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Research Article

Phylogenetic and cytogenetic studies reveal hybrid speciation in *Saxifraga* subsect. *Triplinervium* (Saxifragaceae)

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Abstract Hybridization has played an important role in *Saxifraga* evolution causing reticulation and a high number of described hybrids, but little is known about how hybrid speciation had occurred in the genus. We focus on a group of closely related *Saxifraga* species of the subsection *Triplinervium* from Pyrenees, the phylogenetic relationships of which remain unsolved. Trying to unmask cryptic (or ancient) hybridization processes, we analyze one nuclear (ITS) and three plastid regions (*rpl32-trnL*, *trnS-trnG-trnG*, and *3′trnV-ndhC*), as well as nuclear DNA content. Pollen and seed morphology and viability studies were carried out to evaluate the status of spontaneous hybrids. DNA ploidy levels were also inferred for the two Madeiran taxa (of the same *Saxifraga* subsection), where recent hybridization processes are not expected. Molecular markers revealed multiple reticulation events, which, as suggested by DNA content and chromosome numbers, have occurred in homoploidy (without genome doubling after hybridization). In addition, autopolyploidy has occurred in some species or populations, especially in the Madeiran archipelago colonization. Chromosome number variation appears to be related to centric fission events, which also could lead to the formation of the B chromosomes inferred in some taxa. Spontaneous hybrids do not produce viable seeds and this could be due to differences in parental chromosome numbers. Hybrid speciation has probably been successful by chromosomal arrangements, which also generated new, more or less intermediate, chromosomal numbers in this group of taxa.

Key words: autopolyploidy, B chromosomes, genome size, homoploidy, hybrid speciation, phylogeny.

Hybridization has long been recognized as an important mechanism of plant speciation (Anderson & Stebbins, 1954; Soltis et al., 2009), not only in ancient angiosperm evolution but also in the recent formation of new species (Arnold, 1997). Hybrid speciation can occur at the same ploidy level as parental taxa (homoploid hybrid speciation) or via allopolyploidy (heteroploid hybrid speciation, including hybridization and chromosome doubling, Grant, 1981). Homoploid hybrid speciation has usually been considered as a rare process, because it requires the development of reproductive isolation in sympatry (Rieseberg, 1997). In contrast, allopolyploidy has been supposed to have been a much more common phenomenon, because genome doubling facilitates its identification and reduces or eliminates the possibility of a new polyploid backcrossing with its parents (Soltis & Soltis, 2009). Nevertheless, during the last years the cases of speciation in homoploidy are increasing in the literature (e.g., Marques et al., 2012; Vega et al., 2013), confirming that it is an underestimated process, which usually remains cryptic in the evolutionary history of many plant groups.

Saxifraga L. subsect. *Triplinervium* (Gaudin) Gornall (Saxifragaceae) offers a good species complex to study hybrid speciation. Previous studies have suggested extensive hybridization and reticulation (Vargas, 2000), involving allopolyploidy for a particular closely related species (e.g., *S. osloensis* Knaben, Brochmann et al., 1996). *Saxifraga* is also well known by the occurrence of natural hybrids (Luizet, 1931; Webb & Gornall, 1989) and for being a karyologically complex group. This is due to technical difficulties linked to chromosome size and morphology (Favarger, 1965; Vargas, 1994; Mas de Xaxars et al., 2014) and to the cytotaxonomic mechanisms inferred: (i) dysploidy (Küpfer & Rais, 1983), (ii) aneuploidy (Vargas, 1994), (iii) B chromosomes occurrence (Soltis, 1983), and (iv) polyploidy (Brochmann et al., 1996). These phenomena often generate imprecise counts, heterogeneous putative base

chromosome numbers (Favarger, 1965; Küpfer & Rais, 1983) and a great chromosome number variability between sister species (Vargas & Nieto-Feliner, 1995).

We focus the present work on nine species of *Saxifraga* subsect. *Triplinervium* distributed across the Pyrenees and some neighboring mountains (Montseny-Guilleries massif), in NE Iberian Peninsula. These taxa were selected because they (i) are closely related, but their relationships remain unclear (Vargas, 2000), (ii) are endemic of small territories (Vargas, 1997; with the exception of *S. moschata*), (iii) present a great chromosome number variation, which could be masking either homoploid or allopolyploid speciation processes, and (iv) produce spontaneous hybrids when they cohabite. We carried out genome size measurements in order to establish DNA ploidy levels, use new chloroplast markers to generate a haplotype network and analyze our new populational ITS sequences with the previous published (http://www.ncbi.nlm.nih.gov/genbank/) with the aim of reconstruct species relationship. For comparative purposes, we also obtain the genome size of two closely related subsect. *Triplinervium* species endemic of the Madeiran archipelago (*S. maderensis* and *S. portosanctana*; Vargas et al., 1999; Vargas, 2000). This comparison is interesting because of their high chromosome numbers ($2n$ = ca. 100, ca. 124 and $2n$ = ca. 57, respectively, references in suppl.) and hybridization with other congeners is not expected due to their geographic isolation. Experimental studies on pollen grains and seeds were also used to shed further light in the determination of the viability of three spontaneous hybrids and their introgression capacity. We especially focused on *S.* ×*cadevallii*, because its endemicity to Montseny-Guilleries massif allowed us to study the entire distribution area of this taxon.

