyrenees / RPH	0.08 b / 0.24 a	0.01 / 0.07	0.06 / 0.06	0.06 / 0.08	0.10 / 0.30	0.10 / 0.30	5 / 41
	4x - Alps / RPP	0.20 b / 0.28 a	0.04 / 0.05	0.15 / 0.18	0.15 / 0.18	0.30 / 0.38	0.30 / 0.40	10 / 13
LP	2x - Pyrenees / RPF	1.08 a / 1.10 a	0.20 / 0.13	0.50 / 0.90	0.80 / 0.90	1.40 / 1.29	1.80 / 1.30	50 / 11
	4x - Pyrenees / RPH	0.22 b / 0.93 a	0.05 / 0.38	0.15 / 0.15	0.15 / 0.20	0.30 / 1.36	0.30 / 1.80	5 / 41
	4x - Alps / RPP	0.79 ab/ 1.03 a	0.40 / 0.24	0.30 / 0.50	0.30 / 0.54	1.30 / 1.47	1.30 / 1.50	10 / 13
WP	2x - Pyrenees / RPF	0.80 a / 0.78 a	0.18 / 0.13	0.40 / 0.60	0.50 / 0.60	1.10 / 0.99	1.20 / 1.00	50 / 11
	4x - Pyrenees / RPH	0.18 b / 0.69 a	0.06 / 0.30	0.15 / 0.15	0.15 / 0.15	0.30 / 1.16	0.30 / 1.20	5 / 41
	4x - Alps / RPP	0.47 b / 0.69 a	0.23 / 0.18	0.25 / 0.40	0.25 / 0.41	0.90 / 1.01	0.90 / 1.03	10 / 13
LA	2x - Pyrenees / RPF	0.24 a / 0.24 a	0.03 / 0.01	0.19 / 0.23	0.19 / 0.23	0.31 / 0.26	0.32 / 0.26	33 / 4
	4x - Pyrenees / RPH	0.23 a / 0.27 a	0.03 / 0.05	0.16 / 0.16	0.16 / 0.19	0.27 / 0.40	0.28 / 0.40	13 / 53
	4x - Alps / RPP	0.31 b / 0.23 a	0.05 / 0.04	0.20 / 0.19	0.20 / 0.19	0.40 / 0.31	0.40 / 0.31	20 / 9
WA	2x - Pyrenees / RPF	0.14 a / 0.13 a	0.02 / 0.01	0.10 / 0.12	0.12 / 0.12	0.18 / 0.15	0.19 / 0.15	33 / 4
	4x - Pyrenees / RPH	0.14 a / 0.15 a	0.02 / 0.02	0.11 / 0.10	0.11 / 0.12	0.18 / 0.20	0.19 / 0.20	13 / 53
	4x - Alps / RPP	0.17 b / 0.14 a	0.03 / 0.02	0.10 / 0.10	0.12 / 0.10	0.20 / 0.18	0.20 / 0.18	20 / 9
NF	2x - Pyrenees / RPF	1.68 a / 1.76 a	1.12 / 0.86	1.00 / 1.00	1.00 / 1.00	4.00 / 3.20	8.00 / 4.00	105 / 26
	4x - Pyrenees / RPH	1.61 a / 2.31 a	0.76 / 2.32	1.00 / 1.00	1.00 / 1.00	3.00 / 8.00	3.00 / 13.00	13 / 87
	4x - Alps / RPP	4.71 b / 1.92 a	3.31 / 1.09	1.00 / 1.00	1.00 / 1.00	11.35 / 4.00	13.00 / 4.00	21 / 26
NS	2x - Pyrenees / RPF	5.00 a / 5.00 a	0.00 / 0.00	5.00 / 5.00	5.00 / 5.00	5.00 / 5.00	5.00 / 5.00	50 / 11
	4x - Pyrenees / RPH	5.00 a / 5.00 a	0.00 / 0.00	5.00 / 5.00	5.00 / 5.00	5.00 / 5.00	5.00 / 5.00	5 / 41
	4x - Alps / RPP	5.00 a / 5.00 a	0.00 / 0.00	5.00 / 5.00	5.00 / 5.00	5.00 / 5.00	5.00 / 5.00	10 / 13
NP	2x - Pyrenees / RPF	5.00 a / 5.00 a	0.00 / 0.00	5.00 / 5.00	5.00 / 5.00	5.00 / 5.00	5.00 / 5.00	50 / 11
	4x - Pyrenees / RPH	5.00 a / 5.09 a	0.00 / 0.43	5.00 / 5.00	5.00 / 5.00	5.00 / 5.90	5.00 / 7.00	5 / 41
	4x - Alps / RPP	5.40 a / 5.00 a	0.84 / 0.00	5.00 / 5.00	5.00 / 5.00	7.00 / 5.00	7.00 / 5.00	10 / 13
LLLW	2x - Pyrenees / RPF	1.37 ab / 1.34 ab	0.25 / 0.21	0.72 / 0.95	1.00 / 1.01	1.81 / 1.75	2.28 / 1.90	138 / 34
	4x - Pyrenees / RPH	1.53 a / 1.43 a	0.33 / 0.28	1.09 / 0.72	1.12 / 1.05	2.35 / 1.99	2.41 / 2.41	20 / 111
	4x - Alps / RPP	1.29 b / 1.24 b	0.21 / 0.18	1.00 / 0.93	1.00 / 0.96	1.68 / 1.56	1.70 / 1.59	22 / 35
LSWS	2x - Pyrenees / RPF	2.59 a / 2.55 a	0.54 / 0.40	1.66 / 1.80	1.66 / 1.82	3.60 / 3.45	4.50 / 3.50	50 / 11
	4x - Pyrenees / RPH	1.11 b / 2.25 a	0.13 / 0.85	1.00 / 1.00	1.00 / 1.00	1.33 / 3.42	1.33 / 4.50	5 / 41
	4x - Alps / RPP	1.61 b / 2.44 a	0.78 / 0.54	1.00 / 1.66	1.00 / 1.66	3.00 / 3.54	3.00 / 3.61	10 / 13
LPWP	2x - Pyrenees / RPF	1.39 a / 1.43 a	0.30 / 0.15	0.90 / 1.20	1.00 / 1.20	1.87 / 1.70	2.57 / 1.71	50 / 11
	4x - Pyrenees / RPH	1.26 a / 1.40 a	0.41 / 0.55	0.83 / 0.83	0.83 / 0.91	1.87 / 2.75	1.87 / 3.70	5 / 41
	4x - Alps / RPP	1.81 a / 1.52 a	1.05 / 0.33	1.00 / 1.01	1.00 / 1.01	3.70 / 2.15	3.70 / 2.20	10 / 13
LAWA	2x - Pyrenees / RPF	1.71 a / 1.84 a	0.24 / 0.14	1.11 / 1.71	1.26 / 1.71	2.06 / 2.00	2.08 / 2.00	33 / 4
	4x - Pyrenees / RPH	1.64 a / 1.74 a	0.35 / 0.35	1.12 / 1.12	1.14 / 1.23	2.18 / 2.31	2.18 / 3.00	13 / 53
	4x - Alps / RPP	1.87 a / 1.70 a	0.44 / 0.30	1.33 / 1.11	1.33 / 1.11	2.83 / 2.06	3.00 / 2.06	20 / 9

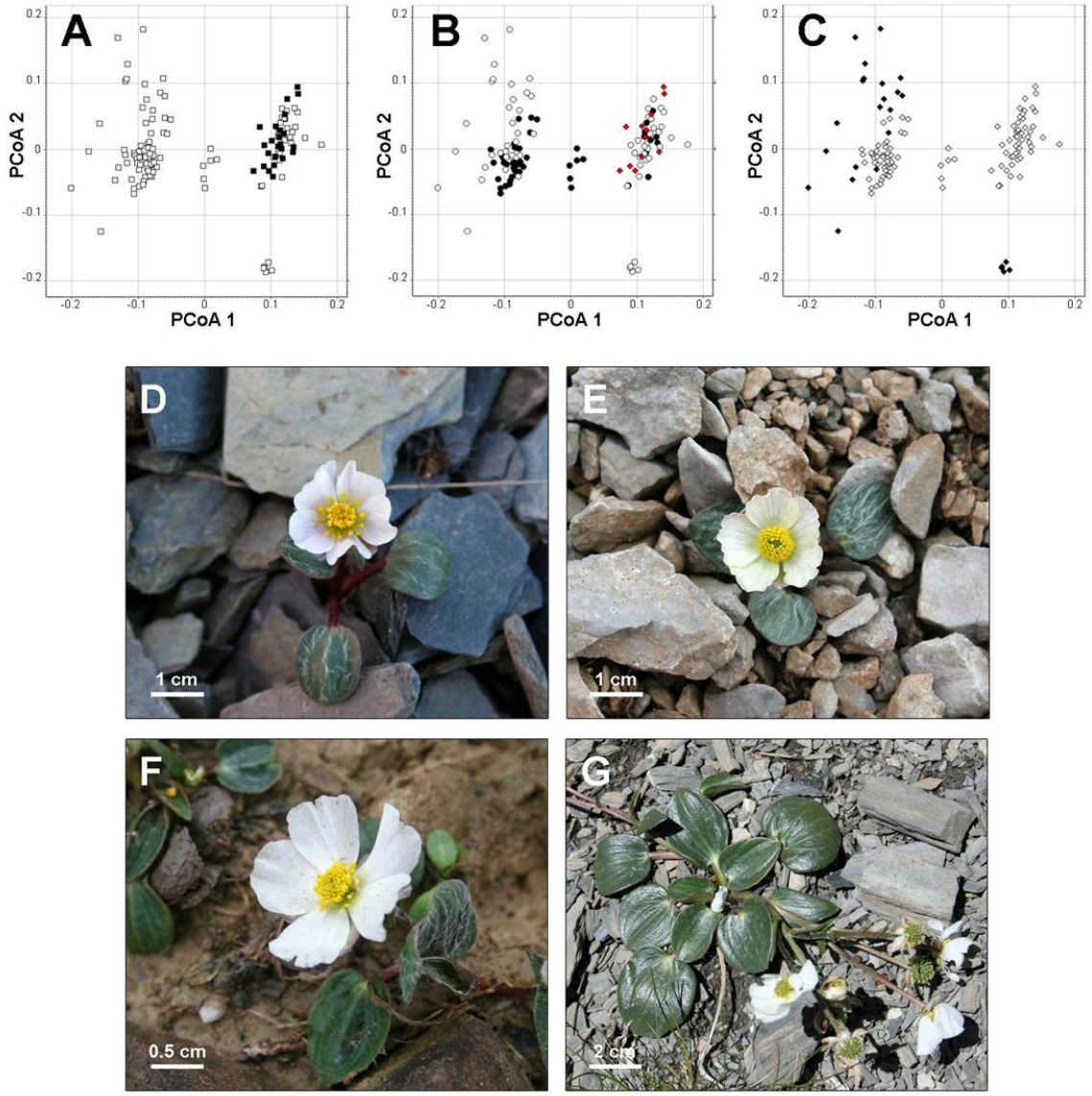


Figure 4. Principal Coordinates Analysis of *R. parnassiifolius s.l.* based on morphological characters (quantitative, ratio, and binary), and taking into account A) the bedrock type: calcareous = open squares (□), siliceous = filled squares (■); B) the different previous subspecific identification: *R. parnassiifolius* subsp. *heterocarpus* = open circles (○), *R. parnassiifolius* subsp. *favargeri* = filled circles (●),*R. parnassiifolius* subsp. *parnassiifolius* = filled diamonds (◆); and C) the cytotypes: diploid = open diamonds (◇), tetraploid = filled diamonds (◆). General appearance of *R. parnassiifolius s.l.* plants from different localities: D) EIX; E) CAD; F) ARL; G) ABP.

On the other hand, on performing the analysis according to biogeographical territories (Figure 5A-F), we found a clear division in the clustering. The first three axes accounted for 65.20% of the total variance (38.36, 19.13, and 7.70%, respectively) in the vegetative matrix (Figure 5A) and 55.48% of the total variance (35.36, 11.59, and 8.53%, respectively) in the vegetative-floral matrix (Figure 5B). Furthermore, similar values were obtained with PCoA based on all vegetative, fructification, and floral characters, including their possible combinations (data not shown).

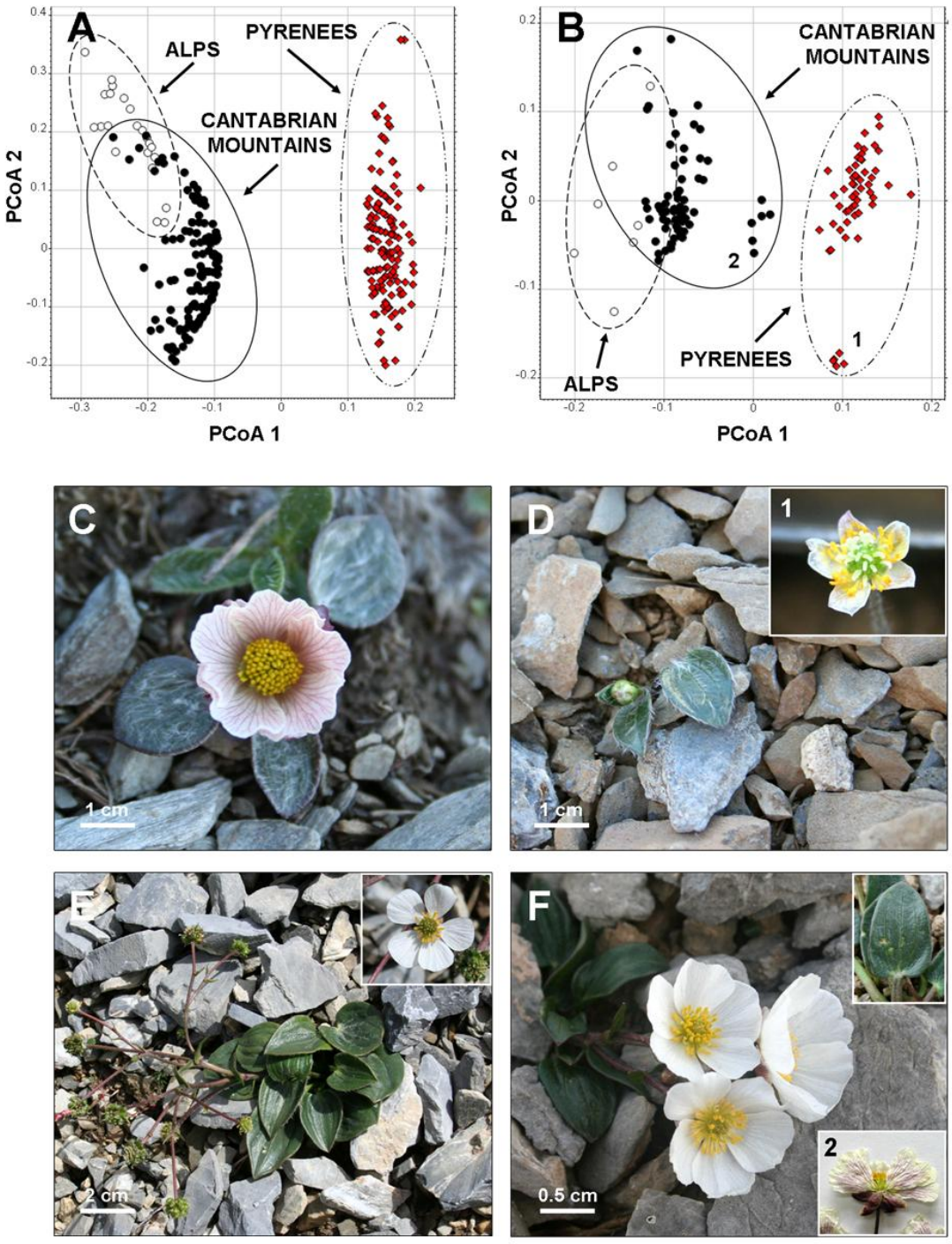


Figure 5. Principal Coordinates Analysis of *R. parnassiifolius s.l.* based on morphological characters and taking into account the biogeographical territories: A) vegetative characters; B) floral and vegetative characters. General appearance of *R. parnassiifolius s.l.* plants from different localities: C) FON; D) CHI, number 1: individuals with very reduced flower petals in tetraploid; E) GCH; F) CAN, number 2: pinkish petals in the Cantabrian Mountains.

Thus, we were able to distinguish at least 3 morphotypes within the *R. parnassiifolius* group from the Orocantabrian, the Pyrenean and the Alpine territories studied. The first morphotype included plants from the Pyrenees (Figure

4D-F & 5C-D), which were characterized by basal leaves that were subcordate-truncate, ovate with a hairy lamina along the adaxial and abaxial surface; glabrous or sparsely hairy sepals; in general pinkish white petals (occasionally white); smooth achenes; and sometimes in tetraploid individuals we observed very reduced flower petals (Figure 5D, see detail number 1). Moreover, no morphological differences were found depending on the substrate or ploidy level. The second morphotype was represented by plants of the Alps (Figure 4G & 5E) with: subcordate, ovate-suborbicular and glabrous basal leaves, except on the adaxial surface where they were densely hairy on the veins and sometimes at the base; glabrous or sparsely hairy sepals; white petals and more or less regular corolla (Figure 5E detail); and smooth achenes. Note that this is the morphotype with the largest leaves. Finally, the last morphotype, that of the Cantabrian Mountains (Figure 5F), where plants were characterized by: basal leaves that were subcordate-truncate, ovate and glabrous lamina (except sometimes at the base and/or on the veins of the adaxial surface) (Figure 5E upper right detail); glabrous or sparsely hairy sepals; smooth achenes; and, in 96% of cases, the petals were white instead of pinkish white (Figure 5F see detail number 2). In this territory, as in the Pyrenees, tetraploid individuals with reduced petals were also found.

Geographical scale of genome size variation

It follows that knowledge of the geographical distribution of cytotypes and their ecological correlates can be useful to give insight into speciation. Combining cytotype (diploid and tetraploid) and biogeographical territory resulted in five groups (presented in Figure 6A-B) that were subjected to ANOVA for an assessment of holoploid and monoploid genome size variation (for these analyses data from Cires *et al.* (2009a) were also included).

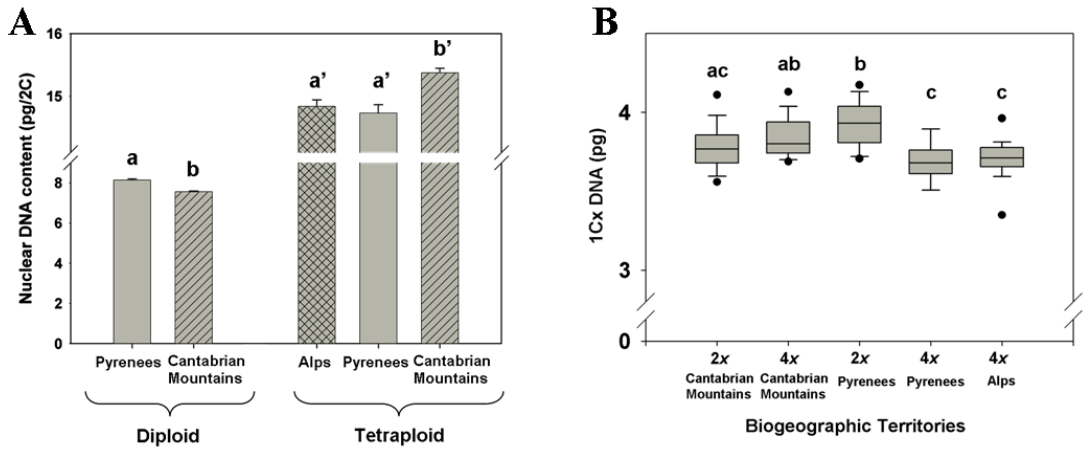


Figure 6. Holoploid (A) and monoploid (B) DNA amount in different cytotypes of *R. parnassiifolius s.l.* from three biogeographical territories. Means which are significantly different at $P < 0.001$ are indicated by different letters (Dunn's test). In box-plot representation, horizontal lines represent the median, and boxes and whiskers, respectively, the interquartile range and the non-outlier ranges. Circles denote outliers.

The holoploid genome size (2C) ranged between 7.80 pg and 8.47 pg in diploid, and 14.72 pg and 14.91 pg in tetraploid. Examining the statistical analyses, post-hoc tests revealed significant differences (P < 0.001) between tetraploid Pyrenees-Alps and Cantabrian populations, and also between diploid samples of the Pyrenees and the Cantabrian Mountains (Figure 6A). The monoploid genome size (1Cx) ranged from 3.68 pg (AMP and CHI) to 3.96. pg (ARL and ALB: massif of Picos Albos, Northwest of Spain) (1.07-fold difference), and statistical analyses showed significant differences (Figure 6B). In this case, so as not to mask effects of other diploid populations, plants from CAD and EIX of the Pyrenees were excluded from the analysis due to their confirmed genome size variation. To obtain insights into the relationships between populations characterized by genome size (population means), a cluster analysis was performed (Figure 7). Populations were clustered in two non-overlapping groups (diploid-tetraploid), differing nearly 2.13-fold in genome size. Additionally, within the tetraploid ploidy level, two main groups were observed: the first composed of the Pyrenean plants together with the Alpine ones and the other formed by the Cantabrian populations (except CM13). Regarding the diploids, three groups with non-random distribution were also distinguished where, in general, most of the populations within a cluster corresponded to specific biogeographical territories (Figure 7). The Spearman correlation coefficient (r_s) did not reveal significant correlations between genome size and altitude, longitude or latitude for accessions of *R. parnassiifolius s.l.* from the Cantabrian Mountains, the Pyrenees or the Alps (r_s = -0.0941, P = 0.721, n = 16; r_s = -0,0176, P = 0,943, n = 16; r_s = 0.124, P = 0.640, n = 16 respectively).

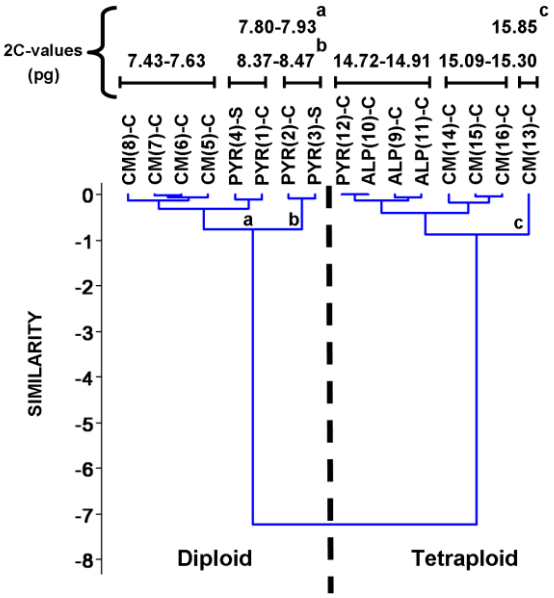


Figure 7. Cluster analysis (UPGMA: unweighted pair group method with arithmetic mean) of genome size data (population means) for 16 populations of *R. parnassiifolius s.l.* from throughout the Northwest Iberian Peninsula, the Pyrenees and the Alps. Two major clusters are delimited. Triploid and pentaploid plants were omitted from the analysis. Abbreviations: CM: Cantabrian Mountains; PYR: Pyrenees; ALP: Alps. [a,b]Genome size variation in diploid populations from the Pyrenees, for which introgressive hybridization has been suggested as a possible explanation. [c]Genome size in the tetraploid CM(13)-C population.

Discussion

Flow cytometry (FCM) is now the prevailing method for the measurement of genome size variation in plants. It can be used to screen many plants in a short time and can be applied to any plant tissue (Doležel & Bartoš 2005). Furthermore, FCM analysis offers an alternative to classic microscopic chromosome counting which is time consuming and also requires samples containing mitotically active cells. FCM can also be applied in a non-destructive way to rare species, including endemics with extremely narrow ranges of distribution (e.g. Castro *et al.* 2007). In some cases, difficulties with taxonomic classification may stem from undetected polyploidy, and for years ploidy level has been used as one of the main criteria for discrimination of closely related species (e.g. Španiel *et al.* 2008) or even at subspecific levels (Besnard *et al*. 2008). In addition to describing variation across entire geographical ranges, FCM has enabled researchers to map the fine-scale distribution of ploidies within individual populations (e.g. Baack 2004; Suda *et al.* 2004).

Intraspecific genome size variation

Genome size has also attracted an ever increasing amount of attention from plant taxonomists. In this field, nuclear DNA content may often facilitate the delimitation of taxa in various taxonomic ranks and may therefore influence taxonomic decisions (Kron *et al.* 2007). However, although divergences in nuclear DNA content among different species/subspecies within a genus are widely acknowledged (see the Plant DNA C-values database; Bennett & Leitch 2004), and in recent years there has been an increase in the number of studies reporting genome size variation, even at intrapopulational level (e.g. Schmuths *et al.* 2004; Suda *et al.* 2007a; Slovák *et al.* 2009), the presence and extent of intraspecific variation in genome size remains a controversial issue. Over time, several sources of artifactual variation in genome size, such as instrumental or methodological error or the interference of secondary metabolites in DNA staining have been identified (Doležel *et al.* 2007b).

Although numerous early reports of genome size variation below species level have been dismissed by subsequent investigations using best practice methodology (Greilhuber 2005), we are convinced that the divergence observed in this study, in selected *R. parnassiifolius* populations, is genuine: low coefficients of variation were achieved, arguing against a potential inhibitory secondary metabolites effect; and samples with different genome sizes analyzed together showed two distinct peaks (Figure 2) which, as Greilhuber *et al*. (2007) argued, is the most compelling evidence for true differences in the amount of nuclear DNA.

This difference in genome size in the diploid cytotype could be explained by the phenomena of aneuploidy or hybridization. In addition, several studies have found associations between genome size and environmental parameters, including temperature and precipitation (Knight *et al*. 2005; Knight & Beaulieu 2008). We are

of the opinion that this change in nuclear DNA variation could indeed be caused in greater part by hybridization phenomena, as we have found the hybrid *Ranunculus* ×*luizetii* Rouy (*R. parnassiifolius* L. x *R. pyrenaeus* L.) in one of the two populations, (EIX), where these differences in genome size were detected. However, we cannot rule out the combined effect of the above mentioned phenomena. In *R. parnassiifolius s.l.*, the maximum difference in genome size variation (within each cytotype) was detected in the diploid population of the Pyrenees (20.27%). Considering the maximum genome size heterogeneity reported in other species, for example 7.4% in *Hieracium pilosselliflorum* Nägeli & Peter (Suda *et al*. 2007a), 16.6% in *Festuca pallens* Host (Šmarda & Bureš 2006) and 23% in *Picris hieracioides* L. (Slovák *et al.* 2009), the variation revealed in *R. parnassiifolius s.l.* is rather larger.

Furthermore, it is relevant to note that it is the first time that a triploid cytotype has been observed. Until now, only diploid, tetraploid and pentaploid cytotypes had been described (Küpfer 1974; Vuille & Küpfer 1985; Vuille 1987; Cires *et al*. 2009a). The most plausible mechanism for triploid genesis is a fusion of unreduced (n = 2x) and reduced gametes (n = 1x). The somatic DNA content (2C-value) of any given polyploid is expected to exceed its diploid progenitor(s) in proportion to its ploidy; however, its genome size (Cx-value) would be expected to be similar (Kron *et al*. 2007). Variations in genome size (Cx-value) have been found in a number of plant groups though, either between species (e.g. Mishiba *et al*. 2000) or within species (e.g. Pecinka *et al*. 2006) and highly valuable information of taxonomic significance is stored in the Plant DNA C-values database (Bennett & Leitch 2004).

Genome downsizing following polyploidization has been reported quite frequently, and appears to be more common than the opposite process, i.e. genome expansion (Leitch & Bennett 2004). The Pyrenean populations of *R. parnassiifolius* studied show a clear decrease in monoploid genome size of polyploids (as occurs in Alpine populations) in comparison to diploids. A similar decrease has been observed in the mean population 2C-values between the diploid and tetraploid cytotype (ratio 1:1.8), and for other species within the genus *Ranunculus* (e.g. Hörandl & Greilhuber 2002). This could probably be explained by a partial elimination of repetitive DNA sequences in the polyploid genomes. Additionally, the Cx-value of tetraploid populations from the Cantabrian Mountains (Northwest Iberian Peninsula) appears to be equal or slightly greater than the diploid one. These slight changes could be attributed to genome duplications and movements of transposable elements which can quickly inflate genome sizes (e.g. Kapralov *et al*. 2009). Indeed, we should not exclude the possibility that within the total amount of genomic DNA, this also has a considerable influence (biological and otherwise) on the organism. That is to say, the nuclear DNA affects the phenotype independently of its encoded informational content: the "nucleotype theory" (Bennett 1971). These nucleotypic correlations were reviewed recently by Leitch & Bennett (2007). Whatever the origin of the

variation in genome sizes of *R. parnassiifolius s.l.*, such differences may give us an idea of independent polyploidization events within the group.

Comparison of different DNA reference standards

For quite some time, there has been debate on the use of animal reference standards. Although opinions are varied, a rather limited advantage of animal over plant standards has been indicated. With the exception of *Caenorhabditis elegans*, for which 1C = 100.25 Mbp (http://wormbase.org/) has been established, most of the animal exact genome sizes are not known, and thus they do not offer any advantage over plants as primary reference standards. Chicken red blood cells (CRBC) are frequently used as reference standard in FCM of nuclear DNA content in higher plants (Loureiro *et al.* 2008), as they are commercially available or can be self-prepared, fixed and stored, often for years, at low temperatures. Johnston *et al*. (1999) demonstrated difficulties when using CRBC as reference standard, as problems with variability were observed.

Based on previous FCM analyses from herbarium voucher, fresh material and frozen material of *R. parnasssifolius s.l.* from the Northwest Iberian Peninsula (Cires *et al*. 2009a), a new value for the chicken genome size has been established for use as an internal reference standard (2C = 3.14 ± 0.155 pg). This value is higher than the commonly accepted values of between 2.33 and 2.50 pg (cf. Bennett *et al*. 2003). To re-test our value, we decided to analyze the samples from the Alps, using CRBC and *P. sativum* cv. 'Ctirad' as internal standards. No significant differences were detected (Figure 3, Table 3). In any case, if CRBC is to be used in a study of genome size estimation, we would recommend a pilot study to define the "true value" for the CRBC employed, and that the same single blood sample be used for all runs. In the case of failing to verify the exact genome size for the CBRC, we would strongly suggest the use of plant standards.

Morphometric analyses

Generally, there is no consensus in recent literature on the question of taxonomic treatments of taxa including multiple cytotypes, but autopolyploids have traditionally been considered conspecific (mostly just as cytotypes of a single species, without recognition of infraspecific units). This fact has been pointed out in the recent review by Soltis *et al*. (2007), who focused on the taxonomic significance of autopolyploidy and argued for its acceptance as a significant mechanism of speciation. They stressed that even if morphological variation does not allow reliable cytotype identification, taxa should be considered as distinct species on the basis of geographical and ecological differences and reproductive isolation (meeting the criteria of multiple species concepts), because of the evolutionary significance of such plants as well as for practical reasons (e.g. conservation of rare cytotypes). Rowley (2007), however, contradicted their arguments and argued for a more flexible approach to taxonomic classification, employing infraspecific categories as well. Our taxonomic decision also relies on other numerous cases that document the

presence of intraspecific cytotypes with similar variation patterns (e.g. *Cardamine pratensis* L., Lihová *et al.* 2003; *Senecio jacobea* L., Hodálová *et al.* 2007; *Juncus biglumis* L., Schönswetter *et al.* 2007). Some authors have preferred a concept of a single species (e.g *Centaurea stoebe* L., Španiel *et al*. 2008) with two cytotypes, without any infraspecific division. A similar case to that documented in this work occured with the polyploid complex *Festuca airoides* Lam. (Šmarda 2008), and nowadays modern European floras include all plants of this complex (the diploid *F. airoides*, the tetraploids *F. supina* Schur and *F. vivipara* (L.) Sm. and mixed populations of tetraploid and pentaploid plants) under the oldest name *F. arioides*.

To resolve heteroploid taxonomies, the combined use of FCM and multivariate morphometrics has proven particularly beneficial. Together, these analyses have provided reliable insights into the range and organization of phenotypic variation and have often allowed researchers to distinguish morphologically indiscernible taxa (Suda *et al.* 2007b). Until now, there have been no clear criteria to identify the infraspecific taxa within *R. parnassiifolius s.l.* For instance, Baudere & Gauquelin (2005) quoted the *R. parnassiifolius* subsp. *parnassiifolius* and *R. parnassiifolius* subsp. *heterocarpus* in the Pyrenees, but differentiation of these two subspecies is mainly based on the type of substrate (i.e. "*R. p.* ssp. *heterocarpus* Küfer le taxon tétraploïde des substratums calcaires, *l.c.* pp. 10"), but our observations have not always concurred with these assertions.

Referring to the record of Puigllançada (CAD), the population has been attributed several times to the tetraploid subspecies *R. parnassiifolius* subsp. *heterocarpus* (Vigo i Bonada 1983), because of being placed in a limestone substrate. However cytometric data obtained in this work indicates that this is a diploid population. Similarly, the population of Coll de la Creu de l'Eixol (EIX) has also been treated as *R. parnassiifolius* subsp. *heterocarpus* (Carrillo i Ortuño & Ninot i Sugranyes 1992), whereas in our case we identified it as a diploid population located in a siliceous area. In this sense, similar studies with unclear morphological differentiation of individual taxa and contradictory distribution data for some taxa are common (e.g. Koutecký 2007). In summary, in the sampling range of this work, we define three different morphotypes (see Results).

Geographical scale of genome size variation

Our results contradict the data published by Küpfer (1974, 1980), where the author set out that the three diploid races of *R. parnassiifolius* can be considered schizoendemics derived from a primitive singameon. Furthermore, in the cited work, Küpfer considers the *R. parnassiifolius* subsp. *favargeri* to be a Cantabrian-Pyrenean patroendemic, from which the polyploid *R. parnassiifolius* subsp. *heterocarpus* emerged. Küpfer suggests that the existing morphological evolution does not depend on geographical or genetic isolation; rather, he proposes that it could be due to differences in environmental performance. Our data indicate that plants from distinct biogeographical territories do show clear-cut differences as regards morphological

characters and genome size (Figure 5, 6 & 7). The difference in genome size we observed in the Pyrenees between diploid populations contrasts with the uniformity of their morphological characters. This inter-population variation in genome size has also been reported for more-or-less distant populations of other genus *Hordeum* L. (Jakob *et al*. 2004) or *Koeleria* Pers. (Pecinka *et al*. 2006) for instance.

It has long been recognized that related asexual and sexual taxa have different distributional patterns. This phenomenon, summarized under the term "geographical parthenogenesis" was coined by Vandel (1928) and has been described in both animals and plants (see reviews in Koltunow & Grossniklaus 2003; Ozias-Akins 2006; Hörandl 2006, 2009; Hörandl *et al.* 2008). Focusing on plant species with geographical parthenogenesis, asexual organisms (apomictic) frequently occupy larger and more northern distribution areas, range to higher latitudes and altitudes than their sexual relatives, and tend to occupy previously glaciated areas (Asker & Jerling 1992; Hörandl 2009). However, each taxon has its own specific pattern and not all the above characteristics are necessarily present. Although several nonexclusive hypotheses have been proposed to explain the widespread distribution of apomictic groups (e.g. Haag & Ebert 2004; Hörandl 2009), geographical parthenogenesis cannot be explained by a single scheme, and many aspects of this hypothetical framework need to be further analyzed. Previous studies suggested an autopolyploid origin in *R. parnassiifolius* (see Diosdado 1991); and the present work confirmed those results reported by Vuille & Küpfer (1985) and Hüber (1988) suggesting that the mode of reproduction in tetraploid individuals is apomictic.

According to Hörandl (2009), there is recent evidence to support the idea of the natural origin of apomixis in flowering plants, which seems to be related to a combination of hybridization and polyploidy. However, apomixis in cytotypes of autopolyploid origin has also been demonstrated (reviewed by Gornall 1999; Grimanelli *et al*. 2001), inferring that hybrid origin is not a prerequisite for asexual reproduction. It may be that genome duplication *per se* causes a "genomic shock" such as hybridization, resulting in the deregulation of reproductive pathways (Grimanelli *et al*. 2001). However, an alternative explanation suggests that polyploidy is required for the inheritance of genetic factors controlling apomixis (Hörandl 2009). Furthermore, glaciations may cause area fragmentation and geographical isolation of sexual populations (Hewitt 1996, 2000). During cold periods and glacial maxima (in the Northern hemisphere), the distribution range of most species would have pushed southwards or to lower elevations in high mountain regions. In warmer interglacial periods, distribution ranges would have expanded again northwards and upwards. Then, during re-colonization, asexual populations would have dispersed rapidly as a result of uniparental reproduction (low cost of dispersal) while sexual populations would have remained restricted because of the density-dependence of sex. This "opportunistic model" of geographical parthenogenesis (Hörandl 2009) to describe the hybrid origin of asexuality, could also be applied to *R. parnassiifolius s.l.*

Recently, a geographical parthenogenesis hypothesis and a colonization edge syndrome due to postglacial dispersals have been demonstrated in the *R. kuepferi* complex Greuter & Burdet (Burnier *et al.* 2009; Cosendai & Hörandl 2010). Similarly, looking globally at the *R. parnassiifolius s.l.* complex it could be considered a clear-cut example of geographical parthenogenesis. However, if we focus only on the high mountains of South-West Europe (the Pyreenes and the Cantabrian Mountains) this geographical parthenogenesis cannot be fully explained by a single scheme, and many aspects of this hypothetical framework need to be analyzed further (e.g. Mráz *et al.* 2009). In this South-West region of the distribution there appears to be a zone where both cytotypes frequently occur, sometimes even with mixed populations, and occupy low and high altitude areas interchangeably.

Concluding remarks

In the present study, FCM allowed the analysis of a large number of plants from many populations throughout the wide distribution range in the Pyrenees and the Alps. This investigation has thus shed light on the geographical distribution of ploidy levels in the *R. parnassiifolius* complex through its detailed examination. The knowledge that the amount of nuclear DNA may vary considerably even among closely related taxa, whilst showing remarkable constancy within most species, constitutes a rationale for employing genome size as an important taxonomic criterion. This difference in DNA also concurs with differences in morphology, which might indicate different evolutionary histories. The *R. parnassiifolius* complex probably underwent extensive range fragmentation during the Pleistocene, and secondary contact has probably led to the origin of tetraploid apomicts. Most of the endemic alpine plants of mountain environments are characterised by historical fragmentation and disjunction in their population systems. It is possible that several different evolutionary lineages may be hidden within each morphological group.

In light of the results obtained in this work, together with previous knowledge, we would conclude that the taxonomic treatment of *R. parnassiifolius s.l.* remains controversial. A multimethod approach, with an emphasis on molecular analyses, will be necessary to gain further insight into the evolutionary history of *R. parnassiifolius s.l.* and to elucidate its taxonomy. These taxa may provide indirect evidence of its population history, and therefore contribute to a more general understanding of evolutionary processes in isolated populations. Moreover, comparative analysis of a large body of phylogeographical data focused on the Iberian Peninsula, one of the most important glacial refugia in the European subcontinent, will enable the identification and refined delineation of multiple coincidental refugial areas, that is, the "refugia within refugia" scenario described by Gómez & Lunt (2007), and therefore facilitate the establishment of priority strategies for conservation programmes.

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CAPÍTULO 3

42º 14' 33.8'' Lat. N
6º 44' 40.0'' Long. W

The Iberian endemic species *Ranunculus cabrerensis* Rothm.: an intricate history in the *Ranunculus parnassiifolius* L. polyploid complex

Summary

The orophilous plant, *Ranunculus parnassiifolius* L., is a polyploid complex which is widespread throughout the Southern European mountains, where at least five taxa have traditionally been recognized. The aim of this study is to test whether *R. parnassiifolius* subsp. *cabrerensis* should be treated as an independent species of the *R. parnassiifolius* polyploid complex and constitutes, therefore, an evolutionary line in itself. To disentangle its evolutionary history and taxonomy, we used genome size estimation based on flow cytometric measurements, multivariate morphometric analyses, polymerase chain reaction–restriction fragment length polymorphisms and subsequent sequencing of cpDNA regions (*trnS*, *trnR-atpA*, *atpH-atpI*).

