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Unravelling the evolutionary history of the polyploid complex *Ranunculus parnassiifolius*† (Ranunculaceae)

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Ranunculus L. represents the largest genus within Ranunculaceae, comprising more than 600 species with a worldwide distribution. However, there are still many gaps in our knowledge of the infrageneric taxonomy and evolution of *Ranunculus*. In this regard, intraspecific variation of the polyploid complex *Ranunculus parnassiifolius* remains under discussion. To reconstruct the biogeographical history of the polyploid complex *R. parnassiifolius*, 20 populations distributed throughout the Cantabrian Mountains, Pyrenees, and Alps were investigated. Phylogenetic studies were based on nuclear internal transcribed spacers (ITS) and plastid (*rpl32-trnL*, *rps16-trnQ*) sequence data, analysed using Bayesian approaches as well as the evolution of morphological characters. Additionally, biogeographical patterns were conducted using statistical dispersal–vicariance analysis. The analyses presented here support the recognition of two evolutionary independent units: *R. cabrerensis sensu lato* (*s.l.*) and *R. parnassiifolius s.l.* Furthermore gradual speciation depending on the biogeographical territory is proposed, and optimal reconstructions have probably favoured the ancestor of *Ranunculus parnassiifolius* as originating in the Iberian Peninsula. *Biological Journal of the Linnean Society*, 2012, **107**, 477–493.

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INTRODUCTION

The largest genus within Ranunculaceae is *Ranunculus* L., which comprises approximately 600 species (Tamura, 1993, 1995) and numerous apomictic races (Hörandl *et al*., 2005, Hörandl, Cosendai & Temsch, 2008). Its distribution is almost worldwide, the largest number of species occurring from temperate to artic/subantarctic zones (Ziman & Keener, 1989), and being rare in the tropics where it is restricted to high mountain areas. *Ranunculus* chromosome number is usually $x = 7$ or $x = 8$, with the latter being much more frequent and regarded as the basic state (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 spacer (ITS) sequences (Hörandl *et al*., 2005), *matK*/*trnK* plus ITS (Paun *et al*., 2005; Gehrke & Linder, 2009; Hoffmann *et al*., 2010), and *matK*/*trnK*, ITS plus *psbJ-petA* (Emadzade *et al*., 2010) have

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†The authors recognise that there is disagreement over the spelling of the specific name, with some authorities preferring '*parnassifolius*' (following original description spelling) and others '*parnassiifolius*' (following Division II – Rules and Recommendations – Chapter VII – Section 1 – Orthography – Article 60 and Recommendation 60G; International Code of Botanical Nomenclature, Vienna Code, 2006: http://ibot.sav.sk/icbn/main.htm).

supported the monophyly of *Ranunculus*, showing that the genus is subdivided into several well-supported clades that correspond 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). Recent complete biogeographical studies of the tribe Ranunculeae have been conducted (Emadzade & Hörandl, 2011; Emadzade *et al*., 2011) but relationships between several small and distinctive groups of *Ranunculus*, mainly distributed in, or even endemic to, the mountains of South-Central Europe (e.g. *R. kuepferi*) have been controversial, and need to be evaluated in a phylogenetic context (e.g. Burnier *et al*., 2009).

Here we reassesses the species aggregate *Ranunculus parnassiifolius* (= *R.* grex *parnassiifolius*), a polyploid complex belonging to section *Ranuncella* (Spach) Freyn, which is widespread throughout the Central-Southern European mountains. Küpfer (1974) and Bueno Sánchez, Fernández Casado & Fernández Prieto (1992) proposed to recognize five taxa within *R. parnassiifolius* L., treated at the subspecies level, although some of them have been suggested as different species (see Rothmaler, 1934; Guinea López, 1953). These are: *R. parnassiifolius* subsp. *parnassiifolius* (endemic to the eastern Pyrenees), *R. parnassiifolius* subsp. *cabrerensis* Rothm. (endemic to the northwestern mountains of Spain), *R. parnassiifolius* subsp. *muniellensis* Bueno, Fern.Casado & Fern.Prieto (endemic to the western Cantabrian Mountains, Muniellos Biosphere Reserve), *R. parnassiifolius* subsp. *favargeri* P. Küpfer (endemic to 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, 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, after taking into consideration our initial results (Cires *et al*., 2009, 2010; Cires & Fernández Prieto, 2012), this classification is not entirely clear, and it is therefore essential to gain deeper insight into the evolutionary history of this group to elucidate its taxonomy. For instance, our previous studies revealed the separation of the *R. parnassiifolius* subsp. *cabrerensis* and *R. parnassiifolius* subsp. *muniellensis* from the *R. parnassiifolius sensu lato* (*s.l.*) polyploid complex, and that they should consequently be treated as an independent species (*R. cabrerensis* vs. *R. parnassiifolius*), constituting an evolutionary line in itself (Cires & Fernández Prieto, 2012). However, taxonomic rearrangements of the group lack a phylogenetic context and a complete biogeographical study.

To tackle these problems, here we have used the nuclear ribosomal DNA (nrDNA) ITS and the chloroplast DNA (cpDNA) regions for phylogenetic reconstruction. The analysis of both plastid 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 recently 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). In fact, ribosomal genes are subjected to concerted evolution, potentially leading to uniformity of ITS sequences at the individual, population, and species levels (Rauscher, Doyle & Brown, 2004; Kovarik *et al*., 2005). Maternal cpDNA lineages in natural populations 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 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).

In this study different data sets (plastid and nuclear DNA markers) have been combined to analyse the systematics of *Ranunculus* grex *parnassiifolius*. We assess the validity of the current classification and examine the evolution of several key characters. Three objectives are addressed: (1) to infer the phylogenetic relationships in the *R. parnassiifolius* complex by means of nuclear (ITS) and plastid (*rpl32-trnL*; *rps16-trnQ*) sequences and identify any infraspecific polymorphisms; (2) to clarify the biogeographical background and propose a scenario of dispersal/vicariance events in the divergence of the *R. parnassiifolius* species aggregate; and (3), on the basis of our results, to suggest a new classification of the group.