Material and Methods

Plant material

A total of 49 populations of 14 taxa of *Saxifraga* subsect. *Triplinervium* were collected for this study (Appendix I). Population sampling was carried out covering the entire distribution area in the studied region. For each population, leaves, flowers, and seeds were collected from 1 to 28 individuals depending on the study performed (Appendix I). Material from young leaves was dried and preserved in silica gel. For *S. maderensis* and *S. portosanctana* only material for genome size measurements was collected. Voucher specimens for each population sampled were deposited in the herbarium BCN (Centre de Documentació de Biodiversitat Vegetal, Universitat de Barcelona).

Pollen and seed study

Pollen grains from each of all 12 Pyrenean-Montseny-Guilleries (hereafter Pyrenean) taxa were acetolyzed for light microscopy following the micromethod of Avetissian (1950) and for scanning electron microscopy (hereafter SEM) observation using gold for sputter coating to study exine surface and pollen ornamentation. Size variation of pollen was estimated by measuring the polar axis (P) and the equatorial diameter (E) of 30 grains per population by light microscopy with the AxioCam software (Carl Zeiss, Jena, Germany). Alexander's (1969) test was performed for the analyses of pollen grain viability (detection of aborted and non-aborted pollen grains).

Seed ornamentation of five samples from each Pyrenean taxon (Appendix I, except *S. praetermissa*) was also observed by SEM following the procedure described above for the pollen grain treatment. SEM observations were carried out at the Centres Científics i Tecnològics, Universitat de Barcelona. Seeds were kept at 4 °C for several months, and a growth chamber was used to test germination in constant conditions of 20 °C and 12 h photoperiod.

DNA content assessment

Fresh young leaves of each collected plant (275 individuals, Appendix I and Table 1) were co-chopped with the appropriate standard using a razor blade with an internal standard in the proportions 2:1 in 1200 μL of LB01 buffer (Doležel et al., 1989) with 8% of Triton X-100 and supplemented with 100 μg/mL ribonuclease A (RNase A, Boehringer, Meylan, France) in a plastic Petri dish. *Lycopersicon esculentum* Mill. "Montfavet 63-5" (2C = 1.99 pg), *Petunia hybrida* Vilm. "PxPc6" (2C = 2.85 pg), *Pisum sativum* L. "Express long" (2C = 8.37 pg) were used as internal standards (Marie & Brown, 1993; Lepers-Andrzejewski et al., 2011) and were first analyzed separately in 600 μL of LB01 buffer to locate their peak positions. Nuclei were filtered through a 70-μm nylon filter in order to eliminate cell debris before the addition of 36 μL of propidium iodide (1 mg/mL, solution in water; Invitrogen, Eugene, OR, USA). For each individual, two samples were extracted, kept on ice and analyzed independently. Fluorescence analysis was carried out using an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL, USA) at the Centres Científics i Tecnològics, Universitat de Barcelona, with the standard configuration as described in Garnatje et al. (2004). Acquisition was stopped at 8000 nuclei. The DNA content was calculated for 10 of the aforementioned runs, assuming a linear correlation between the fluorescence signals (of the stained nuclei) and DNA amount. Mean and standard deviations were calculated for 2C values of each population based on five studied individuals. The mean of the half-peak coefficient of variation (HPCV) was also calculated for both target plant and internal standard.

Differences in DNA content between and within species and populations were tested with a General Linear Model, considering species as a fixed factor and the origin population as random. All statistical analyses were performed using SPSS package 19.0 (SPSS, Chicago, IL, USA).

DNA extraction, amplification, and sequencing

DNA from at least one individual per population (total 54, see Appendix I) was extracted using the CTAB method (Doyle & Doyle, 1987) as modified by Soltis et al. (1991) from silica gel-dried leaves collected in the field. In the case of *S. praetermissa*, herbarium material was used, but the protocol for DNA extraction was similar to that for silica gel-dried tissues. A total of 42 individuals from *S. genesiana*, *S. geranioides*, *S. vayredana*, and *S.* ×*cadevallii* (*S. genesiana* × *S. vayredana*) populations were added and extracted for haplotype networks.

Amplification of ITS regions by PCR was performed using primers ITS1 and ITS4 developed by White et al. (1990). The PCR

Table 1 Values (mean ± standard deviation) of genome size (in pg) obtained by flow cytometry for the taxa studied (Anglos population of *Saxifraga moschata* (Smos4, Appendix I) is indicated separately)