This study provides molecular and morphological evidence for the recognition of *R. cabrerensis* at species level, rather than as an intraspecific taxon of *R. parnassiifolius*. Furthermore, it is concluded that those plants previously known as *R. parnassiifolius* subsp. *muniellensis* should be systematized at the subspecies level as *R. cabrerensis* subsp. *muniellensis*. This contribution highlights the benefit of combining diverse approaches to obtain knowledge about relict populations and for the implementation of suitable conservation measures.

Este capítulo se corresponde con la publicación:

- Cires, E., Fernández Prieto, J.A. (2011). The Iberian endemic species *Ranunculus cabrerensis* Rothm.: an intricate history in the *Ranunculus parnassiifolius* L. polyploid complex (enviado).

Introduction

Phylogeography is the phylogenetic analysis of geographically contextualized genetic data for the testing of hypotheses regarding the causal relationships between geographical phenomena, species distributions and the mechanisms driving speciation (Avise 2000, 2009). Molecular phylogeographical studies of plant species from European mountains have increased in number in recent years (e.g. reviewed in Comes & Kadereit 2003; Tribsch & Schönswetter 2003; Schönswetter *et al.* 2005, 2009; Kropf *et al.* 2008). Most of these studies are focussed on the Alps and neighboring high mountain ranges, such as the Pyrenees or the Carpathians; while molecular analyses in the Southwestern European mountains are centred on the Mediterranean Basin (e.g. Vargas 2003; Kropf *et al.* 2006, 2008). However, phylogeographical studies considering the Northwest Iberian Peninsula, and particularly the Cantabrian Mountains and surroundings (e.g. "Montes de León"), are still limited (but see Kropf *et al.* 2003; Vargas 2003; Dixon *et al.* 2007, 2008; Terrab *et al.* 2008; Peredo *et al.* 2009). This although, according to Schmitt *et al.* (2010), the Cantabrian Mountains represent an important mountain area for the survival of old lineages which can be interpreted as relics of the cold stages of a more distant past.

Species groups with complicated phylogenetic relationships, evolutionary histories and intraspecific variations are not rare among vascular plants (see review Hickerson *et al.* 2009). A good example is the alpine buttercup *Ranunculus parnassiifolius* L., a polyploid complex widespread throughout the Southern European mountains with five subspecies recognized (Küpfer 1974; Bueno Sánchez *et al.* 1992): *R. parnassiifolius* subsp. *parnassiifolius*: diploid, silicicole, endemic in the Eastern Pyrenees; *R. parnassiifolius* subsp. *cabrerensis* Rothm.: diploid, silicicole, endemic in the Northwestern mountains of Spain; *R. parnassiifolius* subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto: diploid, silicicole, endemic in the Western Cantabrian Mountains (Muniellos Biosphere Reserve) with a single population; *R. parnassiifolius* subsp. *favargeri* P.Küpfer: diploid, calcicole, endemic in the Cantabrian Mountains and the Western Pyrenees; and finally, *R. parnassiifolius* subsp. *heterocarpus* P.Küpfer: tetraploid, calcicole, spread throughout the Cantabrian Mountains, the Central Pyrenees and the Alps. In previous papers we have proved that this classification is not entirely clear (Cires *et al.* 2009, 2010), and it has therefore become essential to gain deeper insight into the evolutionary history of *R. parnassiifolius* group and to elucidate its taxonomy.

We consider there are numerous arguments to support the separation of the *R. parnassiifolius* subsp. *cabrerensis* from the *R. parnassiifolius s.l.* polyploid complex, and that it should consequently be treated as an independent species (*R. cabrerensis* versus *R. parnassiifolius*) and that in fact it constitutes an evolutionary line in itself. This treatment was adopted by Rothmaler (1934) when he described the taxon "*Ranunculus cabrerensis* Rothmaler nov. sp. (...) Ex affinitate

R. parnassifolii L., a quo differt habitu robustiore, foliis utrinque lanatis, petiolis lanatis, rostro carpelli longiore spiram formante" (*l.c.* p. 148). However, later in the same paper Rothmaler (1934) concluded that there was insufficient reason to describe the taxon as a species, and it was therefore included as a geographical race of the *R. parnassiifolius* complex: "Est forma geographica et melius *R. parnassifolius* L. ssp. *R. cabrerensis* Rothm. nominanda" (*l.c.* p. 148). Additionally, as was indicated by Bueno Sánchez *et al.* (1992), *R. parnassiifolius* subsp. *muniellensis* has stronger similarities (morphological, ecological, etc.) with *R. parnassiifolius* subsp. *cabrerensis*, which leads us to hypothesize that it could be a lineage within of *R. cabrerensis* rather than of *R. parnassiifolius*.

To explore this main hypothesis, we supported our study by the use of morphological data, flow cytometry (FCM) analysis and plastid genome (cpDNA) data. In fact, the use of morphological data together with genome size variation to resolve complex groups (e.g. Suda *et al.* 2007), have been frequently used. The estimation of nuclear DNA content in absolute terms or in relative units as an indicator of ploidy level is by far the dominant use of FCM: it enables cytotype surveys over large spatial scales and involves large population samples. It has consequently revolutionized many fields of polyploid plant biology and changed the perception of the magnitude of ploidy variation and its dynamics in natural conditions (Doležel *et al.* 2007). Similarly, the use of cpDNA regions which are structurally stable, haploid, non-recombinant, and maternally inherited in the majority of angiosperms studied has proved valuable in resolving closely related taxa (e.g. Mort *et al.* 2007; Hufbauer & Sforza 2008).

Within this framework, we addressed the following objectives: (1) to resolve taxonomic uncertainties among the orophilous taxa *R. parnassiifolius* subsp. *cabrerensis* and *R. parnassiifolius* subsp. *muniellensis* versus the *R. parnassiifolius s.l.* polyploid complex. That is to say, determine whether they might be considered subspecies of the same taxon or otherwise have their own identity at the species level; and (2) to infer, for the first time, the phylogeography and population structure within the *R. parnassiifolius* group. Finally, taking into account the results achieved, a renewed taxonomic treatment is presented.

Materials and methods

Study area

As a rule, 15-20 plants of *R. parnassiifolius* polyploid complex were collected from each sample site throughout the whole distribution range between 2006 and 2008 (Table 1).

Table 1. Locality of sampled populations of *Ranunculus* and outgroup plants used in the molecular analyses. The populations incorporated into the sequencing study are given at the bottom of the table. Voucher specimens are kept in the Herbarium of the University of Oviedo (FCO).

Acronym	Subsp. Syst.[†]	Locality, altitude, collector/s and date	Country	Latitude/Longitude	Voucher specimens	GenBank accession (*trnS*, *trnR-atpA* and *atpH-atpI*)
ABE	RPC	Abelgas de Luna. Near the stream Valverde, north of Peña Piquera (Sena de Luna, León); 1956 m; *CC & EC*; 2008/06/29	E	42º 53' 32.7'' N 6º 4' 59.0'' W	31368	HM026686, HM026708, HM026730
AMP	RPH	Behind the Refuge Auronzo, Tre Cime di Lavaredo (Véneto, Belluno, Auronzo di Cadore); 2353 m; *CC, EC, JAFP & MC*; 2008/07/18	I	46º 36' 53.0'' N 12º 17' 48.2'' E	31354	HM026687, HM026709, HM026731
ARL	RPF	Col d'Arlas (Aquitaine); 1971 m; *EC & JAFP*; 2007/07/27, 2008/06/10	F	42º 58' 10.9'' N 0º 45' 4.5'' W	31355; 31356; 31357	HM026688, HM026710, HM026732
BAÑ*	RPC	Lago de la Baña, Sierra Cabrera (Encinedo, León); 2000 m ; *BJA, EC & LG*; 2007/06/14	E	42º 14' 33.8'' N 6º 44' 40.0'' W	31369	HM026689, HM026711, HM026733
CAN*	RPF	Central Massif of the Picos de Europa, Collado de la Canalona (Camaleño, Cantabria); 2455 m; *AF & EC*; 2007/07/11, 2008/07/07	E	43º 10' 46.0'' N 4º 48' 53.7'' W	31107; 31108; 31109; 31110	HM026690, HM026712, HM026734
CHI	RPH	End of the track that begins in Chisagües, south of Sierra de Liena (Bielsa, Huesca); 2346 m; *EC & JAFP*; 2007/07/25, 2008/06/27	E	42º 41' 17.3'' N 0º 9' 26.4'' E	31360	HM026691, HM026713, HM026735
FON*	RPP	Collada de Fontalba (Queralbs, Gerona); 2223 m; *EC & JAFP*; 2007/06/27, 2008/06/08	E	42º 22' 2.0'' N 2º 8' 29.1'' E	31363; 31365; 31364	HM026692, HM026714, HM026736
MOL	RPH	Western Massif of the Picos de Europa, Los Moledizos (Posada de Valdeón, León); 2043 m; *EC*; 2008/07/30	E	43º 10' 35.3' 'N 4º 57' 47.9'' W	31114	HM026693, HM026715, HM026737
MUN*	RPM	Peña Velosa, Reserva Natural Integral de Muniellos (Cangas del Narcea, Asturias); 1442 m; *EC*; 2007/07/18	E	43º 1' 9.0'' N 6º 44' 45.4'' W	31370	HM026694, HM026716, HM026738
YEG	RPC	Portillo de las Yeguas, San Glorio (Vega de Liébana, Cantabria); 2094 m; *AB & EC*; 2007/06/19	E	43º 3' 17.6'' N 4º 44' 20.4'' W	31371	HM026695, HM026717, HM026739
Outgroup samples						
OUT 1	RP	Pas de la Casa (Encamp); 2579 m; *EC & JAFP*; 2007/06/28	AND	42º 33' 16.6'' N 1º 43' 7.2'' E	31372	HM026696, HM026718, HM026740
OUT 2	CP	From the Alto de la Farrapona to Lago Cerveriz, opposite Collado Balbarán (Somiedo, Asturias); 1649 m; *EC & JAFP*; 2007/06/12	E	43º 3' 31.5'' N 6º 5' 40.0'' W	31373	HM026697, HM026719, HM026741
Samples added to DNA sequencing						
ABP	RPH	Albulapass, Blais Cuorta (Graubünden); 2507 m; *CC, EC, JAFP & MC*; 2008/07/19	CH	46º 35' 22.1'' N 9º 50' 28.7'' E	31353	HM026698, HM026720, HM026742
ALB	RPH	Massif of Los Picos Albos, above Lago Cerveriz, Lagos de Saliencia (Somiedo, Asturias); 1946 m; *EC & JAFP*; 2007/06/18, 2008/07/01	E	43º 2' 51.8'' N 6º 7' 23.4'' W	31103; 31104	HM026699, HM026721, HM026743
BOC	RPF	Central Massif of the Picos de Europa, Jou de los Boches (Cabrales, Asturias); 2136 m; *AF & EC*; 2007/07/13, 2008/07/10	E	43º 11' 9.8'' N 4º 49' 40.0 '' W	31105; 31106	HM026700, HM026722, HM026744
CAD	RPH	Top of Puigllançada, Parc Natural Cadí-Moixeró (Bagà, Barcelona); 2165 m; *EC & JAFP*; 2007/06/28, 2008/06/08	E	42º 18' 19.2'' N 1º 55' 36.8'' E	31358; 31359	HM026701, HM026723, HM026745

Acronym	Subsp. Syst.†	Locality, altitude, collector/s and date	Country	Latitude/Longitude	Voucher specimens	GenBank accession (*trnS*, *trnR-atpA* and *atpH-atpI*)
CAR	RPH	Eastern Massif of the Picos de Europa, Collado de San Carlos (Camaleño; Cantabria); 2050 m; *CC & EC*; 2008/07/30, 2009/07/05	E	43º 12' 22.9'' N 4º 41' 48.8'' W	31111; 31112; 31374; 31375	HM026702, HM026724, HM026746
EIX	RPH	Coll de la Creu de l'Eixol (Espot, Lérida); 2246 m; *EC & JAFP*; 2007/06/29, 2008/06/09	E	42º 32' 24.9'' N 1º 5' 5.5'' E	31361; 31362	HM026703, HM026725, HM026747
GCH*	RPH	Grand Chavalard, west face (Valais); 2071 m; *CC, EC, JAFP & MC*; 2008/07/21	CH	46º 10' 10.7'' N 7º 6' 15.6'' E	31366; 31367	HM026704, HM026726, HM026748
GRA	RPH	Eastern Massif of the Picos de Europa, Canal de las Grajas (Camaleño, Cantabria); 1688 m; *AF & EC*; 2008/07/30	E	43º 10' 46.8'' N 4º 45' 19.6'' W	31113	HM026705, HM026727, HM026749
PAN	RPF	Central Massif of the Picos de Europa, pathway from Pandébano to Vega de Urriellu, opposite Jou Lluengu (Cabrales, Asturias); 1632 m; *AF & EC*; 2007/07/12, 2008/05/20	E	43º 12' 49.0'' N 4º 48' 51.9'' W	31115; 31116	HM026706, HM026728, HM026750
URR	RPF	Central Massif of the Picos de Europa, Vega de Urriellu once left behind the refuge J.D. Ubeda in the direction of Horcados Rojos (Cabrales, Asturias); 1967 m; *AF & EC*; 2007/07/12, 2008/07/10	E	43º 12' 12.1'' N 4º 49' 15.1'' W	31117; 31118; 31119	HM026707, HM026729, HM026751

* *Locus classicus*: localities where plants were first collected for the original taxa descriptions (in the case of FON, although the exact "locality type" of *R. parnassiifolius* subsp. *parnassiifolius* is unknown, Küpfer (1974) states that the type locality is in the Eastern Pyrenees: "ainsi is est hors de doute que le type du *R. parnassifolius* appartient à une population diploïde des Pyrénées orientales" *l.c.*, p. 184).

† Subspecific systematization of samples according to previous studies [RPC: *Ranunculus parnassiifolius* subsp. *cabrerensis*; RPF: *R. parnassiifolius* subsp. *favargeri*; RPH: *R. parnassiifolius* subsp. *heterocarpus*; RPM: *R. parnassiifolius* subsp. *muniellensis* and RPP: *R. parnassiifolius* subsp. *parnassiifolius*] (see Rothmaler 1934; Küpfer 1974; Vigo i Bonada 1983; Cook *et al.* 1986; Jalas & Suominen 1989; Bueno Sánchez *et al.* 1992; Carrillo i Ortuño & Ninot i Sugranyes 1992) and herbarium material (ABH, AH, ALME, ARAN, B, BC, BCN, BHUPM, BIO, BOLO, BOZ, CCEC, CLF, COA, DR, EMMA, FCO, FR, GDA, GDAC, GJO, HEID, HJBS, HSS, JACA, JAEN, JBAG, KL, LEB, LYJB, MA, MACB, MAF, MSTR, OSN, P, PAD, PAMP, REG, RO, ROST, ROV, SALA, SANT, SEV, STR, TFC, UPNA, VAL, VIT, WFBVA, WU, Z, ZT and GBIF (2010), http://www.gbif.org/).

Outgroup samples: RP: *Ranunculus pyrenaeus*; CP: *Caltha palustris*.

Collector abbreviations: *AB* = A. Bueno; *AF* = A. Fernández; *BJA* = B. Jiménez-Alfaro; *CC* = C. Cuesta; *EC* = E. Cires; *JAFP* = J.A. Fernández Prieto; *LG* = L. González; *MC* = M. Ceballos.

Country abbreviations: AND, Andorra; CH, Switzerland; E, Spain; F, France; I, Italy.

Field-collected specimens were potted and kept in a greenhouse at room temperature for cytometric and morphometric analysis. Furthermore, fresh leaf material was collected for molecular analysis: leaves taken from plants at the site were wrapped in moistened paper enclosed in a plastic bag and transported in a cool box to avoid high temperatures during transportation; then plant tissues were frozen and stored at -80 ºC. The material also included samples from the *locus classicus* of all subspecies of the *R. parnassiifolius* complex. In addition, one other European species belonging to the genus *Ranunculus* (*R. pyrenaeus* L.) and also other species of the family Ranunculaceae (*Caltha palustris* L.) were sampled as outgroups (Table 1). The distributions of species in the European Mountain System as defined here include the Cantabrian Mountains and range from there over the Pyrenees and the Alps. Some of the populations designated by acronyms had been previously studied with respect to morphology and ploidy levels by Cires *et al.* (2009, 2010). Additionally, voucher specimens were collected and kept in the Herbarium of the University of Oviedo (FCO).

Genome size estimation

Samples for FCM measurements were prepared from fresh tissues of young leaves. Nuclear DNA amounts were estimated with propidium iodide staining, using a Cytomics FC 500 (Beckman Coulter) with 488-nm excitation from an argon ion laser. Data analysis was carried out using Cytomics RXP Analysis (Beckman Coulter, Inc. 2006), following the protocol described by Cires *et al.* (2009, 2010). At least 3 individuals from each population were analyzed on 3 different days to avoid errors due to instrumental drift, and at least 5000 nuclei were analyzed per sample. The position of nuclei G0/G1 peaks in histograms of relative fluorescence intensity was used to estimate nuclear DNA content. The 2C DNA content of *R.* grex *parnassiifolius* (2C = holoploid genome size; sensu Greilhuber *et al.* 2005) was calculated as:

$$= \frac{\textit{Ranunculus}\ \text{sp.G0/G1 peak mean}}{\text{reference standard G0/G1 peak mean}} \text{ x nuclear DNA content of reference standard}$$

The monoploid genome size (1Cx; sensu Greilhuber *et al.* 2005) of all plants was also calculated in mass values (pg).

Morphometric analyses

Twenty-three characters successfully employed in previous works were measured for plants collected (20 plants per population) (see Table 2 in Chapter 1 for abbreviations, p. 32). These included quantitative and qualitative characters, as well as ratios. The character set was chosen on the basis of published determination keys, flora handbooks, and our own observations. Principal Coordinates Analysis (PCoA) together with Gower similarity coefficient (Gower 1971) were conducted. Descriptive statistical parameters of the measured characters (mean, median, standard deviation and percentiles) for the different taxa of *R.* grex *parnassiifolius*

are presented in box-plot graphics.

DNA extraction and polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP)

DNA was isolated applying an extraction method based on CTAB (cetyl trimethyl ammonium bromide) buffer (Doyle & Doyle 1987) with slight modifications (see Appendix I). Six samples were used in a pilot study to test universal primers of 57 cpDNA regions (Demesure *et al.* 1995; Powell *et al.* 1995; Bryan *et al.* 1999; Weising & Gardner 1999; Grivet *et al.* 2001; Chung & Staub 2003), excluding loci ccmp 8 and ccmp 9 from the analyses, as they have already been shown by the above-cited authors to be poorly conserved in angiosperms. Annealing temperatures (Ta) were tested in a gradient between 50-59 ºC using a gradient thermal cycler (MyCycler™, Bio-Rad). Twenty-five cpDNA regions were successfully amplified and used for subsequent screening of polymorphism (Table 2).

Table 2. cpDNA fragments successfully amplified during the study. Empirically selected annealing temperatures (Ta) are given for each fragment.

cpDNA region	Consensus Primer	Annealing temperature (ºC)	Approx. size (bp)*	Reference#
trnS	ccmp 2	58	189	a
trnG	ccmp 3	50	112	a
rps2	ccmp 5	58	121	a
orf77-orf82	ccmp 6	58	103	a
trnK	ccSSR 1	58	174	b
trnG	ccSSR 3	58	107	b
trnR-atpA	ccSSR 4	58	205	b
rps2-rpoC2	ccSSR 5	58	270	b
rpoB	ccSSR 6	58	299	b
psbC-trnS	ccSSR 7	58	349	b
ycf3	ccSSR 8	58	249	b
ycf3	ccSSR 9	58	173	b
rpl20-clpP	ccSSR 11	50	165	b
psbB-psbT	ccSSR 12	58	249	b
rps3-rpl22	ccSSR 13	50	264	b
rpl2-rpl23	ccSSR 15	58	264	b
trnR-rrn5	ccSSR 21	58	280	b
orf98-trnS	NTCP 7	58	175	c
trnG-trnR	NTCP 9	58	237	c
rrn5-trnR	NTCP 37	58	143	c
trnR-rrn5	NTCP 39	58	156	c
rp12-trnH	NTCP 40	50	163	c
atpH-atpI	*atpH-atpI*	58	1228	d
trnS-trnf$_M$	Sf$_M$	58	1254	d
trnD-trnT	DT	58	1800	e

*Expected size according to the tobacco chloroplast genome.

#: a: Weising & Gardner (1999); b: Chung & Staub (2003); c: Bryan *et al.* (1999); d: Grivet *et al.* (2001); e: Demesure *et al.* (1995).

Unamplified fragments or unclear amplifications: ccmp 1, ccmp 4, ccmp 7, ccmp 10, ccSSR 2, ccSSR 10, ccSSR 14, ccSSR 16, ccSSR 17, ccSSR 18, ccSSR 19, ccSSR 20, ccSSR 22, ccSSR 23, NTCP 2, NTCP 4, NTCP 5, NTCP 6, NTCP 13, NTCP 16, NTCP 18, NTCP 19, NTCP 20, NTCP 24, NTCP 26, NTCP 27, NTCP 30, NTCP 32, NTCP 34, RP 19, SOYCP, QS.

Amplification of selected regions was carried out in 25 µL of reaction mixture containing 25 ng template DNA, 2.5 µL of 10x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.08 µM of each primer and 1 U of Taq DNA polymerase (Invitrogen). Cycling conditions were as follows: initial template denaturation for 5 min at 94 ºC, followed by 35 cycles of 1 min at 94 ºC, 1 min at annealing temperature (Table 2), 1 min at 72 ºC, plus a final extension of 10 min at 72 ºC.

All successfully amplified regions were tested for restriction polymorphisms, spanning major parts of the species distribution range. These samples (represented by 48 individuals) included different populations, representing both environmental performance (calcicolous and siliceous) and different ploidy level (diploids and tetraploids): 10 individuals per population of *R. parnassiifolius* subsp. *cabrerensis* (ABE, BAN, YEG); 10 individuals of *R. parnassiifolius* subsp. *muniellensis* (MUN); 1 individual from the different populations of other *R. parnassiifolius* subspecies (AMP, ARL, CAN, CHI, FON, MOL); and finally 2 outgroups (OUT 1, OUT 2).

PCR products were digested with five restriction enzymes (*Eco*RI, *Hae*III, *Hinf*I, *Mse*I, *Taqa*I: New England Biolabs, Inc.). Reactions were carried out according to the manufacturers' suggestions in a total volume of 10 µL. The restriction probes were incubated at 65 ºC for *Taqa*I, and for all other enzymes at 37 ºC, for 4 h. Products were separated on 2.5% agarose and stained with ethidium bromide.

In the PCR–RFLP data analysis, the lengths of uncut PCR products and restricted fragments were estimated using 100-bp DNA ladder (Fermentas, Canada, Inc.) and lambda phage DNA (New England Biolabs, Inc.) digested with *Eco*RI and *Hind*III as the size marker. The amplification products were photographed on a UV transilluminator (GelLogic 100, Kodak). Restriction-site polymorphisms and length polymorphisms (indels) were treated as equally weighted single polymorphic sites, scored as present (1) or absent (0), and included in a binary matrix. A minimum spanning tree of haplotypes was constructed in Arlequin 3.1 (Excoffier *et al.* 2005).

DNA sequencing

In a pilot study, 6 plastid regions (*trnS*, *trnR-atpA*, *rps3-rpl22*, *atpH-atpI*, *trnS-trnf*$_M$; *trnD-trnT*; see Table 2) used in the PCR-RFLP analyses were amplified and sequenced to find the most variable sequences and to test the reliability of the PCR-RFLP.

The same DNA regions were also sequenced from the other species of *R. parnassiifolius* complex (see bottom part of the Table 1) to reconstruct phylogenetic relationships of the cpDNA haplotypes and survey interspecific variation. Finally, 3 plastid regions (*trnS*, *trnR-atpA*, *atpH-atpI*) were amplified and sequenced for all 22 populations (one individual per population), using direct sequencing. Both strands were sequenced to check for the reliability of detected differences.

PCRs were performed as in the previous section. Amplified products were

treated with ExoSAP-IT (USB Corporation, Ohio) following the manufacturers' protocols. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator v. 2.0, Applied Biosystems, Little Chalfont, UK). All chromatograms were visually examined to correct possible misinterpretations of the computational routine. DNA sequences were aligned using ClustalX 2.0.10 within Geneious Pro 4.7.6 (Biomatters, Auckland, New Zealand). In addition, all the sequences were checked with Muscle 4.0 in order to assure the accuracy of results.

Sequences from the 3 plastid regions were concatenated based on the assumption that the plastid genome forms a single linkage group; therefore the 3 regions were combined into a multilocus dataset (total evidence approach), and IUPAC (International Union of Pure and Applied Chemistry) symbols were used to represent nucleotide ambiguities.

We analyzed the nucleotide diversity π (Nei 1987) as the number of nucleotide differences per site, and also the average number of nucleotide differences k (Tajima 1983). These two measures were calculated between sequences of the multilocus region (total evidence approach) and *trnS*, *trnR-atpA* and *atpH-atpI* separately for the whole matrix, the *R. parnassiifolius* group matrix, and finally for the diploid populations matrix in DnaSP 5.10 (Librado & Rozas 2009).

In order to visualize the genetic relationships between the observed haplotypes, we adopted a network-based rather than a tree-based approach because networks can take into account intraspecific processes leading to divergence of multiple descendant haplotypes from a single ancestral haplotype, as well as coexistence of descendant and ancestral haplotypes, or reticulate evolution (Posada & Crandall 2001).

Two different networks for the cpDNA were conducted. Firstly, the statistical parsimony algorithm (Templeton *et al.* 1992), implemented in TCS 1.21 (Clement *et al.* 2000), was used to infer relationships based on nucleotide substitutions. The maximum number of differences resulting from single substitutions among haplotypes was calculated with 95% confidence limits, treating gaps as missing data.

Secondly, the median-joining networks (MJN), particularly suitable for inferring intraspecific phylogenies and to display all potential evolutionary paths for a set of haplotypes (Bandelt *et al.* 1999) and implemented in Network 4.5.1.6 (www.fluxus-engineering.com), which allowed the analysis of both nucleotide substitutions and indels. As indels are generally considered to be less reliable characters than substitutions, and since homology is especially uncertain in poly-A/T microsatellites (Kelchner 2000, 2009), each type of sequence mutation was therefore given a different weight in the analysis: 10 for substitutions, 5 for several-nucleotide indels, and 1 for differences in number of repeats within microsatellites (Besnard *et al.* 2007; Naciri & Gaudel 2007). Several-nucleotide indels, most likely resulting from a single event, were coded following the "simple indel coding" approach (Simmons & Ochotorena 2000), while each mononucleotide repeated in a

microsatellites was coded independently as a character, following Naciri & Gaudeul (2007). The parameter epsilon was set to its default value (ε = 0) in all the analyses. A maximum parsimony (MP) calculation was finally performed to eliminate non-parsimonious links and median vectors (putative haplotypes) from the resulting network, as recommended in the Network 4.5.1.6 manual.

Results

To provide a clearer framework within which to consider the results, these are organized below in keeping with the hypothesis of this study, that is: *R. cabrerensis s.l.* (*R. cabrerensis* subsp. *cabrerensis* and *R. cabrerensis* subsp. *muniellensis*) should be treated as an independent taxon of the *R. parnassiifolius* polyploid complex.

The authors would assert that evidence for this taxonomic proposal is provided by the results themselves.

Genome size estimation

FCM analyses yielded high-resolution histograms with CVs of G0/G1 peaks for *R. cabrerensis* subsp. *cabrerensis* and *R. cabrerensis* subsp. *muniellensis* ranging from 3.07 to 5.94% (mean 4.04%). Among the 29 samples collected at 4 localities only diploid plants were detected in *R. cabrerensis s.l.*

The 2C-values (2C/pg) and standard deviation of the mean for the populations analysed were: 9.93 ± 0.45 in ABE (1Cx = 4.96 pg; *n* = 12); 9.88 ± 0.72 in BAÑ (1Cx = 4.94 pg; *n* = 7); 9.70 ± 0.52 in MUN (1Cx = 4.85 pg; *n* = 5), and finally 9.62 ± 0.59 in YEG (1Cx = 4.81 pg; *n* = 6). Differences in nuclear DNA content among *R. cabrerensis s.l.* (2x) and *R. parnassiifolius s.l.* (2x, 4x) populations are shown in Figure 1.

The monoploid genome size (1Cx) ranged between 4.89 ± 0.28 pg (mean of all populations) for *R. cabrerensis s.l.* and 3.86 ± 0.17 pg for *R. parnassiifolius s.l.* (mean of all populations), i.e. a 1.26-fold difference, and statistical differences were reported.

On examining the statistical analyses, post-hoc tests revealed significant differences ($P \leq 0.001$) between the Pyrenees-Alps and the Cantabrian populations in tetraploids, and also between diploid samples of the Pyrenees and the Cantabrian Mountains (Figure 2). Moreover, we found a clear division in diploid populations from the Cantabrian Mountains (groups I and II), corresponding to the species *R. parnassiifolius s.l.* and *R. cabrerensis s.l.* respectively.

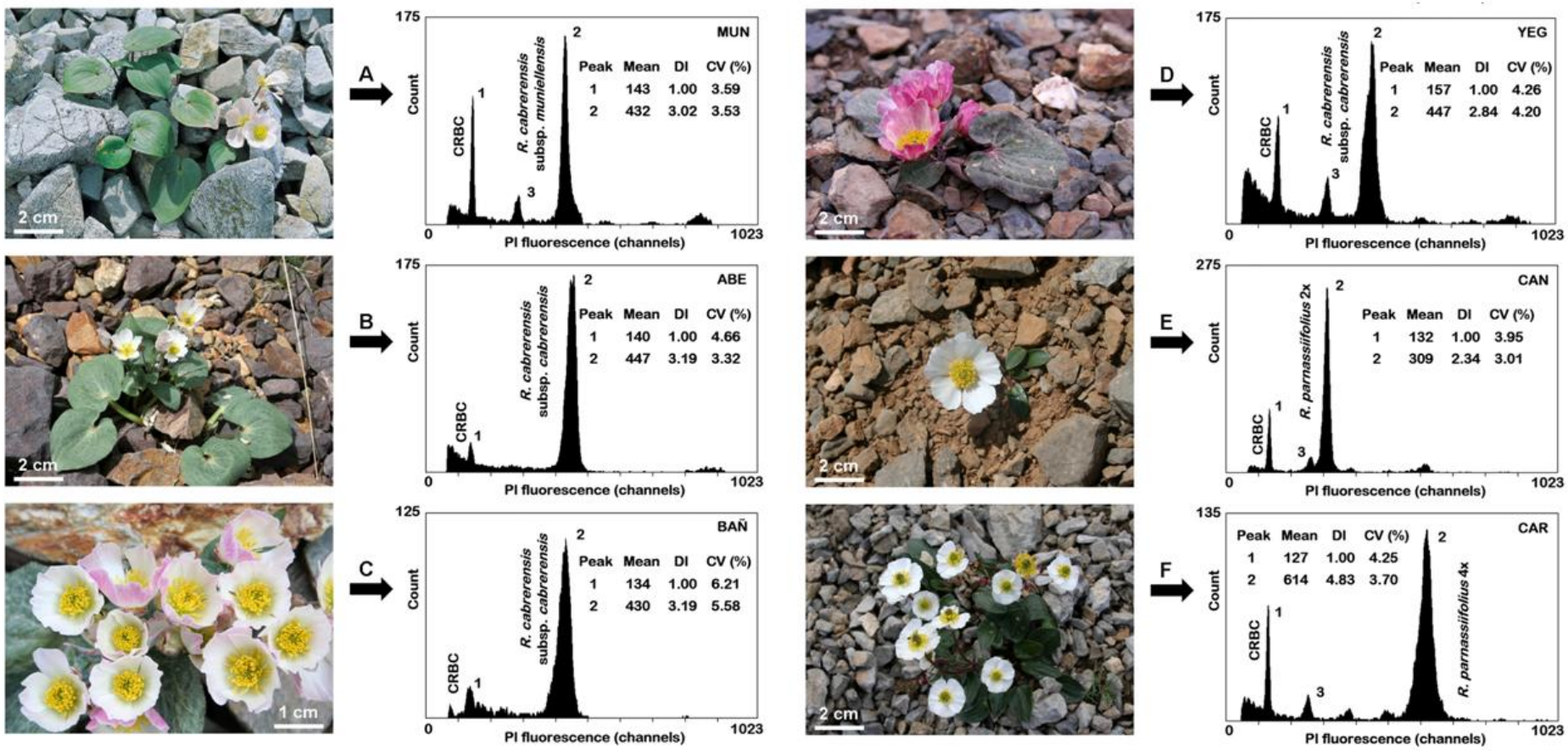

Figure 1. Morphological aspect and flow cytometric histograms obtained for *Ranunculus cabrerensis s.l.* (A-D) and *R. parnassiifolius s.l.* (E-F). The peaks marked with 1 and 2 indicate nuclei at the G0/G1 phase of the internal standard and the G0/G1 phase of the sample, respectively. The mean channel number (PI fluorescence), DNA index (DI = mean channel number of sample/mean channel number of reference standard) and coefficient of variation value (CV, %) of each peak are also given. The number 3 corresponds to G2/M peaks. Internal reference standard: CRBC: chicken red blood cells, 2C = 3.14 pg of DNA (Cires *et al.* 2009).

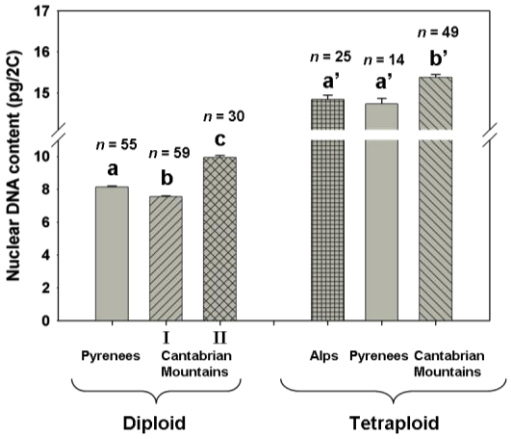

Figure 2. Holoploid DNA amount in different cytotypes of *Ranunculus* grex *parnassiifolius* studied from three biogeographical territories. Means not significantly different at $P \leq 0.001$ are indicated by the same letter (Dunn's test). All data correspond to samples of *R. parnassiifolius s.l.* of the mentioned territories, with the exception of the Cantabrian Mountains II, whose samples correspond to *R. cabrerensis s.l.*; i.e. including both subspecies.

Morphometric analyses

Using an ordination diagram of PCoA with qualitative and quantitative characters based on Gower's similarity coefficient, and taking into account biogeographical territories, clear models of clustering were detected. The first three axes accounted for 75.92% of the total variance (47.87, 23.95, and 4.09%) in vegetative matrix (Figure 3A). Furthermore, similar values were obtained with PCoA based on all vegetative, fructification and floral characters, including their possible combinations (data not shown). Clear morphological differences were found between *R. parnassiifolius s.l.* (Cantabrian Mountains I) and *R. cabrerensis s.l.* (Cantabrian Mountains II). Furthermore, within *R. cabrerensis s.l.*, we noted a clear division between the two subspecies at the morphological level (Figure 3B-C).

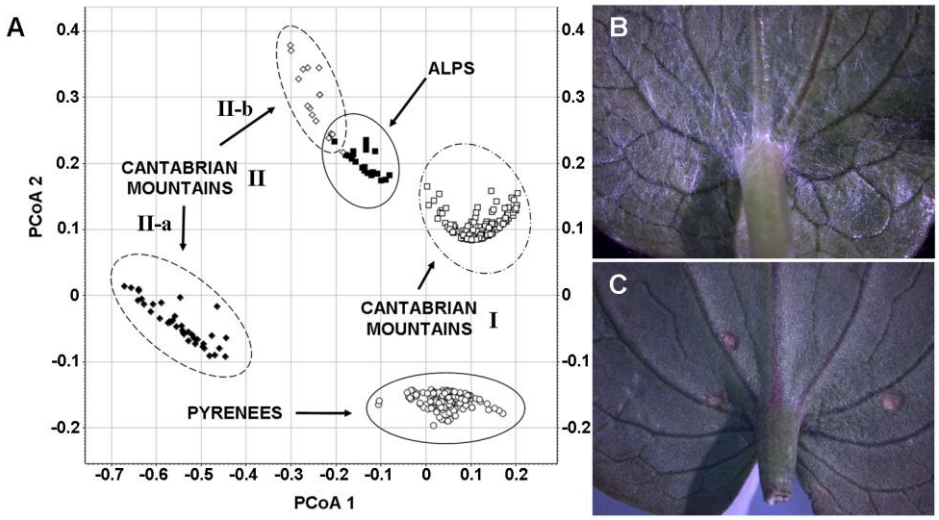

Figure 3. Multivariate analyses and morphological aspect of *Ranunculus* grex *parnassiifolius* samples. A) Principal Coordinates Analysis based on morphological characters (quantitative, ratio, and binary), considering the biogeographical territories (*R. parnassiifolius s.l.*: Alps, Pyrenees, Cantabrian Mountains I; *R. cabrerensis*: Cantabrian Mountains II.a; *R. cabrerensis* subsp. *muniellensis*: Cantabrian Mountains II.b. B-C) General appearance of abaxial indumentum surface from *R. cabrerensis* subsp. *cabrerensis* (B) and *R. cabrerensis* subsp. *muniellensis* (C).

In addition, quantitative variation in selected morphological characters of *R. cabrerensis s.l.* and *R. parnassiifolius s.l.* were apparent and are shown in Figure 4.

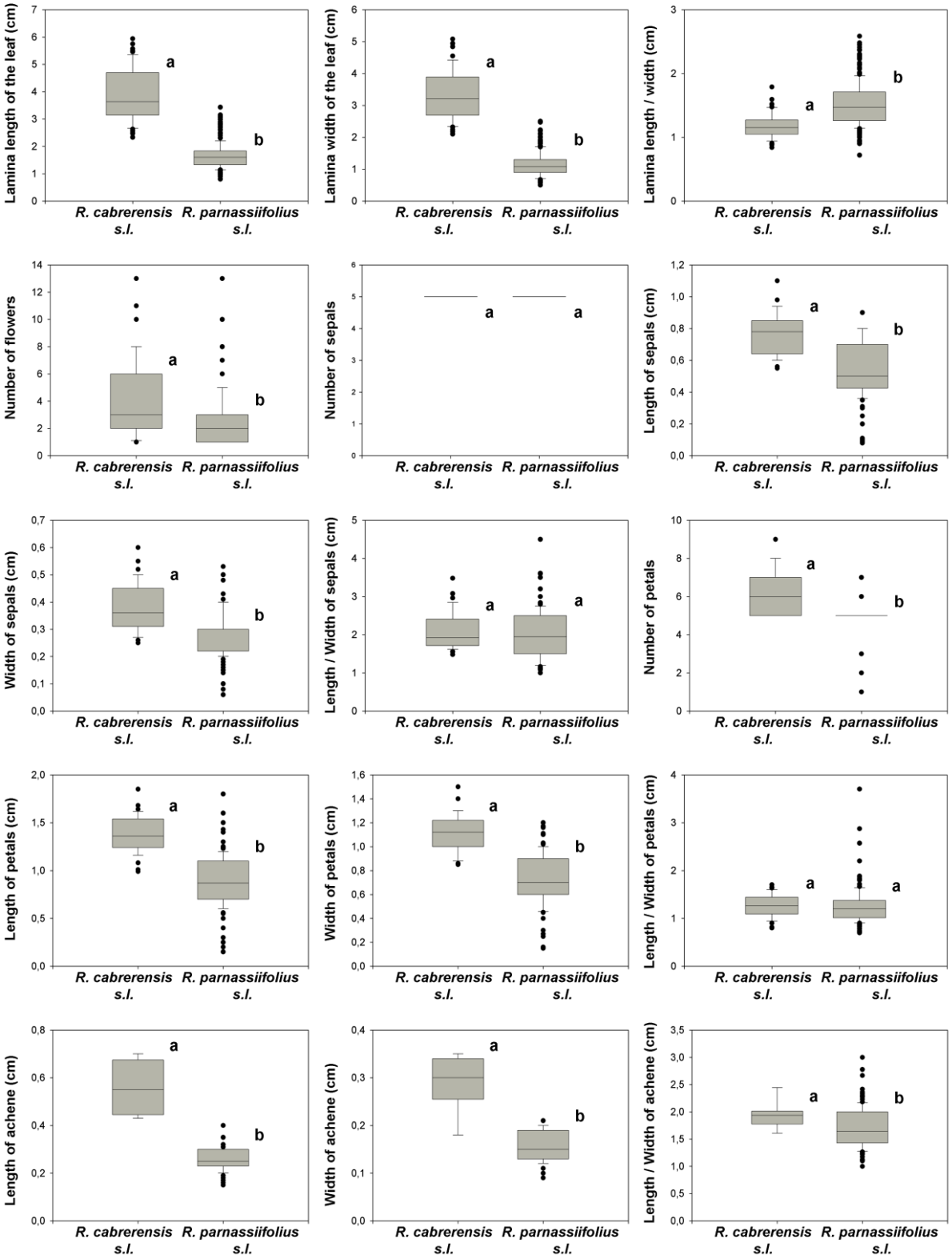

Figure 4. Descriptive statistics in selected quantitative morphological characters of *Ranunculus cabrerensis s.l.* and *R. parnassiifolius s.l.* Rectangles define 25th and 75th percentiles; horizontal lines show median, and dots show extreme values. Letters indicate statistical differences ($P < 0.05$; Dunn's test).