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),

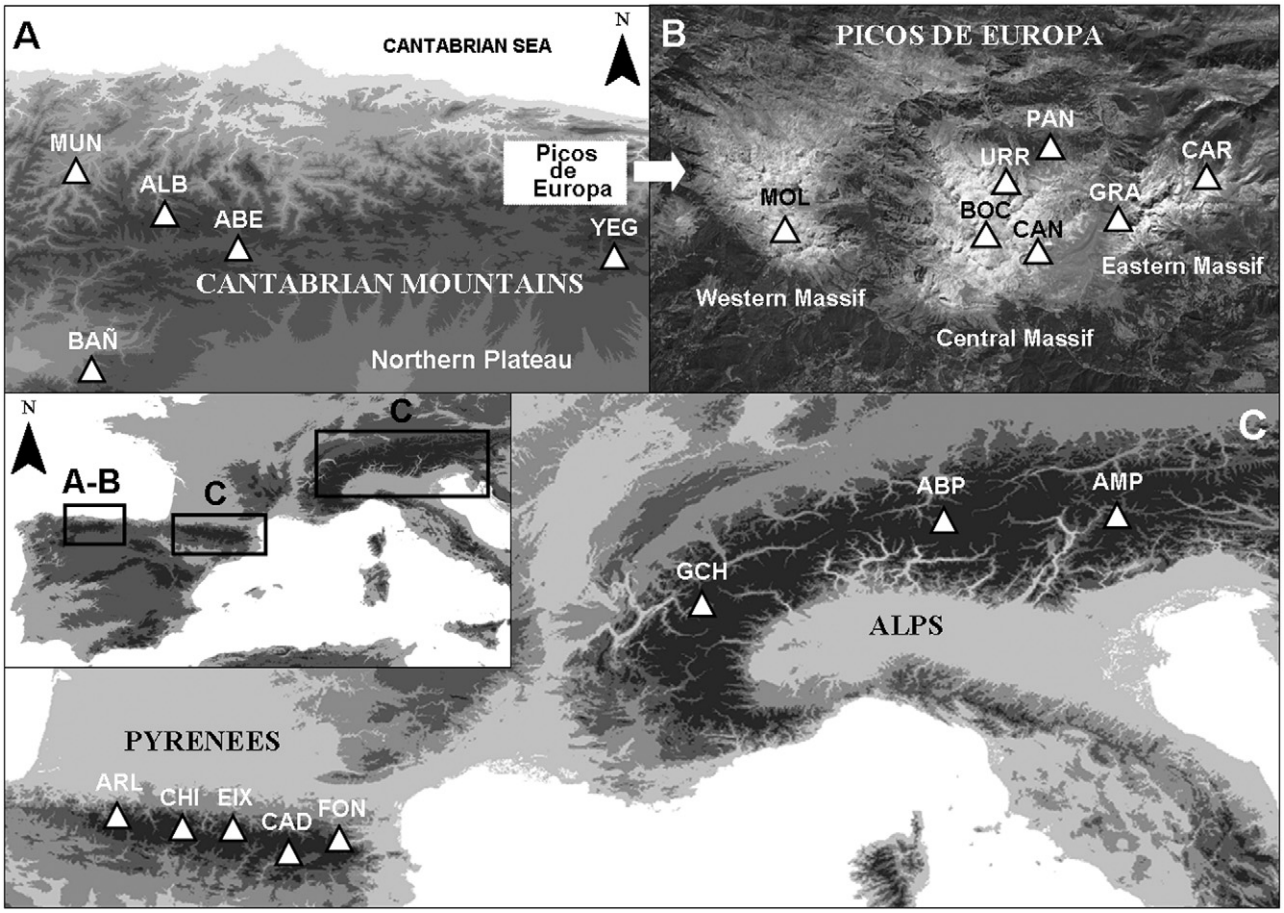

Figure 1. Map of the study area. A, geographical distribution of *Ranunculus* grex *parnassiifolius* in the north-western mountains of the Iberian Peninsula. B, sampled territories in the Picos de Europa. C, sampled territories in the Pyrenees and the Alps. Open triangles indicate sample collection localities. Populations are coded as in the Appendix.

representing the whole diversity of *Ranunculus* grex *parnassiifolius* (Appendix, Fig. 1). In addition, ten other closely related populations belonging to section *Ranuncella* were sampled: *R. amplexicaulis* L., *R. gramineus* L., *R. kuepferi* Greuter & Burdet, *R. pyrenaeus* L.; and also other related species of the genus *Ranunculus*: *R. aconitifolius* L., *Ficaria verna* Huds. (= *R. ficaria* L.). The plant material included samples from the locus classicus of all subspecies of *R.* grex *parnassiifolius*. Particular effort was made 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 occurring in the area (Küpfer, 1974; Cook, Grau & López González, 1986; Bueno Sánchez *et al*., 1992). Additionally, it is worth noting that *R. parnassiifolius* behaves like an apomictic taxon with geographical parthenogenesis (Hörandl *et al*., 2008; Hörandl, 2009; Cires *et al*., 2010). Indeed, diploid sexuals are only localized in the Cantabrian Mountains and the Pyrenees; tetraploids are found 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 the Appendix. 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 was collected for molecular analysis. DNA was isolated applying an extraction method based on the CTAB procedure (Doyle & Doyle, 1987) with slight modifications (see Cires & Fernández

Prieto, 2012) and using a Dneasy Plant Mini Kit (Qiagen, CA, USA). 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). In the case of plastid sequences, a pilot study using one to three samples of each species from distant geographical areas was performed to find the most variable sequences among the seven primer pairs from the chloroplast genome: *ndhF-rpl32R* (Shaw *et al*., 2007); *rps16x1-trnQr* (Dumolin-Lapègue, Pemonge & Petit, 1997; Shaw *et al*., 2007); *trnH*^{GUG}*-psbA* (Sang, Crawford & Stuessy, 1997; Tate & Simpson, 2003); *rpl32F-trnL*^{UAG} (Shaw *et al*., 2007); *trnS*^{GCU}*-trnG*^{UUC} (Shaw *et al*., 2005); and *trnV*^{UAC}*x2-ndhC* (Shaw *et al*., 2007). Finally, two 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 in an Eppendorf Mastercycler Epgradient S (Westbury, NY, USA). After 5 min pre-treatment at 94 °C, PCR conditions were: 40 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C; plus a final extension of 10 min at 72 °C. 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 the 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

Phylogenetic reconstruction was 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 5000 000 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 'burn-in'. A majority-rule consensus tree was constructed and posterior probabilities (PP) of nodes were calculated from the remaining sample. Moreover, the software TCS v.1.21 (Clement, Posada & Crandall, 2000), which implements a statistical parsimony approach using the algorithm described in Templeton, Crandall & Sing (1992), was used to construct haplotype networks. The maximum number of differences among haplotypes was calculated with 95% confidence limits, and treating gaps as missing data.