Section	Subsection	Taxon	N	2C ± SD (pg)	2C (Mbp)†	HPCV‡ samples (%)	HPCV‡ standards (%)	DNA ploidy level	Chromosome numbers
Saxifraga	*Triplinervium*	*S. conifera*¶	8	1.310 ± 0.02	1281			2*x*	42
		S. moschata	14	1.310 ± 0.045	1281	3.14	4.94	2*x*	22–28, 44–48, 52, ca. 56
		S. vayredana	30	1.360 ± 0.037	1329	3.53	3.54	2*x*	62, 64
		S. ×*jeanpertii*	8	1.380 ± 0.044	1351	2.89	3.63	2*x*	–
		S. pubescens subsp. *pubescens*	23	1.420 ± 0.033	1387	2.59	4.28	2*x*	26, 28
		S. intricata	22	1.430 ± 0.057	1399	3.40	4.46	2*x*	32, 34
		S. pentadactylis subsp. *pentadactylis*	20	1.490 ± 0.033	1461	3.90	4.50	2*x*	32 + 5B
		S. ×*cadevallii*	10	1.530 ± 0.035	1500	3.35	3.95	2*x*	–
		S. ×*bubaniana*	12	1.560 ± 0.036	1524	2.84	5.03	2*x*	–
		S. geranioides	55	1.700 ± 0.135	1667	2.81	4.49	2*x*	ca. 44, ca. 52
		S. fragilis	36	1.740 ± 0.060	1706	3.51	3.71	2*x*	64
		S. genesiana	30	1.750 ± 0.043	1709	2.54	4.05	2*x*	ca. 44
		S. moschata (Anglos)	5	2.590 ± 0.086	2531	3.24	3.22	4*x*	ca. 56
		S. portosanctana	5	2.92 ± 0.08	2856	5.84	1.41	4*x*	ca. 54, ca. 60
		S. maderensis	5	7.52 ± 0.23	7355	0.38	4.16	8*x*	ca. 100, ca. 108, ca. 124
	Saxifraga	*S. granulata*§		1.35 ± 0.03	1320			2*x*	22
		S. granulata§		3.54 ± 0.08	3462			4*x*	44
		S. granulata§		4.76	4655			5*x*	52
Porphyrion		*S. oppositifolia* L. subsp. *oppositifolia*¶	5	2.93 ± 0.02	2866			2*x*	26, 52
Ligulatae Haworth		*S. paniculata* Mill.¶	5	3.20 ± 0.02	3130			2*x*	28
Gymnopera		*S. hirsuta* subsp. *paucicrenata* (Gillot) D. A. Webb¶	5	3.21 ± 0.06	3139			2*x*	28

Chromosome numbers reported in literature are provided (no reports are indicated with "–"). N, number of individuals sampled per taxa.

†1 pg = 978 Mbp (Doležel et al., 2003); ‡Half-peak coefficient of variation; cDNA amount from §Redondo et al. (1996) and ¶Loureiro et al. (2013). References for chromosome numbers in supplementary information (Table S1).

profile began with 4 min at 95 °C followed by 30 cycles of 1 min 30 s denaturing at 94 °C, 2 min annealing at 55 °C and 3 min extension at 72 °C, with a final extension step of 15 min at 72 °C. Furthermore, three plastid regions were also amplified: *rpl32-trnL*, primers rpl32-F and trnL^{UAG} (Shaw et al., 2007), *trnS-trnG-trnG*, primers trnS^{GCU} and 3′trnG^{UUC} (Shaw et al., 2005), and *3′trnV-ndhC*, primers trnV^{UAC}x2 and ndhC (Shaw et al., 2007). The PCR protocols for these regions were carried out as follow: *rpl32-trnL*: 94 °C at 3 min; 34 cycles of 40 s at 95 °C, 40 s for annealing at 54 °C and 1 min 40 s extension at 72 °C, with a final step of 10 min at 72 °C. *trnS-trnG-trnG*: 80 °C, 5 min; 30× (95 °C, 1 min; 66 °C, 4 min); 66 °C, 10 min. *3′trnV-ndhC*: 80 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 1 min, primer annealing at 50 °C for 1 min, followed by a ramp of 0.3 °C/s to 65 °C, and primer extension at 65 °C for 4 min; followed by a final extension step of 5 min at 65 °C. PCR were performed using a Thermocycler (MJ Research PTC 200, Basel, Switzerland). Final PCR products were checked on 1.2% agarose gels. Direct sequencing of the amplified DNA segments was performed using BigDye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, CA), following the protocol recommended by the manufacturer. ITS1, ITS4, rpl32-F, trnL^{UAG}, 3′trnG^{UUC}, and ndhC were used as primers for amplification of the respective regions. Nucleotide sequencing was carried out at the Centres Científics i Tecnològics, Universitat de Barcelona, on an ABI PRISM 3730 DNA Analyzer (PE Biosystems).

Phylogenetic analyses and genetic differentiation

For the phylogenetic analyses, 85 sequences of ITS region (49 new from this study) and 222 plastid sequences were used (3 plastid regions from 74 individuals, see Appendix I for the accession numbers). Nucleotide sequences were edited with Chromas 1.56 (Technelysium Pty, Tewantin, Australia). DNA sequences were aligned visually by sequential pair-wise comparison using BioEdit 7.0.9 (Hall, 1999). Indels were treated as binary characters following the "simple indel coding method" (Simmons & Ochoterena, 2000). *Saxifraga spathularis* (sect. *Gymnopera* D. Don) and *S. aizoides* (sect. *Porphyrion* Tausch.) were used as outgroup.

Data sets were analyzed using jModeltest (Darriba et al., 2012) to determine the sequence evolution model that best described the present data according to the AIC criterion. This model (SYM + G) was used to perform a Bayesian analysis using the program Mr. Bayes 3.2.1 (Huelsenbeck et al., 2001). Four Markov chains were run simultaneously for 10 000 000 generations, and these were sampled every 1000 generations. Data from the first 1000 generations were discarded as the "burn-in" period, after confirming that likelihood values had stabilized prior to the 1000th generation. The 50% majority rule consensus phylogeny and posterior probability (PP) of nodes were calculated from the remaining sample.

TCS 1.21 (Clement et al., 2000) was used to estimate relationships among haplotypes for the three plastid regions concatenated, applying the method of Templeton et al. (1992).

Only nucleotide substitutions were analyzed to this end. Ribotype and haplotype variation was evaluated using DnaSp v5 (Librado & Rozas, 2009).