Differences between *R. cabrerensis* subsp. *cabrerensis* and *R. cabrerensis* subsp. *muniellensis* have not been analyzed in the present work as they have been previously published (Bueno Sánchez *et al.* 1992).

Polymerase chain reaction–restriction fragment length polymorphisms (PCR-RFLP)

Of the 125 DNA region/restriction enzyme combinations surveyed, 74 did not yield any digestion. Among the digestion patterns obtained, only 6 regions showed polymorphisms (Appendix II). Altogether twenty-seven polymorphisms were detected across the whole data set excluding *Caltha palustris* (157 polymorphisms if we consider the latter species).

They allowed the recognition of 10 haplotypes within *R. cabrerensis s.l.* and *R. parnassiifolius s.l.* (A to L; see Appendix III); and 2 more haplotypes, one for each outgroup (K and L; see Appendix III). The distribution of haplotypes clearly lent itself to a structure with 3 main groups (Figure 5), with the two main haplotype groups separated by 19 mutational steps.

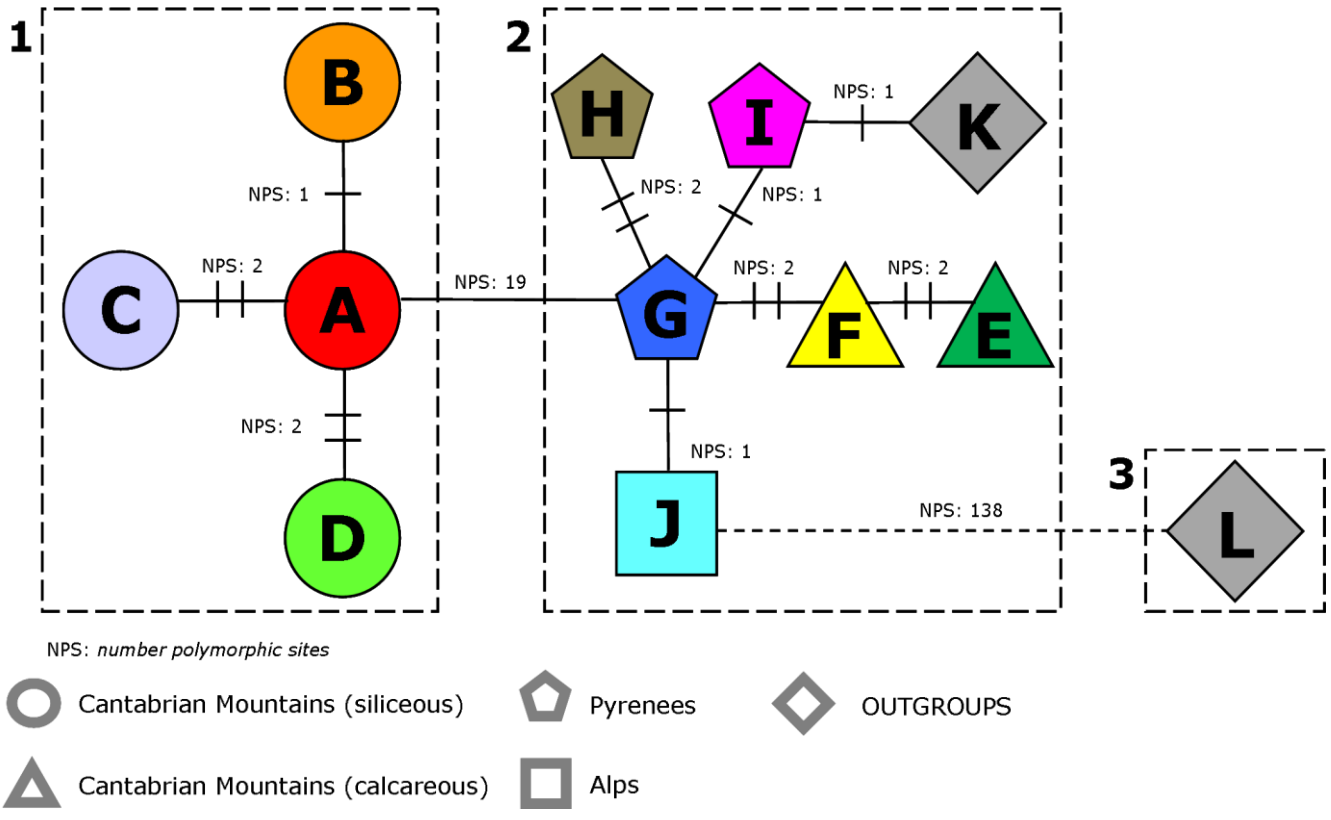

Figure 5. Minimum spanning tree of plastid DNA haplotypes in *Ranunculus cabrerensis s.l.* and *R. parnassiifolius s.l.* based on the PCR–RFLP analysis, with the three major groups of haplotypes indicated by dashed lines (group 1: *R. cabrerensis s.l.*; group 2: *R. parnassiifolius s.l.* and *R. pyrenaeus*; group 3: *Caltha palustris*). Haplotype A (ABE, BAÑ, YEG, MUN); B (YEG); C-D (MUN); E (CAN); F (MOL); G (ARL); H (CHI); I (FON); J (AMP); K (OUT1); L (OUT2).

One group (haplotypes A to D) was composed of siliceous populations of the Cantabrian Mountains, that is to say, exclusively by the populations of *R. cabrerensis* subsp. *cabrerensis* and *R. cabrerensis* subsp. *muniellensis* (ABE, BAN, YEG and MUN). These populations shared the most common haplotype, namely A. Moreover, haplotype B was characteristic of YEG and occurred in 25% of this population. This locality includes the natural hybrid *R.* ×*peredae* M.Laínz (*R. cabrerensis* Rothm. x *R. amplexicaulis* L.), in which introgressive hybridization has been suggested to be the trigger for this haplotype variation. Haplotype C and D were unique to *R. cabrerensis* subsp. *muniellensis* (occurring in 25 and 12.5% of the population, respectively).

The second main group of haplotypes (E-K), occurs in the remaining *R. parnassiifolius* populations (calcareous Cantabrian Mountains, Pyrenees and Alps) and in outgroup 1 (*R. pyrenaeus*). Finally the third group (haplotype L), far removed from haplotype J with 138 polymorphic sites, was characterized in outgroup 2 (*C. palustris*).

DNA sequencing

The characteristics of the three data sets are summarized in Table 3.

Table 3. Phylogenetic characteristics and nucleotide diversity obtained from the analyses of *trnS*, *trnR-atpA* and *atpH-atpI* sequences of *Ranunculus* grex *parnassiifolius* and outgroup plants.

	trnS	*trnR-atpA*	*atpH-atpI*	Total evidence
Whole matrix				
Number of sequences	22	22	22	22
Aligned length (bp)	175	99	701	975
Mean G + C content (%)	29.6	27.4	32.8	31.7
Nº of indels	27	4	73	104
Polymorphic sites	16	24	102	142
Parsimony informative sites	3	1	3	7
Average number of nucleotide differences k	2.10823	2.45887	9.98300	14.5500
Nucleotide diversity π	0.01424	0.02588	0.01590	0.01670
R. cabrerensis s.l. - R. parnassiifolius s.l. matrix				
Number of sequences	20	20	20	20
Aligned length (bp)	171	96	665	932
Mean G + C content (%)	29.8	27.2	32.4	31.4
Nº of indels	20	1	11	32
Polymorphic sites	3	1	4	8
Parsimony informative sites	3	1	4	8
Average number of nucleotide differences k	0.94211	0.33684	1.20000	2.47895
Nucleotide diversity π	0.00624	0.00355	0.00183	0.00275
Diploids matrix (excluding outgroups)				
Number of sequences	12	12	12	12
Aligned length (bp)	170	96	665	931
Mean G + C content (%)	29.7	27.0	32.3	31.3
Nº of indels	19	1	10	30
Polymorphic sites	3	1	4	8
Parsimony informative sites	3	1	4	8
Average number of nucleotide differences k	1.37879	0.48485	1.75758	3.62121
Nucleotide diversity π	0.00913	0.00510	0.00268	0.00402

Length variation for the entire region *trnS*, *trnR-atpA* and *atpH-atpI* (total evidence approach) in the populations studied ranged from 931 to 975 bp. One hundred and two polymorphic sites were detected, of which 7 were parsimony informative sites, and the GC-content for the whole matrix was 31.7%. In all cases the highest average number of nucleotide differences (k) was found in the region *atpH-atpI*; while the *trnS* region showed the highest nucleotide diversity (π) values (with the exception of the whole matrix). All sequences are available from GenBank with accession numbers HM026686-HM026751 (see Table 1).

Alignment of polymorphic fragments of the *trnS*, *trnR-atpA* and *atpH-atpI* sequences between *R. cabrerensis s.l* and *R. parnassiifolius s.l.* are given in Table 4.

The sequences of the *trnS* plastid region were 171 bp long, and contained 4 single nucleotide polymorphisms (point mutations). Two of these mutations (positions 5 and 22) differentiate *R. cabrerensis s.l* and *R. parnassiifolius s.l.* An insertion (19 bp) in all populations from the Cantabrian Mountains and in a single diploid population of the Pyrenees (EIX) should also be noted.

The sequences of the *trnR-atpA* plastid region were 96 bp long. In total, 2 polymorphic sites were found in this region: The first point mutation, the insertion of a cytosine in ARL, was located at position 17 of the sequence; and the second was located in the recognition site of the TaqαI enzyme [T↓CGA], at position 57-61 of the sequence. This second mutation distinguished *R. cabrerensis s.l* from *R. parnassiifolius s.l.*, and was reliably detected in PCR–RFLP (Appendix III).

Finally, the sequences of the *atpH-atpI* plastid region were 665 bp long. Three single nucleotide polymorphisms (positions 224, 238 and 254), a deletion (position 537) and several nucleotide insertions (from position 18 to 24, most likely resulting from a single event) reconfirmed the differentiation between *R. cabrerensis s.l* and *R. parnassiifolius s.l.*

Table 4. Position of polymorphisms in the alignment (bp) of the *trnS*, *trnR-atpA* and *atpH-atpI* in *Ranunculus* grex *parnassiifolius* and outgroup1 (*R. pyrenaeus*). Outgroup 2 has not been incorporated because of its high number of polymorphic sites. Intraspecific nucleotide substitutions/indels between *R. cabrerensis s.l.* and *R. parnassiifolius s.l.* are marked in bold.

Species	Locations	Region and position																											
		trnS																										*trnR-atpA*	
		5	22	27	40	81																			99	135	17	58
R. cabrerensis s.l.	ABE	**A**	**A**	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	**A**
	BAÑ	**A**	**A**	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	**A**
	MUN	**A**	**A**	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	**A**
	YEG	**A**	**A**	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	**A**
R. parnassiifolius s.l.	ABP	**C**	**T**	C	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	**C**
	ALB	**C**	**T**	-	T	T	C	G	A	G	A	T	T	T	T	C	T	T	T	C	G	A	A	T	A	-	**C**
	AMP	**C**	**T**	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	**C**
	ARL	**C**	**T**	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	C	**C**
	BOC	**C**	**T**	-	T	T	C	G	A	G	A	T	T	T	T	C	T	T	T	C	G	A	A	T	A	-	**C**
	CAD	**C**	**T**	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	**C**
	CAN	**C**	**T**	-	T	T	C	G	A	G	A	T	T	T	T	C	T	T	T	C	G	A	A	T	A	-	**C**
	CAR	**C**	**T**	-	T	T	C	G	A	G	A	T	T	T	T	C	T	T	T	C	G	A	A	T	A	-	**C**
	CHI	**C**	**T**	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	**C**
	EIX	**C**	**T**	-	T	T	C	G	A	G	A	T	T	T	T	C	T	T	T	C	G	A	A	T	A	-	**C**
	FON	**C**	**T**	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	**C**
	GCH	**C**	**T**	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	**C**
	GRA	**C**	**T**	-	T	T	C	G	A	G	A	T	T	T	T	C	T	T	T	C	G	A	A	T	A	-	**C**
	MOL	**C**	**T**	-	T	T	C	G	A	G	A	T	T	T	T	C	T	T	T	C	G	A	A	T	A	-	**C**
	PAN	**C**	**T**	-	T	T	C	G	A	G	A	T	T	T	T	C	T	T	T	C	G	A	A	T	A	-	**C**
	URR	**C**	**T**	-	T	T	C	G	A	G	A	T	T	T	T	C	T	T	T	C	G	A	A	T	A	-	**C**
R. pyrenaeus	OUT1	**C**	**T**	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	**C**

Table 4. (continuation)

Species	Locations	Region and position														
		atpH-atpI														
		18						24	224	238	254	322	537	610	623	634
R. cabrerensis s.l.	ABE	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**T**	**T**	**T**	C	**-**	T	A	-
	BAÑ	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**T**	**T**	**T**	C	**-**	T	A	T
	MUN	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**T**	**T**	**T**	C	**-**	T	A	T
	YEG	**T**	**A**	**A**	**A**	**A**	**A**	**T**	**T**	**T**	**T**	C	**-**	T	A	T
R. parnassiifolius s.l.	ABP	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	A	-	T
	ALB	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	-	A	-
	AMP	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	-	A	T
	ARL	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	A	A	T
	BOC	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	A	A	T
	CAD	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	A	**T**	A	A	T
	CAN	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	-	A	-
	CAR	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	-	-	-
	CHI	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	A	A	-
	EIX	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	A	A	-
	FON	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	A	**T**	-	A	-
	GCH	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	A	A	-
	GRA	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	-	A	-
	MOL	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	-	A	T
	PAN	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	-	A	T
	URR	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	C	**T**	-	A	T
R. pyrenaeus	OUT1	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**A**	**C**	**A**	A	**T**	A	A	T

Similarly, statistical parsimony analysis of the combined matrix allowed the recognition of these 2 species. The network constructed by TCS (Figure 6A) shows 2 *R. cabrerensis s.l.* haplotypes connected to *R. parnassiifolius s.l.* through 5 missing haplotypes (extinct or not found). In addition, the MJN algorithm constructed a network (Figure 6B) depicting a topology congruent with that based on nucleotide substitutions.

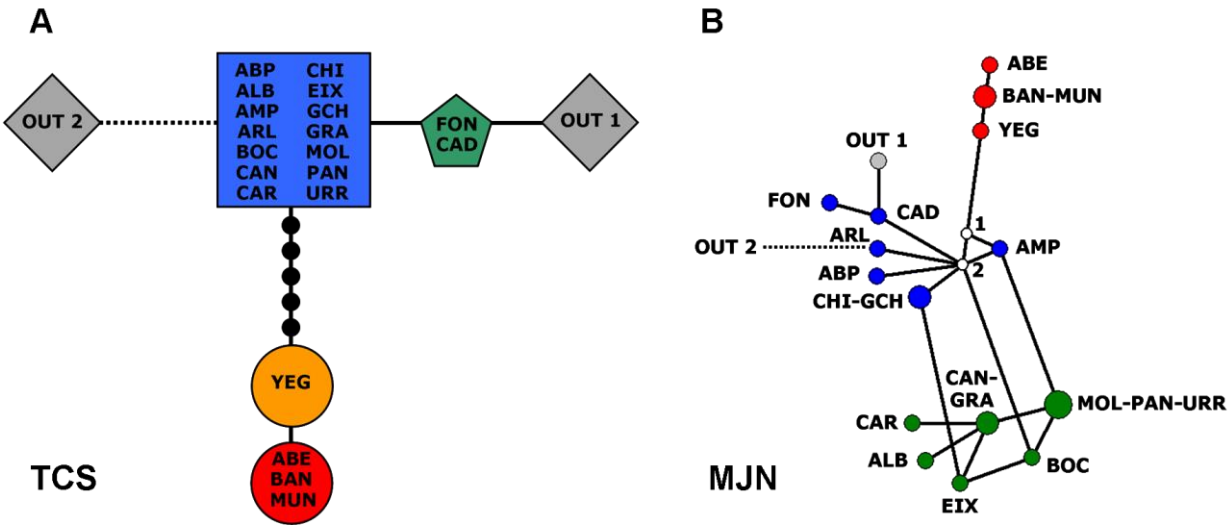

Figure 6. Phylogenetic networks analysis based on *trnS/trnR-atpA/atpH-atpI* sequences in different populations of *Ranunculus* grex *parnassiifolius* and outgroups. A) Statistical parsimony network. Lines (–) indicate a single nucleotide substitution, and black dots (●) represent 5 haplotypes (extinct or not detected). B) Median-joining network. Lines (–) indicate nucleotide substitutions and/or indels, and white dots (○; numbers 1 and 2) represent median vectors in the data set. Weights of all nucleotide positions are: 10 for substitutions, 5 for several-nucleotide indels and 1 for differences in number of repeats within microsatellites.

Discussion

In the introduction to this work, the currently distinguished taxonomic groups of *R. parnassiifolius* have been referred to. However, for convenience and consistency between the results and discussion they will henceforth be referred to as *R. cabrerensis s.l.* (including the infraspecific taxon *R. cabrerensis* subsp. *muniellensis*), and *R. parnassiifolius s.l.*, with the first two being formally named later in this work. Previous studies by the same authors (Cires *et al.* 2009, 2010) had highlighted the need for deeper insight into the evolutionary history of the *R. parnassiifolius* group and the elucidation of its taxonomy. For example, data based on estimation of nuclear DNA content by FCM together with morphological characters, had revealed that plants from distinct biogeographical territories do show clear-cut differences, regardless of the subspecific systematization of samples according to previous studies or geological substrates (Cires *et al.* 2010). In the current study, particular attention has therefore been paid to covering a representative sample of the cytotaxonomical diversity and distribution of the taxa. Hence, with the aim of clarifying the taxonomic treatment of *R. cabrerensis s.l.,* closely related species have also been included.

Information on genetic diversity and differentiation between populations using morphological and molecular markers provides initial guidance for conservation and can contribute to set conservation priorities between populations (Carroll & Fox 2008). Such results can provide valuable information for conservation biologists because they allow the estimation of population genetic differentiation between and within populations and support the design of sampling strategies for *ex situ* collections (Iriondo *et al.* 2008). In fact, FCM has allowed the analysis of a large number of plants from many populations throughout a wide distribution range and has also been used to examine the systematics and taxonomy of a number of plant groups, and more specifically has been frequently used in ploidy analysis (e.g. Kolář *et al.* 2009). Clear differences of 2C nuclear DNA content were found between *R. cabrerensis s.l.* and *R. parnassiifolius s.l.* (Figure 1 & 2), supporting the karyological observations reported by Diosdado & Pastor (1991), which indicated a large difference in chromosome size of *R. cabrerensis* subsp. *cabrerensis* (4.10-8.90 µm) in regard to different subspecies of *R. parnassiifolius s.l.* (i.e. 2.86-5.72 µm for *R. parnassiifolius* subsp. *parnassiifolius*; and 2.14-6.43 µm for *R. parnassiifolius* subsp. *heterocarpus*). Therefore, our work represents the first study dealing with FCM in *R. cabrerensis s.l.* and also represents an example of the use of the nuclear DNA content as a taxonomically informative marker, highlighting its value in resolving complex groups. In the same way, morphometric and cluster analyses showed a well-supported distinction between *R. cabrerensis s.l.* and *R. parnassiifolius s.l.* (Figure 3 & 4).

During the past decade, several new DNA-markers have emerged, and these have been rapidly integrated into the arsenal of common routine laboratory tools available for genome analysis. The advantages and disadvantages of these markers

have been widely debated (e.g Nybom 2004; Bussell *et al.* 2005; Agarwal *et al.* 2008). In particular, taxonomic, evolutionary and ecological studies on plant populations and species have benefited tremendously as a result of these molecular marker techniques. The analysis of plant organelle genomes such as cpDNA, has been increasingly applied to study population genetic structure and phylogenetic relationships in plants and provides information on population dynamics that is complementary to that obtained from the nuclear genome (Agarwal *et al.* 2008). As studies turn to closely-related species, phylogeographical approaches become more appropriate (Avise 2000). This is related to the possibility of revealing population-level historical shifts in distributions inferred from gene genealogies and not just synapomorphies characterizing monophyletic groups.

In the PCR-RFLP data analysis, three main groups of haplotypes were distinguished (Figure 5). One of them, consisted of 4 haplotypes restricted to *R. cabrerensis s.l.* (haplotypes A-D), was separated by 19 mutational steps from the *R. parnassiifolius* group. Given the low mutation rates in cpDNA (Provan *et al.* 1999), the genetic distance between these two main haplotype groups (excluding the group pertaining to the outgroup 2) may be the result of a relatively ancient differentiation predating the last glacial stage of the Quaternary glaciations. As regards, siliceous plants of the Muniellos Biosphere Reserve (*R. cabrerensis* subsp. *muniellensis*) were characterized by the most common haplotype (A) and 2 specific haplotypes (C-D). Such data, as well as the fact of the existence of only a single known population; support the idea that Muniellos plants may represent a relict of a geographical race of *R. cabrerensis*.

It is worth mentioning that relicts are distinctive populations or species, typically small in size or severely restricted in geographical range (Habel *et al.* 2010a), that mostly occur in small, isolated habitats, which are remnants of wider distributions in the past (Habel *et al.* 2010b). A similar example has been documented by Nève & Verlaque (2010) in the orophyte *Ranunculus kuepferi* Greuter & Burdet of Corsica and the Alps, where the sexual diploid relict *R. kuepferi* subsp. *kuepferi* is restricted to the Southwestern margin, whereas the apomictic tetraploid *R. kuepferi* subsp. *orientalis* is widespread in the rest of the species range (Küpfer 1974). Consequently, we infer here that *R. cabrerensis s.l.* is an endemic species of the Northwestern Iberian Peninsula (see Appendix IV). In addition to the above, the network analysis of three concatenated cpDNA sequenced regions supports this monophyly. The same scenario has been obtained using Internal Transcribed Spacer (ITS) sequences: both subspecies share a very similar ribosomal ITS sequences, completely different to that of *R. parnassiifolius s.l.* (see Chapter 6).

Interestingly, the distribution of the haplotypes obtained in the second main group by PCR-RFLP analysis do not coincide with the infraspecific taxonomic treatment distinguished in *R. parnassiifolius s.l.* In fact, in the comparison of cpDNA sequences across representatives of *R. parnassiifolius s.l.*, low genetic divergence was observed among all taxa. Network approaches are, however, appropriate

methods for within-species phylogeny reconstructions as they incorporate population processes in the construction and refinement of haplotype relationships (Gemeinholzer 2008). Furthermore, the statistical parsimony analysis of *trnS*/*trnR*-*atpA*/*atpH*-*atpI* sequences yielded a single network including only two plastid haplotypes; one of them unique to the Eastern populations of the Pyrenees (Figure 6A). Nevertheless, focusing on a median-joining network a phylogeographical structure was found. Unexpectedly, with the exception of a Pyrenean population (EIX) characterized by the presence of the hybrid *R.* ×*luizetii* Rouy (*R. parnassiifolius* L. x *R. pyrenaeus* L.), populations of the Cantabrian Mountains were isolated from other populations as a result of an insertion event (19 bp).

In light of the results obtained in this work, together with previous knowledge, we conclude that the taxonomic treatment of *R. parnassiifolius s.l.* still remains controversial. A larger comparative analysis of all taxa, beyond the scope of this study, would be necessary to reconstruct the phylogenetic relationships between the subspecies of *R. parnassiifolius* using both ITS nuclear region as well as the best non-coding cpDNA regions (e.g. Small *et al.* 1998; Shaw *et al.* 2005, 2007) for molecular studies at low taxonomic levels.

The combination of analyses presented here supports the recognition of two evolutionary units in the traditional *R. parnassiifolius* polyploid complex (sensu Küpfer 1974). The separation of *R. cabrerensis s.l.* and *R. parnassiifolius s.l.* from the remaining units is supported by all the analyses (morphology, FCM, PCR-RFLP and DNA sequencing). Therefore, we suggest the following groups as two taxa at the rank of species:

● *Ranunculus cabrerensis* Rothm., *Bol. Soc. Esp. Hist. Nat.* 34: 148 (1934).

(≡ *Ranunculus parnassiifolius* subsp. *cabrerensis* Rothm., *Bol. Soc. Esp. Hist. Nat.* 34: 148 (1934).

● *Ranunculus parnassiifolius* L., *Sp. Pl.*: 549 (1753).

In the same way, the plants described as *R. parnassiifolius* subsp. *muniellensis* should be systematized as a race of *R. cabrerensis*:

● ***Ranunculus cabrerensis*** subsp. ***muniellensis*** (Bueno, Fern.Casado & Fern.Prieto) Fern.Prieto & Cires **comb. nov.**

Basionymon: *Ranunculus parnassiifolius* subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto, *Bot. J. Linn. Soc.* 109: 365, fig. 366 (1992).

Natural hybrids:

● *R.* ×*peredae* M.Laínz, *Bol. Inst. Estud. Asturianos, Supl. Ci.* 10: 187 (1964).

[*R. cabrerensis* Rothm. x *R. amplexicaulis* L.]

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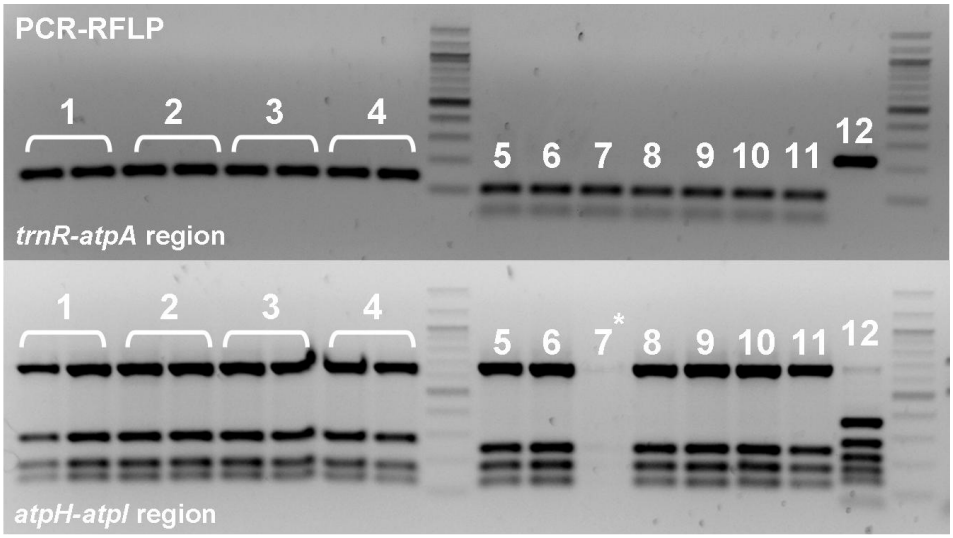
Appendices

Appendix I: DNA extraction protocol from Ranunculus *tissues*

Total genomic DNA was extracted from each individual applying an extraction method based on Cetyl Trimethylammonium Bromide (CTAB) buffer. 100-150 mg of tissue were ground to a fine powder in liquid nitrogen and transferred to a cold microcentrifuge tube. Then, 750 µL of preheated (60 °C) extraction buffer [2% CTAB, 10mM Tris-HCl pH 8.0, 2% w/v PVP 40, 20 mM EDTA pH 8.0, 2 M NaCl, 4mM DIECA] and 15 µL of 14.3 M β- mercaptoethanol were added. Samples were gently mixed and incubated at 55 °C for 30-45 min with sporadic mixing by inversion. After the incubation period, cell debris was removed by centrifugation at 13000 rpm (24500 g) for 10 min. The supernatant was transferred to a new microcentrifuge tube and 750 µL of choloroform:isoamylalcohol (24:1) was added and mixed by inversion to form an emulsion. After 10 min of centrifugation at 13000 rpm (24500 g) the aqueous phase (on the top) was transferred to a new tube. Samples were washed at least twice. DNA was precipitated by the addition of 1 volume of isopropanol and at least 30 min of incubation at room temperature or overnight at 4 ºC. DNA was pelleted by centrifugation at 13000 rpm (24500 g) for 10 min. The supernatant was carefully decanted and the pellet was cleaned by the addition of 500 µL of cold (-20 °C) ethanol 70%. DNA was recovered by centrifugation at 11000 rpm (24500 g) at 4 °C for 5 min and the pellet was dried. After this, the supernatant was carefully poured off and the tubes inverted on a towel and air dried for at least 20 min. The DNA was then re-suspended in 60 µL on TE buffer (1 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and 40 µL of RNase (1 mg mL^{-1}) followed by incubation at 37 ºC for 1 h. Then 50µL of 3M sodium acetate was added to each sample and afterwards gently mixed by inversion. DNA was precipitated by adding 2 ½ volumes of ice-cold ethanol 96%, followed by gentle mixing. Samples were centrifuged at 13000 rpm (24500 g) for 10 min in order to collect the DNA pellet. The supernatant was poured away and samples air dried for 20 min, then re-suspended in 50-100 µL TE buffer. DNA concentration was measured by spectrophotometer (Beckman-Coulter DU800®).

Appendix II: PCR-RFLPs profile

PCR-RFLPs with *Taqa*I enzyme digestion of *trnR–atpA* and *atpH-atpI* plastid regions in different populations of *Ranunculus* grex *parnassiifolius* and outgroups. Numbers 1-3: *R. cabrerensis* subsp. *cabrerensis* (ABE, BAÑ, YEG, respectively); 4: *R. cabrerensis* subsp. *muniellensis* (MUN); 5-6: *R. parnassiifolius* subsp. *favargeri* (CAN, ARL respectively); 7-9: *R. parnassiifolius* subsp. *heterocarpus* (MOL, CHI, AMP, respectively); 10: *R. parnassiifolius* subsp. *parnassiifolius* (FON); 11: *Ranunculus pyrenaeus* (OUT1); 12: *Caltha palustris* (OUT2). The lengths of restricted fragments were estimated using a 100-bp DNA ladder marker. Asterisk: negative control with H_2O.



Appendix III: PCR-RFLPs analysis of the cpDNA

PCR–RFLP analysis of the cpDNA in *Ranunculus cabrerensis s.l.* and *R. parnassiifolius s.l.* The recognition sequence for each respective enzyme is given at the end of the table. The haplotype L belonging to *Caltha palustris* has not been incorporated because of its high number of polymorphic sites (157).

			Haplotype										
cpDNA region	Enzyme	Size (bp)	A	B	C	D	E	F	G	H	I	J	K
trnS	*Taqα*I	175	0	0	1	0	0	0	0	0	0	0	0
	*Eco*RI	225	0	0	1	0	0	0	0	0	0	0	1
	*Eco*RI	25	0	1	0	0	0	0	0	0	0	0	0
	*Mse*I	135	0	0	0	0	1	1	0	0	0	1	0
	*Mse*I	125	1	1	1	1	0	0	1	1	1	1	1
trnR-atpA	*Taqα*I	150	1	1	1	1	0	0	0	0	0	0	0
	*Taqα*I	100 + 50	0	0	0	0	1	1	1	1	1	1	1
rps3-rpl22	*Hae*III	375	1	1	1	0	0	0	0	1	0	0	0
	*Hinf*I	300 + 75	0	0	0	0	1	0	0	0	0	0	0
	*Taqα*I	375	0	0	0	1	1	0	0	1	0	0	0
	*Mse*I	375	1	1	1	1	0	0	0	0	0	0	0
atpH-atpI	*Hae*III	825	1	1	1	1	0	0	0	0	0	0	0
	*Hae*III	800	0	0	0	0	1	1	1	1	1	1	1
	*Hinf*I	150	1	1	1	1	0	0	0	0	0	0	0
	*Hinf*I	75	0	0	0	0	1	1	1	1	1	1	1
	*Taqα*I	300	1	1	1	1	0	0	0	0	0	0	0
	*Taqα*I	250	0	0	0	0	1	1	1	1	1	1	1
	*Eco*RI	350	1	1	1	1	0	0	0	0	0	0	0
	*Eco*RI	325	0	0	0	0	1	1	1	1	1	1	1
	*Mse*I	300	0	0	0	0	0	0	0	0	1	0	1
trnD-trnT	*Eco*RI	450	0	0	0	0	1	1	1	1	1	1	1
	*Eco*RI	250	1	1	1	1	0	0	0	0	0	0	0
	*Eco*RI	200	1	1	1	1	0	0	0	0	0	0	0
	*Mse*I	500	0	0	0	0	1	1	1	1	1	1	1
	*Mse*I	450	1	1	1	1	0	0	0	0	0	0	0
trnS-trnf$_M$	*Mse*I	500	0	0	0	0	1	1	1	1	1	1	1
	*Mse*I	450	1	1	1	1	0	0	0	0	0	0	0

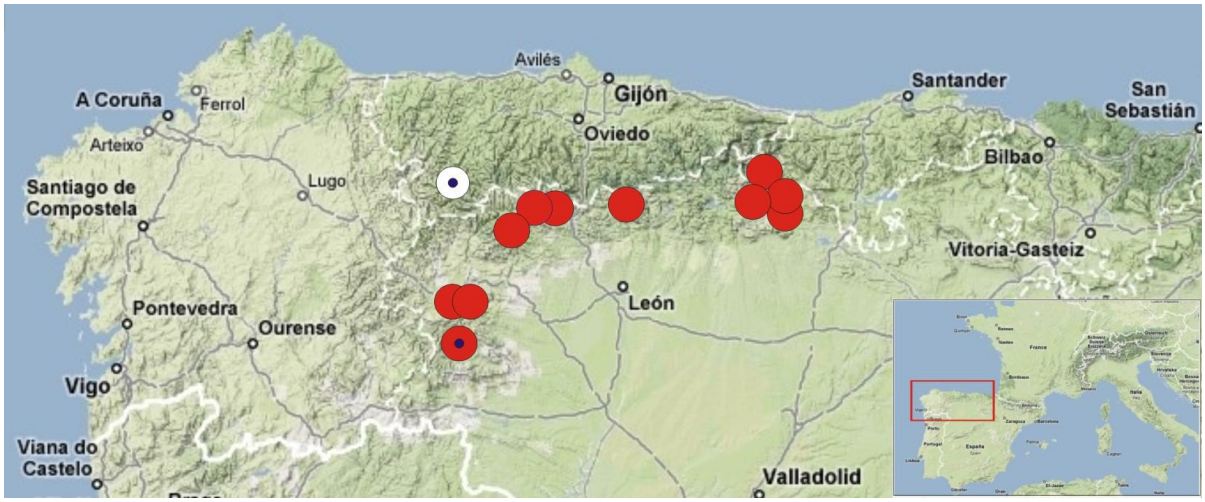
Recognition site:

*Eco*RI [G↓AATTC]; *Hae*III [GG↓CC]; *Hinf*I [G↓ANTC]; *Mse*I [TT↓AA]; *Taqα*I [T↓CGA]

Appendix IV: Distribution of Ranunculus cabrerensis s.l.

Distribution of *Ranunculus cabrerensis* subsp. *cabrerensis* and *R. cabrerensis* subsp. *muniellensis*. A) Map of localities. B) List of all populations quoted of *R. cabrerensis* group in the Iberian Peninsula. Localities are arranged according to taxa, and within taxa, in alphabetical order according to the population abbreviation. Each record is given as follows on the basis of available information: scientific name, country, state/province/county, description of the locality, altitude (m a.s.l.), geographic coordinates, data collected, collector (leg.), identifier (det.), institution code, catalogue number, chromosome number, observations and, finally, reference. Abbreviations: ES = Spain; O = Asturias; S = Cantabria; Le = León; P = Palencia.

A



Open circles (○): *R. cabrerensis* subsp. *muniellensis*
Filled circles (●): *R. cabrerensis* subsp. *cabrerensis*
The black dot (•) indicates the *locus classicus*

B

Ranunculus cabrerensis Rothm. subsp. ***cabrerensis***, *Bol. Soc. Esp. Hist. Nat.* 34: 148 (1934).

- ES, S, Vega de Liébana, del Mojón de las Tres Provincias a Peña Prieta, 2450 m, 30TUN56, Rivas Martínez *et al.* (1984).
- ES, S, Vega de Liébana, Macizo de Peña Prieta, pedregal de la umbría en el circo entre la cumbre y Tres Provincias, 2280 m, 30TUN5864, 1990-VII-16, leg. M.L. Gil Zúrriga & J.A. Alejandre, MA-493493, MACB-42722.
- ES, S, Vega de Liébana, Macizo de Peña Prieta, amplias crestas pedregosas, collados y laderas del contrafuerte de Pico Cuartas hacia el Valle de Lechada, 2050-2200 m, 30TUN5665, 30TUN5664, 1990-VII-15, leg. M.L. Gil Zúrriga & J.A. Alejandre, MACB-42723.

- ES, S, Vega de Liébana, non longe a Peña Prieta, pr. locum dictum Cubil de Can (ditione santanderiensi), 2000 m, 30TUN56, 1962-VI-30, leg. M.Laínz, JBAG-Laínz-2789.

- ES, S, Vega de Liébana, Peña Prieta, 2300-2400 m, 30TUN56, 1952-VIII-01, leg. Losa & P.Monts., BCN-45707.

- ES, S, Vega de Liébana, Peña Prieta, 2400 m, 30TUN56, 1987-VIII-14, leg. C. Aedo, MA-609173.

- ES, S, Vega de Liébana, subida al Pico Tres Provincias, 2300 m, 30TUN56, 2005-VII-22, leg. B. Jiménez Alfaro & S. Rivas Martínez, JBAG-244.

- ES, Le, Boca de Huérgano, bajando Las Lomas (cerca del Lago), 2230 m, 30TUN5664, 1997-IX-10, Alonso Redondo (2000).

- ES, Le, Boca de Huérgano, base de Pico Murcia, 1910-1930 m, 30TUN5361, 1997-VIII-16, Alonso Redondo (2000).

- ES, Le, Boca de Huérgano, 30TUN4977, 1990-VII-03, leg. M.L. Gil Zúrriga & J.A. Alejandre, LEB-47395.

- ES, Le, Boca de Huérgano, Fuentes Carrionas, Pico las Lomas, 2350 m, 30TUN56, 1981-VII-19, leg. M.L. Vera, FCO-14820, Bueno Sánchez *et al.* (1992).

- ES, Le, Boca de Huérgano, laderas del Pico Las Lomas, 2340 m, 30TUN56, 1997-VIII-16, leg. J. A. Fernández Prieto, Fernández Prieto (1983).

- ES, Le, Boca de Huérgano, Las Lomas, 2230 m, 30TUN56, 1997-IX-10, leg. Alonso Redondo, LEB-67619, Alonso Redondo (2000).

- ES, Le, Boca de Huérgano, Macizo de Peña Prieta, amplias crestas pedregosas, collados y laderas del contrafuerte de Pico Cuartas hacia el Valle de Lechada, 2050 m, 30TUN56, 1990-VII-15, leg. M.L. Gil Zúñiga & J.A. Alejandre, MA-493494.

- ES, Le, Boca de Huérgano, Macizo de Peña Prieta, Hoyo de Vargas, gleras de fuerte pendiente al W del Pico Cuartas, 2220 m, 30TUN56, 1990-VII-15, leg. M.L. Gil Zúñiga & J.A. Alejandre, MA-493495.

- ES, Le, Boca de Huérgano, Macizo de Peña Prieta, pedregales de comglomerados y pizarras en la fuerte pendiente N de Los Altares, 2100 m, 30TUN56, 1990-VII-03, leg. M.L. Gil Zúñiga & J.A. Alejandre, MA-493497.