Character evolution

Character tracing was performed on traits generally used in taxonomic studies of *R.* grex *parnassiifolius*. On the basis of the topology of the 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://www.tropicos.org/Project/IPCN).

Biogeographical analyses

To infer vicariance and dispersal events, a Bayesian-based method (MCMC = 1000 000; nchains = 10; temp. = 0.1) implemented in RASP (Reconstruct Ancestral State in Phylogenies; Yu, Harris & He, 2011) was employed. The Bayesian method in RASP (version 2.0 of S-DIVA: Statistical Dispersal-Vicariance Analysis; Yu, Harris & He, 2010) extends Olsson *et al*.'s (2006) and Sanmartín, Mark & Ronquist's (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-neighbour 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 (north-western Iberian Peninsula, Pyrenees, and Alps). This statistic is a measure of how often the nearest neighbours of sequences are from the same locality in geographical space (Hudson,

Table 1. 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*			
No. 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 DNA G + C content (mol%)	54.9%	28.3%	28.4%
Ranunculus grex *parnassiifolius*			
No. 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 DNA G + C content (mol%)	54.9%	28.3%	28.4%

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 means of different statistical tests based on DNA sequences: K_{ST}, K_S, and Z calculated according to Hudson, Boos & Kaplan (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; and 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, the coefficient of gene differentiation F_{ST} was also estimated for all populations and loci (Hudson, Slatkin & Maddison, 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 the significance of the values obtained. Gaps were treated as missing data and nucleotide ambiguities as indeterminate.

RESULTS

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

The characteristics of ITS, *rpl32-trnL*, and *trnQ-rps16* sequences are summarized in Table 1. Visual inspection of ITS chromatograms of the core *R.* 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.

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 majority-rule consensus of 75 002 trees derived from Bayesian analysis with accompanying PP values expressed as percentages is presented in Figure 2. The topology provided by the consensus tree displayed four well-supported clades within *R.* grex *parnassiifolius*: clade A (76 PP) comprised two subspecies, *R. parnassiifolius* subsp. *cabrerensis* and *R. parnassiifolius* subsp. *muniellensis* (*R. cabrerensis s.l.* according to Cires & Fernández Prieto, 2012) plus a sample of *R.* × *peredae* (= *R. parnassiifolius* × *R. amplexicaulis*); clade B (80 PP) included samples of *R. parnassiifolius s.l.* from the Cantabrian Mountains (including all diploids and the only tetraploid without nucleotide additivity: ALB); clade C (76 PP) comprised samples of *R. amplexicaulis*; and finally, the rest of samples contained a large polytomy (clade D) with different samples of *R. parnassiifolius s.l.*

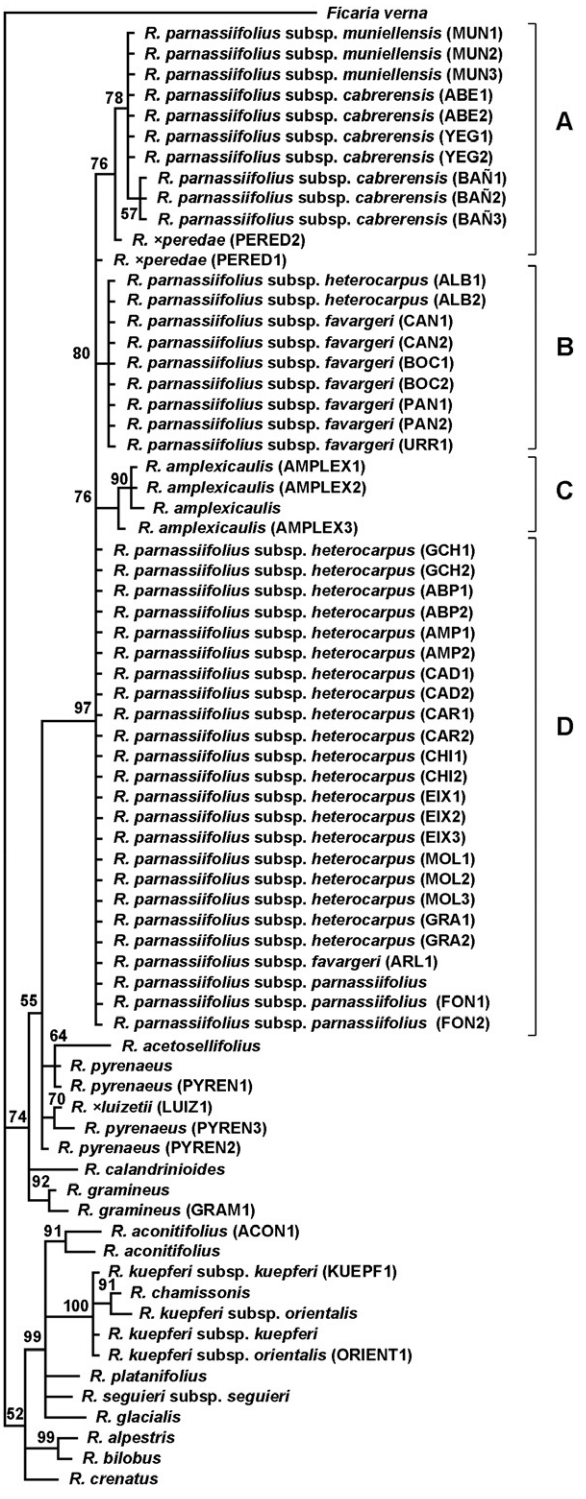

Figure 2. Majority-rule consensus of 75 002 trees derived from Bayesian inference analysis of nrDNA ITS sequences of 20 populations, representing the whole diversity of the *Ranunculus* grex *parnassiifolius*. Numbers above branches are posterior probability values presented as percentages. Population codes are as shown in the Appendix.