Results

Palynology and seed morphometry

Pollen viability, assessed by Alexander's test (Alexander, 1969), was >90% in all analyzed populations. For *Saxifraga* ×*cadevallii*, *S.* ×*bubaniana* (*S. geranioides* × *S. pubescens* subsp. *pubescens*) and *S.* ×*jeanpertii* (*S. moschata* × *S. pubescens* subsp. *pubescens*), for which no previous information was available, SEM images revealed that hybrids' seeds were malformed and without the ornamentation showed by the other studied taxa (Mas de Xaxars et al., 2014). Although *S.* ×*cadevallii* pollen grains were as fertile as those of the other species, according to this staining method, its seeds were significantly smaller and did not germinate at all, whereas those of the parental taxa looked normal and germinated in considerable proportions (45–70%). SEM images of seed covers of all pure species confirmed previously described secondary ornamentation (Fernández Areces et al., 1988; Vargas, 1997).

DNA content assessment

The mean of 2C values are shown in Table 1 for all the studied populations. Values ranged from 1.31 pg (*S. moschata*) to 2.59 pg (*S. moschata*, Anglos population). The highest standard deviation (0.135) was found in *S. geranioides*. Natural hybrids analyzed (*S.* ×*cadevallii*, *S.* ×*bubaniana*, and *S.* ×*jeanpertii*) showed intermediate values for DNA content with respect to their parental taxa (Table 1). We found a positive correlation between DNA content (2C, pg) and pollen volume (Spearman correlation coefficient, $r_S = 0.496$, $P = 0.001$). No significant correlation ($r_S = 0.471$, $P = 0.201$) was found between DNA content and the number of chromosomes reported for Pyrenean taxa (Table 1).

ITS phylogeny

The length of the ITS1-5.8S-ITS2 ranged from 663 to 667 bp. Alignment required the introduction of indel characters, and the aligned matrix was 694 bp long. Thirty-six variable sites were detected, 29 of which were parsimony-informative. *Saxifraga* ×*bubaniana* and *S.* ×*jeanpertii* ITS sequences where discarded for analysis and unpublished due to heterozygous gap presence that hampers chromatograms' reading. The tree based on Bayesian inference is shown in Fig. 1. The correspondence between supported clades and geographical areas occurs only in a few cases. Individuals from the Pyrenees are grouped in a single clade with a PP of 100%, with the exception of *S. aquatica*. *Saxifraga fragilis* accessions are the only ones that cluster in a monophyletic clade (PP = 100%). Three of the studied taxa (*S. intricata*, *S. moschata*, and *S. pubescens*) are clearly polyphyletic since they were placed in different and well-supported clades (PP = 100%). The populations of the remaining taxa showed short branches and low support (PP < 85%).

Plastid phylogeny and haplotype network

The aligned matrix of the three plastid regions (*rpl32-trnL*, *trnS-trnG-trnG*, and *3′trnV-ndhC*) was 1813 bp long (554, 606, and 653 bp, respectively). Sixteen variable sites were detected, 12 of them being parsimony-informative. The tree based on 15 different sequences of the 10 taxa sampled showed well-supported clades in the Bayesian inference reconstruction. The network showed a considerable number (27) of missing (extinct or not found) haplotypes. Only one loop was retrieved, which indicated low homoplasy of the plastid sequences and two well-defined clades. Also, the plastid network revealed the complex evolutionary history of the group because there is no clear relationship between haplotype clades and species (Fig. 2). No geographic pattern was detected in the haplotype network. *Saxifraga geranioides* was central to the network with four haplotypes (H1, H5, H12, and H13, Fig. 2) of different lineages. In contrast, we only found one haplotype (H1) for the 13 accessions of the morphologically similar *S. genesiana*, which was also present in two individuals of *S. geranioides*, which is distributed in the eastern Pyrenees (Sger4 and Sger7, Appendix I). Accessions of *S. fragilis* had an internal position, which contrast with their position in the ITS tree (Fig. 1).

Discussion

Interpreting high variation in chromosome numbers with little variation of DNA content

All genome size values provided here are new records for the taxa examined (according to the RBG Kew C-values database, Bennett & Leitch, 2012) and provide a significant increase in nuclear DNA amount information on the genus *Saxifraga*. The rapid estimation of ploidy level using flow cytometry is increasingly gaining appreciation in biosystematic studies (Lysak & Lexer, 2006 and references therein), adopting the concept of DNA ploidy level (Suda & Trávníček, 2006). Comparing with the previous DNA amounts from other sections (Loureiro et al., 2013; Table 1) and the polyploid series of *S. granulata* L. (Redondo et al., 1996; Table 1), Pyrenean *Saxifraga* measured show a diploid DNA level with the exception of one tetraploid population of *S. moschata*, which presents a 2C value of 2.59 pg, that is, 1.9 times higher than the mean value of the species (1.31 pg). Interestingly, this mean value is the minimum observed among the studied taxa and coincides with the value of *S. conifera* (Loureiro et al., 2013; Table 1), which is the sister species to the rest of the subsect. *Triplinervium* in the ITS phylogeny (PP = 100, Fig. 1).