- ES, Le, Boca de Huérgano, Macizo de Peña Prieta, pedregales de la umbría del circo de Hoyo de Vargas, 2270 m, 30TUN56, 1990-VII-29, leg. M.L. Gil Zúñiga & J.A. Alejandre, MA-493496.

- ES, Le, Boca de Huérgano, Peñas Zahurdias, 1940-2070 m, 30TUN5361, 1997-VIII-29, Alonso Redondo (2000).

- ES, Le, Boca de Huérgano, Peñas Zahurdias, proximidades del Pico Murcia, 1925 m, 30TUN5361, 1997-VIII-16, leg. Alonso Redondo, LEB-68159, Alonso Redondo (2000).

- ES, Le, Boca de Huérgano, proximidades de Las Lomas, 2440 m, 30TUN5764, 1997-IX-10, Alonso Redondo (2000).

- ES, Le, Boca de Huérgano, proximidades de Pico Murcia, hacia Peñas Matas, 2150 m, 30TUN5361, 1997-VIII-29, Alonso Redondo (2000).

- ES, Le, Boca de Huérgano, S du Puerto de San Glorio, Portillo de las Yeguas, 2010 m, 30TUN56, leg. Küpfer, NEU-K02217, 2n = 16, Küpfer (1974).

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CAPÍTULO 4

Conservation genetics of the endangered endemic *Ranunculus cabrerensis* subsp. *muniellensis* (Ranunculaceae)

Summary

Genetic diversity and population structure within and between two subpopulations of *Ranunculus cabrerensis* subsp. *muniellensis*, an endangered plant endemic to the Iberian Peninsula with only a single known population, were investigated using Amplified Fragment Length Polymorphism (AFLP) and Random Amplified Polymorphic DNA (RAPD) markers. Polymorphisms per loci were detected more frequently with AFLP (84.11%) compared to RAPD (65.08%). Relatively high genetic diversity at subspecies level was revealed with RAPD (percentage of polymorphic bands PPB = 65.08%, Nei heterozygosity H_E = 0.217, Nei unbiased heterozygosity UH_E = 0.220 and Shannon information index I = 0.326) and also with AFLP markers (PPB = 84.11%, H_E = 0.232, UH_E = 0.239 and I = 0.360).

Furthermore, analysis of molecular variance (AMOVA) was used to evaluate the variation within and between subpopulations. The proportion of variation attributable to within-subpopulation differences was very high (94% by RAPD; 96% by AFLP). Principal Coordinates and Bayesian analyses revealed a weak but significant spatial genetic structure in *R. cabrerensis* subsp. *muniellensis*. These results therefore suggest that conservation of each subpopulation is important for maintaining genetic diversity of the endangered species *R. cabrerensis* subsp. *muniellensis*.

Este capítulo se corresponde con la publicación:

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Introduction

The survival of plant populations, their evolutionary potential, and their ability to adapt to changing environmental conditions are determined by their genetic variability especially at the intraspecific level (Falk & Holsinger 1991). Many endangered species distributed in small populations (often with patchy or island-like distributions) are susceptible to genetic processes that reduce intraspecific variation, such as genetic drift and inbreeding depression (Ellstrand & Elam 1993; Reed & Frankham 2003; Frankham 2005). Indeed, rare and endangered species often show lower levels of genetic diversity than widespread species, maybe as a consequence of their small population size and population isolation (Falk & Holsinger 1991; Ellstrand & Elam 1993; Höglund 2009). Such characteristics, by increasing genetic drift and inbreeding, and also reducing gene flow, can therefore lead to the erosion of genetic variation and to an increase in genetic divergence between populations (Young *et al.* 1996; Aguilar *et al.* 2008).

However, the lack of information about the genetic diversity of endangered species can hamper conservation efforts, hence studies on the conservation genetics of rare and endangered plant species are necessary when establishing management plans to preserve their biodiversity (Smith & Wayne 1996; Raphael & Molina 2007; Höglund 2009). In fact, the efficient design of conservation strategies for endangered species largely depends on knowledge of the levels of genetic diversity of the target species (e.g. Ramp Neale *et al.* 2008).

Ranunculus cabrerensis subsp. *muniellensis* (Bueno, Fern.Casado & Fern.Prieto) Fern.Prieto & Cires (= *R. parnassiifolius* subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto; see Chapter 3), occurs naturally in the Muniellos Biosphere Reserve (Western Cantabrian Mountains, Iberian Peninsula). At the present time, only a single population composed of a small number of individuals (779 and 448 individuals counted in the year 2007 and 2008 respectively) is known. It covers an area of 4655 m^2 and is fragmented into two nuclei, or subpopulations. The population is evenly distributed across siliceous scree, included in plant communities of the alliance *Linario saxatilis-Senecion carpetani* Rivas Martínez 1968 (Bueno Sánchez *et al.* 1992; Fernández Prieto & Bueno Sánchez 1996), and it may be that the main factor limiting the expansion of the population is the lack of potential habitat.

In regard to its protection status, *R. cabrerensis* subsp. *muniellensis* does not appear in the regional catalogue of threatened species (Catálogo Regional de Especies Amenazadas de la Flora del Principado de Asturias; Decreto 65/1995), while in the Red List of Spanish Vascular Flora (VV.AA. 2000) it appears under the category of "data deficient". Bañares *et al.* (2004) and Moreno (2008) reported the cited plant as "vulnerable", and Fernández Prieto *et al.* (2007) also proposed its inclusion in the catalogue of threatened vascular plants of Asturias under the same category. Furthermore, this taxon, like most Cantabrian endemic species typical of

habitats exposed to risk of fragmentation, are of a great interest in the field of conservation biology.

Molecular markers are now widely used to detect the genetic diversity and population structure of endangered species (e.g. Cieślak *et al.* 2007; González-Pérez *et al.* 2009). Two such markers are: Amplified Fragment Length Polymorphisms (AFLP), which have been used as a DNA fingerprinting technique to assess genome-wide diversity for individuals, populations and species, and also to analyze population genetic structure (Vos *et al.* 1995); and Randomly Amplified Polymorphic DNA (RAPD), another popular DNA fingerprinting method because of its low development costs and the fact that the laboratory procedures are easily transferable to any plant species (Wesh & McClelland 1990; Williams *et al.* 1990).

These marker techniques are being widely used to assess genetic relationship between different groups of taxa since many polymorphic loci can be obtained fairly easy, in a relatively short time, and without any prior knowledge of the genome of the species under study (e.g. Nybom & Bartish 2000; Nybom 2004; Meudt & Clarke 2007).

In this study, we used AFLP and RAPD markers to characterize genetic diversity and genetic differentiation within and between subpopulations of endangered species *Ranunculus cabrerensis* subsp. *muniellensis*. Thus, the aim of the study is to explore possible underlying causes which have led to its endangered status in order to establish conservation recommendations based on the genetic data.

Materials and methods

Plant material

Ranunculus cabrerensis subsp. *muniellensis* (Figure 1A), is a geophyte of 15-25 cm tall with basal leaves cordate at the base, abaxial and adaxial surface glabrous or scarcely villose only on the base of the adaxial surface, petals usually pinkish in thick umbeliform cymes and achenes smooth or nearly smooth with lateral fruit-bearing peduncles longer than 15 mm.

It grows in siliceous gravels on mountain slopes (Figure 1B), with entomophilous pollination like other *Ranunculus* species. It is a diploid species of 2n=16 (Bueno Sánchez *et al.* 1992) and the estimation of nuclear DNA content (2C/pg) is 9.70 pg (1Cx = 4.85 pg) (see Chapter 3).

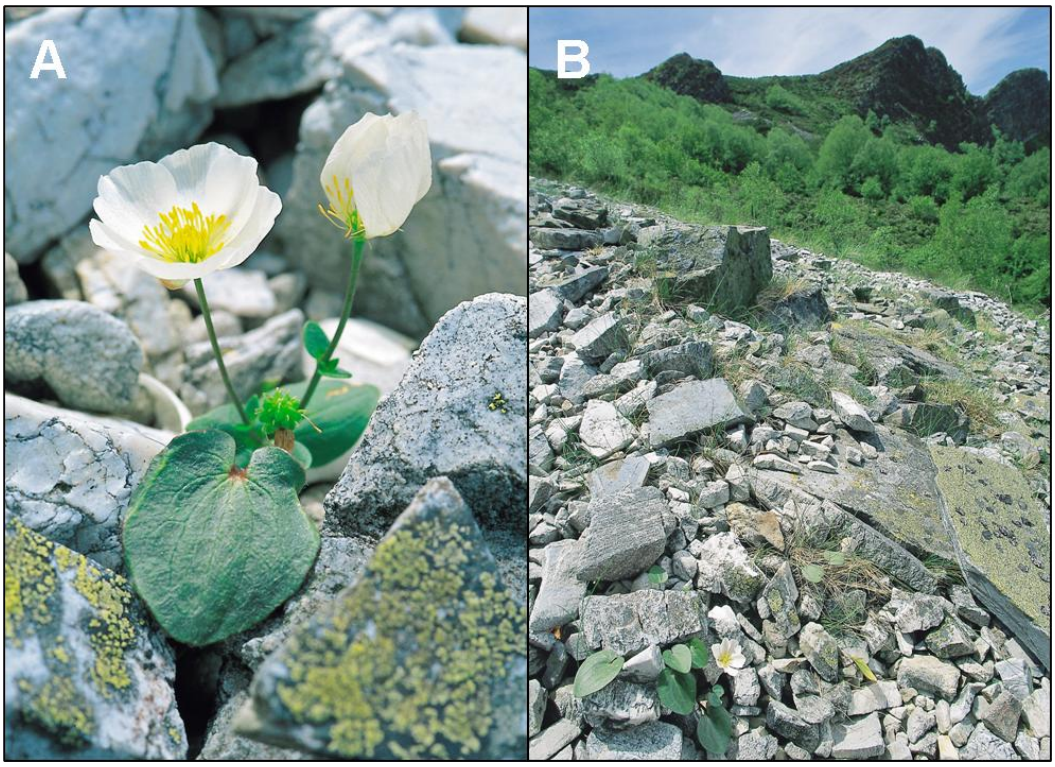

Figure 1. *Ranunculus cabrerensis* subsp. *muniellensis*, an endangered endemism. A) General appearance between blocks of siliceous (silicate-dwelling). B) Coarse and angular rocks in the alpine rock-field habitat in the Muniellos Biosphere Reserve, Asturias. Note the patches of *R. cabrerensis* subsp. *muniellensis* in the foreground.

Sampling design and DNA extraction

The natural population of *Ranunculus cabrerensis* subsp. *muniellensis* is divided into two main subpopulations separated by about 500 m, which we named "Subpopulation 1" and "Subpopulation 2" (Figure 2).

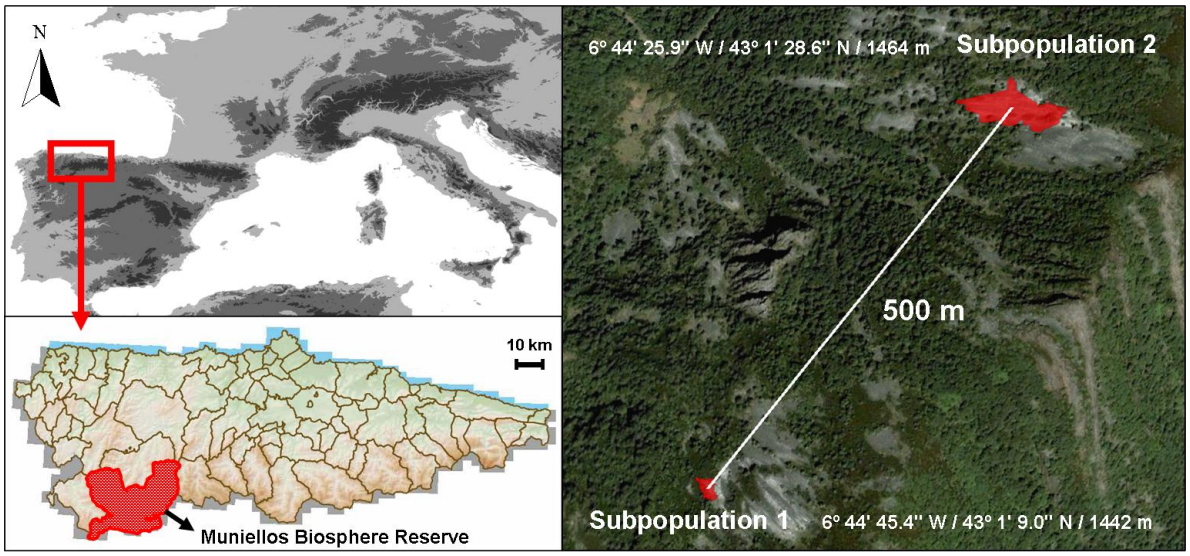

Figure 2. Location of the unique known population of *Ranunculus cabrerensis* subsp. *muniellensis* in the Muniellos Biosphere Reserve (Asturias, Spain). Population area = 4655.32 m^2 (Subpopulation 1 = 1335.73 m^2 + Subpopulation 2 = 3319.59 m^2).

Leaves were randomly collected from each subpopulation in 2007, with sampled individuals being selected at minimum intervals of 3 m from each other to increase the area of sampling and to avoid subsampling of each population. A total of 16 individuals per subpopulation were sampled, 8 of which were used for AFLP and 16 for RAPD. Leaves were stored on moistened paper enclosed in a plastic bag and kept at low temperatures (4 ºC) before and during transportation, and on arrival at the laboratory the plant tissue was frozen at -80 ºC until analysis. In addition, voucher specimens were collected, dried by pressing in absorbent paper, stored at room temperature, and kept in the Herbarium of the University of Oviedo (FCO).

DNA extraction was carried out using the CTAB method described by Doyle & Doyle (1987), with minor modifications. DNA concentration was measured by a Beckman-Coulter DU800® spectrophotometer (Fullerton, CA, USA).

RAPD amplification

Sixty primers were used for the RAPD analysis (Kit: A, C, H; Operon Technologies Inc., Huntsville, AL, USA). Thirty-five of them which generated clear patterns, were selected for this study (Table 1). Amplification reactions were set in a 25 µL final volume reaction mixture containing 25 ng of DNA, 2.5 µL of 10x PCR buffer, 2 mM $MgCl_2$, 0.2 µM of primer, 0.05 mM dNTPs, and 0.5 U of Taq DNA polymerase (Invitrogen).

In order to prevent unspecific band patterns and to guarantee reproducibility, a gradient of annealing temperatures (between 36 and 41 °C) was assayed, and the most effective one was selected for subsequent analyses. Furthermore, the reproducibility of the RAPD was improved by maintaining standardized conditions with regard to all possible sources of variation: for instance always using the same thermocycler and Taq polymerase. Amplifications were carried out in a gradient thermal cycler (MyCycler™, Bio-Rad), programmed at the following thermal conditions: an initial cycle of 5 min at 94 °C, followed by 45 cycles of 1 min at 94 ºC, 1 min at 41 ºC, and 2 min at 72 ºC. The final step was 5 min at 72 ºC before cooling down to 4 °C.

Amplification products were resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide, and then photographed on a GelLogic 100 Kodak UV transilluminator (Eastman Kodak, Rochester, NY, USA). In all cases lambda phage DNA digested with *Eco*RI and *Hin*dIII (New England Biolabs, Inc.) was used as size marker. In order to ensure the reliability of the results obtained, reactions were performed at least twice, including internal controls (replicates of the same sample in each combination, and different DNA extractions of the same individual). Only the consistently reproduced, distinguishable bands were considered.

Table 1. Polymorphic bands generated by RAPD and AFLP primers in two subpopulations of *Ranunculus cabrerensis* subsp. *muniellensis*. NB: number of bands; NPB: number of polymorphic bands; PPB: percentage of polymorphic bands.

RAPD Primer name	NB	NPB	PPB (%)	**RAPD Primer name**	NB	NPB	PPB (%)
OPA-1	4	3	75.00	OPC-6	2	0	0.00
OPA-2	2	2	100.00	OPC-7	3	2	66.67
OPA-3	2	1	50.00	OPC-9	6	4	66.67
OPA-4	7	7	100.00	OPC-13	5	2	40.00
OPA-7	6	6	100.00	OPC-14	4	4	100.00
OPA-8	5	5	100.00	OPC-16	2	1	50.00
OPA-10	6	6	100.00	OPH-1	2	1	50.00
OPA-11	5	5	100.00	OPH-2	3	3	100.00
OPA-12	3	2	66.67	OPH-3	2	0	0.00
OPA-13	5	1	20.00	OPH-4	2	1	50.00
OPA-15	4	2	50.00	OPH-7	4	1	25.00
OPA-17	6	3	50.00	OPH-8	2	0	0.00
OPA-18	2	2	100.00	OPH-9	2	1	50.00
OPA-20	4	4	100.00	OPH-12	3	2	66.67
OPC-1	2	1	50.00	OPH-13	4	1	25.00
OPC-3	4	4	100.00	OPH-14	2	0	0.00
OPC-4	2	1	50.00	OPH-18	3	1	33.33
OPC-5	6	3	50.00				
Mean	3.60	2.34	65.08				
Total	126	82					

AFLP Primer name	NB	NPB	PPB (%)	**AFLP Primer name**	NB	NPB	PPB (%)
*Eco*RI-AAC / *Mse*I-CAT	117	104	88.89	*Eco*RI-ACG / *Mse*I-CCAG	49	39	79.59
*Eco*RI-AAC / *Mse*I-CTT	109	98	89.91	*Eco*RI-ACG / *Mse*I-CCTA	115	93	80.87
*Eco*RI-AAC / *Mse*I-CCAC	69	52	75.36	*Eco*RI-ACT / *Mse*I-CAT	183	154	84.15
*Eco*RI-AAC / *Mse*I-CCAG	96	83	86.46	*Eco*RI-ACT / *Mse*I-CTT	149	138	92.62
*Eco*RI-AAC / *Mse*I-CCTA	116	91	78.45	*Eco*RI-ACT / *Mse*I-CCAC	133	106	79.70
*Eco*RI-ACG / *Mse*I-CAT	208	179	86.06	*Eco*RI-ACT / *Mse*I-CCAG	111	89	80.18
*Eco*RI-ACG / *Mse*I-CTT	131	122	93.13	*Eco*RI-ACT / *Mse*I-CCTA	120	84	70.00
*Eco*RI-ACG / *Mse*I-CCAC	113	98	86.73				
Mean	121.27	102.00	84.11				
Total	1819	1530					

Combined AFLP + RAPD	NB	NPB	PPB (%)
Mean	38.90	32.24	82.88
Total	1945	1612	

AFLP procedure

The AFLP protocol was carried out following the procedure described by Vos *et al.* (1995) with minor modifications. Marker reproducibility was tested by the random addition of four replicated individuals in the PCR plates: the mean error statistics for duplicate samples was 1.9%, and therefore is not likely to have significantly affected the results. Double digestion of DNA was performed using *Eco*RI and *Mse*I enzymes at 37 ºC for 2 h. This reaction contained 5 U *Eco*RI (New England Biolabs, Inc.), 5 U *Mse*I (New England Biolabs, Inc.), 3.5 µL 10x Restriction/Ligation buffer (RL: 100 mM Tris HAc pH=7.5; 100 mM MgAc; 500 mM KAc; 50 mM DTT) and 200 ng of DNA, in a final volume of 35 µL. Subsequently, a volume of 10 µL of ligation mixture was prepared (5 µM of *Mse*I adapter, 0.5 µM of *Eco*RI adapter, 1.2 mM ATP, 1 U of T4 DNA ligase (Roche Diagnostics) and 1 µL of RL buffer), added to the restriction reaction, and incubated for 5 h at 37 ºC. The pre-selective amplification reaction contained 5 µL of 10x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each pre-selective primer (*Eco*RI+A and *Mse*I+C; Applied Biosystems), 1.25 U Taq DNA

polymerase (Invitrogen), and 5 µL of restriction-ligation product (final volume of 50 µL). For pre-selective amplification in the thermal cycler, the following programme was used: an initial cycle of 5 min at 94 °C, followed by 26 cycles of 1 min at 94 ºC, 45 s 60 ºC, and 1 min at 72 ºC. The final step was 10 min at 72 ºC before cooling to 4 °C. The product of pre-selective amplification was checked by electrophoresis in 1.5% agarose gel and then diluted (1:10) in sterile de-ionised H_2O. A preliminary screening of 69 selective primer pair combinations (MWG Laboratories) was done for four samples, and the 15 primer pair combinations that gave the best results with respect to polymorphism and clarity of AFLP profiles were used in all 16 samples (Table 1).

The reaction mix of selective amplifications (20 µL reaction volume) contained 2 µL of 10x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each *Eco*RI selective primer (5'-fluorescent labelled), 0.2 µM of each *Mse*I selective primer, 0.75 U Taq DNA polymerase (Invitrogen) and 5 µL of diluted pre-selective PCR product. The PCR cycle profile was a single cycle at 92 ºC (5 min); 9 cycles at 92 ºC (1 min), 65 ºC (each cycle of 45 s, gradually decreasing temperature by 1 ºC per cycle), 72 ºC (1 min); 24 cycles at 92 ºC (1 min), 56 ºC (45 s), 72 ºC (1 min); followed by a final extension at 72 ºC (10 min). Selective amplification products were sent to the Sequencing Services of the University of Oviedo for fragment analysis: samples were run on an automated DNA sequencer (ABI PRISM® 3100, Applied Biosystems) with an internal size standard GeneScan 500 (ROX™, Applied Biosystems). Raw AFLP data were collected and sized using the Genemapper 4.0 software (Applied Biosystems), analyzing the AFLP fragments following the recommendations provided by Applied Biosystems (2007) and Holland *et al.* (2008). Preliminary tests were performed to determine the optimal parameter settings: peak height threshold (PHT) of 50, minimum fragment length (MFL) of 50, and bin width (BW) of 0.5.

Data analysis

The presence or absence of each band was recorded in a binary data matrix for each individual, assigning a value of 1 or 0 depending on band presence or absence, respectively. The binary data matrix obtained was used to calculate the following parameters assuming Hardy–Weinberg equilibrium: observed number of bands (NB), number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), mean observed number of alleles (A_O), mean effective number of alleles (A_E), observed heterozygosity (H_E), unbiased heterozygosity (UH_E) and, lastly, Shannon diversity index (I).

Molecular variance analysis (AMOVA) was used to calculate variance components and the significance levels of variation among and within subpopulations; a Mantel test (9999 permutations) was also performed to test significance in the isolation by distance relationships. In addition, a multivariate representation of individuals from each analyzed subpopulation was obtained by subjecting a pairwise distance matrix to Principal Coordinates Analysis (PCoA). All

the above-mentioned tests were performed using the programme GenAlEx 6.4 (Peakall & Smouse 2006).

To further substantiate the assessment of population genetic structure, a model-based Bayesian inference clustering was run using Structure 2.3 (Pritchard *et al.* 2000; Falush *et al.* 2007). The analysis assumed an admixture model and uncorrelated allele frequencies between clusters. Ten independent runs were carried out for each value of K ranging from 1 to 6, with a burn-in period of 2×10^5 and 1×10^5 Markov Chain Monte Carlo replicates after burn-in. The estimated mean logarithmic likelihood of K values and the optimal K was determined.

Results

RAPD and AFLP polymorphisms

To assess the genetic variation and population structure of *R. cabrerensis* subsp. *muniellensis* in the Muniellos Biosphere Reserve, AFLP and RAPD analyses were conducted. From RAPD analysis, 16 individuals per subpopulation were used, with 35 primers employed and a total of 126 bands ranging from 300 to 2050 bp, corresponding to an average of 3.60 bands per primer. The number of bands and the percentage of polymorphic fragments produced by each primer were varied (Table 1). The AFLP analysis was conducted on 8 individuals per each subpopulation, and a total of 1819 bands were identified by using 15 primer combinations, i.e., 121.27 bands per combination. Polymorphic products in these combinations ranged from 39 to 179 bands, with an average of 102.00 per combination (Table 1).

Genetic diversity within subpopulations

Genetic diversity in *R. cabrerensis* subsp. *muniellensis* based on RAPD and AFLP markers, is summarized in Table 2.

Table 2. Genetics diversity within subpopulations of *Ranunculus cabrerensis* subsp. *muniellensis*, based on RAPD and AFLP markers.

			RAPD				
Level	N	PPB (%)	A_O ± SE	A_E ± SE	H_E ± SE	UH_E ± SE	I ± SE
Subpop. 1	16	53.97	1.524 ± 0.047	1.319 ± 0.033	0.187 ± 0.018	0.193 ± 0.018	0.279 ± 0.026
Subpop. 2	16	50.79	1.460 ± 0.052	1.325 ± 0.034	0.187 ± 0.018	0.193 ± 0.019	0.276 ± 0.026
Subespecies	32	65.08	1.651 ± 0.043	1.366 ± 0.033	0.217 ± 0.017	0.220 ± 0.018	0.326 ± 0.025
			AFLP				
Level	N	PPB (%)	A_O ± SE	A_E ± SE	H_E ± SE	UH_E ± SE	I ± SE
Subpop. 1	8	68.39	1.568 ± 0.016	1.358 ± 0.008	0.214 ± 0.004	0.228 ± 0.005	0.327 ± 0.006
Subpop. 2	8	66.36	1.522 ± 0.017	1.343 ± 0.008	0.206 ± 0.004	0.220 ± 0.005	0.316 ± 0.006
Subespecies	16	84.11	1.841 ± 0.009	1.382 ± 0.008	0.232 ± 0.004	0.239 ± 0.004	0.360 ± 0.006

N, sample size; PPB, percentage of polymorphic bands; A_O, mean observed number of alleles; A_E, mean effective number of alleles; H_E, observed heterozygosity; UH_E, unbiased heterozygosity; I, Shannon diversity index.

With regard to RAPD markers, percentage of polymorphic bands (PPB) at the subpopulation level was moderate, ranging from 53.97% (subpopulation 1) to 50.79% (subpopulation 2). At subspecies level, the average number of alleles per locus (A_O) was 1.651, while the effective number of alleles per locus (A_E) was found to be 1.366. Values for Nei heterozygosity (H_E), Nei unbiased heterozygosity (UH_E) and Shannon diversity index (I) were 0.187, 0.193 and 0.279 respectively for the subpopulation 1; and 0.187, 0.193 and 0.276 for the subpopulation 2. However, relatively high levels of genetic diversity were revealed at subspecies level (PPB = 65.08%, H_E = 0.217, UH_E = 0.220 and I = 0.326).

In the case of AFLP markers, the PPB percentages ranged from 68.39% (subpopulation 1) to 66.36% (subpopulation 2) and A_O and A_E average values at subspecies level were 1.841 and 1.382 respectively. Values for H_E, UH_E and I were 0.214, 0.228 and 0.327 respectively for subpopulation 1, and 0.206, 0.220 and 0.316 for subpopulation 2. As found with RAPD, high levels of genetic diversity were revealed at subspecies level (PPB = 84.11%, H_E = 0.232, UH_E = 0.239 and I = 0.360), demonstrating, therefore, that measures of genetic diversity within subpopulations were similar using either RAPD and AFLP markers (Table 2).

Genetic differentiation among subpopulations

The AMOVA analysis from the distance matrices permitted partitioning of the overall variation into two levels (Table 3). The proportion of variation attributable to within-subpopulation was very high (94 and 96% by RAPD and AFLP respectively). The genetic differentiation among subpopulations detected by RAPD was 6% (P < 0.001) and a similarly small amount of variation (4%) was detected with AFLP (P < 0.05).

Table 3. Analysis of molecular variance (AMOVA) based on RAPD and AFLP markers for two subpopulations of *Ranunculus cabrerensis* subsp. *muniellensis* (d.f.: degrees of freedom; SS: sums of squares; MS: mean sums of squares; VC: variance components). Level of significance are based on 9999 iteration steps.

	RAPD						**AFLP**					
Source variation	d.f.	SS	MS	VC	%	p	d.f.	SS	MS	VC	%	p
Among subpop.	1	22.78	22.78	0.74	6	<0.001	1	326.31	326.31	10.75	4	<0.05
Within subpop.	30	328.06	10.93	10.93	94	<0.001	14	3363.37	240.24	240.24	96	<0.05
Total	31	350.84	33.71	11.67			15	3689.68		251.00		

The Mantel test revealed a low but significant positive correlation between matrices of genetic and geographic distances based on RAPD analysis (r = 0.177; P = 0.023). However, after performing an exhaustive analysis based on AFLP markers, no correlations were detected between genetic and geographic distances (Mantel's test: r = 0.106; P = 0.137), therefore the isolation of the subpopulations based on distances is not supported.

The first two principal coordinates of PCoA (Figure 3) showed a weak structure of clusters. In the case of RAPD markers, the first and second axes explained 22.14 and 20.15% of the total variation, respectively (18.99% in the case

of axis 3, not shown). Furthermore, the first and second axes from AFLP data explained 24.47 and 19.46% of the total variation respectively (axis 3 explained 16.43%, not shown).

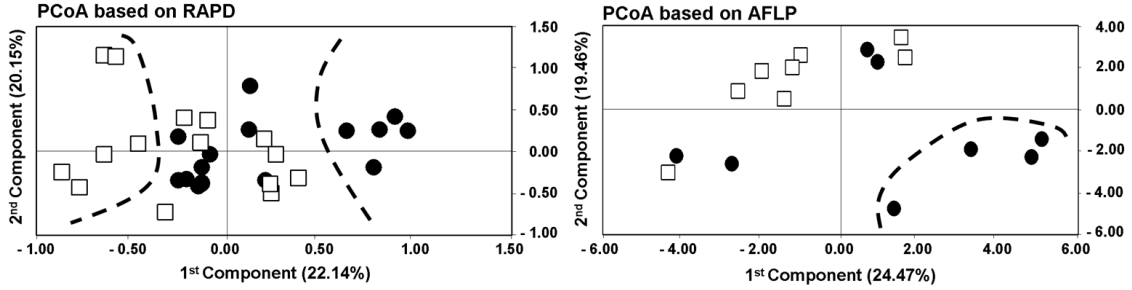

Figure 3. Principal Coordinates Analysis from *Ranunculus cabrerensis* subsp. *muniellensis* subpopulations based on the correlation matrix of presence/absence of RAPD and AFLP data: Subpopulation 1 = filled circles (●); Subpopulation 2 = open squares (□). The discontinuous lines of points emphasize the differences found between both subpopulations.

According to these data, the PCoA analysis can therefore suggest a possible slight structure based on the genetic data. This subpopulation structure pattern was also supported by the genetic admixture analysis based on AFLP data: the best mean likelihood value for 10 independent runs was at K = 2 (-18187.19), which identified two distinct geographical groups (Table 4). However, if considering the mean likelihood values in RAPD data (K = 1; -2071.61), none population genetic structure was found.

Table 4. Number of clusters inferred by Structure software for RAPD and AFLP analyses. Mean of ten independent runs for each value of K ranging from 1 to 6 is presented. In bold: most probable number of clusters (highest ln Pr(X/K)). Var: variance of the likelihood.

	RAPD		AFLP	
K	ln Pr(X/K)	Var[LnP(X/K)]	ln Pr(X/K)	Var[LnP(X/K)]
1	**-2071.61**	513.66	-18232.79	2293.98
2	-2081.44	538.12	**-18187.19**	2376.43
3	-2084.43	545.84	-18198.63	2417.98
4	-2085.59	548.53	-18201.33	2426.12
5	-2086.27	548.86	-18200.17	2426.51
6	-2088.51	552.36	-18199.40	2421.97

Discussion

In endangered species of high conservation or restoration priority, knowledge of genetic variation can guide future management decisions. Thus, information about genetic variation within and between populations of endangered plant species is essential for strategies based on such decisions.

The application of polymorphic markers is therefore an important, useful tool for planning strategies of collection and *in situ* and *ex situ* conservation. The present study is focused on the only known population of *R. cabrerensis* subsp. *muniellensis*, where 102.00 polymorphisms were generated per primer in the case of AFLP but

only 2.34 reported by RAPD (Table 1). As well as detecting polymorphisms per primer more frequently, AFLP also detected more polymorphism per locus (84.11%) than the RAPD (65.08%). These results demonstrate the greater discriminating power of AFLP analysis, whose superiority has also been reported in studies of other endangered species (e.g. Palacios & González-Candelas 1999).

Given the endemic character, the small population size and the very restricted geographical distribution of *R. cabrerensis* subsp. *muniellensis*, we would expect a genetically impoverished population, as has been shown in other rare species (Frankham *et al.* 2004). Many investigations have demonstrated that rare and endemic species have low genetic diversity (e.g. Ellstrand & Elam 1993; Swensen *et al.* 1995), but recently others have shown that some endemic and endangered species in fact present high levels of genetic diversity (e.g. Gaudeul *et al.* 2000; Crema *et al.* 2009). Indeed, genetic diversity values detected in natural populations of *R. cabrerensis* subsp. *muniellensis* (H_E = 0.217 by RAPDs; H_E = 0.232 by AFLPs) were similar or slightly higher than most of those different authors have described in small populations of endangered, rare species using RAPD markers, such as *Anagyris latifolia* (H_E = 0.200; González-Pérez *et al.* 2009), *Antirrhinum microphyllum* (H_E = 0.188; Torres *et al.* 2003), *Gnaphalium teydeum* (H_E = 0.140; González-Pérez *et al.* 2008) as well as with AFLP markers, such as *Olearia gardneri* (H_E = 0.17; Barnaud & Houliston 2010), *Silene tatarica* (H_E = 0.127; Tero *et al.* 2003). Additionally, Bayesian clustering (Table 4) and PCoA (Figure 3) based on AFLP data (and also according to RAPD data, although to a lesser extent), proposed a slight different genetic structure between *R. cabrerensis* subsp. *muniellensis* subpopulations.

Relatively high genetic diversity in plants characterized by a small number of populations and few individuals within each population may be an indication that the number of individuals of this species was larger in the past (Ellstrand & Elam 1993). Another explanation for the relatively high genetic diversity of *R. cabrerensis* subsp. *muniellensis* may be the existence of outcrossing: commonly, outcrossing species have higher levels of genetic diversity and lower differentiation between populations than selfing species (Rossetto *et al.* 1995; Muir *et al.* 2004). Finally, an alternative hypothesis could be based on high levels of genetic drift and mutations occurring over a very long period after the subpopulations separated.

Implications for conservation

Our analysis based on molecular markers is the first attempt to reveal the levels of genetic variability and differentiation of the natural populations of *R. cabrerensis* subsp. *muniellensis*, and provides important guidance toward the conservation of this taxon. These results also highlight the pertinent role of small remnant populations as important sources of genetic variation.

Currently, only a single population of *R. cabrerensis* subsp. *muniellensis* is known, an uncommon phenomenon according to herbarium collections and field

observations. This population appears in the Muniellos Biosphere Reserve, a Site of Community Importance (SCI) as defined in the European Commission Habitats Directive (92/43/EEC), where access, even to walkers, is controlled and very limited. However, the potential threat for this population with such a small number of individuals is the low probability of the occurrence of new populations, along with the presence of natural enemies such as pathogens, herbivores, and seed predators.

Genetic diversity and population structure results suggest that a strategy for conserving *R. cabrerensis* subsp. *muniellensis* must focus on both subpopulations. In the short term, priority should thus be given to protecting the two existing populations *in situ*, and also to avoiding any population decline. In the long term, a good conservation strategy might be (1) to maximize the protection of the contiguous landscape in which selection and migration can interact to maintain *R. cabrerensis* subsp. *muniellensis* evolution; and (2) to develop *ex situ* conservation strategies: samples should be collected from both subpopulations to increase genetic diversity; pollination biology and reproductive studies should be conducted, leading to a better understanding of those mechanisms that maintain genetic variation in these populations, and thereby ensuring successful conservation of this threatened taxon can be achieved. Finally, it should be highlighted that *R. cabrerensis* subsp. *muniellensis* requires urgent attention and special consideration in future reviews of the regional and national protection lists.

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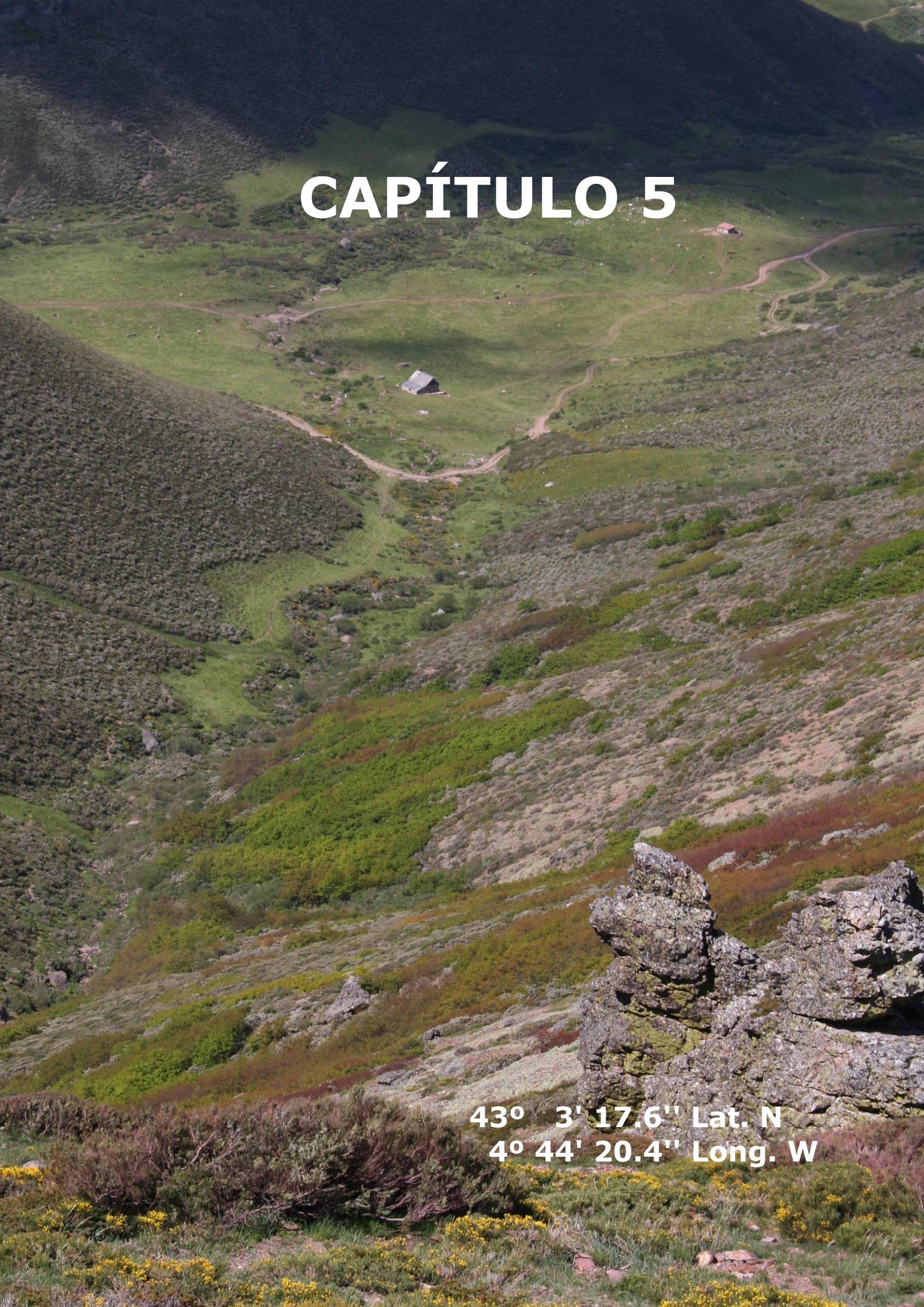
CAPÍTULO 5



Genetic diversity and structure in fragmented populations of the endangered species *Ranunculus cabrerensis* (Ranunculaceae): implications for conservation

Summary

Ranunculus cabrerensis is an endemic and endangered species of the Northwestern Iberian Peninsula. The molecular markers Amplified Fragment Length Polymorphism (AFLP) and Inter Simple Sequence Repeat (ISSR) were used to investigate the genetic diversity and population structure of four populations across its known distribution. Fifteen selective primer combinations of AFLP and seventeen ISSR primer combinations produced a total of 2830 and 103 unambiguous repeatable fragments respectively, of which 97.57 and 81.38% were polymorphic for both markers.