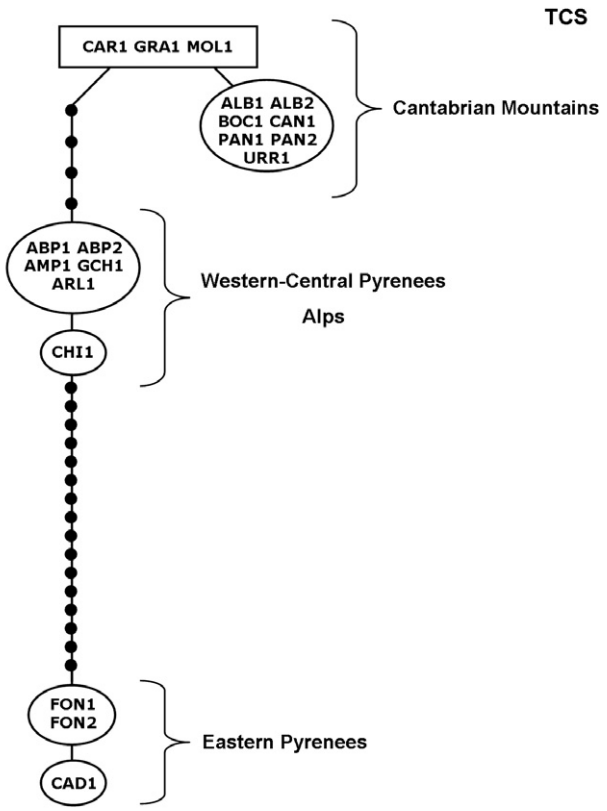

Figure 3. Statistical parsimony network based on combined ITS, *rpl32-trnL*, and *trnQ-rps16* sequences of *Ranunculus* grex *parnassiifolius* and other closely related species. Lines (–) indicate a single nucleotide substitution, and black dots (•) represent haplotypes extinct or not detected.

(*R. parnassiifolius sensu* Küpfer, 1974; excluding *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. Bayesian analysis of combined plastid data on the complex *R. parnassiifolius* (data not shown) revealed the same tree topology and PP, supporting those clades found in the ITS analysis. However, it should be noted that a well-supported clade (100 PP) was found in the eastern populations of *R.* grex *parnassiifolius* from the Pyrenees, together with samples of *R. pyrenaeus* from the same territories. The TCS analysis based on ITS and plastid concatenated sequences within *R.* grex *parnassiifolius* produced a single network with six haplotypes, three haplotype clades, and no loops (Fig. 3). It appears that TCS analysis is only useful for studying data sets where the sequences are not significantly divergent from each other. In this sense, and to avoid a possible influence of hybridization, the natural hybrid popula-

tions were excluded from the network analysis. Indeed, identification of hybrids individuals in the field is relatively easy, because they display intermediate characteristics, i.e. between their putative progenitors.

Character evolution

The reconstruction of ancestral states for the four studied characters is shown in Figure 4. 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 (Fig. 4C, D). The remaining characters (ploidy level, Fig. 4A; bedrock type, Fig. 4B) showed a pattern of multiple independent events and were much less informative at the 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 sequence-based statistics (K_{ST}, K_S and Z). The last statistic was S_{nn}, referred to as the nearest-neighbour statistic, a measure of how often the nearest neighbours 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 ($S_{nn} = 0.584$). 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 vs. ALP). F_{ST} values calculated for each pair of geographical groups are given in Table 2.

The optimal reconstruction in dispersal–vicariance scenarios (Fig. 5A, B) revealed that the ancestor of *R.* grex *parnassiifolius* (primitive singameon) originated on 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 taxon as having 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%). Based on the results obtained here and in previous studies (Cires *et al*., 2009, 2010; Cires & Fernández Prieto, 2012), a new evolutionary scenario is therefore proposed (Fig. 5C).

DISCUSSION

The history of *R. parnassiifolius* dates back to Linnaeus (1753), who described the species. The protologue is short indicating only 'habitat in Europa australi', and the only literature cited is 'Institutione rei herbariae' of Tournefort (1700), where there is no chorological information. According to Küpfer (1974: 181–184, PL VIb), and considering the regularity of the pollen from the 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 corolla, and 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).

The results obtained here (ITS, *rpl32-trnL*, *trnQ-rps16* sequences), and also in previous studies based on cytotype distribution, morphological characters and plastid DNA sequences (Cires *et al*., 2009, 2010; Cires & Fernández Prieto, 2012), indicate that the current classification is controversial. For instance, in many cases the only criteria to identify the infraspecific taxon 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 attributed several times to the tetraploid subspecies *R. parnassiifolius* subsp. *heterocarpus* (e.g. Vigo i Bonada, 1983) because of being placed in a limestone substrate, whereas cytometric data (Cires *et al*., 2010) indicate 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 an area of siliceous bedrock (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 appears to depend mainly on the sampling period (our personal observations) and thus should no longer be considered. Even Küpfer (1974: 187) mentions that some Swiss populations (including the type locality of *R. parnassiifolius* subsp. *heterocarpus*: Grand Chavalard) are characterized by regular corollas. In addition, no morphological character or combination of