Changes in chromosome numbers between sister lineages not entailing important variations in DNA content are attributed to centric fusions or fissions processes (fusions or centric fissions, e.g., Weiss-Schneeweiss & Schneeweiss, 2013). Meanwhile doubling or near-doubling in chromosome number are often assumed to result from tetraploidy events (Jones, 1998; Wood et al., 2009). Here, we observe homogeneous 2C values in Pyrenean taxa (1.3-fold variation, Table 1), but the maximum chromosome number near-triplicates the minimum (64 vs. 26, Table 1). In a recent study, Fishman et al. (2014) point that chromosome number increase by fission has been relatively unexplored and can be difficult to distinguish from polyploidy when gaps in chromosome number are large. This could explain some important changes in chromosome numbers in the species studied not accompanied by relevant genome size variation (Table 1). In any case, such explanation

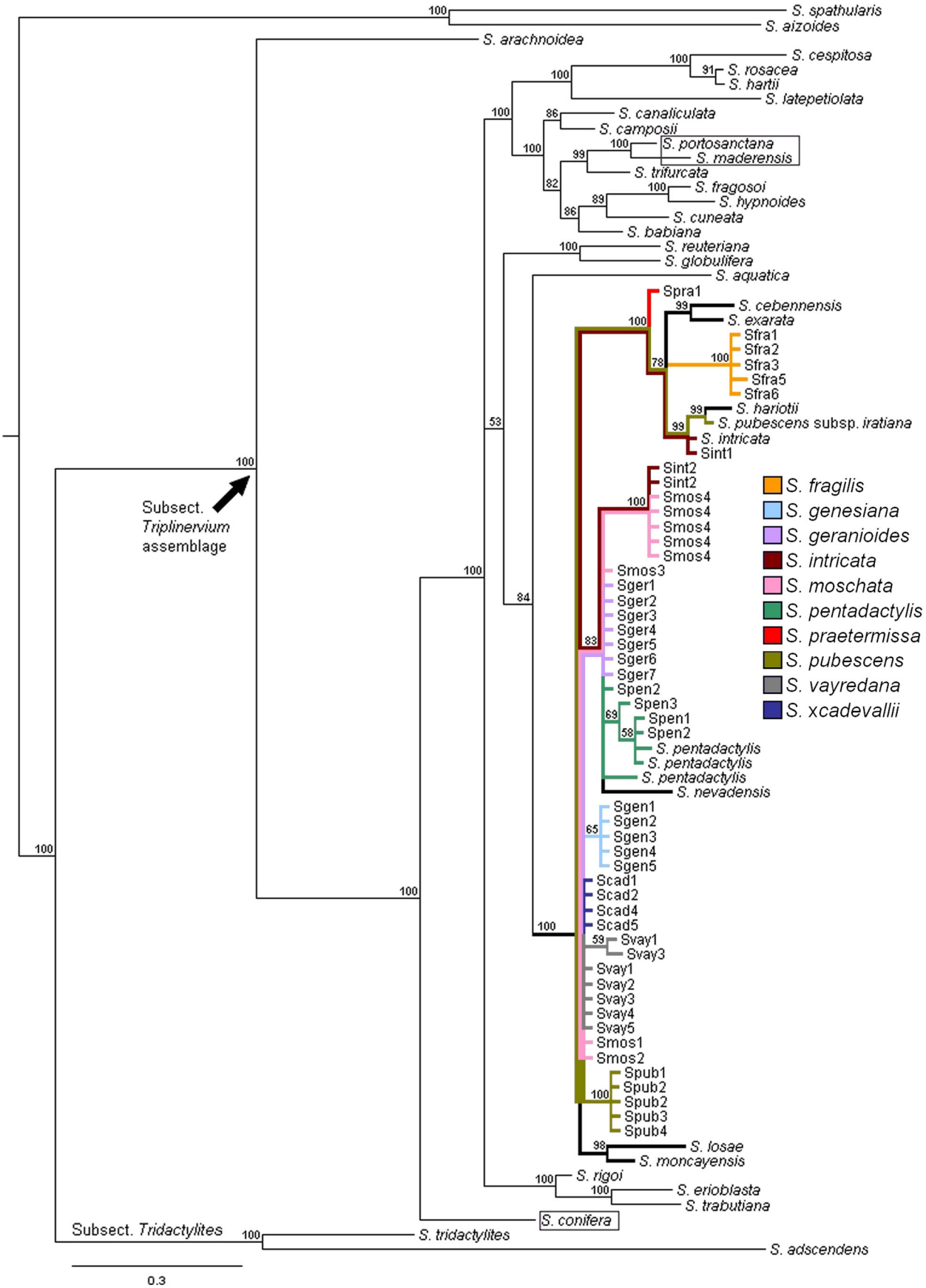

Fig. 1. Continued

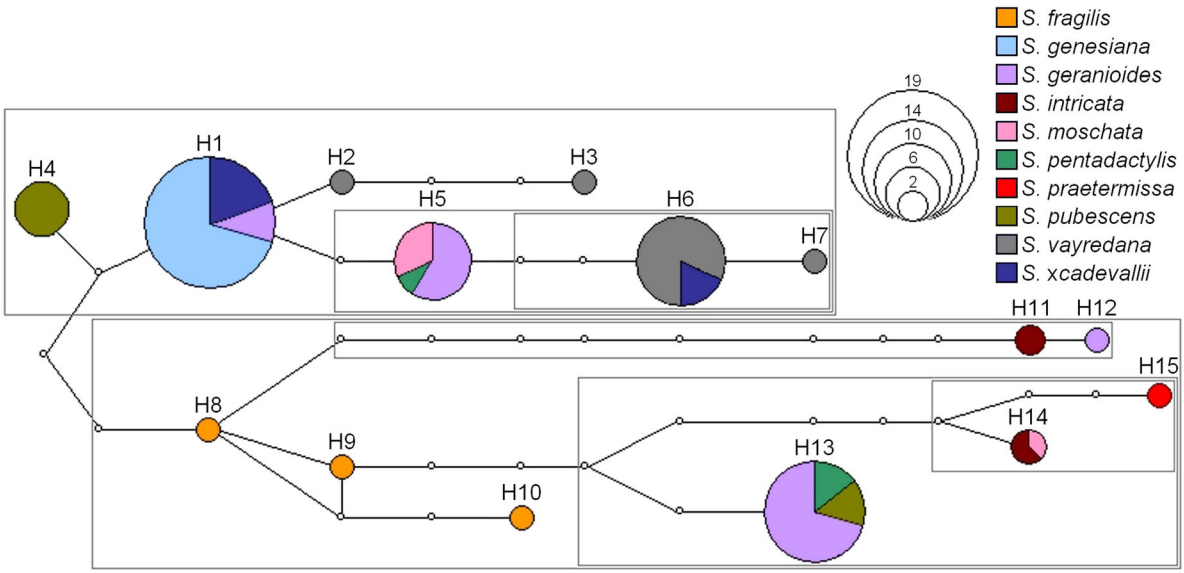

Fig. 2. Haplotype network from three plastid regions (*rpl32-trnL*, *trnS-trnG-trnG*, and *3′trnV-ndhC*). Circle sizes are proportional to haplotype frequencies. Small circles represent single mutational steps. The gray squares correspond to lineages with a PP $\geq$ 0.95 in the tree obtained by Bayesian inference.

would entail the centric fission of all the chromosomes at least one time, which can be considered a rather dramatic, not highly probable cytogenetic event. Further studies are needed in *Saxifraga* to determine the importance of centric fissions, since it can neither be appreciated in chromosomal preparations (due to the above-mentioned technical difficulties) nor be inferred only with genome size data.

Autopolyploidy also seems to be a prevalent phenomenon in the Saxifragaceae (Soltis et al., 2007, supported by *S. moschata*, this study). In addition to the mentioned *S. moschata* population, we detected increase in DNA content in the Madeiran *Saxifraga* taxa. Colonization of the Madeiran archipelago by this genus appears to have occurred from a Northern-Iberian ancestor in a single event (Vargas et al., 1999). DNA ploidy levels suggest that colonization started in Porto Santo island, where a first autopolyploidization was experienced (*S. portosanctana*, 2.92 pg, Table 1), and a second autopolyploidization was experimented in the colonization of Madeira island (*S. maderensis*, 7.52 pg, Table 1). In this case, the increase of ploidy level can be correlated with the polyploid series of *S. granulata* ($R^2 = 0.8544$). Autopolyploidization could have played an important role in the adaptation of *Saxifraga* to the new climatic conditions and lowers altitudes of the Madeiran archipelago in comparison with their Iberian ancestors' habitat.