The genetic diversity of *R. cabrerensis* at species level was high (H_E = 0.294 by ISSR and H_E = 0.191 by AFLP) and differentiation between sampled locations was also relatively high (G_{ST} = 0.316 and 0.158 by ISSR and AFLP analysis respectively) compared to other studies of endangered and rare species using the same techniques. The analysis of molecular variance (AMOVA) indicated the main genetic variation was within sampled locations (73% by AFLP; 52% by ISSR), even though the variation among locations was also significant. Principal Coordinates, NeighborNet and Bayesian analyses revealed a weak but significant relationship between the genetic structures of different populations in *R. cabrerensis*, with gene flow acting as a homogenizing force that prevents stronger differentiation of populations. Finally, suggestions for conservation strategies to preserve the genetic resources of this species are outlined.

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Introduction

In the last decade, an increasing number of studies have demonstrated the value of genetic data in addressing issues of plant conservation biology, especially in rare and narrowly endemic species (e.g. Gaudeul *et al.* 2000; Bellusci *et al.* 2008; González-Pérez *et al.* 2009). Genetic variation within a taxon is thought to be crucial for the long-term survival and continued evolution of populations or species (Frankham *et al.* 2004). Experimental and field investigations have demonstrated that fragmented populations may lose allelic richness or genetic diversity, and have increased population differentiation due to genetic drift and inbreeding depression (e.g. Buza *et al.* 2000; Tomimatsu & Ohara 2003). Thus, an accurate estimate of the level and distribution of genetic diversity of threatened and endangered species is an important element in designing conservation programmes (Smith & Wayne 1996; Höglund 2009).

In the present study, an attempt to examine the impact of habitat fragmentation on the genetic diversity and population structure of *Ranunculus cabrerensis* Rothm., an endangered plant with a narrow distribution in the Northwestern Iberian Peninsula, was conducted. Such population genetic information is a prerequisite to understanding its species survival possibility in the short-term, so that an effective conservation strategy for long-term survival can be formulated and implemented.

In the past, *R. cabrerensis* was treated as a subspecies of the *R. parnassiifolius s.l.* polyploid complex. However, as a result of recent studies, we have put forward numerous arguments to support the separation of these taxa, and consequently for them to be treated as independent species (see Chapter 3). In addition to this, within *R. cabrerensis* it is possible to differentiate two subspecies: *R. cabrerensis* subsp. *cabrerensis* and *R. cabrerensis* subsp. *muniellensis* (Bueno, Fern.Casado & Fern.Prieto) Fern.Prieto & Cires (for more details on the subspecies differentiation at morphological and molecular level see Bueno *et al.* 1992; Chapter 3).

The first subspecies grows in small, localised populations on rock-fields and coarse rocky screes (rock-slides) at altitudes of 1750 to 2450 m, in the region of Castile and Leon (Northwest Iberian Peninsula). Currently, Spanish national legislation lists *R. parnassiifolius s.l.* (i.e. not specifying any particular subspecies) without any specific category (Real Decreto 139/2011); nevertheless, previous legislation catalogued *R. parnassiifolius* subsp. *cabrerensis* (= *R. cabrerensis* subsp. *cabrerensis*) under the category of "special interest" (Orden MAM/2734/2002). Additionally, it should be noted that this plant is considered "rare" by several regional catalogues (Llamas *et al.* 2007) and has not been included in the National Red List of threatened plants for Spain (see VV.AA. 2000; Bañares *et al.* 2004; Moreno 2008).

The second subspecies occurs naturally in siliceous screes in the Muniellos Biosphere Reserve (Western Cantabrian Mountains, Asturias, Iberian Peninsula), and at the moment, only a single population is known. In regard to its protected status, this subspecies does not appear in the regional catalogue of threatened species for Asturias. Indeed, in the Red List of Spanish Vascular Flora (VV.AA. 2000) it appears under the category of "data deficient", while in Bañares *et al.* (2004) and Moreno (2008) it is listed as "vulnerable". Fernández Prieto *et al.* (2007) proposed the inclusion of this plant in the catalogue of threatened vascular plants of Asturias under the category of "vulnerable".

Despite its rarity, its patchy distribution and the endangered status of this endemic taxon, there is no population genetic study and conservation management plan for the species to date. As an initial step in developing such a plan, we have assessed the genetic diversity and population structure of four natural populations of *R. cabrerensis s.l.* using Inter Simple Sequence Repeat (ISSR) (Zietkiewicz *et al.* 1994) and Amplified Fragment Length Polymorphisms (AFLP) (Vos *et al.* 1995).

ISSR and AFLP have advantages over other DNA polymorphism analysis methods, as they do not require prior sequence knowledge, cloning procedures or characterized probes. It is generally accepted that they have a comparatively high reproducibility (Jones *et al.* 1997), and as a result, AFLP and ISSR have both been used to investigate within-species genetic variation (e.g. Ci *et al.* 2008; Cuesta *et al.* 2010). Moreover, both techniques have been successfully used in plant population genetic studies, especially for endangered species (e.g. Li & Jin 2007; Gong *et al.* 2010).

Here, we report a genetic variability analysis on *Ranunculus cabrerensis*, performed from a conservation perspective. The following questions were examined: (1) what is the level of genetic diversity in populations of *R. cabrerensis s.l.*? (2) how is genetic diversity distributed within and between populations? And finally, (3) how are the different populations related, and is there a geographical correlation? In addition to assess the levels of genetic variability in the endangered species *R. cabrerensis*, the results of this study are used to propose conservation and management strategies.

Materials and methods

Sample collection and DNA extraction

Four natural populations of *R. cabrerensis s.l.*, covering the whole range of the subspecies (Figure 1), were collected between 2007 and 2008, and mapped using GPS (Garmin) (Table 1).

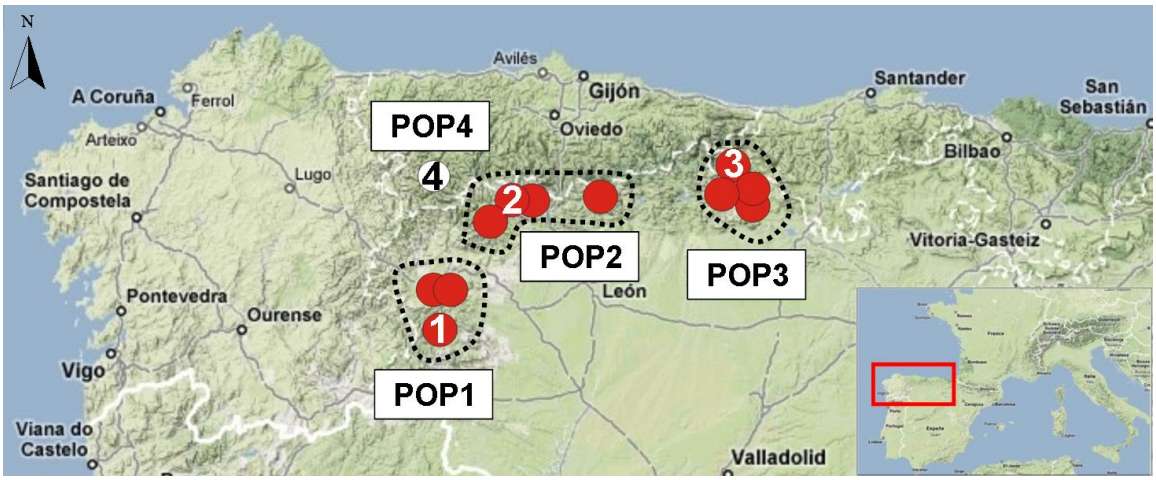


Figure 1. Locations of four populations of *Ranunculus cabrerensis s.l.* in the Northwest of the Iberian Peninsula. POP1: Lago de la Baña; POP2: Abelgas de Luna; POP3: Portillo de las Yeguas; POP4: Muniellos Biosphere Reserve. Open circles (○): distribution of *R. cabrerensis* subsp. *muniellensis* (a single population is known). Filled circles (●): global distribution of *R. cabrerensis* subsp. *cabrerensis*.

From each site, plants were randomly sampled and leaves were taken from individuals that were at least 3 m apart, to avoid duplicate sampling. Leaves were stored on moistened paper, enclosed in a plastic bag and kept at low temperatures (4 ºC) before and during transportation, and on arrival at the laboratory the plant tissue was frozen at -80 ºC until analysis. In addition, voucher specimens were collected, dried by pressing in absorbent paper, stored at room temperature, and kept in the Herbarium of the University of Oviedo (FCO) (Table 1).

Table 1. Code, location, population and sample size of the three examined populations of *Ranunculus cabrerensis s.l.*

Code	Subsp. Syst.†	Population	Coordinates	Sample size AFLP / ISSR	Voucher specimens
POP1	RCC	*Lago de la Baña, Sierra Cabrera (Encinedo, León, Spain); 2000 m; *BJA, EC & LG*; 2007/06/14	42º 14' 33.8'' N 6º 44' 40.0'' W	8 / 10	FCO: 31369
POP2	RCC	Abelgas de Luna, north of Peña Piquera (Sena de Luna, León, Spain); 1956 m; *CC & EC*; 2008/06/29	42º 53' 32.7'' N 6º 4' 59.0'' W	8 / 10	FCO: 31368
POP3	RCC	Portillo de las Yeguas, San Glorio (Boca de Huérgano, León, Spain); 2094 m; *AB & EC*; 2007/06/19	43º 3' 17.6'' N 4º 44' 20.4'' W	8 / 10	FCO: 31371
POP4	RCM	*Muniellos Biosphere Reserve (Cangas del Narcea, Asturias, Spain); 1464 m; *EC*; 2007/07/18	43º 1' 28.6'' N 6º 44' 25.9'' W	8 / 10	FCO: 31370

* *Locus classicus*

† Subspecific systematization of samples according to previous studies (RCC: *Ranunculus cabrerensis* subsp. *cabrerensis*; RCM: *R. cabrerensis* subsp. *muniellensis*) (see Chapter 3 for more details).

Collector abbreviations: *AB* = A. Bueno; *BJA* = B. Jiménez-Alfaro; *CC* = C. Cuesta; *EC* = E. Cires; *LG* = L. González.

Finally, to provide a more complete overview of the study of *R. cabrerensis s.l.*, information related to AFLP in *R. cabrerensis* subsp. *muniellensis* (POP4) (see Chapter 4) has been included in the present work, and we also performed new ISSR

analysis of the latter subspecies. The specimens collected belong to the central population of *R. cabrerensis* subsp. *muniellensis* (the biggest in terms of number of plants and area), since the isolation and smaller size of peripheral populations could lead to less variation than in central populations because of genetic drift. Even under similar selection procedures in central and peripheral populations, isolation would increase genetic divergence at the range margin. Consequently, geographically peripheral populations may differ considerably from core populations and hence contribute significantly to geographical variation.

DNA extraction was carried out using the CTAB method described by Doyle & Doyle (1987) with minor modifications. DNA concentration was measured by a Beckman-Coulter DU800® spectrophotometer (Fullerton, CA, USA).

ISSR amplification

Twenty-one ISSR primers (MWG-Biotech AG, Ebersberg, Germany) were used for the analysis. A preliminary screening was carried out using gradient annealing temperatures (Ta) in order to select primers that could give reliable patterns of amplification and also to choose a suitable annealing temperature (Table 2).

Table 2. Primers, melting temperatures (Tm), annealing temperatures (Ta), range of molecular weight of bands per primer expressed in base pairs (bp), and number of repeatable bands scored for each ISSR used to amplify *R. cabrerensis s.l.* NB: number of bands; NPB: number of polymorphic bands; PPB: the average percentage of polymorphic bands.

Primers	Sequence (5′ – 3′)[a]	Tm (ºC)	Ta (ºC)	Size range (bp)	NB	NPB	PPB (%)
UBC-807	$(AG)_8T$	50.4	49	700-1450	5	4	80.00
UBC-811	$(GA)_8C$	52.8	49	600-1500	9	8	88.89
UBC-812	$(GA)_8A$	50.4	49	650-1200	8	8	100.00
UBC-813	$(CT)_8T$	50.4	49	700-1800	7	6	85.71
UBC-818	$(CA)_8G$	52.8	49	700-1500	8	6	75.00
UBC-825	$(AC)_8T$	50.4	49	550-1200	5	3	60.00
UBC-828	$(TG)_8A$	50.4	49	650-1500	7	5	71.43
UBC-834	$(AG)_8YC$	52.6	52	275-1500	8	7	87.50
UBC-842	$(GA)_8YG$	54.8	52	350-1000	4	4	100.00
UBC-844	$(CT)_8RC$	54.8	52	400-1400	6	5	83.33
UBC-855	$(AC)_8YT$	52.6	55	750-1550	5	4	80.00
UBC-857	$(AC)_8YG$	54.8	55	300-1800	10	10	100.00
UBC-864	$(ATG)_5$	46.9	49	950-1800	5	4	80.00
UBC-886	$VDV(TC)_7$	51.9	52	550-850	4	3	75.00
LL1	$(CAA)_5$	39.6	49	700-1800	6	4	66.67
LL2	$(CAG)_5$	53.3	62	750-850	2	2	100.00
LL4	$(GACA)_4$	49.2	52	1000-1500	4	2	50.00
Total					103	85	

Unamplified fragments or unclear amplifications: UBC-808 $(AG)_8C$; UBC-820 $(GT)_8C$; UBC-840 $(GA)_8YT$; LL3 $(GATA)_4$.

[a] D = A or G or T; R = A or G; Y = C or T; V = A or C or G.

The PCR reaction mixture (25 µL) consisted of 25 ng of DNA, 2.5 µL of 10x PCR buffer, 2 mM $MgCl_2$, 0.2 µM of primer, 0.1 mM dNTPs, and 1 U of Taq DNA polymerase (Biotools). PCR amplifications were performed using a gradient thermal cycler (MyCycler™, Bio-Rad) with initial denaturation at 94 ºC for 1 min followed by 40 cycles of 1 min at 94 ºC, 1 min at the annealing temperature (Ta), 2 min

elongation at 72 ºC; and a final extension at 72 ºC for 10 min. Amplification products were resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide, and then photographed on a UV transilluminator (GelLogic 100, Kodak). Molecular size of the fragments was estimated using a 100-bp DNA ladder (Biolabs®, New England, USA). In order to ensure the reliability of the results obtained, reactions were performed at least twice, including internal controls (replicates of the same sample in each combination, and different DNA extractions of the same individual). Only consistently reproduced, distinguishable bands were considered.

AFLP amplification

The AFLP-based PCR was carried out as has been previously described (see Chapter 4). The genomic DNA was digested with *Eco*RI and *Mse*I restriction enzymes (New England Biolabs, Inc.). In the following step, double-strand adapters were ligated to *Eco*RI and *Mse*I specific ends by T4 DNA Ligase (Roche Diagnostics). Products of digestion/ligation were checked by electrophoresis in 1.5% agarose. The pre-selective amplification was performed using primers with single selective nucleotides (*Eco*RI+A and *Mse*I+C), checked by electrophoresis in 1.5% agarose gels and subsequently diluted (1:10) in sterile de-ionised H_2O. Then selective amplifications were performed using *Eco*RI and *Mse*I primers with three selective nucleotides (Table 3). The *Eco*RI-selective primers were 5'-fluorescent labelled.

Table 3. Pairs of primers used for AFLP amplification of *R. cabrerensis s.l.* and summary of amplified bands. NB: number of bands; NPB: number of polymorphic bands; PPB: the average percentage of polymorphic bands.

Primer pairs	NB	NPB	PPB (%)
*Eco*RI-AAC / *Mse*I-CAT	267	264	98.88
*Eco*RI-AAC / *Mse*I-CTT	154	142	92.21
*Eco*RI-AAC / *Mse*I-CCAC	111	103	92.79
*Eco*RI-AAC / *Mse*I-CCAG	145	144	99.31
*Eco*RI-AAC / *Mse*I-CCTA	143	142	99.30
*Eco*RI-ACG / *Mse*I-CAT	297	297	100.00
*Eco*RI-ACG / *Mse*I-CTT	192	189	98.44
*Eco*RI-ACG / *Mse*I-CCAC	166	165	99.40
*Eco*RI-ACG / *Mse*I-CCAG	101	97	96.04
*Eco*RI-ACG / *Mse*I-CCTA	169	163	96.45
*Eco*RI-ACT / *Mse*I-CAT	320	317	99.06
*Eco*RI-ACT / *Mse*I-CTT	241	236	97.92
*Eco*RI-ACT / *Mse*I-CCAC	184	180	97.83
*Eco*RI-ACT / *Mse*I-CCAG	172	169	98.25
*Eco*RI-ACT / *Mse*I-CCTA	168	164	97.62
Total	2830	2772	

Selective amplification products were submitted to the Sequencing Services from the University of Oviedo for fragment analysis: samples were run on an automated DNA sequencer (ABI PRISM® 3100, Applied Biosystems) with an internal size standard GeneScan 500 (ROX™, Applied Biosystems). Five randomly chosen samples were analyzed in duplicate and used as positive controls to verify the reproducibility of AFLP analyses. Raw AFLP data were collected and sized using the Genemapper 4.0 software (Applied Biosystems).

Genetic diversity analysis

Since ISSR and AFLP are dominant markers, and given that the species is diploid, we assumed that each band represented the phenotype at a single biallelic locus. Amplified fragments were scored for presence (1) or absence (0) of comigrating bands. The resulting binary data matrices of AFLP and ISSR phenotypes were analyzed using GenAlEx 6.4 (Peakall & Smouse 2006) to estimate the following genetic diversity parameters, assuming Hardy-Weinberg equilibrium: observed number of bands (NB), number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), mean observed number of alleles (A_O), mean effective number of alleles (A_E), number of private alleles (i.e. unique for respective population but not common for all of its samples, A_P), number of discriminating alleles (i.e. present in all analysed samples of a respective population and absent elsewhere, A_D), Nei's gene diversity (H_E), and finally Shannon diversity index (I). The population genetic structure G_{ST} was computed using AFLP-SURV 1.0 (Vekemans 2002) which estimates allele frequencies at each locus in each population for dominant markers, assuming two alleles per locus. We used Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999). Gene flow (Nm) among populations was estimated indirectly from the equation $Nm = (1 - G_{ST})/4G_{ST}$ (Nei 1977; Slatkin & Barton 1989).

An analysis of molecular variance (AMOVA) was performed to further explore the genetic structure of the studied populations. AMOVA works with the squared Euclidian genetic distances between all individuals and allows the partitioning of variance among and within populations. In addition, a Mantel test was performed to test the correlation between geographical distance between pairs of populations and Nei's genetic distance (Nei 1972) (computing 9999 permutations). The AMOVA and Mantel test were performed using the program GenAlEx 6.4 (Peakall & Smouse 2006). In addition to this, the relationship between genetic distance, expressed as F_{ST} and geographic distance was further examined using the software BARRIER 2.2 (Manni *et al.* 2004) which implements Monmonier's maximum difference algorithm to test the presence of genetic barriers among groups.

Principal Coordinates Analysis (PCoA) was performed on the Jaccard similarity matrix to visualize the genetic relationships among all individuals analyzed with ISSR and AFLP markers using Past 1.89 (Hammer *et al.* 2001). To further substantiate the assessment of population genetic structure, a model-based Bayesian inference clustering was run using Structure 2.3 (Pritchard *et al.* 2000; Falush *et al.* 2007). For the Structure analyses, we used an admixture model and uncorrelated allele frequencies between clusters. Nine independent runs were carried out for each value of K ranging from 1 to 10, with a burn-in period of 2×10^5 and 1×10^5 Markov Chain Monte Carlo replicates after burn-in. To infer the number of genetic groups in our data set, we used the ΔK method of Evanno *et al.* (2005), which consists in finding the breakpoint in the slope of the distribution of ln Pr(X/K) for the different K values tested, where ln Pr(X/K) is an estimate of the posterior probability of the data

for a given K. Evanno *et al.* (2005) showed that this ΔK method actually detects the uppermost level of population structure when several hierarchical levels exist.

Finally, NeighborNet analysis was performed in SplitsTree 4.11 (Huson & Bryant 2006) to construct an unrooted network (uncorrected P, NeighborNet distance) of relationships among individuals based on the presence-absence matrix for all populations. Bootstrap analysis was performed on ISSR and AFLP dataset using 5000 replicates.

Results

ISSR and AFLP polymorphism

Seventeen ISSR primers that produced clear and repeatable fragments were chosen for subsequent analysis (Table 2). These primers consistently amplified a total of 103 scored bands that ranged in size from 275 to 1800 bp. The average number of bands produced by each primer was 6.06. Furthermore, 85 bands were polymorphic at the species level (81.38%).

The fifteen selective primer combinations used for AFLP analysis generated 2830 fragments ranging from 50 to 500 bp, with an average of 188.67 bands per primer combination (Table 3). Polymorphic products in these combinations ranged from 97 to 317, with an average of 184.80 per combination (97.57%). The mean error statistic for duplicate samples was 1.6% and therefore not likely to significantly affect the results. Hence the reliability of the AFLP technique at all its different steps was confirmed.

Genetic diversity within populations

A summary of the genetic within-population diversity for each of the four populations of *R. cabrerensis s.l.*, based on ISSR and AFLP markers, is given in Table 4.

According to ISSR analysis, the percentage of polymorphic loci at the population level was low, ranging from 31.07% (POP2) to 54.37% (POP4), with an average of 38.59%. The number of alleles per locus ranged from 1.058 to 1.427, with an average value of 1.211, while the effective number of alleles per locus ranged from 1.229 to 1.404, with an average value of 1.283. The Nei's gene diversity ranged from 0.130 to 0.223 with an average value of 0.157. The Shannon's information index ranged from 0.188 to 0.323 with an average value of 0.228, and finally the genetic diversity at population level was relatively low, whereas the total genetic diversity at the species level was high (PPB = 82.52%; H_E = 0.294; I = 0.439).

In the case of AFLP analysis, the percentage of polymorphic loci at the population level was moderately low and widely variable among populations, with a minimum value of 36.64% (POP2), a maximum of 62.61% (POP3), and an average of 49.03%. The number of alleles per locus ranged from 0.873 to 1.300 with an average value of 1.069; while the effective number of alleles per locus ranged from 1.224 to 1.284, with an average value of 1.233. The Nei's gene diversity ranged from 0.110 to 0.175 and the Shannon's index ranged from 0.169 to 0.274, with mean values of 0.143 and 0.221 respectively. As was the case with ISSR, the genetic diversity at population level was relatively low, while the total genetic diversity at the species level was high (PPB = 97.95%; H_E = 0.191; I = 0.312).

Table 4. Genetic diversity in *R. cabrerensis s.l.* determined by ISSR and AFLP markers at population, subspecies and species level. Population codes are as shown in Table 1.

	ISSR								
Level	PPB	A_O ± SE	A_E ± SE	H_E ± SE	I ± SE	A_P	A_D	G_{ST}	Nm
Populations									
POP1	36.89	1.223 ± 0.067	1.260 ± 0.037	0.146 ± 0.020	0.213 ± 0.029	3	0		
POP2	31.07	1.136 ± 0.068	1.237 ± 0.038	0.130 ± 0.020	0.188 ± 0.028	2	1		
POP3	32.04	1.058 ± 0.075	1.229 ± 0.036	0.130 ± 0.019	0.189 ± 0.028	2	1		
POP4	54.37	1.427 ± 0.068	1.404 ± 0.041	0.223 ± 0.022	0.323 ± 0.031	3	0		
Average	38.59	1.211	1.283	0.157	0.228	2.5	0.5		
Subspecies									
RCC	69.90	1.670 ± 0.052	1.462 ± 0.039	0.262 ± 0.020	0.386 ± 0.028	12	0		
RCM	54.37	1.427 ± 0.068	1.404 ± 0.041	0.223 ± 0.022	0.323 ± 0.031	3	0		
Average	62.13	1.549	1.433	0.243	0.355	7.5	0		
Species	82.52	1.825 ± 0.038	1.508 ± 0.036	0.294 ± 0.018	0.439 ± 0.024			0.316	0.541

	AFLP								
Level	PPB	A_O ± SE	A_E ± SE	H_E ± SE	I ± SE	A_P	A_D	G_{ST}	Nm
Populations									
POP1	52.16	1.105 ± 0.018	1.242 ± 0.006	0.149 ± 0.003	0.232 ± 0.005	213	0		
POP2	36.64	0.873 ± 0.017	1.182 ± 0.006	0.110 ± 0.003	0.169 ± 0.005	125	0		
POP3	62.61	1.300 ± 0.017	1.284 ± 0.006	0.175 ± 0.003	0.274 ± 0.005	515	0		
POP4	44.73	0.997 ± 0.018	1.224 ± 0.006	0.136 ± 0.003	0.209 ± 0.005	213	7		
Average	49.03	1.069	1.233	0.143	0.221	266.5	1.7		
Subspecies									
RCC	89.68	1.822 ± 0.010	1.289 ± 0.006	0.185 ± 0.003	0.301 ± 0.004	1274	3		
RCM	44.73	0.997 ± 0.018	1.224 ± 0.006	0.136 ± 0.003	0.209 ± 0.005	213	7		
Average	67.20	1.410	1.257	0.161	0.255	743.5	5		
Species	97.95	1.980 ± 0.003	1.298 ± 0.006	0.191 ± 0.003	0.312 ± 0.004			0.158	1.332

RCC: *Ranunculus cabrerensis* subsp. *cabrerensis*; RCM: *R. cabrerensis* subsp. *muniellensis*

PPB, percentage of polymorphic bands; A_O, observed mean number of alleles per locus; A_E, effective mean of alleles per locus; H_E, observed heterozygosity; I, Shannon diversity index; A_P, number of private alleles; A_D, number of discriminating alleles; G_{ST}, coefficient of genetic differentiation among populations; Nm, gene flow.

Both in the ISSR and AFLP analysis, the genetic diversity among subspecies was much higher than within populations. Furthermore, a high number of private fragments were found between the subspecies. In addition to this, *R. cabrerensis* subsp. *cabrerensis* and *R. cabrerensis* subsp. *muniellensis* were differentiated by three and seven discriminating fragments respectively.

Genetic diversity among populations

The AMOVA analysis from the distance matrices for the individuals studied permitted partitioning of the overall variation into two levels (Table 5). The proportion of variation attributable to within-population differences was relatively high (52% by ISSR; 73% by AFLP). The genetic differentiation among populations detected by ISSR was 48% ($P < 0.001$), while only a small amount of variation (27%) was detected among populations with AFLP ($P < 0.001$).

Table 5. Analysis of molecular variance among and within six populations of *R. cabrerensis s.l.* based on ISSR and AFLP data.

			ISSR			
Source of variation	df	SS	MS	VC	%	*P* value*
Among populations	3	233.000	77.667	7.009	48	<0.001
Within populations	36	272.800	7.578	7.578	52	<0.001
Total	39	505.800		14.587	100	
			AFLP			
Source of variation	df	SS	MS	VC	%	*P* value*
Among populations	3	3142.094	1047.365	97.237	27	<0.001
Within populations	28	7545.125	269.469	269.469	73	<0.001
Total	31	10687.219		366.706	100	

df, degree of freedom; SS, sum of squares; MS, mean of squares; VC, variance component; %, total variation contributed by each component.

**P* value of fixation index after 9999 random permutations.

The coefficient of genetic differentiation between populations (G_{ST}, the proportion of the interpopulation gene diversity) was 0.316 with ISSR, and 0.158 with AFLP (Table 4). All the estimates of genetic differentiation indicated a relatively high level of genetic differentiation among populations.

The estimated number of migrants per generation (*Nm*) from G_{ST} was 0.541 (ISSR analysis) and 1.332 (AFLP analysis) (Table 4), which suggested a moderate-high rate of gene flow among populations. The Mantel test showed that there was significant correlation between geographical distance and pairwise genetic distance based on ISSR data ($r = 0.7269$, $P = 0.0001$). However, AFLP data did not show such a correlation ($r = 0.0441$, $P = 0.2989$).

Genetic clustering

Principal Coordinates Analysis based on ISSR and AFLP data revealed four main groups (Figure 2).

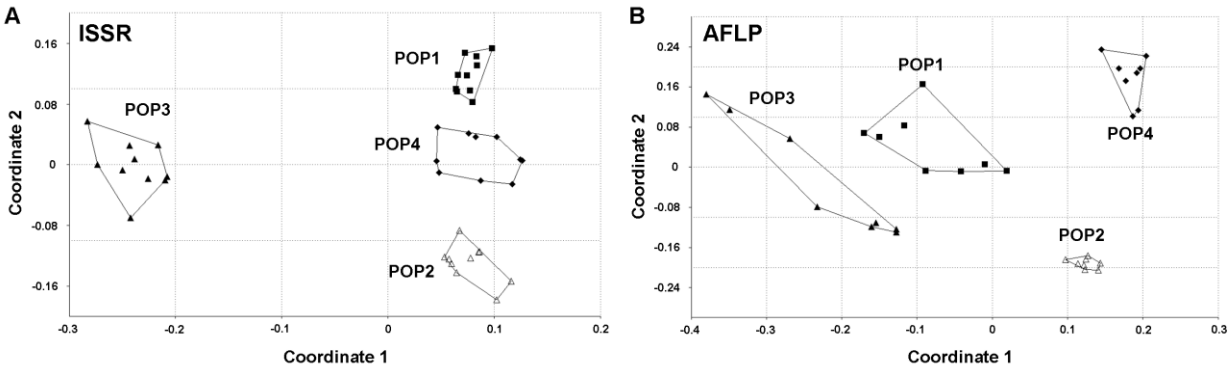


Figure 2. Principal Coordinates Analysis from four populations of *R. cabrerensis s.l.* based on the correlation matrix of presence/absence of ISSR and AFLP fragments. A) ISSR analysis: the first two coordinates explain 46.71% of the total variance. B) AFLP analysis: the first two coordinates explain 28.02% of the total variance. Individuals belonging to the same population are enclosed in convex hull. POP1 = filled squares (■); POP2 = open triangle (△); POP3 = filled triangle (▲); POP4 = filled diamonds (◆). Populations are coded as in Table 1.

In accordance with the PCoA analysis, the NeighborNet (Figure 3) illustrates four major splits corresponding to the number of geographical regions.

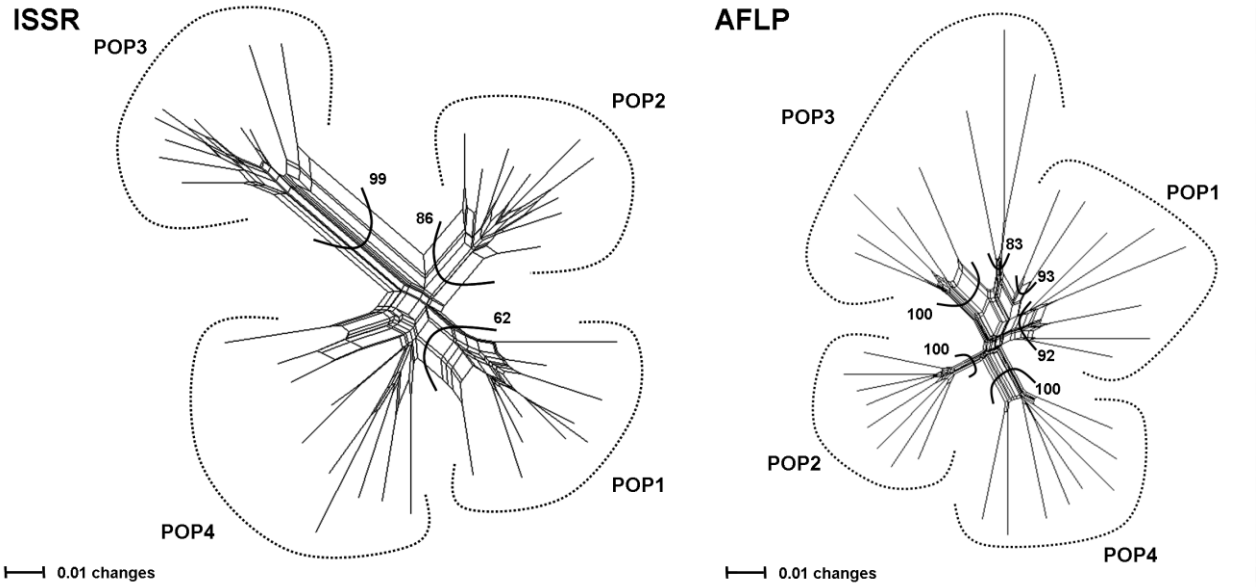


Figure 3. NeighborNet derived from ISSR and AFLP data of *R. cabrerensis s.l.* Bootstrap support values over 50% (analysis of 5000 replicates) are shown on the relevant branches. The least squares fit indexes for the split network were 93.75 and 97.38% for ISSR and AFLP respectively. Population numbers correspond to the populations as mapped in Figure 1 and listed in Table 1.

Additionally, Bayesian clustering of individuals from the four populations was run nine times for every value of K (from 1 to 10). At each round of the process, the consistency of the ln Pr(X/K) values among the different runs with the same K, compared to the variability observed among runs with different Ks, indicated that runs used were sufficiently long (data not shown). The results of the different rounds of the process are summarized in Figure 4A-A'.

In the case of ISSR analysis, the estimator of posterior probabilities of K indicated that the most likely K was 4 (-2070.14), so four groups were inferred, corresponding to the populations studied. However, applying the method of Evanno *et al.* (2005) to the Structure results provided evidence for two genetic clusters (Figure 4B & 5A).

Therefore, ΔK is highest for K = 2 because there is a hierarchical structure in the data set. In such situations, the uppermost hierarchical level of population structure is detected. In the case of AFLP analysis, both method (mean likelihood values and Evanno) for 9 independent runs was K = 2 (Figure 4B-B'). The two genetically different groups (Figure 5B) corresponded closely to the groups recognized by the PCoA (Figure 2).

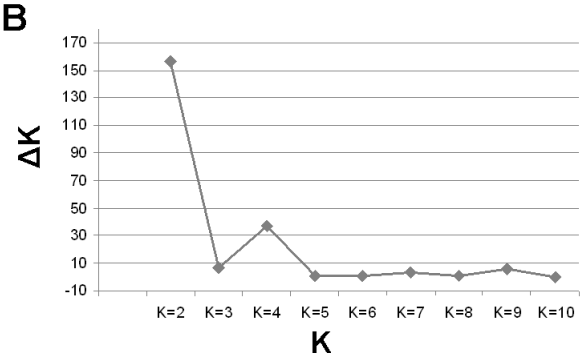
Mean likelihood values **Evanno method**

ISSR

A

	ISSR Structure	
K	ln Pr(X/K)	Var[LnP(X/K)]
1	- 2597.94	383.53
2	- 2256.26	512.78
3	- 2146.13	601.40
4	**- 2070.14**	**694.92**
5	- 2074.06	699.41
6	- 2075.32	698.70
7	- 2079.68	703.21
8	- 2093.90	704.24
9	- 2086.46	710.07
10	- 2100.47	710.03

B



AFLP

A'

	AFLP Structure	
K	ln Pr(X/K)	Var[LnP(X/K)]
1	- 48143.53	2755.37
2	**- 47793.06**	**3660.92**
3	- 47801.51	3678.40
4	- 47802.00	3677.30
5	- 47807.08	3686.14
6	- 47796.93	3659.47
7	- 47797.65	3658.57
8	- 47799.71	3661.14
9	- 47801.15	3663.43
10	- 47811.46	3683.93

B'

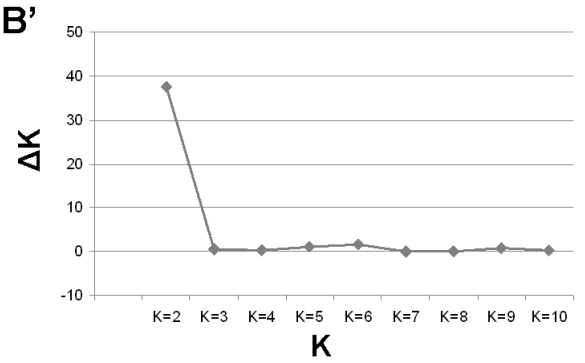


Figure 4. Bayesian maximum likelihood approach in *R. cabrerensis s.l.* based on ISSR and AFLP data. A-A') Mean of ten independent runs for each value of K ranging from 1 to 10. In bold: most probable number of clusters (highest ln Pr(X/K); Var: variance of the likelihood). B-B') Plot of ΔK for each K value calculated as described in Evanno *et al.* (2005) where K is the number of clusters.

Moreover, both ISSR and AFLP analysis showed a clear barrier separating POP3 from the other populations when Monmonier's maximum difference was used as implemented in the BARRIER 2.2 software in a comparison of F_{ST} values and geographic distances.

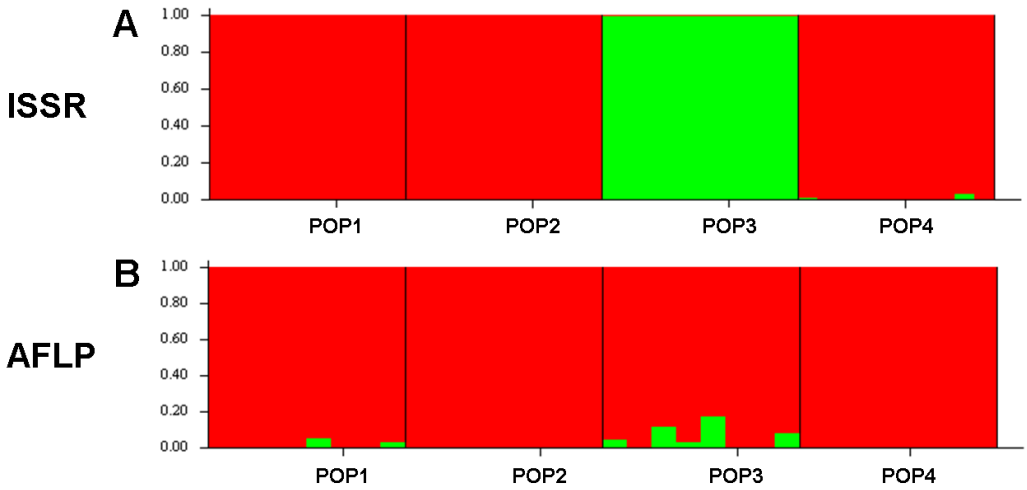


Figure 5. Bar plot of population assignment proportions according to Evanno's statistic (ΔK). Bayesian approach in *R. cabrerensis s.l.* based on ISSR (A) and AFLP (B) data. Vertical lines corresponding to the populations studied. Each individual is represented by a column filled with different colours.

Discussion

Genetic diversity

The documentation of genetic diversity and differentiation between populations using molecular markers provides initial guidance for conservation and can contribute to the setting of conservation priorities among populations (e.g. Neel & Ellstrand 2003). Estimates of genetic diversity derived by dominant molecular markers (i.e. ISSR and AFLP) are usually similar and are generally directly comparable (Nybom 2004). These dominant markers have been used to study a large number of endemic and/or endangered species from different plant families in order to develop conservation strategies, as well as in testing genetic relationships between species (e.g. González-Pérez *et al.* 2009). Our results from AFLP and ISSR show similar overall trends for genetic diversity and population structure. Nevertheless, the genetic diversity indices from ISSR are higher than those from AFLP due to ISSR tending to produce somewhat high estimates of within-population variation (Nybom 2004).

According to the characteristics of *R. cabrerensis s.l.* populations (i.e. fragmented, endemic and rare) we had predicted that there should be low genetic diversity, but generally speaking, it seems that the total genetic diversity based on ISSR and AFLP markers is similar to, or slightly higher, than most of those described by different authors in other species. In fact, Nei's gene diversity estimates in other AFLP studies ranged from 0.07-0.26 (Gaudeul *et al.* 2000; Rottenberg & Parker 2003; Barnaud & Houliston 2010; Chen *et al.* 2010; Gong *et al.* 2010) and in the case of ISSR analysis ranged from 0.10-0.28 (Qiu *et al.* 2004; Xiao *et al.* 2004; Wu

et al. 2004; Shao *et al.* 2009; Jeong *et al.* 2010). In addition, determining levels of diversity within species and populations, as well as ascertaining how species diversity is apportioned within and among populations, is useful in formulating strategies for conserving diversity within taxa (Hamrick *et al.* 1991). The present study indicates that the majority of genetic diversity of *R. cabrerensis s.l.* populations is contained within populations, as indicated by the G_{ST} value of 0.316 and 0.158 in the ISSR and AFLP analysis respectively. This result agrees with those observed in a compilation of studies using RAPD markers (Nybom 2004) for other long-lived perennials (G_{ST} = 0.27), endemic species (G_{ST} = 0.18), and plants whose seed dispersal mechanism is based on gravity (G_{ST} = 0.32). Moreover, literature values obtained with allozymes in different combinations of life history traits (life form, seed dispersal mechanism and geographical range) by Hamrick & Godt (1996) are also consistent with our results.