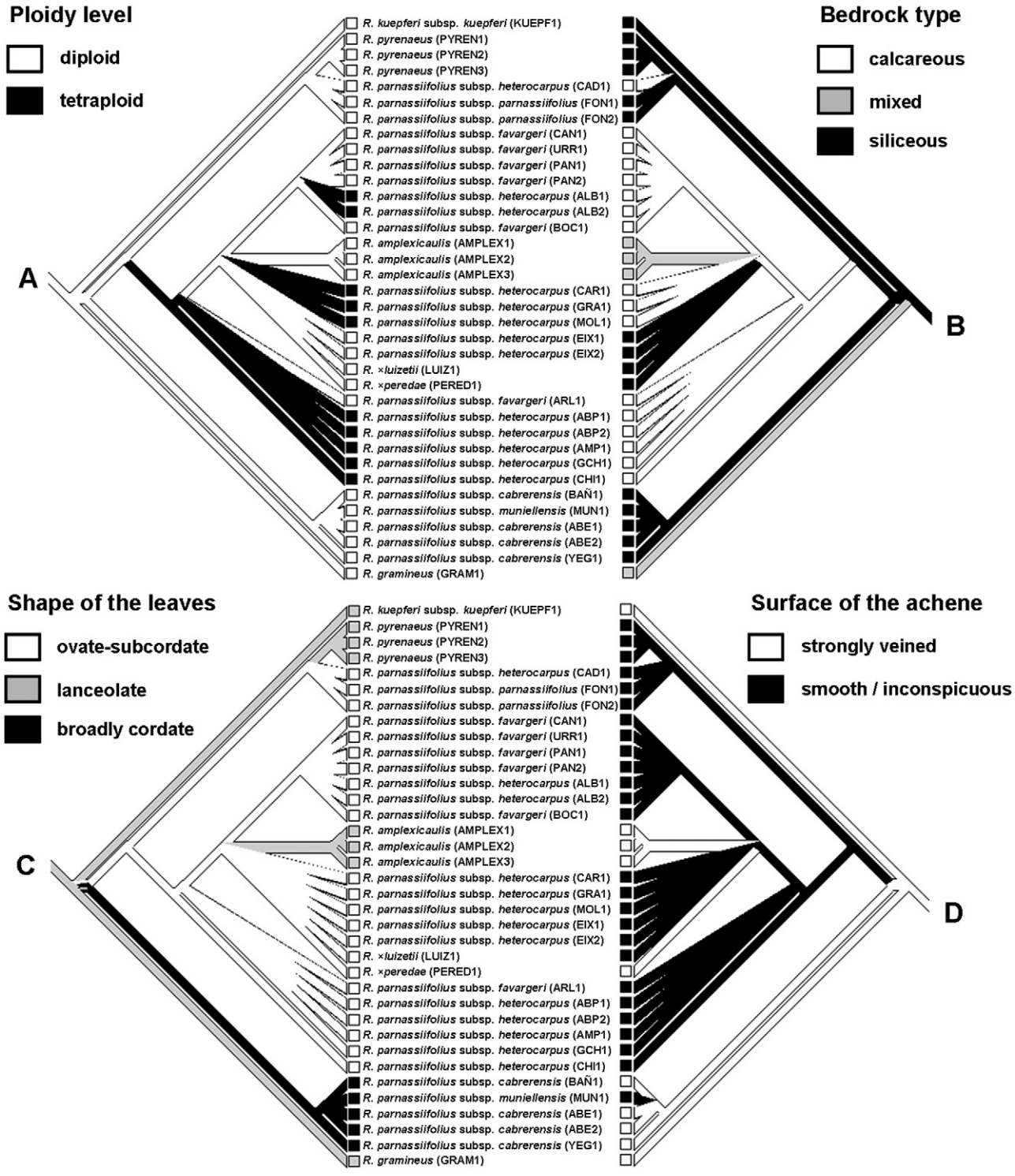

Figure 4. Evolution of categorical characters on the maximum parsimony topology: A, ploidy level (2*x*, 4*x*); B, bedrock type (calcareous, siliceous, mixed); C, base shape of the basal leaves (ovate-subcordate, broadly cordate, lanceolate); D, surface of the achenes (achenes strongly veined, achenes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