These results suggest a combination of multiple cytogenetic mechanisms (mainly centric fissions and autopolyploidy) operating in karyotype evolution in this group of *Saxifraga*. All these in concert with other sources of chromosome variation as the hybridization and the B chromosomes presence (see next subheadings). Nevertheless, we can infer that the ancestor of subsection *Triplinervium* was a diploid, with small DNA amount (2C value about 1.30 pg) and with a basic chromosome number most likely of $x = 11$ as *S. granulata* (Redondo et al., 1996) and the members of the sister subsection *Tridactylites* (Haw.) Gornall (Brochmann et al., 1996; Fig. 1).

Hybrid speciation in Pyrenean *Saxifraga* occurred mostly in homoploidy

Studied Pyrenean taxa form a well-supported clade in ITS Bayesian tree (PP = 100%, Fig. 1), which reinforces that the Pyrenees could have acted as a refuge for these *Saxifraga* species. However, the relationships within the clade remain unclear. Lack of resolution is due in part to MrBayes treatment of polymorphism as uncertainty (Huelsenbeck et al., 2001) so that rDNA loci intragenomic variability cannot be discerned. The short length of most branches (Fig. 1) suggests that these taxa might have arisen recently.

The phylogenetic distance observed between some western Pyrenean specimens (*S. intricata* [Sint1, Appendix I] and *S. pubescens* subsp. *iratiana*, Fig. 1) and the eastern specimens from the same taxa (*S. intricata*: Sint2, Appendix I; and *S. pubescens* subsp. *pubescens*: Spub1, 2, 3, and 4, Table 1) could reveal an hybridization event followed by bidirectional concerted evolution. In the case of *S. pubescens*, the geographical disjunction is related with some morphological differentiation that allows defining the two subspecies (Vargas, 1997). Thus, *S. intricata* and *S. pubescens* have probably arisen from the hybridization between Pyrenean

Fig. 1. Majority rule consensus tree from Bayesian inference based on sequences of the internal transcribed spacer (ITS) of nuclear ribosomal DNA. The figures above the branches are the posterior probabilities. In box non-Pyrenean species with known genome size data (Table 1). Accession numbers and population code of new samples in Appendix I.

taxa on the one side and a *S. fragilis* ancestor (or a related taxon) on the other side, resulting in species with similar DNA content (Table 1). Interior (ancient) haplotypes in the network support this statement.

Moreover, *S. moschata* seems to have played an important role in Pyrenean *Saxifraga* hybrid speciation. This is supported by the shared ribotypes, its wide distribution, its great morphological polymorphism, and the ease of hybridization with other section congeners (Vargas, 1997). DNA content variation also agrees with a central role of *S. moschata* with the lowest values and *S. fragilis* with one of the highest.