Gene flow among populations is one of the factors involved in maintaining genetic diversity within a species and depends on dispersal rates (Slatkin 1994). Species with a limited potential for gene flow show more differentiation among populations than do species with high levels of gene flow (Hamrick *et al.* 1991). A migration rate of 0.5 was considered sufficient by Ellstrand & Elam (1993) to overcome the diversifying effects of random drift. For *R. cabrerensis s.l.*, the effective gene flow per generation (*Nm* of 0.541 and 1.332 in the ISSR and AFLP analysis respectively), was slightly higher than one successful migrant per generation, indicating considerable gene flow among populations that has effectively homogenized populations to some degree (Slatkin 1987), and that genetic drift has not yet had a major influence on these *R. cabrerensis s.l.* populations. Indeed, this homogenization of the population is reflected in the Bayesian analyses (Figure 4 & 5). The relatively high *Nm* value for the studied populations is probably attributable to the smaller adjacent range of sampling, and also to the similar ecogeographical environment with frequent wind.

It is worth noting the high genetic diversity among subspecies, also reflected in the presence of private and discriminating fragments. This variability is considered of great importance for possible adaptation to environmental changes and, consequently, for the long-term survival of a species (Sosa *et al.* 2002). In addition, both taxa grow in a relatively specialised alpine habitat of coarse rock-fields, areas which seem to be especially sensitive to global environmental change.

Conservation implications

Successful management and preservation of populations of rare, threatened or endangered species depends on knowledge of the levels of genetic diversity of the target species (Frankham *et al.* 2004). *In situ* conservation is usually the preferred strategy for most wild plant species, because its dynamic nature enables ongoing evolution. Another criterion for conservation is maintaining geographical connectivity to ensure gene flow and preserve genetic diversity. Based on genetic diversity and

differentiation, proposed efficient and practical conservation strategies for the endangered species *Ranunculus cabrerensis* are as follows.

First, since there is relatively high differentiation among populations, the loss of populations at certain locations may not only cause immediate loss in genetic diversity, but it will also cause considerable damage in terms of long-term genetic consequences due to the reduced number of populations and the small population size. Thus, the priority must be to protect all the existing populations *in situ* and prevent anthropogenic destruction, allowing them to propagate and increase in size through natural regeneration. In addition to this, due to the close proximity between populations and the relatively high rate of gene flow among them, the conservation of all these patches is required to maintain high genetic variability. This is of great importance from a conservation point of view, because a network of small, interconnected demes could maintain more genetic variability than a single, large, random-mating population (Fischer & Matthies 1998), and hence it could be less susceptible to genetic drift although more vulnerable to demographic stochasticity (Gliddon & Goudet 1994).

Second, although it does not appear that *ex situ* conservation is required at present, it might be necessary in the future, so we wish to highlight that samples should be collected from as many populations as possible, especially the eastern population (POP3), characterized by its relatively high genetic diversity. Because of the long life-cycle of *R. cabrerensis*, the seed and germplasm collections in botanical gardens or other institutions could be of practical value for the conservation of its genetic diversity. At this point, we would also like to note that a special strategy in the short and long term should be followed for the only known population of *R. cabrerensis* subsp. *muniellensis*, as has already been suggested for this highly endangered taxon (see Chapter 4).

Finally, periodic visits to the natural locations of *R. cabrerensis s.l.* are also recommended in order to register demographic patterns. As there is no historic data on the size and dynamics of these patches of plants, we suggest that ecological data such as number of plants, pollination biology and seed dispersal should be gathered, as well as searching for new locations of this species. The information obtained in this study provides the basis of a clear framework for developing a conservation programme for the endangered species *R. cabrerensis*.

Acknowledgements

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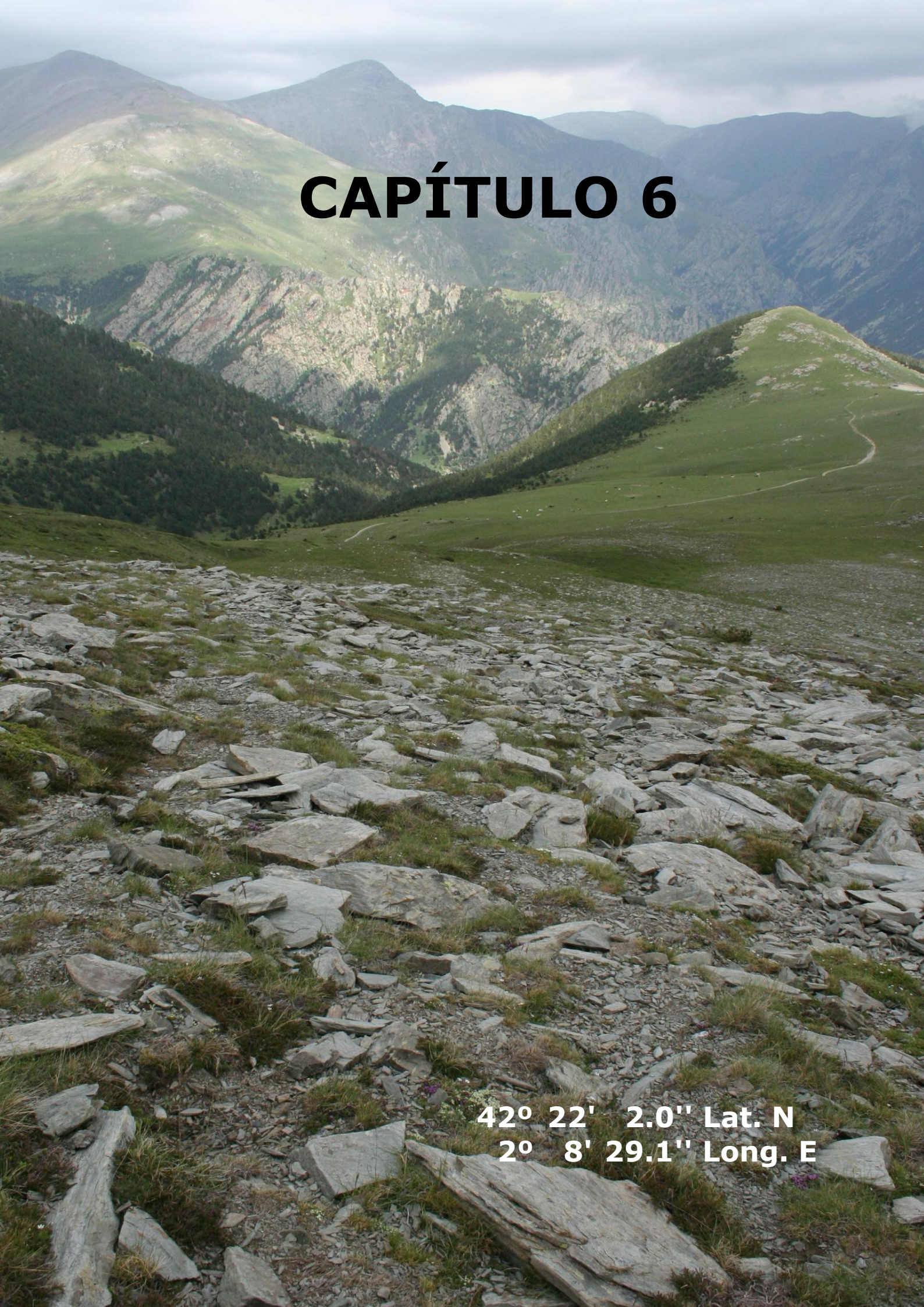

CAPÍTULO 6

42º 22' 2.0'' Lat. N
2º 8' 29.1'' Long. E

New insights into the phylogenetics and biogeography of *Ranunculus* grex *parnassiifolius* (Ranunculaceae): unravelling its evolutionary history

Summary

Ranunculus represents the largest genus within Ranunculaceae, comprising more than 600 species with a worldwide distribution. Previous studies of *Ranunculus* have been useful in identifying and assessing its evolutionary lineages. However, there are still many gaps in our knowledge on infrageneric taxonomy and evolution of *Ranunculus*. In this sense, the intraspecific variation of the polyploid complex *R.* grex *parnassiifolius* is still under discussion. Currently, the group is systematized as a single species, widespread throughout the central and southern mountains of Western Europe, and comprises five subspecies depending primarily on the ploidy level (diploid, tetraploid) and the bedrock type (calcareous, siliceous).

To reconstruct the biogeographical history of *Ranunculus* grex *parnassiifolius*, phylogenetic studies were based on nuclear Internal Transcribed Spacers (ITS) and plastid (*rpl32-trnL*, *rps16-trnQ*) sequence data, analysed using parsimony and Bayesian approaches, and the evolution of several morphological characters. Additionally, biogeographical patterns were conducted using statistical dispersal–vicariance analysis. Our results are not in agreement with the current infrageneric classification of the complex. The combination of analyses presented here supports the recognition of two evolutionary independent units: *R. cabrerensis s.l.* and *R. parnassiifolius s.l.* Furthermore gradual speciation depending on the biogeographical territory is proposed, and optimal reconstructions have likely favoured the ancestor of *Ranunculus* grex *parnassiifolius* as originating in the Iberian Peninsula. A new scenario summarizing our present understanding of the evolution within *Ranunculus* grex *parnassiifolius* is then presented.

Este capítulo se corresponde con la publicación:

- Cires, E., Cuesta, C., Vargas, P., Fernández Prieto, J.A. (2011). New insights into the phylogenetics and biogeography of *Ranunculus* grex *parnassiifolius* (Ranunculaceae): unravelling its evolutionary history (en preparación).

Introduction

The family Ranunculaceae, or buttercup family, consists of about 2500 described species within 59 genera (Tamura 1993) with a worldwide distribution. This family has been considered as one of the most basal families within the eudicots (APG III 2009). In this way, tremendous progress has been made in understanding the relationships within Ranunculaceae family, being resolved the positions of some of these monotypic genera by the employment of molecular data (e.g. Johansson & Jansen 1993; Johansson 1995; Hoot 1995; Ro *et al.* 1997, 1999; Wang *et al.* 2005, 2009; Cai *et al.* 2009, 2010; Emadzade *et al.* 2010).

The largest genus within Ranunculaceae family is *Ranunculus* L., which comprises approximately 600 species (Tamura 1993, 1995) and numerous apomictic races (Hörandl *et al.* 2005, 2008). Its distribution is almost worldwide, and the largest number of species occurs from temperate to artic/subantarctic zones (Ziman & Keener 1989), being rare in the tropics where it is restricted to high mountain areas. The *Ranunculus* chromosome number is usually x = 7 or x = 8, with the latter being much more frequent and regarded as the basic chromosome number (Goepfert 1974). Polyploidy is frequent, and variation in ploidy levels may even occur within species (e.g. Küpfer 1974; Baltisberger & Widmer 2009; Cires *et al.* 2009, 2010). Karyotypes vary considerably within the genus (Goepfert 1974), and even species that are not closely related can hybridize, at least under experimental conditions (Hüber 1988). Therefore, hybridization and polyploidy, often connected with apomixis, may play an important role in *Ranunculus* speciation (Baack 2005; Hörandl *et al.* 2005).

Molecular phylogenetic studies using plastid DNA (cpDNA) restriction sites (Johansson 1998), Internal Transcribed Spacers (ITS) sequences (Hörandl *et al.* 2005), *mat*K/*trn*K plus ITS (Paun *et al.* 2005; Gehrke & Linder 2009; Hoffmann *et al.* 2010), and *matK*/*trn*K, ITS plus *psb*J-*pet*A (Emadzade *et al.* 2010) have assumed the monophyly of *Ranunculus*, showing that the core of *Ranunculus* clade is subdivided into several well-supported clades that corresponds to widespread ecological groups (e.g. wetland and aquatic species) or to regional geographical groups (e.g. in the European mountain system: Hörandl *et al.* 2005; Paun *et al.* 2005). It is interesting to note that according to the published phylogenies of *Ranunculus* (Hörandl *et al.* 2005; Paun *et al.* 2005), the "white-flowered Europe an alpines" (clades IX and X in the former, II and III in the latter study) are situated on rather basal branches of the respective trees. Indeed, recent complete biogeographical studies of all genera of the tribe Ranunculeae DC. and the genus *Ranunculus* have been conducted (Emadzade & Hörandl 2011; Emadzade *et al.* 2011). Despite all the studies mentioned, relationships between several small and unique groups of *Ranunculus* mainly distributed, or even endemic, in the mountains of South-Central Europe (e.g. *R. küepferi*) have been controversial, and need therefore to be evaluated in a phylogenetic context (e.g. Burnier *et al.* 2009).

In such framework, the alpine buttercup *Ranunculus* grex *parnassiifolius*, a polyploid complex belonging to section *Ranuncella*, and widespread throughout the Central-Southern European mountains, should also be reassessed. Our starting hypothesis has been based on the systematics proposed by Küpfer (1974) and Bueno Sánchez *et al.* (1992) (Figure 1), with five taxa recognized within *R.* grex *parnassiifolius*, and treated at the subspecies level, although some of them have been proposed to be considered as different species (see Rothmaler 1934; Guinea López 1953).

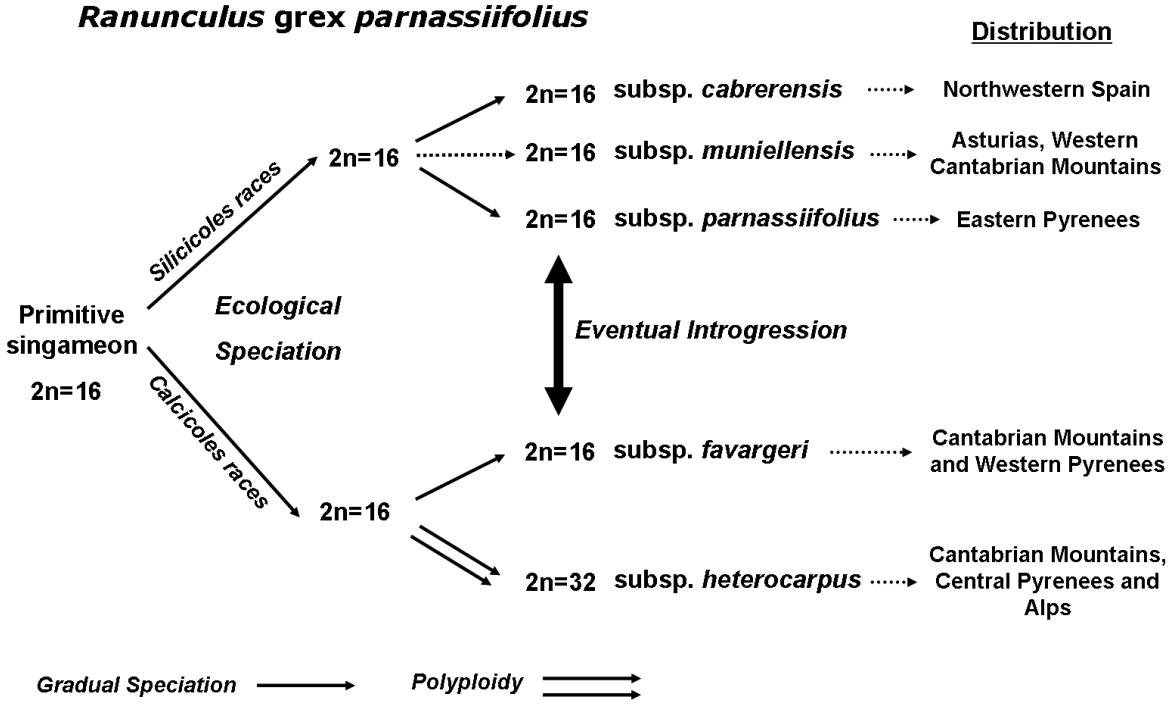

Figure 1. Systematics of *Ranunculus* grex *parnassiifolius* based on Küpfer (1974) with slight modifications (Bueno Sánchez *et al.* 1992).

However, and after taking into consideration our initial results (Cires *et al.* 2009, 2010; see Chapter 3), this classification is not entirely clear, and it has therefore become essential to gain deeper insight the evolutionary history of this group to elucidate its taxonomy. For instance, we have already presented numerous arguments to support the separation of the *R. parnassiifolius* subsp. *cabrerensis* and *R. parnassiifolius* subsp. *muniellensis* from the *R. parnassiifolius s.l.* polyploid complex, and then they should consequently be treated as an independent species (*R. cabrerensis* versus *R. parnassiifolius*), that even constitutes an evolutionary line in itself (see Chapter 3). However, none new systematics of the group based on a phylogenetic context has been proposed to date and a complete biogeographical study is still lacking.

In order to tackle these problems, in the present study we have employed the nuclear ribosomal DNA (nrDNA) ITS and the cpDNA regions for phylogenetic reconstruction, achieving a better exploit of the power of this intriguing complex.

The analysis of both cytoplasmic and nuclear markers can help to detect hybridization phenomena between differentiated populations, and to reconstruct ancestral lineage-sorting events during species formation (Comes & Abbott 2001; Hörandl *et al.* 2005).

The ITS of nrDNA are biparentally-inherited markers, and have been extensively used in the past decade in the analysis of angiosperms, because they display informative polymorphism and can be readily amplified and sequenced even from poorly preserved material (Baldwin *et al.* 1995; Álvarez & Wendel 2003). Actually, ribosomal genes are subjected to concerted evolution, potentially leading to uniformity of ITS sequences at the individual, population and species levels (Rauscher *et al.* 2004; Kovarik *et al.* 2005). Concerning to the maternal cpDNA lineages in natural populations, they often display distinct geographical distributions (Avise 2000), and non-coding regions of cpDNA have been successfully used in phylogeography studies (e.g. Puşcaş *et al.* 2008; Burnier *et al.* 2009). Indeed, and due to its maternal inheritance in angiosperms, the cpDNA transmitted by seeds has less gene flow than nuclear DNA transmitted by pollen dispersal, so the coding regions of cpDNA of higher plants are highly conserved. This fact has led to the design of universal primers that can amplify intergenic regions in most plants. The non-coding sequences, such as *rpl32-trnL* and *rps16-trnQ*, have been frequently used to survey plant intraspecific phylogeny, population genetic structure and phylogeography (Small *et al.* 1998; Saltonstall 2001; Shaw *et al.* 2005, 2007).

Therefore, in this study different data sets (plastid and nuclear DNA markers) have been combined to clarify the systematics of *Ranunculus* grex *parnassiifolius*. This will allow us to assess the validity of the current classification and to examine the evolution of several key characters. In this sense, four basic objectives are addressed: (1) to evaluate congruence between nuclear (ITS) and plastid (*rpl32-trnL*; *rps16-trnQ*) sequences in the *R. parnassiifolius* complex and identify any infraspecific polymorphisms; (2) to infer the phylogenetic relationships between the different taxa; (3) to clarify the biogeographical background and propose a scenario of dispersal/vicariance events to the divergence of *R.* grex *parnassiifolius*; and finally, on the basis of our results, (4) to suggest guidelines for a new classification.

Materials and methods

Plant material and sampling

Field work was carried out between 2006 and 2008 in a total of 20 populations (including natural hybrids), representing the whole diversity of *R.* grex *parnassiifolius* (Table 1, Figure 2).

Table 1. Plant materials, voucher information and GenBank accessions for DNA sequences used in this Chapter. Sequences directly retrieved from GenBank (ITS) are in italics. Systematics of *Ranunculus* grex *parnassiifolius* is based on Küpfer (1974), with slight modifications (Bueno Sánchez *et al.* 1992) (see Figure 1). Details of the sampling sites (i.e. locality, collectors, ploidy level, GPS coordinates, altitude) are provided in Appendix I.

		GenBank accession Nos.		
Ranunculus taxa (population code)	Locality; collector, collection No.; Herbar	ITS	*rpl32-trnL*	*trnQ-rps16*
R. acetosellifolius Boiss.	cult. Gothenburg BG; J.T. Johansson s.n.; GB	*AY680075*	–	–
R. aconitifolius L.	cult. Copenhagen BG; J.T. Johansson 274; LD	*AY680081*	–	–
R. aconitifolius L. (ACON1)	Somiedo (Asturias, Spain); Cires & Fdez. Prieto 31859; FCO	forthcoming	forthcoming	forthcoming
R. alpestris L.	cult. Rezia BG; J.T. Johansson 242; LD	*AY680078*	–	–
R. amplexicaulis L.	cult. Lund BG; J.T. Johansson 222; LD	*AY680071*	–	–
R. amplexicaulis L. (AMPLEX1)	Portillo de las Yeguas (Cantabria, Spain); Bueno & Cires 31962; FCO	forthcoming	forthcoming	forthcoming
R. amplexicaulis L. (AMPLEX2)	Somiedo (Asturias, Spain); Cires & Fdez. Prieto 31963; FCO	forthcoming	forthcoming	forthcoming
R. amplexicaulis L. (AMPLEX3)	Somiedo (Asturias, Spain); Cires & Fdez. Prieto 31964; FCO	forthcoming	forthcoming	forthcoming
R. bilobus Bertol.	Italy; E. Hörandl 4574; WU	*AY680077*	–	–
R. calandrinioides Oliver	cult. Gothenburg BG; J.T. Johansson 240; LD	*AY680073*	–	–
R. chamissonis Aucl.	U.S.S.R.; R. Koropewa s.n.; W	*AY680083*	–	–
R. crenatus Waldst. & Kit.	Austria; E. Hörandl 2818; WU	*AY680086*	–	–
R. ficaria L.	Villaviciosa (Asturias, Spain); Fdez. Prieto & Homet 31967; FCO	forthcoming	forthcoming	forthcoming
R. glacialis L.	Sweden; J.T. Johansson s.n.; -	*AY680082*	–	–
R. gramineus L.	cult. Krefeld BG; J.T. Johansson s.n.; -	*AY680076*	–	–
R. gramineus L. (GRAM1)	Somiedo (Asturias, Spain); Cires & Fdez. Prieto 31968; FCO	forthcoming	forthcoming	forthcoming
R. kuepferi subsp. *kuepferi* Greuter & Burdet	Italy; E. Hörandl 9525; WU	*AY954241*	–	–
R. kuepferi subsp. *kuepferi* Greuter & Burdet (KUEPF1)	Col de Vars (Hautes-Alpes, France); Cires & Fdez. Prieto 31970; FCO	forthcoming	forthcoming	forthcoming
R. kuepferi subsp. *orientalis* W.Huber	Italy; P. Schönswetter & A. Tribsch 2213; WU	*AY680084*	–	–
R. kuepferi subsp. *orientalis* W.Huber (ORIENT1)	Austria; E. Hörandl 4336; WU	*AY680085*	–	–
R. parnassiifolius subsp. cabrerensis Rothm. (BAÑ1)	Lago de la Baña (León, Spain); Cires *et al.* 31369; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. cabrerensis Rothm. (BAÑ2)	Lago de la Baña (León, Spain); Cires *et al.* s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. cabrerensis Rothm. (BAÑ3)	Lago de la Baña (León, Spain); Cires *et al.* s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. cabrerensis Rothm. (ABE1)	Abelgas de Luna (León, Spain); Cuesta & Cires 31368; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. cabrerensis Rothm. (ABE2)	Abelgas de Luna (León, Spain); Cuesta & Cires s.n.; -	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. cabrerensis Rothm. (YEG1)	Portillo de las Yeguas (Cantabria, Spain); Bueno & Cires 31371; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. cabrerensis Rothm. (YEG2)	Portillo de las Yeguas (Cantabria, Spain); Bueno & Cires s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (GCH1)	Grand Chavalard (Valais, Switzerland); Cires *et al.* 31366; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (GCH2)	Grand Chavalard (Valais, Switzerland); Cires *et al.* 31367; FCO	forthcoming	–	–
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (ABP1)	Albulapass (Graubünden, Switzerland); Cires *et al.* 31353; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (ABP2)	Albulapass (Graubünden, Switzerland); Cires *et al.* s.n.; -	forthcoming	forthcoming	forthcoming

Ranunculus taxa (population code)	Locality; collector, collection No.; Herbar	GenBank accession Nos. ITS	*rpl32-trnL*	*trnQ-rps16*
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (AMP1)	Cortina d'Ampezzo (Belluno, Italy); Cires *et al.* 31354; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (AMP2)	Cortina d'Ampezzo (Belluno, Italy); Cires *et al.* s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (ALB1)	Somiedo (Asturias, Spain); Cires & Fdez. Prieto 31103; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (ALB2)	Somiedo (Asturias, Spain); Cires & Fdez. Prieto 31104; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (CAD1)	Cadí-Moixeró (Barcelona, Spain); Cires & Fdez. Prieto 31358; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (CAD2)	Cadí-Moixeró (Barcelona, Spain); Cires & Fdez. Prieto 31359; FCO	forthcoming	–	–
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (CAR1)	San Carlos (Cantabria, Spain); Cires & Cuesta 31111; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (CAR2)	San Carlos (Cantabria, Spain); Cires & Cuesta s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (CHI1)	Chisagües (Huesca, Spain); Cires & Fdez. Prieto 31360; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (CHI2)	Chisagües (Huesca, Spain); Cires & Fdez. Prieto s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (EIX1)	Creu de l'Eixol (Lérida, Spain); Cires & Fdez. Prieto 31361; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (EIX2)	Creu de l'Eixol (Lérida, Spain); Cires & Fdez. Prieto 31362; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (EIX3)	Creu de l'Eixol (Lérida, Spain); Cires & Fdez. Prieto s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (MOL1)	Los Moledizos (León, Spain); Cires 31114; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (MOL2)	Los Moledizos (León, Spain); Cires s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (MOL3)	Los Moledizos (León, Spain); Cires s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (GRA1)	Las Grajas (Cantabria, Spain); Cires 31113; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *heterocarpus* P.Küpfer (GRA2)	Las Grajas (Cantabria, Spain); Cires s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. *favargeri* P.Küpfer (CAN1)	La Canalona (Cantabria, Spain); Fernández & Cires 31107; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *favargeri* P.Küpfer (CAN2)	La Canalona (Cantabria, Spain); Fernández & Cires 31108; FCO	forthcoming	–	–
R. parnassiifolius subsp. *favargeri* P.Küpfer (ARL1)	Col d'Arlas (Aquitaine, France); Cires & Fdez. Prieto 31355; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *favargeri* P.Küpfer (BOC1)	Jou de los Boches (Asturias, Spain); Fernández & Cires 31105; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *favargeri* P.Küpfer (BOC2)	Jou de los Boches (Asturias, Spain); Fernández & Cires 31106; FCO	forthcoming	–	–
R. parnassiifolius subsp. *favargeri* P.Küpfer (PAN1)	Pandébano (Asturias, Spain); Fernández & Cires 31115; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *favargeri* P.Küpfer (PAN2)	Pandébano (Asturias, Spain); Fernández & Cires 31116; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *favargeri* P.Küpfer (URR1)	Urriellu (Asturias, Spain); Fernández & Cires 31117; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto (MUN1)	Muniellos Biosphere Reserve (Asturias, Spain); Cires 31370; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto (MUN2)	Muniellos Biosphere Reserve (Asturias, Spain); Cires s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto (MUN3)	Muniellos Biosphere Reserve (Asturias, Spain); Cires s.n.; -	forthcoming	–	–
R. parnassiifolius subsp. *parnassiifolius* L.	France/Spain; G. Schneeweiss *et al.* 6509; WU	*AY680072*	–	–
R. parnassiifolius subsp. *parnassiifolius* L. (FON1)	Fontalba (Gerona, Spain); Cires & Fdez. Prieto 31363; FCO	forthcoming	forthcoming	forthcoming
R. parnassiifolius subsp. *parnassiifolius* L. (FON2)	Fontalba (Gerona, Spain); Cires & Fdez. Prieto 31364; FCO	forthcoming	forthcoming	forthcoming
R. platanifolius L.	Norway; J.T. Johansson 277; LD	*AY680080*	–	–

Ranunculus taxa (population code)	Locality; collector, collection No.; Herbar	GenBank accession Nos.		
		ITS	*rpl32-trnL*	*trnQ-rps16*
R. pyrenaeus L.	Spain; G. Schneeweiss *et al.* 6498; WU	*AY680074*	–	–
R. pyrenaeus L. (PYREN1)	Chisagües (Huesca, Spain); Cires & Fdez. Prieto 31971; FCO	forthcoming	forthcoming	forthcoming
R. pyrenaeus L. (PYREN2)	Pas de la Casa (Encamp, Andorra); Cires & Fdez. Prieto 31372; FCO	forthcoming	forthcoming	forthcoming
R. pyrenaeus L. (PYREN3)	Fontalba (Gerona, Spain); Cires & Fdez. Prieto 31972; FCO	forthcoming	forthcoming	forthcoming
R. seguieri subsp. *seguieri* Vill.	cult. Gothenburg BG; J.T. Johansson 226; LD	*AY680079*	–	–
R. ×*luizetii* Rouy (LUIZ1)	Creu de l'Eixol (Lérida, Spain); Cires & Fdez. Prieto 31975; FCO	forthcoming	forthcoming	forthcoming
R. ×*peredae* M.Laínz (PERED1)	Portillo de las Yeguas (Cantabria, Spain); Bueno & Cires 31976; FCO	forthcoming	forthcoming	forthcoming
R. ×*peredae* M.Laínz (PERED2)	Portillo de las Yeguas (Cantabria, Spain); Bueno & Cires s.n.;-	forthcoming	–	–

Subspecific systematization of *Ranunculus parnassiifolius* samples according to previous studies (see Rothmaler 1934; Küpfer 1974; Vigo i Bonada 1983; Cook *et al.* 1986; Jalas & Suominen 1989; Bueno Sánchez *et al.* 1992; Carrillo i Ortuño & Ninot i Sugranyes 1992) and herbarium material (ABH, AH, ALME, ARAN, B, BC, BCN, BHUPM, BIO, BOLO, BOZ, CCEC, CLF, COA, DR, EMMA, FCO, FR, GDA, GDAC, GJO, HEID, HJBS, HSS, JACA, JAEN, JBAG, KL, LEB, LYJB, MA, MACB, MAF, MSTR, OSN, P, PAD, PAMP, REG, RO, ROST, ROV, SALA, SANT, SEV, STR, TFC, UPNA, VAL, VIT, WFBVA, WU, Z, ZT and GBIF (2011), http://www.gbif.org/).

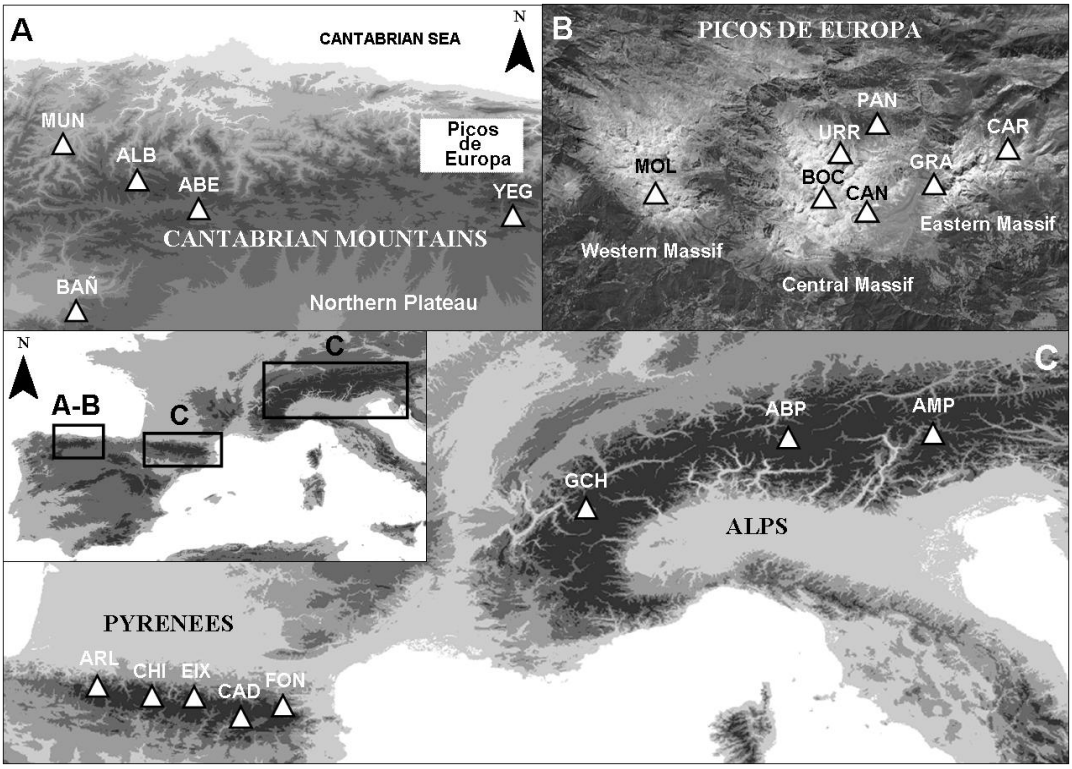

Figure 2. Map of the study area. A) Geographical distribution of *Ranunculus* grex *parnassiifolius* in the Northwestern mountains of the Iberian Peninsula. B) Sampled territories in the Picos de Europa. C) Sampled territories in the Pyrenees and the Alps. Triangles (△) refer to localities of sample collection. Populations are coded as in Table 1.

In addition, 10 other closely related populations belonging to the section *Ranuncella*: *R. amplexicaulis* L., *R. gramineus* L., *R. kuepferi* Greuter & Burdet, *R. pyrenaeus* L.; and also other species of the genus *Ranunculus*: *R. aconitifolius* L., *R. ficaria* L. (= *Ficaria verna* Huds.), were sampled. The plant material also included samples from the *locus classicus* of all subspecies of *R.* grex *parnassiifolius*, and therefore the sampling collection covered all the distribution range. A particular effort was done to include a good representation of the Cantabrian and Pyrenean populations. Despite the relatively small area covered by the Cantabrian Mountains, it may be considered to be the area with the highest global diversity of *R.* grex *parnassiifolius*, with four out of five of the recognized subspecies (Küpfer 1974; Cook *et al.* 1986; Bueno Sánchez *et al.* 1992). Additionally, it is worth noting that *R. parnassiifolius* behaves like an apomictic taxa with geographical parthenogenesis (Hörandl *et al.* 2008; Hörandl 2009; Cires *et al.* 2010). Indeed, diploids sexuals are only localized in the Cantabrian Mountains and the Pyrenees; meanwhile tetraploids are in the Alps, where uniparental apomictic reproduction is expected to result in the formation of large clones. Therefore, the sampling in the Alps was carried out with the intention of collecting those populations most distant among them, taking into account the three alpine sectors (Western, Central and Eastern Alps). Voucher information and GenBank accession numbers are provided in Table 1 (details of the sampling sites are included in Appendix I). Some accessions from GenBank of the ITS region of nrDNA from previous studies (Hörandl *et al.* 2005) were also employed.

DNA extraction, amplification and sequencing

Total genomic DNA from fresh material and silica gel dried leaf tissue were collected for molecular analysis. DNA was isolated applying an extraction method based on CTAB procedure (Doyle & Doyle 1987) with slight modifications (see Chapter 3) and using Kneasy Plant Mini Kit (Qiagen, California). The whole ITS (including ITS1, the 5.8S rDNA and ITS2) was amplified as a single piece with primers 17SE and 26SE (Sun *et al.* 1994; Hörandl *et al.* 2005). Taking into account the presence of nucleotide additivity (nucleotide ambiguities) in some ITS sequences, the analysis was reinforced in some of the sampling locations to determine whether double-peaks were due to sequencing artefacts or to different ITS copies. In the case of plastid sequences, a pilot study using 1 to 3 samples of each species from distant geographical areas was performed to find the most variable sequences among the 7 primer pairs from chloroplast genome: *ndhF* - *rpl32R* (Shaw *et al.* 2007); *rps16x1* - *trnQr* (Dumolin-Lapègue *et al.* 1997; Shaw *et al.* 2007); $trnH^{GUG}$ - *psbA* (Sang *et al.* 1997; Tate & Simpson 2003); *rpl32F* - $trnL^{UAG}$ (Shaw *et al.* 2007); $trnS^{GCU}$ - $trnG^{UUC}$ (Shaw *et al.* 2005); $trnV^{UAC}$*x2* - *ndhC* (Shaw *et al.* 2007). Finally, 2 plastid regions (*rpl32-trnL* and *trnQ-rps16*) were amplified and sequenced for all populations. Amplification of selected regions was carried out in 25 µL of reaction mixture (25 ng template DNA, 2.5 µL of 10x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.08 µM of each primer and 1 U of Taq DNA polymerase, Invitrogen), in an Eppendorf Mastercycler Epgradient S (Westbury, NY). After 5 min pre-treatment at 94 ºC, PCR conditions were: 40 cycles of 1 min at 94 ºC, 1 min at 56 ºC, 1 min at 72 ºC; plus a final extension of 10 min at 72 ºC. In some cases where we reported an unclear amplification, samples were treated with ExoSAP-IT (USB Corporation, Ohio) following the manufacturers' protocols. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator v. 2.0, Applied Biosystems, Little Chalfont, UK). Both strands were sequenced to check the reliability of detected differences. All chromatograms were visually examined to correct possible misinterpretations of the computational routine. Sequenced data were assembled and edited using ClustalW v.1.83 algorithm implemented in Geneious Pro 5.3 (Biomatters, Auckland, New Zealand). IUPAC (International Union of Pure and Applied Chemistry) symbols were used to represent nucleotide ambiguities.

Phylogenetic analyses

Nuclear and plastid data sets were analyzed separately and in combination using maximum parsimony (MP), Bayesian inference (BI) approaches and Network analysis. Maximum parsimony analyses were undertaken using PAUP* v.4.0b10 (Swofford 2002). Heuristic searches were performed using 1000 replicates, random sequence addition, tree-bisection-reconnection (TBR) branch swapping, Multrees on (saving only 10 trees for each replicate to reduce the time spent in swapping large numbers of suboptimal trees) and Steepest Descent in effect. Each nucleotide position was treated as an unordered, multistate character of equal weight (Fitch

parsimony; Fitch 1971). Coding gaps as a fifth character was preferred over treatment of gaps as missing data, mainly due to indels of our data appear highly informative, resulting overall in higher levels of non-parametric bootstrap support (Felsenstein 1985) at different levels of taxonomic relationship. Indeed, this approach has also been used to increase phylogenetic resolution in studies on the genus *Ranunculus* (see Hörandl *et al.* 2005). Besides standard tree parameters (tree length; consistency index, CI; and retention index, RI), internal node support for clades was assessed using 3 x 10^6 bootstrap replicates (fast stepwise-addition, Mort *et al.* 2000). Bootstrap values (BP) are shown on the corresponding branches of the cladograms.

Phylogenetic reconstruction was also undertaken using Bayesian inference as it was found to be relatively efficient and accurate in analysing large *Ranunculus* data sets (e.g. Hörandl *et al.* 2005). Different partitions of the data set (ITS, *rpl32-trnL* and *trnQ-rps16*) were separately tested using MrModeltest v.2.3 (Nylander 2004) to determine the sequence evolution model that best described the present data. The best fitting models of evolution found were SYM+I for ITS, HKY for *rpl32-trnL* and HKY+I for *trnQ-rps16* using the Akaike Information Criterion (AIC). Bayesian Inference analysis was conducted using MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003), where each model substitution fitted to each molecular partition. Four Markov chains were run simultaneously for 5 x 10^6 generations, and they were sampled every 1000 generations. After the chains had reached stationarity, as judged from plots of likelihood and from split variances being <0.01, data from the first 25% of generations were discarded as the "burn-in". A 50% majority-rule consensus tree was constructed and posterior probabilities (PP) of nodes were calculated from the remaining sample.

Finally, two different networks, in order to display conflicts in data, were conducted. Phylogenetic network methods (Huson *et al.* 2010; Huson & Scornavacca 2010) provide a means of evaluating the extent to which data exhibits a hierachical structure. First, we used the NeighborNet analysis implemented in SplitsTree v.4.11 (Huson & Bryant 2006) to construct an unrooted network, applying uncorrected distances (also known as p-distances or Hamming distances). Bootstrap support for internal splits was calculated with 1000 replicates. NeighborNet uses an algorithm that determines a circular ordering of taxa (i.e. based on the extent of differences between their sequences, the taxa are ordered around a circle). The layout on the circle determines what splits occur in the data, and can be displayed in a planar graph. The support for each of these splits is then measured using a least-squares method, that adjusts the lengths of the splits in the splits graph to minimize the difference with the pairwise distances in the original data matrix (Bryant & Moulton 2004; Huson & Bryant 2006). Non tree-like splits graphs indicate contradictory support for relationships. Fit values ranging from 0 to 100% indicate how well the graph represents the information contained in the data. Second, we used the software TCS v.1.21 (Clement *et al.* 2000) which implements a statistical parsimony approach using the algorithm described in Templeton *et al.* (1992) to construct

haplotype networks. The maximum number of differences among haplotypes was calculated with 95% confidence limits, and treating gaps as a fifth character state.