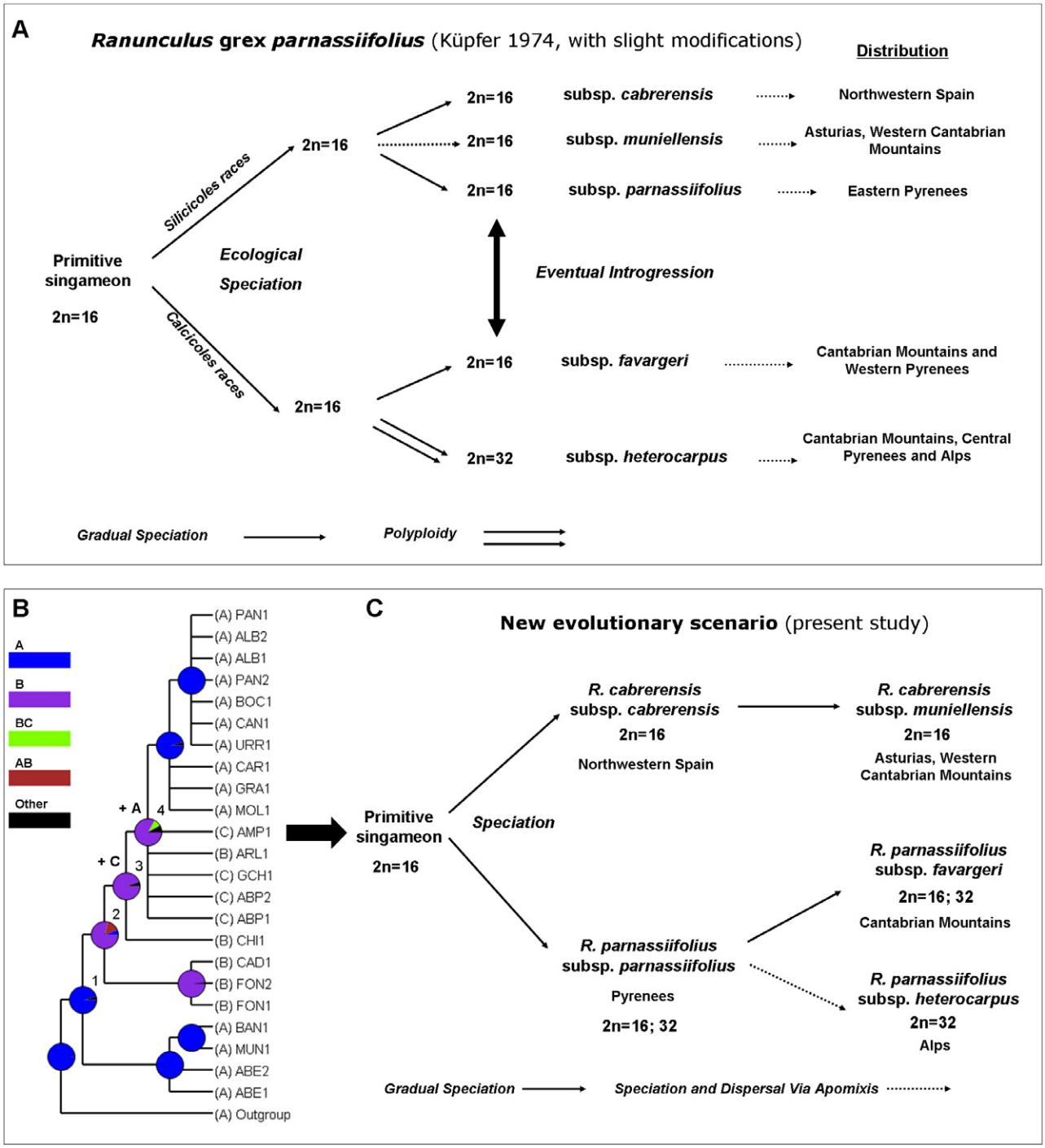

Figure 5. Biogeographical events in *Ranunculus* grex *parnassiifolius*. A, traditional systematics of *Ranunculus* grex *parnassiifolius* based on Küpfer (1974) with slight modifications (Bueno Sánchez *et al*., 1992). B, dispersal–vicariance scenarios reconstructed by RASP (Reconstruct Ancestral State in Phylogenies) with the maximum number of area units set to 3. Pie charts at internal nodes represent the marginal probabilities for each alternative ancestral area. Arrow (+): dispersal event. Letters denote area units (A, north-western Iberian Peninsula; B, Pyrenees; C, Alps; Outgroup: *Ficaria verna*) and the term 'other' (black) denotes the union of other remaining areas with lowest probability. Populations are coded as in the Appendix. C, proposed new evolutionary scenario.

Table 2. 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	0.094ns	25.176	0.155ns	0.584	0.223ns	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	0.060ns	37.486	0.060ns	0.714	0.017*	0.666
	PYR vs. ALP	8.242	0.154	0.087ns	25.176	0.145ns	0.584	0.243ns	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 Fig. 5A). Dataset B: excluding *R. parnassiifolius* subsp. *cabrerensis* and *R. parnassiifolius* subsp. *muniellensis* sequences.
ns, not significant; *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$. K_S, K_{ST}, Z, and S_{nn} are test statistics of genetic differentiation; F_{ST} examines the extent of genetic differentiation between geographical isolates.
†S_{nn} test statistics were performed with excluded gaps (Hudson, 2000).
NIP: north-western Iberian Peninsula; PYR: Pyrenees; ALP: Alps.

characters is able to distinguish the plants as belonging to different ploidy levels (or subspecies), either in the Cantabrian Mountains or 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). A further example highlighting controversy in the current classification appears in the study of Küpfer (1974: 187), who mentions that populations of *R. parnassiifolius* subsp. *favargeri* from the western Pyrenees have larger flowers and leaves compared with the populations from Canalona (Picos de Europa and type locality for this subspecies). This again questions the origin of the type locality for *R. parnassiifolius*, as mentioned above. Therefore, there have been no clear criteria to identify the infraspecific taxon 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 regarding 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 was mentioned by Cires & Fernández Prieto (2012), based on morphological characters and cpDNA regions. Additional to morphological differences and cpDNA sequence variation, *R. cabrerensis* grows in slope deposits of gravel and coarse rocky oucrops, whereas *R. parnassiifolius sensu stricto* grows among fine-grained scree. Indeed, the trees generated by individual and combined 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 analyses of Paun *et al*. (2005), Hoffmann *et al*. (2010), and Emadzade & Hörandl (2011) propose a recent origin for the section *Ranuncella* and *Ranunculus* grex *parnassiifolius*. Age estimates suggest 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 indicates pre-glacial speciation events, 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 (Fig. 5B, C). Our data are consistent with the hypothesis of Paun *et al*. (2005) that 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, Kadereit & Comes, 2002), *Pritzelago alpina* (Kropf, Kadereit & Comes, 2003), and *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, Galen & Stanton, 1994), recent evidence suggests 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) or multiple independent colonizations of the African continent (Gehrke & Linder, 2009). The genus *Ranunculus* otherwise shows many examples of rapid and radiative speciation, for example 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 the systematics of Küpfer (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 & Soltis, 1999, 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 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, has also been described in other Mediterranean polyploid groups (e.g. Balao *et al*., 2010).

At a finer level of taxonomic resolution, our plastid sequence analysis provides a well-supported clade comprising *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). In fact, incongruence between nuclear and chloroplast data, as well as the sharing of plastid haplotypes between species, is usually interpreted as a result of interspecific hybridization, which leads to the replacement of the plastid genome of one species with that 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 has already been suggested by Küpfer (1974: 189). During glaciation (range contractions), diploid species from the Pyrenees could have been isolated at the eastern end 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 to be 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, Comes & Kadereit, 2008). Besides the results presented above, this study has generated unresolved questions, which are beyond the scope of this work. One of them regards 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. In addition, from a standpoint of biological conservation, the natural hybrid populations contain a genetic diversity that does not exist in *R.* grex *parnassiifolius*, which is of great importance as *R.* grex *parnassiifolius* is on Red Lists of protection. Introgression also has important implications for conservation biology, and its consequences in plants have been reviewed several times in the past. For example, Rhymer & Simberloff (1996) showed that hybridization and introgression, due to increased rates of contact and invasions, could lead to the extinction of species.