Spontaneous hybrids are frequent in *Saxifraga*, but, at least in subsect. *Triplinervium*, introgression does not occur. Genome size in all specimens of the three hybrid taxa was close to the expected mean of their putative parents (means in Table 1) and proves the utility of these tool to detect hybridization when DNA amount was significantly different between parental taxa (Table 1, see also Garcia et al., 2008). Seed morphology studies and germination assays in *S.* ×*cadevallii* showed that seeds from hybrid specimens are unviable, because they cannot complete their development. This was also found in *S.* ×*bubaniana* and *S.* ×*jeanpertii*. A general pattern was described for this group of *Saxifraga*, in which barriers are formed at the F2 generation (Vargas & Nieto-Feliner, 1996). Thus, and because pollen seems fertile, hybrids sterility occurs at postzygotic level. No paternal effects were observed in *S.* ×*cadevallii* specimens' morphology with known haplotype (like Vargas & Nieto-Feliner, 1996 in reciprocal crossings experiment). This discards nucleocytoplasmic incompatibility (Levin, 2003) and points toward other mechanisms as being responsible for hybrid sterility, such as chromosomal rearrangements related with the substantial difference in chromosome number of the parental species (Table 1).

Our DNA content assessments suggest that all measured taxa have same DNA ploidy level (except one *S. moschata* population, Table 1). Therefore, hybrid speciation in Pyrenean *Saxifraga* may have occurred in homoploidy (in the sense that there is no change in the hybrid ploidy level in respect to their parental taxa), but involving taxa that display very different chromosome numbers (range from 26 to 64, Table 1). But hybrid speciation without genome doubling and/or ecological differentiation is difficult to be complete (Soltis & Soltis, 2009), especially if hybrids cannot self-perpetuate, as in our case. We propose that homoploid hybrid speciation has been historically successful, probably due to the own cytological instability of the group, which can help hybrids to become stabilized by chromosomal rearrangements (Grant, 1958). Thus, *Saxifraga* spontaneous hybrids constitute a F1 generation that rarely would complete the hybrid speciation process. Therefore, we no longer will use specific nomenclatural binomia when referring to them.

Contacts between the Pyrenees and the neighboring Montseny-Guilleries massif

Contacts between a population of *S. geranioides* from the Eastern Pyrenees (Puigmal, Sger7, Appendix I) and those of the morphologically similar taxon *S. genesiana* from Montseny-Guilleries are suspected based on geographic proximity, similar DNA amount, and the presence of the same chloroplast haplotype (H1, Appendix I). In addition, because Puigmal population contains both haplotypes H1 and H5 (Appendix I), colonization could have occurred in a single or in multiple events, but only a single maternal line (H1 haplotype, Fig. 2) is remaining in *S. genesiana*. Moreover, the high intra- and inter-populational variability found in the DNA amounts of *S. geranioides* ($2C = 1.700 \pm 0.135$ pg) and the higher homogeneity in *S. genesiana* ($2C = 1.750 \pm 0.043$ pg) could also reflect phylogeographic patterns in agreement with a bottleneck experienced during Montseny-Guilleries colonization.

The new chromosome number reported for *S. geranioides* ($2n =$ ca. 44, Mas de Xaxars et al., 2014) also supports this colonization history. The big difference in chromosome number from previous reports ($2n =$ ca. 52, Jones, 1982; Vargas, 1994) could be explained by the presence of B chromosomes, which cannot be discerned from A chromosomes by size as in the case of *S. virginiensis* Michx. (Soltis, 1983). In fact, Küpfer (1971) reported B chromosomes in *S. pentadactylis* subsp. *pentadactylis* (Table 1). Is known that B chromosomes could be spontaneously generated in response to new genomic conditions after a hybridization event (Houben et al., 2013) or could be acting as diploidizing agents in some polyploids (Jones & Houben, 2003). As it has been seen in maize (Lamb et al., 2005) and rice (Houben et al., 2013), B chromosomes are enriched with DNA elements that are normally found at or near A chromosome centromeres. Thus, their presence in *Saxifraga* could be attributed to the centric fissions experienced, supporting the above-stated hypothesis to explain dramatic chromosome number changes without DNA much content variation. Finally, B chromosomes could be one of the sources of the intra-specific variation in DNA amounts (Jones et al., 2008) that we observe in *S. geranioides*.

Conclusions

The taxa of *Saxifraga* subsect. *Triplinervium* endemic to NE Iberian Peninsula appear to be the result of recurrent isolation and hybridization processes and show a great complexity as indicated by numerous cytotaxonomic indicators. Our multiple dataset approach (morphological, hybrid viability, chromosomal, DNA content, molecular) provided significant data to infer how karyological evolution occurred. New genome size data presented in this study prove that all Pyrenean taxa have the same ploidy level and thus, the great chromosome number variability within and between taxa is explained by the conjunction of centric fission processes, B chromosome presence and homoploid hybrid speciation. Autopolyploidy can also occur in some populations (*S. moschata*), but it seems more successful when hybridization is not possible, such as in the case of the Madeiran archipelago and probably has a role in the adaptation of these taxa to the new climatic conditions. In addition, we proved the utility of DNA ploidy estimations by genome size in lineages with small chromosomes and very active dynamic of chromosome number change.

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Appendix 1

Voucher information and GenBank accession numbers for samples obtained in this study following format: taxon name, population code, location, ITS accession number (—, missing data). CatM, Catalanidic Mountains; CP, Central Pyrenees; EP, Eastern Pyrenees; MG, Montseny-Guilleries; TS, Catalan Transversal System; WP, Western Pyrenees.