Statistical tests of incongruence

Homogeneity between the plastid and nuclear data sets was tested under parsimony using partition homogeneity tests and implemented in PAUP* v.4.0b10 (Swofford 2002). This test is equivalent to the incongruence length difference test of Farris *et al.* (1994), and for convenience, it is referred as the ILD test. This test employed 100 replicates, each with 10 random additions using TBR branching swapping. The resulting P value was used to determine whether two data sets had significant incongruence (0.05). In the present study, congruence between data sets was also assessed by comparison of topology and support values of strict consensus trees of data partitions, as unreliability of ILD has been described (Dolphin *et al.* 2000; Barker & Lutzoni 2002).

Character evolution

Character tracing was performed on traits generally used in taxonomic studies of *Ranunculus* grex *parnassiifolius*. On the basis of the topology of the 50% majority-rule parsimony analysis, the following categorical characters were mapped using Mesquite v.2.7 (Maddison & Maddison 2009) with accelerated transformation optimization (ACCTRAN) and unordered parsimony: ploidy level (diploid, tetraploid), bedrock type (i.e. geological substrates in which plants grow: calcareous, siliceous, mixed), base shape of the basal leaves (ovate-subcordate, broadly cordate, lanceolate), and surface of the achenes (achenes strongly veined, achenes smooth or with inconspicuous veins). Characters were obtained from the latest studies performed on the species (Küpfer 1974; Cook *et al.* 1986; Bueno Sánchez *et al.* 1992; Tutin & Akeroyd 1993; Hörandl *et al.* 2005; Cires *et al.* 2009, 2010). Chromosome numbers were taken from the Index to Plant Chromosome Numbers database (http://mobot.mobot.org/W3T/Search/ipcn.html).

Biogeographical analyses

Dispersal-vicariance analysis (Ronquist 1997) is one of the most widely used methods of inferring biogeographical histories. To infer vicariance and dispersal events, a bayesian-based method (MCMC = 1 x 10^6; nchains = 10; temp. = 0.1) implemented in RASP (Reconstruct Ancestral State in Phylogenies, Yu *et al.* 2011) was employed. Bayesian method in RASP (the version 2.0 of S-DIVA: Statistical Dispersal-Vicariance Analysis; Yu *et al.* 2010) extends Olsson *et al.* (2006) and Sanmartín *et al.* (2008) approaches to a more generalized method for statistical analysis of biogeography, based on phylogenies and distributional data (Yu *et al.* 2011). Additionally, the nearest-neighbor statistic (S_{nn}) was calculated to assess genetic differentiation in *R.* grex *parnassiifolius* due to isolation by distance, according to nuclear and plastid sequences. Specimens were assigned to geographical groups based on their distribution areas (Northwestern of the Iberian

Peninsula, the Pyrenees and the Alps). This statistic is a measure of how often the nearest neighbors of sequences are from the same locality in geographical space (Hudson 2000). S_{nn} is expected to approach 1 when two partitions (localities) of a data set form highly differentiated populations, and 0.5 when they are part of a single panmictic population. Moreover, population genetic differentiation was assessed by mean of different statistical test based on DNA sequences: K_{ST}, K_S and Z calculated according to Hudson *et al.* (1992a), where $K_{ST} = 1 - (K_S/K_T)$; K_S = average number of differences between sequences within subpopulations; K_T = average number of differences between sequences regardless of locality; Z = weighted sum of Z_1 and Z_2, where Z_i is the average of the ranks of all the $d_{ij,lk}$ values for pairs of sequences from within locality *i*. Finally, coefficient of gene differentiation F_{ST} was also estimate for all populations and loci (Hudson *et al.* 1992b). F_{ST} measures the amount of interpopulation diversity and takes values between 0 and 1. Permutation tests with 1000 replicates were performed in DnaSP v5 (Librado & Rozas 2009) to evaluate significance of the obtained values. Gaps were treated as fifth character and nucleotide ambiguities as nucleotide indetermination.

Results

Characteristics of ITS, rpl32-trnL *and* trnQ-rps16 *sequences*

The characteristics of ITS, *rpl32-trnL* and *trnQ-rps16* sequences are summarised in Table 2.

Table 2. Summary of phylogenetic results obtained from the analyses of ITS and *rpl32-trnL* and *trnQ-rps16* sequences of the section *Ranuncella* (excluding *R. kuepferi*, see Hörandl *et al.* 2005) and the core *Ranunculus* grex *parnassiifolius* sensu Küpfer (1974) with slight modifications (Bueno Sánchez *et al.* 1992), once hybrids sequences were excluded.

	ITS region	*rpl32-trnL*	*trnQ-rps16*
Section *Ranuncella*			
Numbers of sequences	57	35	35
Length range (bp)	599*-612	911-920	1002-1029
Aligned length (bp)	612	939	1043
Constant characters	580	867	1014
Polymorphic sites	32	112	29
Number of indels (%)	56 (0.2%)	904 (2.8%)	1230 (3.4%)
Number of nucleotide additivities	56	–	–
Mean G + C content (%)	54.9%	28.3%	28.4%
Ranunculus grex *parnassiifolius*			
Numbers of sequences	43	26	26
Length range (bp)	599*-611	911-919	1002-1029
Aligned length (bp)	611	924	1033
Constant characters	606	909	1015
Polymorphic sites	5	15	18
Number of indels	–	273 (1.1%)	621 (2.3%)
Number of nucleotide additivities	44	–	–
Mean G + C content (%)	54.9%	28.3%	28.4%

* Because of sequencing errors in the ITS region (see Figure 3).

Visual inspection of ITS chromatograms of the core *Ranunculus* grex *parnassiifolius* (sensu Küpfer 1974) revealed clear nucleotide additivities (positions containing double nucleotide peaks) in 56 positions.

These additivities have been found in hybrids and (some) polyploids from the Cantabrian Mountains and the Pyrenees. Taking into account the chromatograms, was possible to determine that the double-peaks were due to different ITS copies and not sequencing artefacts.

In the course of the analysis, it was noted that the ITS sequences employed in the study about phylogenetic relationships in the genus *Ranunculus s.l.* by Hörandl *et al.* (2005) did not include the entire ITS region *per se* (Figure 3).

→ **Start ITS region**

```
FONTALBA      TTGTCGAAAC CTGCAAAGCA GACGACCCGC GAACACGTTA AAAAGTACCT AAGCGGATTG  [60]
AY680072         ??????? ????..... ..........  .......... .......... ..........  [60]

FONTALBA      GCGATGGGTG CGAGCTCTGA TCCGTCCGCT TGTCGGGTCG TGGAGTTGGT TGCATCCCTT  [120]
AY680072      .......... .......... .......... .......... .......... ..........  [120]

FONTALBA      GTGGTTGTGG TCGCTTTGCG TTCCTGCACA ACATCAAAAT CCGGCGCGAT TGGCGTCAAG  [180]
AY680072      .......... .......... .......... .......... .......... ..........  [180]

FONTALBA      GAAATCTTAG CGGAAACAAA GCGTTGTGCC TTCACTGGTA CATCGCCAAG AATCCCAATA  [240]
AY680072      .......... .......... .......... .......... .......... ..........  [240]

FONTALBA      CTCAAACGAC TCTCGGCAAC GGATATCTCG GCTCTTGCAT CGATGAAGAA CGTAGCGAAA  [300]
AY680072      .......... .......... .......... .......... .......... ..........  [300]

FONTALBA      TGCGATACTT GGTGTGAATT GCAGAATCCC GTGAACCATC GAGTTTTTGA ACGCAAGTTG  [360]
AY680072      .......... .......... .......... .......... .......... ..........  [360]

FONTALBA      CGCCCGAGAC CTTTAGGTTG AGGGCACGTC TGCCTGGGCG TCACACACAG CGTCGCTCCC  [420]
AY680072      .......... .......... .......... .......... .......... ..........  [420]

FONTALBA      CACCAACCTA GTTGGCGGAG AGCGGAGATT GGCCCCCCGA GTCCTCTGGG CACGGTCGGC  [480]
AY680072      .......... .......... .......... .......... .......... ..........  [480]

FONTALBA      ACAAATATTG GTCCCCGGCA GCGAGTGTCG CGGTCAGCGG TGGTTGTATC TCTTCCTCCA  [540]
AY680072      .......... .......... .......... .......... .......... ..........  [540]

FONTALBA      AAGACAAAAT GACGCGTACC TCGTTGCATG TTGGATCGAA ACGACCCTCG AAGGCCGTTC  [600]
AY680072      .......... .......... .......... .......... .......... ..........  [600]
```

→ **Final ITS region - start 26S rRNA**

```
FONTALBA      GCGGCATTCA CCCTGCG [617]
AY680072      .......... ...?    [617]
```

Figure 3. Multiple alignment and sequencing errors in the ITS region in samples of *Ranunculus parnassiifolius* subsp. *parnassiifolius* from GenBank. As an example, we use the sequences from Fontalba and the GenBank accession *AY680072* (Hörandl *et al.* 2005). The start motif of the ITS region (TTGT) is consistent with the most angiosperms (e.g. Yokota *et al.* 1989), while the start motif of the nuclear 26S ribosomal DNA is based on Ro *et al.* (1997) and the rice sequences (Sugiura *et al.* 1985; GenBank *M11585*). (?) = missing; (.) = identical. For more information on the populations see Appendix I.

In fact, approximately 12 bp (11 bp from ITS1 and 1 bp from ITS2) were not been considered for the study, and neither in further analysis conducted by other researchers (Paun *et al.* 2005; Hoffmann *et al.* 2010; Emadzade *et al.* 2010, 2011; Emadzade & Hörandl 2011).

However, it may have important implications for the study of phylogenetic and biogeographical relationships in the family Ranunculaceae, and more specifically in the genus *Ranunculus*. Therefore, we would like to highlight the importance of not missing parts of the sequences when large phylogenetic studies are performed.

Phylogenetic analyses

Total sequence length for the ITS, *rpl32-trnL* and *trnQ-rps16* regions in *Ranunculus* samples, were 598-612, 911-948 and 968-1029 bp, respectively. We used 2716 aligned nucleotide positions in total: 614 bp in the ITS data set (which included the 5.8S rDNA) and 2102 bp in the chloroplast data set.

The MP analysis of the ITS data set resulted in 4916 most parsimonious trees with a length of 151 steps (CI = 0.79; RI = 0.88). The topology provided by the strict consensus tree displayed two large well-supported clades (Figure 4, clades I–II). However, the relationships of some clades (indicate with asterisks, see Figure 4), were not completely congruent between parsimony analysis and Bayesian inference. Clade I corresponded to *Ranunculus* grex *parnassiifolius*, *R. amplexicaulis* and the hybrid *R.* ×*peredae*. Clade II comprised different species: *R. aconitifolius*, *R. seguieri* and *R. platanifolius* (*R.* sect. *Aconitifolii*); *R. chamissonis* and *R. glacialis* (*R.* subg. *Crymodes*) and finally *R. kuepferi* (*R.* sect. *Ranuncella*, according to Tamura 1995; or *R.* sect. *Aconitifolii*, according to Hörandl *et al.* 2005).

The subclade Ia (74% BS, 76 PP) comprised two subspecies: *R. parnassiifolius* subsp. *cabrerensis* and *R. parnassiifolius* subsp. *muniellensis* (*R. cabrerensis s.l.*; see Chapter 3) plus a sample of *R.* ×*peredae*, and it was found sister to two distinct subclades (Ib and Ic). Subclade Ib (61% BS, 80 PP) included samples of *R. parnassiifolius s.l.* from the Cantabrian Mountains (including all diploids and the only tetraploid without nucleotide additivity: ALB). Subclade Ic (87% BS, 76 PP) comprised samples of *R. amplexicaulis*.

The rest of clade I contained a large polytomy with different samples of *R. parnassiifolius s.l.* (*R. parnassiifolius* sensu Küpfer 1974, excl. *R. parnassiifolius* subsp. *cabrerensis*), corresponding to tetraploids from the Cantabrian Mountains (all of them showed nucleotide additivity), diploids and tetraploids from the Pyrenees, and tretraploids from the Alps.

The MP analysis of combined plastid data (*rpl32-trnL* and *trnQ-rps16*) resulted in 3 most parsimonious trees with a length of 953 steps (CI = 0.96; RI = 0.94). Bayesian inference (BI) revealed the same tree topology, and posterior probability (PP) supported those clades found in the maximum parsimony analysis. The strict consensus tree contained two major clades (I and II) with 100% BS (100 PP) and 96% BS (100 PP) for each clade respectively (Figure 5).

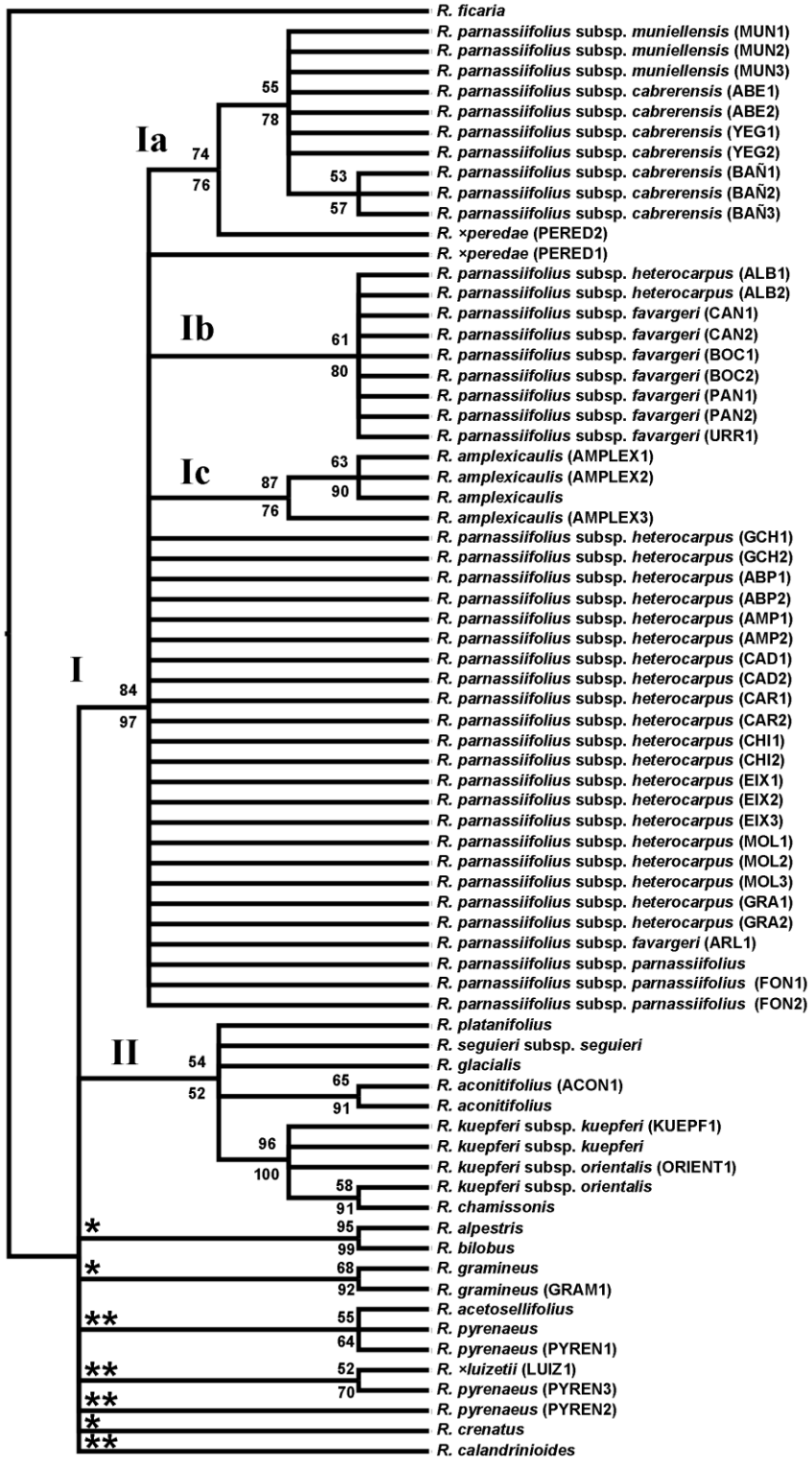

Figure 4. Strict consensus of 4916 most parsimonious trees based on ITS sequences. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities (PP) from the Bayesian analysis. Roman numerals indicate the major clades/subclades. One asterisk indicates branches that were united in one clade in Bayesian analysis. Two asterisks indicate branches belonging to clade I in Bayesian inference analysis (see Appendix II, phylogram obtained from Bayesian analysis, for more information).

The first clade (I) of Figure 5 comprised *R. parnassiifolius* subsp. *cabrerensis* and *R. parnassiifolius* subsp. *muniellensis*. Within clade II, two major subclades were distinguished: subclade IIa, consisted on the eastern populations of *R.* grex *parnassiifolius* from the Pyrenees, together with samples of *R. pyrenaeus* from the same territories (99% BS, 100 PP); and subclade IIb (100% BS, 100 PP), that comprises the rest of taxa of *R.* grex *parnassiifolius* (Pyrenees and Alps).

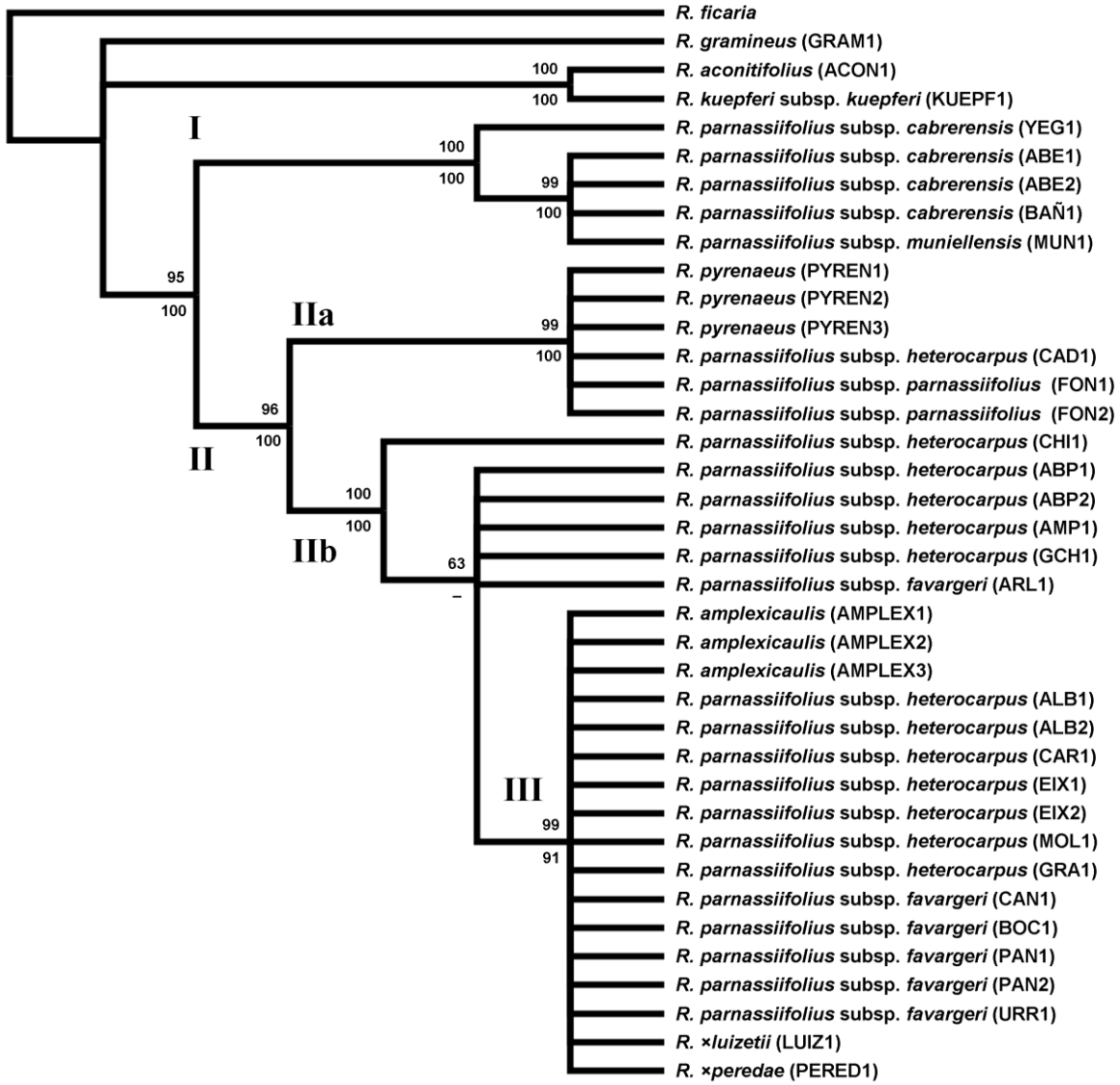

Figure 5. Strict consensus of 3 most parsimonious trees based on *rpl32-trnL* and *trnQ-rps16* sequences. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities (PP) from the Bayesian analysis. Bayesian inference (BI) resolution incongruent with MP clades/subclades as indicated with a hyphen (–) below branches. Roman numerals indicate the major clades/subclades.

Additionally, it is worth noting an additional subclade (subclade III, 100% BS, 100 PP) formed by samples of *R. amplexicaulis* (Cantabrian populations), *R. parnassiifolius s.l.* (diploids and tetraploids from the Cantabrian Mountains together with a hybrid locality from the Pyrenees, EIX) and the natural hybrids (*R. ×luizetii*, *R. ×peredae*).

According to the partition homogeneity test, the nuclear and plastid data sets were significantly incongruent (P = 0.01). However, several studies have shown that significant ILD p-values may not indicate genealogical conflicts but may reflect other issues such as different evolutionary rates between the data partitions (e.g. Hipp *et al.* 2004). In fact, the comparison of the single-gene topologies on a node-by-node basis may give a better assessment of congruence than the sometimes unreliable partition homogeneity test (Dolphin *et al.* 2000; Yoder *et al.* 2001). Examination of the MP strict consensus, bootstrap, and BI trees for the individual data sets showed to be fundamentally congruent, except for some internal clades and polytomies. Therefore, we have adopted the same approach of Paun *et al.* (2005) to increase phylogenetic resolution in the studies of the genus *Ranunculus*, regarding combining data partitions from ITS and plastid sequences.

The NeighborNet analysis (Figure 6A) based on ITS and plastid concatenated sequences, where indels were considered as informative characters, confirmed the basic dichotomy of six major clades found in the parsimony and Bayesian analysis.

In fact, four of the six clusters identified in NeighborNet, supported splits which correspond to different species (i.e. *R. amplexicaulis*, *R. gramineus*, *R. kuepferi*, *R. pyrenaeus*). According to *R.* grex *parnassiifolius*, one of the cluster in the NeighborNet splits graph comprised *R. parnassiifolius* subsp. *cabrerensis* and *R. parnassiifolius* subsp. *muniellensis*, meanwhile a large non-specific cluster comprised the rest of the populations of *R.* grex *parnassiifolius* (populations without a convex hull).

To solve this non-specific cluster, we performed a statistical parsimony approach. It is notable that TCS analysis is only useful for studying data sets where the sequences are not significantly divergent from each other. In this sense, and in order to avoid a possible influence of hybridization, the natural hybrids populations were excluded from the network analysis. Indeed, the identification in the field of those individuals that are hybrids is relatively easy, because they display intermediate characteristics, i.e. between their putative progenitors. TCS constructed a single network with six haplotypes, three haplotype clades and no loops (Figure 6B).

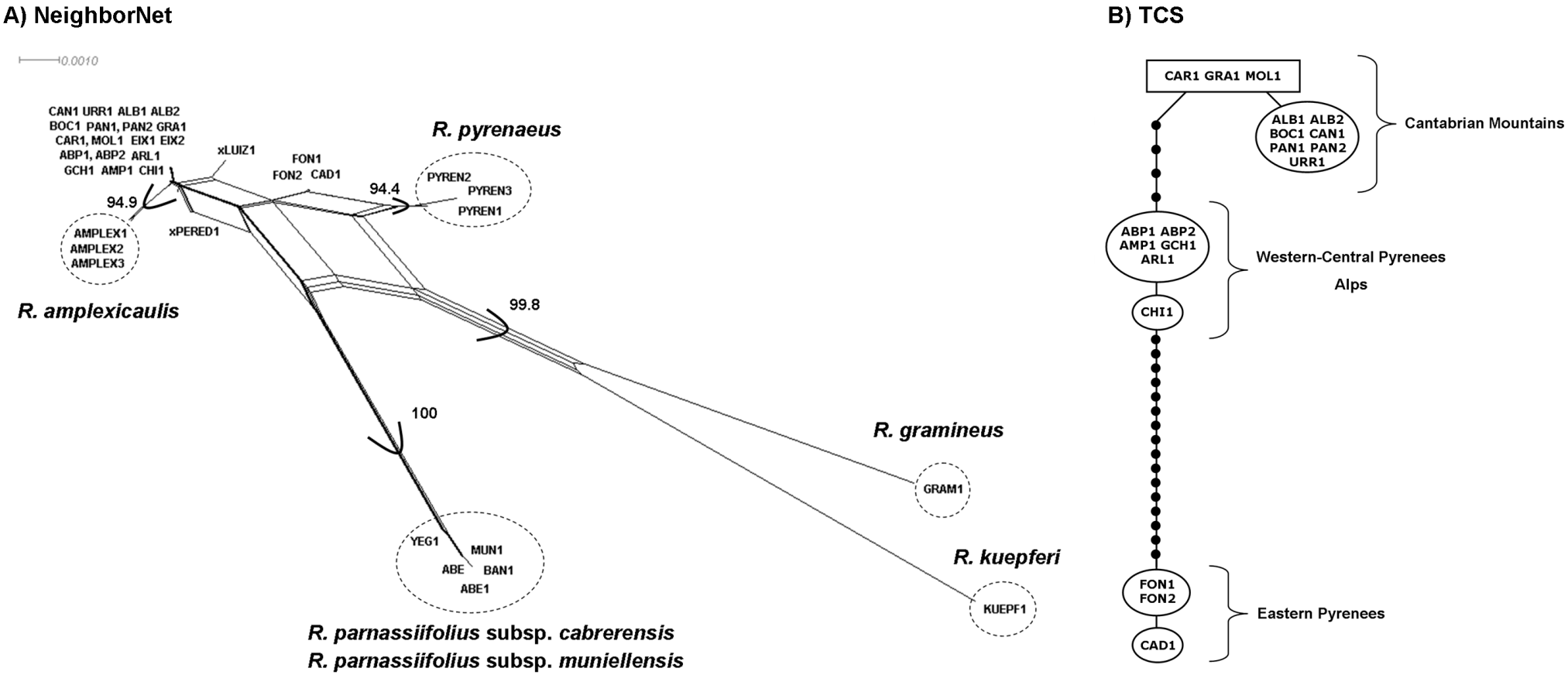

Figure 6. Phylogenetic networks analysis based on combined ITS, *rpl32-trnL* and *trnQ-rps16* sequences of *Ranunculus* grex *parnassiifolius* and other closely related species. A) NeighborNet splits graph. Numbers along branches are bootstrap values from neighbor-joining analysis (1000 replicates). The least squares fit index for the split network has a value of 98.13%. B) Statistical parsimony network. Lines (–) indicate a single nucleotide substitution, and black dots (●) represent haplotypes extinct or not detected.

Character evolution

The reconstruction of ancestral states for the four studied characters is shown in Figure 7A-D.

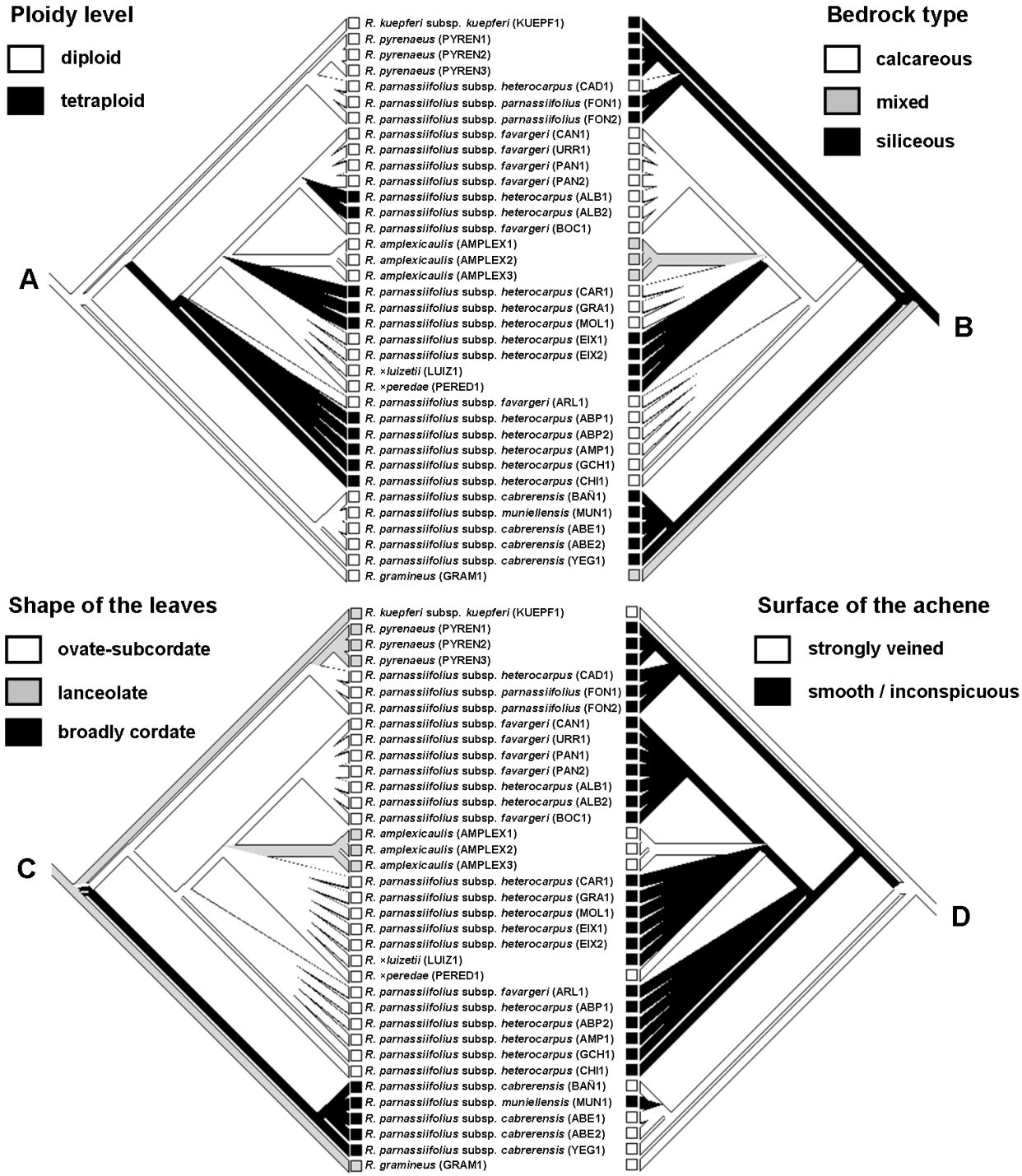

Figure 7. Evolution of categorical characters on the maximum parsimony topology. A) ploidy level (2x, 4x). B) bedrock type (calcareous, siliceous, mixed). C) base shape of the basal leaves (ovate-subcordate, broadly cordate, lanceolate). D) surface of the achenes (achenes strongly veined, achenes smooth or with inconspicuous veins). Colours are explained in the legend of each figure.

The traits that appeared to be most constrained from the phylogenetic reconstruction were the base shape of the basal leaves and the surface of the achenes (Figure 7C-D). The remaining characters (ploidy level, Figure 7A; bedrock type, Figure 7B) showed a pattern of multiple independent events and were much less informative at infraspecific level. It is noteworthy that tetraploidy, a major character in the systematics of Küpfer (1974) evolved several times independently in *R.* grex *parnassiifolius*.

Biogeographical analyses

To test if geographical isolates are genetically differentiated populations, four statistical tests of population differentiation were applied (Hudson *et al.* 1992a; Hudson 2000). The null hypothesis (no genetic differentiation) was rejected for the majority of comparisons, under the sequence-based statistics (K_{ST}, K_S and Z). The last statistic was S_{nn}, referred to as the nearest-neighbor statistic, a measure of how often the nearest neighbors of sequences are found in the same population (Hudson 2000). In our case, only the comparison between the Pyrenees and the Alps showed a value close to 0.5 (S_{nn} statistic = 0.584). Additionally, the coefficient of gene differentiation F_{ST} was used to estimate the extent of genetic differentiation between geographical isolates. The overall values of F_{ST} for the three areas showed relatively low genetic differentiation (except in the dataset B when comparing more distant populations, NIP versus ALP). F_{ST} values calculated for each pair of geographical group are presented in Table 3.

Table 3. Summary of test statistics and parameter estimates examined for population differentiation between geographical isolates in *Ranunculus* grex *parnassiifolius*.

Dataset	Pairwise comparisons	K_S	K_{ST}	*P*	*Z*	*P*	S_{nn}†	*P*	F_{ST}
A	NIP vs PYR	28.926	0.092	0.048*	108.334	0.036*	0.813	0.001**	0.208
	NIP vs ALP	28.721	0.098	0.043*	76.493	0.025*	0.789	0.020*	0.329
	PYR vs ALP	8.242	0.154	0.094 ns	25.176	0.155 ns	0.584	0.223 ns	0.313
	Three areas	24.476	0.140	0.030*	144.197	0.015*	0.691	0.000***	0.271
B	NIP vs PYR	5.607	0.337	0.000***	51.137	0.000***	0.758	0.012*	0.453
	NIP vs ALP	0.333	0.380	0.060 ns	37.486	0.060 ns	0.714	0.017*	0.666
	PYR vs ALP	8.242	0.154	0.087 ns	25.176	0.145 ns	0.584	0.243 ns	0.313
	Three areas	4.539	0.420	0.000***	67.778	0.000***	0.617	0.001**	0.510

Dataset A: considering all described subspecies (see Figure 1). Dataset B: excluding *R. parnassiifolius* subsp. *cabrerensis* and *R. parnassiifolius* subsp. *muniellensis* sequences. NIP: Northwestern of the Iberian Peninsula; PYR: the Pyrenees; ALP: the Alps. † S_{nn} test statistics were performed with excluded gaps (Hudson 2000). ns, not significant; *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

The optimal reconstruction in dispersal-vicariance scenarios (Figure 8A) revealed that the ancestor of *R.* grex *parnassiifolius* (primitive singameon) was originated at the Iberian Peninsula (node 1; A: 96.04%, AB: 3.55%, B: 0.26%, AC: 0.14%, C: 0.01%). Then, after a gradual process of speciation, two evolutionary units were supported: *R. cabrerensis s.l.* and *R. parnassiifolius s.l.* The most favoured reconstructions for *R. parnassiifolius s.l.* indicated two dispersals to explain the present distribution (node 3: +C; node 4: +A), and favoured the ancestor of this taxa as being originated in the Pyrenees (node 2; B: 78.65%, AB: 15.08%,

A: 6.04%, BC: 0.14%, C: 0.06%, ABC: 0.03%, AC: 0.01%). Taking into account all the results obtained in this work and previous studies (Cires *et al.* 2009, 2010; see Chapter 3), a new evolutionary scenario is therefore proposed (Figure 8B).

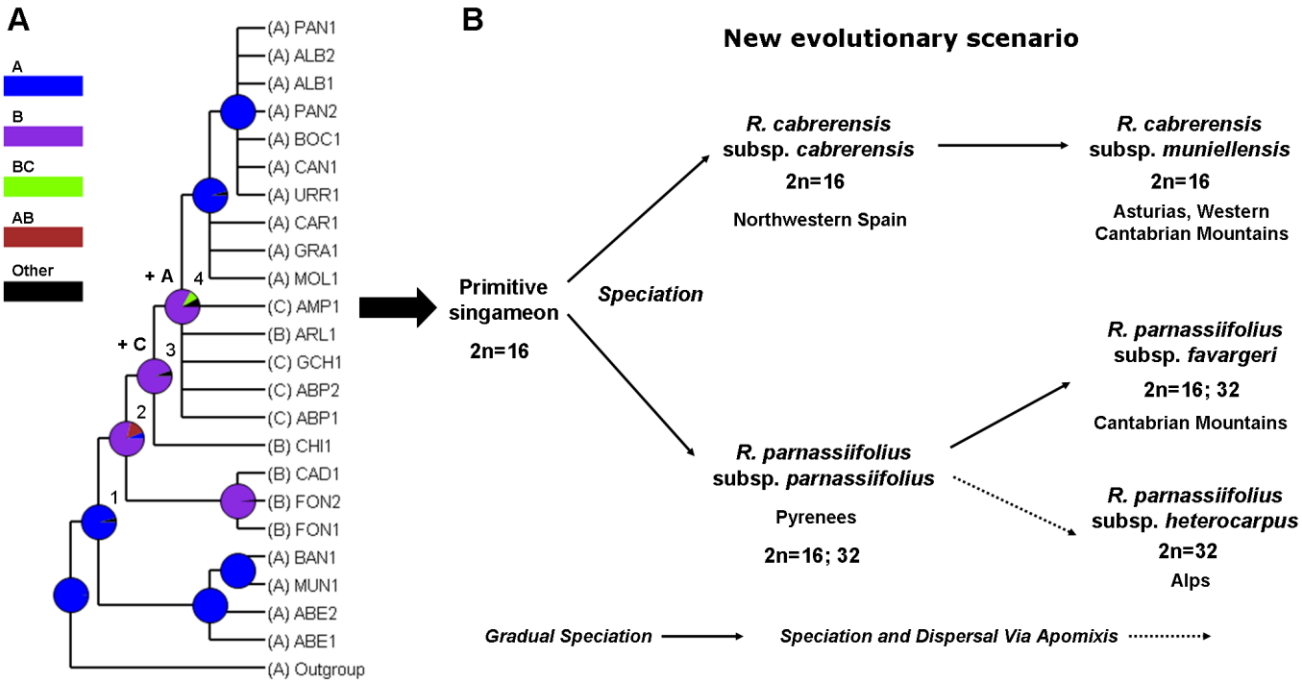

Figure 8. Biogeographical events in *Ranunculus* grex *parnassiifolius*. A) Dispersal–vicariance scenarios reconstructed by RASP (Reconstruct Ancestral State in Phylogenies) with the maximum number of area units set to three. Pie charts at internal nodes represent the marginal probabilities for each alternative ancestral area. Arrow (+): dispersal event. Letters denote area units (A: Northwestern of the Iberian Peninsula; B: the Pyrenees; C: the Alps; Outgroup: *R. ficaria*) and the term "other" (black color) denotes the union of other remaining areas with lowest probability. Populations are coded as in Table 1. B) New evolutionary scenario proposed.

Discussion

The history of *Ranunculus parnassiifolius* dates back to Linnaeus (1753), who described the species. Indications of the protologue are very scarce because only indicates "habitat in Europa australi", and the only literature cited that appears is "Institutione rei herbariae" of Tournefort (1700), where there is no chorological information. According to Küpfer (1974, pp. 181-184, PL VIb), and considering the regularity of the pollen from Linnaean Herbarium (Lectotype: Herb. Linn. No. 715.8; LINN), the type corresponds to a diploid of the Pyrenees (specifically from the Eastern Pyrenees), and not from an Alpine tetraploid. However, with the aforementioned characteristics (relatively robust plant, regular and large enough corolla, and a diploid plant), it cannot be excluded that the locality type comes from the centre or west of the Pyrenees, where diploid populations were found with the above requirements (Cires *et al.* 2010).