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; Cires & Fernández Prieto, 2012). We therefore consider that these morphological and DNA sequence differences are sufficient to treat *R. cabrerensis* at the specific level, and also provide clear evidence that geography has been the major factor in the rest of the *R.* grex *parnassiifolius* complex. Therefore, taking into account all the results obtained in the study of *R.* grex *parnassiifolius*, a new evolutionary scenario is proposed (Fig. 5C):

1. There are arguments to support the separation of *R. parnassiifolius* subsp. *cabrerensis* (Rothmaler, 1934: 148) from *R.* grex *parnassiifolius*, as an independent species: *R. cabrerensis* (Fig. 1A).
2. The plants described as representing *R. parnassiifolius* subsp. *muniellensis* (Bueno Sánchez *et al*., 1992: 365) should be systematically placed as a geographical race of *R. cabrerensis*: *R. cabrerensis* subsp. *muniellensis* (Cires & Fernández Prieto, 2012; Fig. 1A).
3. The original circumscription of *R. parnassiifolius* subsp. *parnassiifolius* (*sensu* Küpfer, 1974: 190) includes diploids from the eastern Pyrenees. Here we expand the use of this name to diploids and tetraploids of the Pyrenees (without geographical restriction; Fig. 1C).
4. The original circumscription of *R. parnassiifolius* subsp. *heterocarpus* (Küpfer, 1974: 192) includes tetraploids from the Cantabrian Mountains, the Pyrenees, and the Alps. Here we expand the use of this name to apomict tetraploids of the Alps (Fig. 1C).
5. The original circumscription of *R. parnassiifolius* subsp. *favargeri* (Küpfer, 1974: 191–192) includes diploids from the Cantabrian Mountains and the western Pyrenees. Here we expand the use of this name to diploids and tetraploids of the Cantabrian Mountains (Fig. 1B). Guinea López (1953: 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 the International Code of Botanical Nomenclature (article 34.2; McNeill *et al*., 2006), alternative names are illegitimate if they were not published before 1 January 1953, which is the case here.

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, *Plant Syst. Evol.* 298: 121–138
≡ *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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APPENDIX

Plant materials, voucher information and GenBank accessions for DNA sequences used in this paper. Sequences retrieved directly from GenBank (ITS) are in italics. The systematics of *Ranunculus* grex *parnassiifolius* is based on Küpfer (1974), with slight modifications (Bueno Sánchez *et al*., 1992) (see Fig. 5).

Ranunculus taxa (population code); Locality; Collector, Collection number and herbarium; ITS GenBank no.; *rpl32-trnL* GenBank no.; *trnQ-rps16* GenBank no.

Ficaria verna Huds. (= *R. ficaria* L.); Villaviciosa (Asturias, Spain); J.A. Fernández Prieto & J. Homet 31967 (FCO); JX025227; JX025320; JX025282. ***Ranunculus acetosellifolius*** Boiss.; cult. Gothenburg BG; J.T. Johansson s.n. (GB); AY680075. ***R. aconitifolius*** L.; cult. Copenhagen BG; J.T. Johansson 274 (LD); AY680081. ACON1; Somiedo (Asturias, Spain); E. Cires & J.A. Fernández Prieto 31859 (FCO); JX025228; JX025321; JX025283. ***R. alpestris*** L.; cult. Rezia BG; J.T. Johansson 242 (LD); AY680078. ***R. amplexicaulis*** L.; cult. Lund BG; J.T. Johansson 222 (LD); AY680071. AMPLEX1; Portillo de las Yeguas (Cantabria, Spain); A. Bueno & E. Cires 31962 (FCO); JX025229; JX025322; JX025284. AMPLEX2; Somiedo (Asturias, Spain); E. Cires & J.A. Fernández Prieto 31963 (FCO); JX025230; JX025323; JX025285. AMPLEX3; Somiedo (Asturias, Spain); E. Cires & J.A. Fernández Prieto 31964 (FCO); JX025231; JX025324; JX025286. ***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. glacialis*** L.; Sweden; J.T. Johansson s.n.; AY680082. ***R. gramineus*** L.; cult. Krefeld BG; J.T. Johansson s.n.; AY680076. GRAM1; Somiedo (Asturias, Spain); E. Cires & J.A. Fernández Prieto 31968 (FCO); JX025232; JX025325; JX025287. ***R. kuepferi*** subsp. ***kuepferi*** Greuter & Burdet; Italy; E. Hörandl 9525 (WU); AY954241. KUEPF1; Col de Vars (Hautes-Alpes, France); E. Cires & J.A. Fernández Prieto 31970 (FCO); JX025233; JX025326; JX025288. ***R. kuepferi*** subsp. ***orientalis*** W.Huber; Italy; P. Schönswetter & A. Tribsch 2213 (WU); AY680084. ORIENT1; Austria; E. Hörandl 4336 (WU); AY680085. ***R. parnassiifolius*** subsp. ***cabrerensis*** Rothm. BAÑ1; Lago de la Baña (León, Spain); E. Cires, B. Jiménez-Alfaro & L. González 31369 (FCO); JX025234; JX025327; JX025289. BAÑ2; Lago de la Baña (León, Spain); E. Cires, B. Jiménez-Alfaro & L. González s.n.; JX025235. BAÑ3; Lago de la Baña (León, Spain); E. Cires, B. Jiménez-Alfaro & L. González s.n.; JX025236. ABE1; Abelgas de Luna (León, Spain); C. Cuesta & E. Cires 31368 (FCO); JX025237; JX025328; JX025290. ABE2; Abelgas de Luna (León, Spain); C. Cuesta & E. Cires s.n.; JX025238; JX025329; JX025291. YEG1; Portillo de las Yeguas (Cantabria, Spain); A. Bueno & E. Cires 31371 (FCO); JX025239; JX025330; JX025292. YEG2; Portillo de las Yeguas (Cantabria, Spain); A. Bueno & E. Cires s.n.; JX025240. ***R. parnassiifolius*** subsp. ***heterocarpus*** P. Küpfer GCH1; Grand Chavalard (Valais, Switzerland); C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto 31366 (FCO); JX025241; JX025331; JX025293. GCH2; Grand Chavalard (Valais, Switzerland); C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto 31367 (FCO); JX025242. ABP1; Albulapass (Graubünden, Switzerland); C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto 31353 (FCO); JX025243; JX025332; JX025294. ABP2; Albulapass (Graubünden, Switzerland); C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto s.n.; JX025244; JX025333; JX025295. AMP1; Cortina d'Ampezzo (Belluno, Italy); C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto 31354 (FCO); JX025245; JX025334; JX025296. AMP2; Cortina d'Ampezzo (Belluno, Italy); C. Cuesta, E. Cires, M. Ceballos & J.A. Fernández Prieto s.n.; JX025246. ALB1; Somiedo (Asturias, Spain); E. Cires & J.A. Fernández Prieto 31103 (FCO); JX025247; JX025335; JX025297. ALB2; Somiedo (Asturias, Spain); E. Cires & J.A. Fernández Prieto 31104 (FCO); JX025248; JX025336; JX025298. CAD1; Cadí-Moixeró (Barcelona, Spain); E. Cires & J.A. Fernández Prieto 31358 (FCO); JX025249; JX025337; JX025299. CAD2; Cadí-Moixeró (Barcelona, Spain); E. Cires & J.A. Fernández Prieto 31359 (FCO); JX025250. CAR1; San Carlos (Cantabria, Spain); E. Cires & C. Cuesta 31111 (FCO); JX025251; JX025338; JX025300. CAR2; San Carlos (Cantabria, Spain); E. Cires & C. Cuesta s.n.; JX025252. CHI1; Chisagües (Huesca, Spain); E. Cires & J.A. Fernández Prieto 31360 (FCO); JX025253; JX025339; JX025301. CHI2; Chisagües (Huesca, Spain); E. Cires & J.A. Fernández Prieto s.n.; JX025254. EIX1; Creu de l'Eixol (Lérida, Spain); E. Cires & J.A. Fernández Prieto 31361 (FCO); JX025255; JX025340; JX025302. EIX2; Creu de l'Eixol (Lérida, Spain); E. Cires & J.A. Fernández Prieto 31362 (FCO); JX025256; JX025341; JX025303. EIX3; Creu de l'Eixol (Lérida, Spain); E. Cires & J.A. Fernández Prieto s.n.; JX025257. MOL1; Los Moledizos (León, Spain); E. Cires 31114 (FCO); JX025258; JX025342; JX025304. MOL2; Los Moledizos (León, Spain); E. Cires s.n.; JX025259. MOL3; Los Moledizos (León, Spain); E. Cires s.n.; JX025260. GRA1; Las Grajas (Cantabria, Spain); E. Cires 31113 (FCO);