To date, the most accepted classification in *R.* grex *parnassiifolius* is the proposal made by Küpfer (1974) with slight modifications (Bueno Sánchez *et al.* 1992) (see Figure 1), which recognizes five subspecies referred as: *R. parnassiifolius* subsp. *parnassiifolius* (endemic in the Eastern Pyrenees), *R. parnassiifolius* subsp. *cabrerensis* (endemic in the Northwestern mountains of Spain), *R. parnassiifolius* subsp. *muniellensis* (endemic in the Western Cantabrian Mountains, Muniellos Biosphere Reserve), *R. parnassiifolius* subsp. *favargeri* (endemic in the Cantabrian Mountains and the Western Pyrenees) and finally, *R. parnassiifolius* subsp. *heterocarpus* (spread throughout the Cantabrian Mountains, the Central Pyrenees and the Alps). The presence in Sierra Nevada (Pico Alcazaba) was reported by Quézel (1953) but without any material support and no recent confirmation (Blanca *et al.* 2002). The first three subspecies live on slope deposits of gravel and siliceous pebbles, whereas the latter two are considered to be from calcareous environments. In addition to the type of substrate, several characters have been reported for the differentiation within subspecies, such as the regularity of the corolla and the presence of aborted carpels (Küpfer 1974). However, in light of the results obtained in this work (ITS, *rpl32-trnL*, *trnQ-rps16* sequences), and also in previous studies based on cytotype distribution, morphological character and plastid DNA sequences (Cires *et al.* 2009, 2010; see Chapter 3), indicate that the current classification is controversial.

For instance, in many cases the only criteria to identify the infraspecific taxa are based on the type of substrate combined with the geographical origin. However, as it has already been demonstrated in the record of Cadí-Moixeró (CAD), this population has been several times attributed to the tetraploid subspecies *R. parnassiifolius* subsp. *heterocarpus* (e.g. Vigo i Bonada 1983) because of being placed in a limestone substrate, but cytometric data (Cires *et al.* 2010) indicates that this is a diploid population. Similarly, the population of Creu de l'Eixol (EIX) has also been treated as *R. parnassiifolius* subsp. *heterocarpus* (e.g. Carrillo i Ortuño & Ninot i Sugranyes 1992), whereas we identified it as a diploid population located in a siliceous area (Cires *et al.* 2010). On other occasions, one of the main criteria for the differentiation within subspecies is the regularity of the corolla. An irregular corolla is related to *R. parnassiifolius* subsp. *heterocarpus*, but this character seems to depend mainly on the sampling period (personal observation) and then should not be considered anymore. Even Küpfer (1974, p. 187) mentions that some swiss populations (including the locality type of *R. parnassiifolius* subsp. *heterocarpus*: Grand Chavalard) are characterized by regular corollas. In addition to this, no morphological character or combination of characters is able to distinguish the plants as belonging to different ploidy levels (or subspecies), neither in the Cantabrian Mountains nor in the Pyrenees (see Cires *et al.* 2009, 2010). Moreover, the altitudinal patterns proposed by Küpfer (1974) for the Picos de Europa (Cantabrian Mountains), in which the highest altitudes were restricted to diploid *R. parnassiifolius* subsp. *favargeri*, were not confirmed (Cires *et al.* 2009). One more example that expresses controversy in the current classification appears in the Küpfer's work (1974, p. 187), who mentions that populations of *R. parnassiifolius* subsp. *favargeri*

from the Western Pyrenees have a bigger size of flower and leaf compared to the populations from Canalona (Picos de Europa and type locality for this subspecies). Additionally, this comment actually induces us to doubt again about the origin of the type locality for *R. parnassiifolius*, as it was mentioned at the beginning of the discussion section. Therefore, there have been no clear criteria to identify the infraspecific taxa within *R.* grex *parnassiifolius* until now.

Our phylogenetic analysis based on nuclear (ITS) and plastid (*rpl32-trnL*; *rps16-trnQ*) sequences, provides the first available phylogenetic framework for relationships within *R.* grex *parnassiifolius*, and offers a new perspective in the current classification. The first notable feature is the separation of *R. cabrerensis s.l.* (including the infraspecific taxon *R. cabrerensis* subsp. *muniellensis*) from the *R. parnassiifolius s.l.* polyploid complex, as it was mentioned in the Chapter 3, based on morphological characters and cpDNA regions. Besides all the differences, we would mention that the species *R. cabrerensis* grows in rock-fields and coarse rocky scree (rock-slides), meanwhile *R. parnassiifolius s.l*. grows in fine-grained scree (shingle-slide). Indeed, the trees generated by individual and combined maximum parsimony, Bayesian inference and networks analysis of nuclear and plastid sequences for *R. parnassiifolius s.l*. (excluding *R. cabrerensis s.l.*) agree with recent morphological and cytometric studies (Cires *et al.* 2009, 2010), where geographically-close members of *R.* grex *parnassiifolius* are grouped together.

Ranunculus-wide phylogenetic analysis reported by Paun *et al.* (2005), Hoffmann *et al.* (2010) and Emadzade & Hörandl (2011), propose a recent origin for the section *Ranuncella* and *Ranunculus* grex *parnassiifolius*. Ages estimates suggested a remarkably young diversification of this clade (3.4-1.02 Mya) in the late Pliocene/early Pleistocene. According to Paun *et al.* (2005), this clearly means pre-glacial speciation events, which is consistent with the idea of polyploidy, a trait often correlated with degree of glaciation (Stebbins 1984) and predominant in endemics of formerly glaciated areas. RASP provides strong support for an Iberian origin in *R.* grex *parnassiifolius*, and the most favoured reconstructions indicate two dispersal events from the Pyrenees to the Alps and the Cantabrian Mountains (Figure 8A). Our data are actually consistent with the hypothesis mentioned by Paun *et al.* (2005), where the only taxon reaching the Alps, *R. parnassiifolius* subsp. *heterocarpus*, is a tetraploid apomict and therefore most likely a derivative of the diploid sexual subspecies of *R. parnassiifolius*, endemic to the Iberian Peninsula. An Iberian origin with subsequent eastward migration has also been inferred for *Anthyllis montana* (Kropf *et al.* 2002), *Pritzelago alpina* (Kropf *et al.* 2003) or *Androsace vitaliana* (Dixon *et al.* 2009). Furthermore, the low variation of ITS sequences detected in the present study supports the hypothesis of a recent origin. Factors influencing the colonization success of plant groups include, among others, seed dispersal, seed germination rate, habitat preference, plant-growth conditions, breeding system and biotic interactions (Wang & Smith 2002). Although the dispersal ability of achenes has been considered limited in some *Ranunculus* species (Scherff *et al.* 1994), recent evidences suggest the contrary (Emadzade & Hörandl

2011). For instance, molecular phylogenetic studies suggest the colonization of Australia and New Zealand by *Ranunculus* species against prevailing winds (Lockhart *et al.* 2001; Winkworth *et al.* 2005). The genus *Ranunculus* otherwise shows many examples of rapid and radiative speciation, e.g. in the Mediterranean, on oceanic islands, and in several high-mountain systems, including a striking radiation in the New Zealand Alps (Lockhart *et al.* 2001; Hörandl *et al.* 2005; Paun *et al.* 2005). In all these radiations, geographical isolation, together with efficient dispersal barriers, has played a major role.

Both plastid and nuclear markers indicate that the tetraploid cytotype, a major character in Küpfer's systematics (1974), is likely to have emerged more than once. Polyploid complexes are often the result of recurrent, independent genome duplication events that frequently lead to spatial coexistence of parental lineages with derived polyploids (Soltis, D.E. & Soltis, P.S. 1999; Soltis, P.S. & Soltis, D.E. 2000). However, in *R.* grex *parnassiifolius*, coexistence of different cytotypes within a population is very rare, which suggests that cytotypes evolved in single events. Furthermore, the putative advantages of polyploids over diploids could include lower rates of population extinction and increased diversification rates in the long term (Soltis *et al.* 2009). Although infrequent, polyploidization events in *R.* grex *parnassiifolius* seem to have had a variety of causes. We hypothesize that this extensive polyploid series has resulted from both autopolyploidy and allopolyploidy (or introgressive hybridization). In our case, ITS data and morphological similarity suggest that the tetraploids were originated by autopolyploidy from diploid plants (with which they share the monoploid DNA complement; Cires *et al.* 2009, 2010). However, the presence of nucleotide additivity in some ITS sequences of the polyploids suggests that hybridization is related to the increment of chromosome complements, and then an allopolyploid origin cannot be ruled out. The same scenario, in which autopolyploidy and allopolyploidy have played prominent roles, have also been described in other Mediterranean polyploid groups (e.g. Balao *et al.* 2010).

At a finer level of taxonomic resolution, this study supports present-day delimitation of some species: *R. amplexicaulis*, *R. gramineus*, *R. kuepferi*, *R. pyrenaeus* and two natural hybrids (*R.* ×*luizetii*, *R.* ×*peredae*). Additionally, our plastid sequence analysis provides a well-supported clade (Figure 5, subclade IIa) between *R. pyrenaeus* and the eastern populations of *R. parnassiifolius s.l.* from the Pyrenees (CAD, FON). One possible explanation for the sharing of plastid haplotypes might be the extensive interspecific introgression within areas of sympatry. Many systematic studies of closely related plant taxa have revealed incongruence between phylogenies inferred from nuclear and chloroplast regions (e.g. Frajman & Oxelman 2007). In angiosperms, with maternally inherited chloroplasts, introgression is generally more frequent in cpDNA markers than in nuclear DNA (Rieseberg & Soltis 1991). Actually, incongruence between nuclear and chloroplast data, as well as the sharing of plastid haplotypes between species, are usually interpreted as a result of interspecific hybridization, which leads to the replacement of the plastid genome of

one species by the one from another species, while the nuclear genome remains more or less unchanged. The close relationship between *R. parnassiifolius s.l.* and *R. pyrenaeus* in the Eastern Pyrenees had already been suggested by Küpfer (1974, p. 189). During glaciation (range contractions), diploid species from the Pyrenees could have been isolated at the eastern of the Pyrenees (Favarger & Küpfer 1968), which would have acted as a refuge for *R. parnassiifolius* and other alpine species (Küpfer 1974; Favarger 1975). Indeed, the Iberian Peninsula is considered as one of the most important glacial refugia in Europe for endemic alpine flora, and acted as geographical settings for speciation and glacial survival during the Quaternary (Comes & Kadereit 2003; Vargas 2003; Kropf *et al.* 2008).

Besides the results presented above, this study generates new questions unresolved, which are beyond the scope of this work. One of them is the origin of *R. amplexicaulis*, because if we consider the plastid sequences studied, a putative interspecific hybridization occurred between *R. amplexicaulis* and *R. parnassiifolius s.l.* from the Cantabrian Mountains (Figure 5, subclade III). In addition to this, from a standpoint of biological conservation, the natural hybrid populations contain a genetic diversity that does not exist in *R. grex parnassiifolius*. This fact can be of great importance since *R. grex parnassiifolius* appears in red lists of protection. Finally, there is a considerable taxonomic confusion regarding an isolated population of *Ranunculus wettsteinii* Dörfler in Korab (Sar Planina, Albania-Macedonia). Meanwhile is often considered as a separate species, it is possible that this plant, only known from the type collection, should perhaps be treated as a subspecies of *R. parnassiifolius* (Tutin & Akeroyd 1993).

Concluding remarks

Because our phylogenetic reconstruction strongly contradicts the current systematics of *Ranunculus* grex *parnassiifolius*, the need of a new classification is evident. The results are consistent with previous studies based on cytotype distribution, morphological characters and plastid DNA sequences (Cires *et al.* 2009, 2010; see Chapter 3). We therefore consider that these morphological and DNA sequence differences are sufficient to warrant the recognition of *R. cabrerensis* at species rank, and also give clear evidences that geography has been the major factor in the rest of *R.* grex *parnassiifolius*. Therefore, taking into account all the results obtained in the study of *R.* grex *parnassiifolius*, a new evolutionary scenario is proposed (Figure 8B):

- There are numerous arguments to support the separation of *R. parnassiifolius* subsp. *cabrerensis* (Rothmaler 1934, p. 148) from *R.* grex *parnassiifolius*, and then it should be consequently treated as an independent species: *R. cabrerensis*.

- In the same way, the plants described as *R. parnassiifolius* subsp. *muniellensis* (Bueno Sánchez *et al.* 1992, p. 365) should be systematized as a

geographical race of *R. cabrerensis*: *R. cabrerensis* subsp. *muniellensis* (see Chapter 3).

- The original circumscription of *R. parnassiifolius* subsp. *parnassiifolius* (sensu Küpfer 1974, p. 190) includes diploids from the Eastern Pyrenees. Here we restrict the use of this name to diploids and tetraploids of the Pyrenees (without geographical restriction).

- The original circumscription of *R. parnassiifolius* subsp. *heterocarpus* (Küpfer 1974, p. 192) includes tetraploids from the Cantabrian Mountains, the Pyrenees and the Alps. Here we restrict the use of this name to apomict tetraploids of the Alps.

- The original circumscription of *R. parnassiifolius* subsp. *favargeri* (Küpfer 1974, pp. 191-192) includes diploids from the Cantabrian Mountains and the Western Pyrenees. Here we restrict the use of this name to diploids and tetraploids of the Cantabrian Mountains. Guinea López (1953, pp. 381-383) proposes two combinations for *R. parnassiifolius* from the Picos de Europa*: R. aloisii-ceballi* and *R. parnassiifolius* subsp. *aloisii-ceballi*. However, according to the rules of International Code of Botanical Nomenclature (article 34.2, Vienna Code 2006; http://ibot.sav.sk/icbn/main.htm), alternative names are illegitimate if they are not published before first day of January 1953, which is the case that here happens.

According to the foregoing, the new systematic proposal for *Ranunculus* grex *parnassiifolius* is as follows:

- ***Ranunculus parnassiifolius*** L., *Sp. Pl.*: 549 (1753)

Ind. loc.: "Habitat in Europa australi"

Lectotypus: "Herb. Linn. No. 715.8 (LINN)" [Designated by: Küpfer in *Boissiera* 23: 181 (1974)]

- ***Ranunculus parnassiifolius*** L. subsp. ***parnassiifolius***

  Distribution: Pyrenees

  Habitat: calcareous and siliceous screes of high mountains

  Ploidy level: diploid and tetraploid (2n = 16, 32)

- ***Ranunculus parnassiifolius*** L. subsp. ***favargeri*** P.Küpfer, *Boissiera* 23: 191 (1974)

  Holotypus: "E., Sa., Picos de Europa, Collado de la Canalona, 2450 m, NEU K02201"

  Distribution: Cantabrian Mountains

  Habitat: calcareous screes of high mountains

  Ploidy level: diploid and tetraploid (2n = 16, 32)

- ***Ranunculus parnassiifolius*** L. subsp. ***heterocarpus*** P.Küpfer, *Boissiera* 23: 192 (1974)

  Holotypus: "CH., Valais, Grand Chavalard, versant ouest, 2100 m, NEU K02208"

  Distribution: Alps

Habitat: calcareous screes of high mountains

Ploidy level: tetraploid (2n = 32)

- ***Ranunculus cabrerensis*** Rothm., *Bol. Soc. Esp. Hist. Nat.* 34: 148 (1934)

= *Ranunculus parnassiifolius* L. subsp. *cabrerensis* Rothm., *Bol. Soc. Esp. Hist. Nat.* 34: 148 (1934)

Ind. loc.: "Hab.: in glareosis regionis nivei montium Sierra Cabrera, prope Lago de la Baña, part. Ponferrada, prov. León"

- ***Ranunculus cabrerensis*** Rothm. subsp. ***cabrerensis***

  Distribution: Mountains of Leon and Cantabrian Mountains

  Habitat: siliceous screes of high mountains

  Ploidy level: diploid (2n = 16)

- ***Ranunculus cabrerensis*** Rothm. subsp. ***muniellensis*** (Bueno, Fern.Casado & Fern.Prieto) Fern.Prieto & Cires (see Chapter 3).

  ≡ *Ranunculus parnassiifolius* Rothm. subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto, *Bot. J. Linn. Soc.* 109(3): 365 (1992) (Basionym)

  Holotypus: "España, Asturias, Ibias, Peñavelosa, 29TPH86, 1450 m, 16.v.1990, A. Bueno & J. A. Fernández Prieto (FCO 18250)"

  Distribution: Muniellos Biosphere Reserve (Western Cantabrian Mountains)

  Habitat: siliceous screes of high mountains

  Ploidy level: diploid (2n = 16)

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Appendices

Appendix I: Details of the sampling sites and voucher information

R. aconitifolius L. (2n = 16), from the Alto de la Farrapona to Lago Cerveriz, opposite Collado Balbarán 1649 m (Somiedo, Asturias, Spain) 43º 3' 31.5'' N / 6º 5' 40.0'' W; E. Cires & J.A. Fernández Prieto 31859 FCO. ***R. amplexicaulis*** L. (2n = 16), Portillo de las Yeguas, San Glorio 2072 m (Vega de Liébana, Cantabria, Spain) 43º 3' 17.6'' N / 4º 44' 20.4'' W; A. Bueno & E. Cires 31962 FCO; near Lago Calabazosa 1687 m (Somiedo, Asturias, Spain) 43º 2' 54.9'' N / 6º 6' 29.4'' W; E. Cires & J.A. Fernández Prieto 31963, 31964 FCO. ***R. ficaria*** L. (2n = 16, 24, 32), edge Ría de Villaviciosa 5 m (Villaviciosa, Asturias, Spain) 43º 29' 58.4'' N / 5º 25' 32.7'' W; E. Cires, J.A. Fernández Prieto & J. Homet 31967 FCO. ***R. gramineus*** L. (2n = 16), Camino Real de Castro 750 m (Somiedo, Asturias, Spain) 43º 6' 24.0'' N / 6º 15' 24.0'' W; E. Cires & J.A. Fernández Prieto 31968 FCO. ***R. kuepferi* subsp. *kuepferi*** Greuter & Burdet (2n = 16), Col de Vars 2118 m (Hautes-Alpes, France) 44º 32' 29.6'' N / 6º 42' 13.7'' E; E. Cires & J.A. Fernández Prieto 31970 FCO. ***R. parnassiifolius* subsp. *cabrerensis*** Rothm. (sensu Rothmaler 1934; Küpfer 1974) (2n = 16), Lago de la Baña, Sierra Cabrera 2000 m (Encinedo, León, Spain) 42º 14' 33.8'' N / 6º 44' 40.0'' W (*loc. class.*); B. Jiménez-Alfaro, E. Cires & L. González 31369 FCO; Abelgas de Luna, near the stream Valverde, north of Peña Piquera 1956 m (Sena de Luna, León, Spain) 42º 53' 32.7'' N / 6º 4' 59.0'' W; C. Cuesta & E. Cires 31368 FCO; Portillo de las Yeguas, San Glorio 2094 m (Vega de Liébana, Cantabria, Spain) 43º 3' 17.6'' N / 4º 44' 20.4'' W; A. Bueno & E. Cires 31371 FCO. ***R. parnassiifolius* subsp. *heterocarpus*** P.Küpfer (sensu Küpfer 1974) (2n = 32, 40), Grand Chavalard, west face 2071 m (Valais, Switzerland) 46º 10' 10.7'' N / 7º 6' 15.6'' E (*loc. class.*); C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto 31366, 31367 FCO; Albulapass, Blais Cuorta 2507 m (Graubünden, Switzerland) 46º 35' 22.1'' N / 9º 50' 28.7'' E; C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto 31353 FCO; Massif of Los Picos Albos, above Lago Cerveriz, Lagos de Saliencia 1946 m (Somiedo, Asturias, Spain) 43º 2' 51.8'' N / 6º 7' 23.4'' W; E. Cires & J.A. Fernández Prieto 31103, 31104 FCO; behind the Refuge Auronzo, Tre Cime di Lavaredo 2353 m (Véneto, Belluno, Auronzo di Cadore, Italy) 46º 36' 53.0'' N / 12º 17' 48.2'' E; C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto 31354 FCO; Top of Puigllançada, Parc Natural Cadí-Moixeró 2165 m (Bagà, Barcelona, Spain) 42º 18' 19.2'' N / 1º 55' 36.8'' E; E. Cires & J.A. Fernández Prieto 31358, 31359 FCO; Eastern Massif of the Picos de Europa, Collado de San Carlos 2050 m (Camaleño; Cantabria, Spain) 43º 12' 22.9'' N / 4º 41' 48.8'' W; E. Cires & J.A. Fernández Prieto 31111, 31112, 31374, 31375 FCO; end of the track that begins in Chisagües, south of Sierra de Liena 2346 m (Bielsa, Huesca, Spain) 42º 41' 17.3'' N / 0º 9' 26.4'' E; E. Cires & J.A. Fernández Prieto 31360 FCO; Coll de la Creu de l'Eixol 2246 m (Espot, Lérida, Spain) 42º 32' 24.9'' N / 1º 5' 5.5'' E; E. Cires & J.A. Fernández Prieto 31361, 31362 FCO; Western Massif of the Picos de Europa, Los Moledizos 2043 m (Posada de Valdeón, León, Spain) 43º 10' 35.3' 'N / 4º 57' 47.9'' W; E. Cires 31114 FCO; Eastern Massif of the Picos de Europa, Canal de las Grajas 1688 m (Camaleño, Cantabria, Spain) 43º 10' 46.8'' N / 4º 45' 19.6'' W; A. Fernández & E. Cires 31113 FCO. ***R. parnassiifolius* subsp. *favargeri*** P. Küpfer (sensu Küpfer 1974) (2n = 16), Central Massif of the Picos de Europa, Collado de la Canalona 2455 m (Camaleño, Cantabria, Spain) 43º 10' 46.0'' N / 4º 48' 53.7'' W (*loc. class.*); A. Fernández & E. Cires 31107, 31108, 31109, 31110 FCO; Col d'Arlas 1971 m (Aquitaine, France) 42º 58' 10.9'' N / 0º 45' 4.5'' W; E. Cires & J.A. Fernández Prieto 31355, 31356, 31357 FCO; Central Massif of the Picos de Europa, Jou de los Boches 2136 m (Cabrales, Asturias, Spain) 43º 11' 9.8'' N / 4º 49' 40.0 '' W; A. Fernández & E. Cires 31105, 31106 FCO; Central Massif of the Picos de Europa, pathway from Pandébano to Vega de Urriellu, opposite Jou Lluengu 1632 m (Cabrales, Asturias, Spain) 43º 12' 49.0'' N / 4º 48' 51.9'' W; A. Fernández & E. Cires 31115, 31116 FCO; Central Massif of the Picos de Europa, Vega de Urriellu once left behind the refuge J.D. Ubeda in the direction of Horcados Rojos 1967 m (Cabrales, Asturias, Spain) 43º 12' 12.1'' N / 4º 49' 15.1'' W; A. Fernández & E. Cires 31117, 31118, 31119 FCO. ***R. parnassiifolius* subsp. *muniellensis*** Bueno, Fern. Casado & Fern. Prieto (sensu Bueno Sánchez *et al.* 1992) (2n = 16), Peña Velosa, Reserva de la Biosfera de Muniellos 1442 m (Cangas del Narcea, Asturias, Spain) 43º 1' 9.0'' N / 6º 44' 45.4'' W (*loc. class.*); E. Cires 31370 FCO.

R. parnassiifolius* subsp. *parnassiifolius L. (sensu Küpfer 1974) (2n = 16), Collada de Fontalba 2223 m (Queralbs, Gerona, Spain) 42º 22' 2.0'' N / 2º 8' 29.1'' E (*loc. class.*); E. Cires & J.A. Fernández Prieto 31363, 31365, 31364 FCO. ***R. pyrenaeus*** L. (2n = 16), end of the track that begins in Chisagües, south of Sierra de Liena 2175 m (Bielsa, Huesca, Spain) 42º 41' 10.2'' N / 0º 8' 53.9'' E; E. Cires & J.A. Fernández Prieto 31971 FCO; Pas de la Casa 2579 m (Encamp, Andorra) 42º 33' 16.6'' N / 1º 43' 7.2'' E; E. Cires & J.A. Fernández Prieto 31372 FCO; Collada de Fontalba 2166 m (Queralbs, Gerona, Spain) 31T 0429439 - 4690737; E. Cires & J.A. Fernández Prieto 31972 FCO. ***R. ×luizetii*** Rouy (*R. parnassiifolius* L. x *R. pyrenaeus* L.) (2n = 16), Coll de la Creu de l'Eixol 2207 m (Espot, Lérida, Spain) 43º 10' 35.4'' N / 4º 49' 24.1'' W; E. Cires & J.A. Fernández Prieto 31975 FCO. ***R. ×peredae*** M.Laínz (*R. parnassiifolius* L. x *R. amplexicaulis* L.) (2n = 16), Portillo de las Yeguas, San Glorio 2095 m (Vega de Liébana, Cantabria, Spain) 43º 3' 15.3'' N / 4º 44' 21.2'' W; A. Bueno & E. Cires 31976 FCO.

References Appendix I

Bueno Sánchez, A., Fernández Casado, M.A., Fernández Prieto, J.A. (1992). A new subspecies of *Ranunculus parnassiifolius* L. (Ranunculaceae) from the Cantabrian Mountains, Spain. *Botanical Journal of the Linnean Society* 109: 359-367.

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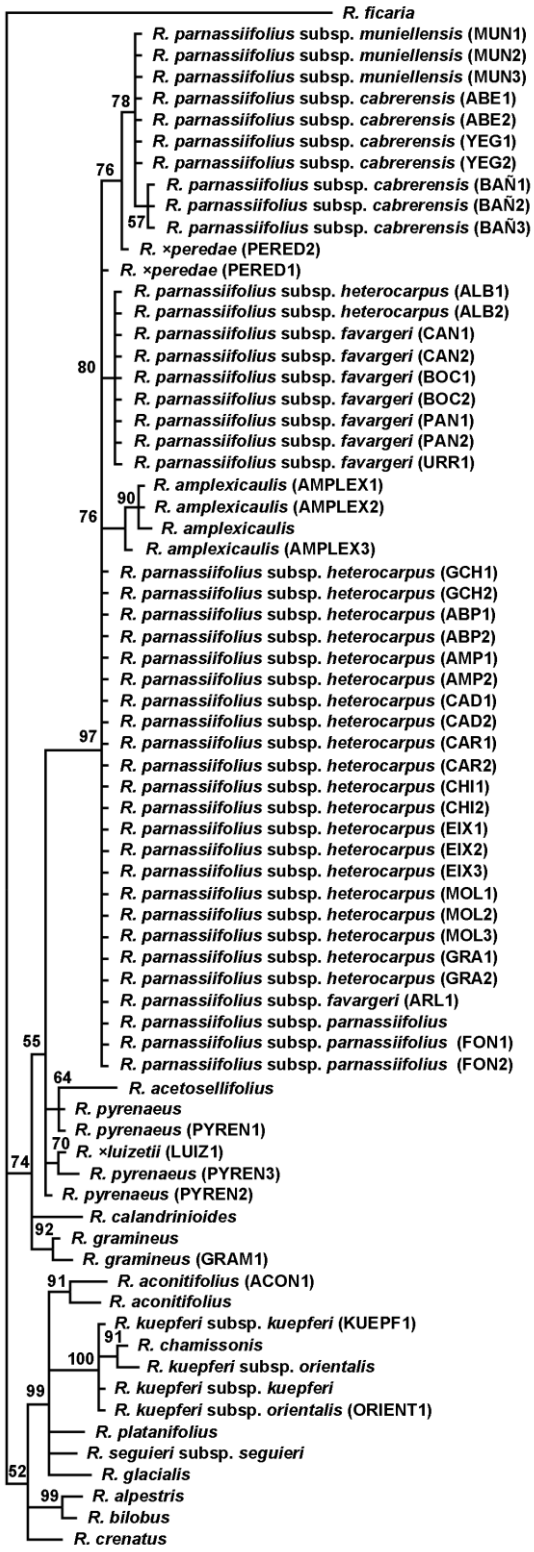
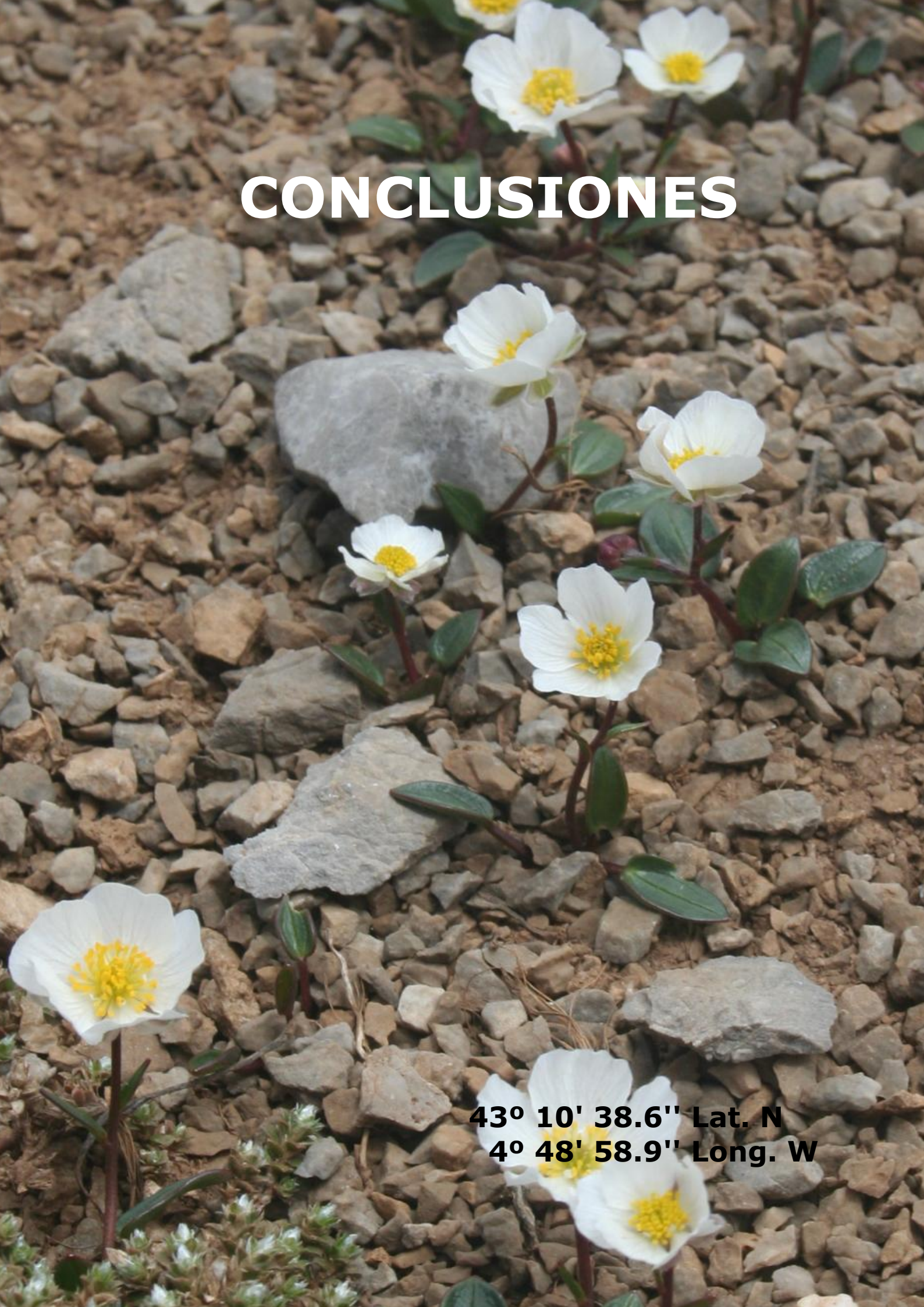
Rothmaler, W. (1934). Species novae vel nomina nova florae hispanicae. *Boletín de la Sociedad Española de Historia Natural* 34(2-3): 147-155.

Appendix II: Phylogram obtained from Bayesian inference analysis

Phylogram obtained from Bayesian inference analysis based on ITS sequences. Numbers above branches are posterior probabilities. Population codes are as shown in Table 1.



CONCLUSIONES



43º 10' 38.6'' Lat. N
4º 48' 58.9'' Long. W

Conclusiones generales y perspectivas futuras

De forma complementaria a las conclusiones parciales detalladas en cada uno de los seis capítulos expuestos, se resumen a continuación las principales aportaciones de esta tesis doctoral, en referencia a los objetivos definidos al comienzo del estudio.

1) La citometría de flujo ha resultado ser una técnica rápida y eficaz para medir la cantidad de ADN e inferir el nivel de ploidía en plantas de *Ranunculus* grex *parnassiifolius*. De entre los distintos protocolos ensayados, el uso de material fresco junto con el tampón de aislamiento nuclear LB01 ofrecieron los resultados más satisfactorios.

2) Un nuevo valor para el estándar interno de eritrocitos de pollo (2C = 3.14 ± 0.155 pg) fue establecido durante el análisis de plantas de *Ranunculus* grex *parnassiifolius* por citometría de flujo. Independientemente del origen del estándar interno empleado (animal o vegetal), se proponen recomendaciones para su uso y validación.

3) A lo largo de toda el área de distribución de *Ranunculus* grex *parnassiifolius*, fueron encontrados cuatro niveles de ploidía (citotipos): diploide, triploide, tetraploide y pentaploide. Cabe destacar la existencia de variación intraespecífica en el tamaño del genoma en las poblaciones diploides de los Pirineos, presumiblemente debido a fenómenos de hibridación introgresiva.

4) Existen numerosos argumentos (caracteres morfológicos y ecológicos, cantidad de ADN, marcadores moleculares nucleares y plastidiales) que avalan la independencia de *Ranunculus cabrerensis* del complejo poliploide *R. parnassiifolius*. De la misma manera, las plantas descritas como la subespecie *R. parnassiifolius* subsp. *muniellensis*, con una única población natural conocida, deben ser sistematizadas como una raza geográfica de *Ranunculus cabrerensis*.

5) Se proponen estrategias específicas de conservación basadas en el análisis de la diversidad genética dentro y entre las poblaciones de *Ranunculus cabrerensis* subsp. *cabrerensis* y *Ranunculus cabrerensis* subsp. *muniellensis*.

6) No se encontraron diferencias morfológicas que nos permitan identificar las distintas subespecies descritas bajo la sistemática aceptada hasta la fecha. Por el contrario, hemos sido capaces de distinguir al menos tres morfotipos dentro de *Ranunculus parnassiifolius s.l.*, que corresponden a los territorios biogeográficos estudiados (cantábrico, pirenaico y alpino).

7) Se propone una nueva clasificación y un nuevo escenario evolutivo para *Ranunculus* grex *parnassiifolius* que refleja las relaciones filogenéticas

existentes. Bajo esta circunscripción, *Ranunculus cabrerensis s.l.* es considerada como una especie independiente del complejo poliploide *R. parnassiifolius s.l.* Restringimos el uso de: *R. parnassiifolius* subsp. *parnassiifolius* a los diploides y tetraploides de los Pirineos; *R. parnassiifolius* subsp. *heterocarpus* a los tetraploides apomícticos de los Alpes, y finalmente, *R. parnassiifolius* subsp. *favargeri* a los diploides y tetraploides de la Cordillera Cantábrica.

Finalmente, procederemos a presentar algunas de las líneas de investigación más relevantes que se derivan de los resultados obtenidos. Las ventajas y características de la metodología empleada en la presente Tesis, hace posible su aplicación al resto de las especies de la sección *Ranuncella* del género *Ranunculus*. Esta sección constituye un grupo taxonómico monofilético, formado en general por especies diploides, endémicas y con comportamientos ecológicos especializados, cuyo origen y diversificación se ha centrado en la Península Ibérica y el norte de África. Asimismo, en el extremo oriental del área de distribución de *Ranunculus parnassiifolius s.l.* (Albania-Macedonia: Macizo del Korab) crece una población aislada de *Ranunculus wettsteinii* Dörfler, una raza tetraploide (2n = 32) cuya sistematización precisa de revisión.

General conclusions and future perspectives

In addition to the specific conclusions within each chapter, a summary of the main contributions of the present PhD Thesis is presented, organized according to the main objectives already defined at the beginning of the study.

1) Flow cytometry has been found to be a fast and efficient technique for measuring the nuclear DNA content, as well as for inferring the ploidy level, of *Ranunculus* grex *parnassiifolius* plants. Within all the protocols assayed, the best results were given by using fresh material combined with the nuclei buffer LB01.

2) A new value for the internal standard of chicken red blood cells (2C = 3.14 ± 0.155 pg) was established for the flow cytometry analysis of *Ranunculus* grex *parnassiifolius* plants. Independent of the type of the internal standard applied (animal or plant origin), some recommendations on their use and validation are proposed.

3) Throughout all the distribution area of *Ranunculus* grex *parnassiifolius*, four ploidy levels (cytotypes) were reported: diploid, triploid, tetraploid and pentaploid. It is worth noting the existence of intraspecific variation in the genome size in the diploid populations from the Pyrenees, probably due to introgressive hybridization phenomena.

4) Numerous arguments (morphological and ecological characters, DNA content, nuclear and plastid molecular markers) support the independence of *Ranunculus cabrerensis* from the polyploid complex *R. parnassiifolius*. Similarly, the subspecies *R. parnassiifolius* subsp. *muniellensis*, with only one natural population known, should be systematized as a geographical race of *Ranunculus cabrerensis*.

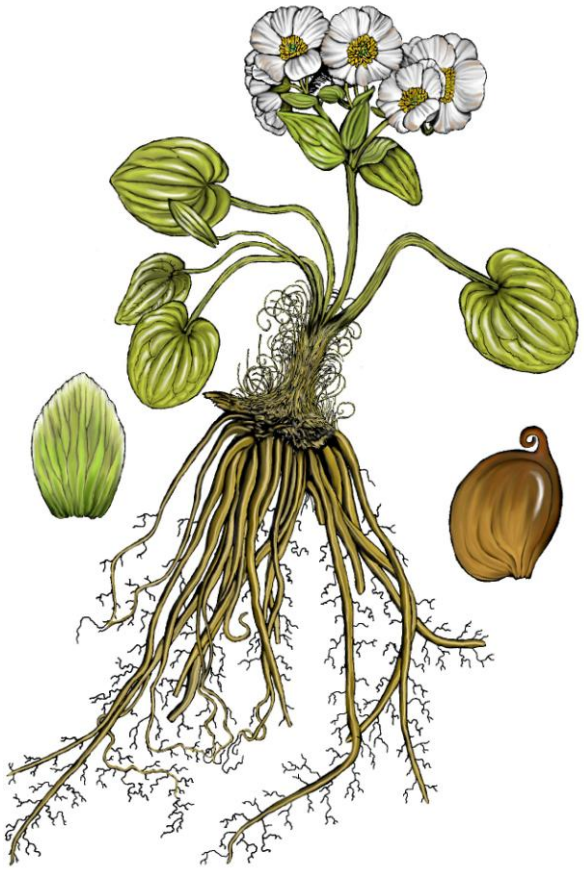
5) Specific conservation strategies, based on the analysis of the genetic diversity within and among *Ranunculus cabrerensis* subsp. *cabrerensis* and *Ranunculus cabrerensis* subsp. *muniellensis* populations, are proposed.

6) There were no morphological differences to distinguish the different subspecies described by the current systematics. On the contrary, at least three morphotypes were distinguished within *Ranunculus parnassiifolius s.l.*, corresponding to the biogeographical territories studied (cantabrian, pyrenean and alpine).

7) A new classification and evolutionary scenario for *Ranunculus* grex *parnassiifolius* is proposed, reflecting phylogenetic relationships. Under these conditions, *Ranunculus cabrerensis s.l.* is considered an independient species

to the polyploid complex *R. parnassiifolius s.l.* Additionally, *R. parnassiifolius* subsp. *parnassiifolius* is restricted to diploids and tetraploids from the Pyrenees; *R. parnassiifolius* subsp. *heterocarpus* to apomict tetraploids from the Alps, and finally, *R. parnassiifolius* subsp. *favargeri* to diploids and tetraploids from the Cantabrian Mountains.

Taking into consideration all the results obtained, some research outlines are presented. The characteristics and advantages of the methodology applied to the present work also support its application to the rest of the species belonging to the section *Ranuncella* from the genus *Ranunculus*. This section represents a monophyletic taxonomic group, mainly constituted by diploid endemic species, with specialized ecological behaviour, and whose origin and diversification is focused in the Iberian Peninsula and North of Africa. Furthermore, in the most eastern distribution area of *Ranunculus parnassiifolius s.l.* (Albania-Macedonia: Korab massif), there is an isolated population of *Ranunculus wettsteinii* Dörfler, a tetraploid race (2n = 32) which systematics needs to be revised.



Ranunculus cabrerensis subsp. *muniellensis*