JX025261; JX025343; JX025305. GRA2; Las Grajas (Cantabria, Spain); E. Cires s.n.; JX025262. ***R. parnassiifolius*** subsp. ***favargeri*** P. Küpfer CAN1; La Canalona (Cantabria, Spain); A. Fernández & E. Cires 31107 (FCO); JX025263; JX025344; JX025306. CAN2; La Canalona (Cantabria, Spain); A. Fernández & E. Cires 31108 (FCO); JX025264. ARL1; Col d'Arlas (Aquitaine, France); E. Cires & J.A. Fernández Prieto 31355 (FCO); JX025265; JX025345; JX025307. BOC1; Jou de los Boches (Asturias, Spain); A. Fernández & E. Cires 31105 (FCO); JX025266; JX025346; JX025308. BOC2; Jou de los Boches (Asturias, Spain); A. Fernández & E. Cires 31106 (FCO); JX025267. PAN1; Pandébano (Asturias, Spain); A. Fernández & E. Cires 31115 (FCO); JX025268; JX025347; JX025309. PAN2; Pandébano (Asturias, Spain); A. Fernández & E. Cires 31116 (FCO); JX025269; JX025348; JX025310. URR1; Urriellu (Asturias, Spain); A. Fernández & E. Cires 31117 (FCO); JX025270; JX025349; JX025311. ***R. parnassiifolius*** subsp. ***muniellensis*** Bueno, Fern.Casado & Fern-.Prieto MUN1; Muniellos Biosphere Reserve (Asturias, Spain); E. Cires 31370 (FCO); JX025271; JX025350; JX025312. MUN2; Muniellos Biosphere Reserve (Asturias, Spain); E. Cires s.n.; JX025272. MUN3; Muniellos Biosphere Reserve (Asturias, Spain); E. Cires s.n.; JX025273. ***R. parnassiifolius*** subsp. ***parnassiifolius*** L.; France/Spain; G. Schneeweiss & al. 6509 (WU); AY680072. FON1; Fontalba (Gerona, Spain); E. Cires & J.A. Fernández Prieto 31363 (FCO); JX025274; JX025351; JX025313. FON2; Fontalba (Gerona, Spain); E. Cires & J.A. Fernández Prieto 31364 (FCO); JX025275; JX025352; JX025314. ***R. platanifolius*** L.; Norway; J.T. Johansson 277 (LD); AY680080. ***R. pyrenaeus*** L.; Spain; G. Schneeweiss & al. 6498 (WU); AY680074. PYREN1; Chisagües (Huesca, Spain); E. Cires & J.A. Fernández Prieto 31971 (FCO); JX025276; JX025353; JX025315. PYREN2; Pas de la Casa (Encamp, Andorra); E. Cires & J.A. Fernández Prieto 31372 (FCO); JX025277; JX025354; JX025316. PYREN3; Fontalba (Gerona, Spain); E. Cires & J.A. Fernández Prieto 31972 (FCO); JX025278; JX025355; JX025317. ***R. seguieri*** subsp. ***seguieri*** Vill.; cult. Gothenburg BG; J.T. Johansson 226 (LD); AY680079. ***R. ×luizetii*** Rouy (= *R. parnassiifolius* L. *x R. pyrenaeus* L.) LUIZ1; Creu de l'Eixol (Lérida, Spain); E. Cires & J.A. Fernández Prieto 31975 (FCO); JX025279; JX025356; JX025318. ***R. ×peredae*** M.Laínz (= *R. parnassiifolius* L. *x R. amplexicaulis* L.) PERED1; Portillo de las Yeguas (Cantabria, Spain); A. Bueno & E. Cires 31976 (FCO); JX025280; JX025357; JX025319. PERED2; Portillo de las Yeguas (Cantabria, Spain); A. Bueno & E. Cires s.n.; JX025281.

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/).