Saxifraga fragilis Schrank, **Sfra1**, TS, El Far (Catalonia, Spain), KJ774137, **Sfra2**, CatM, Sant Sadurní de Gallifa (Catalonia, Spain), KJ774138, **Sfra3**, CatM, Morella (Spain), KJ774139, **Sfra4**, WP, San Juan de la Peña (Spain), —, **Sfra5**, WP, Peña de Oroel (Spain), KJ774140, **Sfra6**, CatM, Prades mountains (Catalonia, Spain), KJ774141, **Sfra7**, EP, Corberes mountains (France), —; ***S. genesiana*** P. Vargas, **Sgen1**, MG, Sant Hilari Sacalm (Catalonia, Spain), KJ774142, **Sgen2**, MG, Coll de Sabènia (Catalonia, Spain), KJ774143, **Sgen3**, MG, Puig Sacarbassa (Catalonia, Spain), KJ774144, **Sgen4**, MG, Turó Gros (Catalonia, Spain), KJ774145, **Sgen5**, MG, Sot de les Cordes (Catalonia, Spain), KJ774146, **Sgen6**, MG, Sant Miquel de Solterra (Catalonia, Spain), —; ***S. geranioides*** L., **Sger1**, EP, Ulldeter (Catalonia, Spain), KJ774147, **Sger2**, CP, Estany de Tristaina (Andorra), KJ774148, **Sger3**, CP, Estret de l'Estanyó (Andorra), KJ774149, **Sger4**, CP, Cim de l'Estanyó (Andorra), KJ774150, **Sger5**, CP, Estanyet de Besiberri (Catalonia, Spain), KJ774151, **Sger6**, CP, Portella d'Orlu (France), KJ774152, **Sger7**, EP, Puigmal 2 (Catalonia, Spain), KJ774153; ***S. intricata*** Lapeyr., **Sint1**, WP, Baños de Panticosa (Spain), KJ774154, **Sint2**, CP, Coth der Estanh (Catalonia, Spain), KJ774155, KJ774156, **Sint3**, CP, Mont Lude (Catalonia, Spain), —; **Sint4**, CP, Estanh Long de Vilamòs (Catalonia, Spain), —; ***S. maderensis*** D. Don, —; ***S. moschata*** Wulfen, **Smos1**, EP, Puigmal 1 (Catalonia, Spain), KJ774157, **Smos2**, EP, Coll de la Marrana (Catalonia, Spain), KJ774158, **Smos3**, CP, Estany de Besiberri (Catalonia, Spain), KJ774159, **Smos4**, CP, Coll d'Anglos (Catalonia, Spain), KJ774160, KJ774161, KJ774162, KJ774163, KJ774164; ***S. pentadactylis*** Lapeyr. subsp. ***pentadactylis***, **Spen1**, EP, Cim de Bastiments (Catalonia, Spain), KJ774165, **Spen2**, CP, Arcalís (Andorra), KJ774166, KJ774167, **Spen3**, CP, Estret de l'Estanyó (Andorra), KJ774168; ***S. portosanctana*** Boiss., —; ***S. praetermissa*** D. A. Webb, **Spra1**, CP, Estanhó d'Escunhau (Catalonia, Spain), KJ774169; ***S. pubescens*** Pourr. subsp. ***pubescens***, **Spub1**, EP, Núria (Catalonia, Spain), KJ774170, **Spub2**, EP, Ulldeter (Catalonia, Spain), KJ774171, **Spub3**, EP, Puigmal 1 (Catalonia, Spain), KJ774172, **Spub4**, EP, Puigmal 2 (Catalonia, Spain), KJ774173; ***S. vayredana*** Luizet, **Svay1**, MG, Sant Hilari Sacalm (Catalonia, Spain), KJ774174, KJ774175, **Svay2**, MG, Coll de Sabènia (Catalonia, Spain), KJ774176, **Svay3**, MG, Puig Sacarbassa (Catalonia, Spain), KJ774177, KJ774178, **Svay4**, MG, Turó Gros (Catalonia, Spain), KJ774179, **Svay5**, MG, Sot de les Cordes

(Catalonia, Spain), KJ774180; ***S.*** ×***bubaniana*** Engl. & Irmsch., **Sbub1**, EP, Ulldeter (Catalonia, Spain), —; ***S.*** ×***cadevallii*** Luizet, **Scad1**, MG, Sant Hilari Sacalm (Catalonia, Spain), KJ774181, **Scad2**, MG, Coll de Sabènia (Catalonia, Spain), KJ774182, **Scad4**, MG, Turó Gros (Catalonia, Spain), KJ774183, **Scad5**, MG, Sot de les Cordes (Catalonia, Spain) KJ774184; ***S.*** ×***jeanpertii*** Luizet, **Sjea1**, EP, Núria (Catalonia, Spain), —.

GenBank accession numbers of the plastid regions used in this study showed by population and haplotype (*rpl32-trnL*, *trnS-trnG-trnG* and *3′trnV-ndhC*).

Sfra1, H9 (KJ774259, KJ774185, KJ774333); Sfra2, —; Sfra3, —; Sfra4, —; **Sfra5**, H10 (KJ774260, KJ774186, KJ774334), **Sfra6**, H8 (KJ774261, KJ774187, KJ774335); Sfra7, —; **Sgen1**, H1 (KJ774262, KJ774188, KJ774336), H1 (KJ774263, KJ774189, KJ774337), H1 (KJ774264, KJ774190, KJ774403); **Sgen2**, H1 (KJ774265, KJ774191, KJ774338), H1 (KJ774266, KJ774192, KJ774339); **Sgen3**, H1 (KJ774267, KJ774193, KJ774340), H1 (KJ774268, KJ774194, KJ774341), H1 (KJ774269, KJ774195, KJ774342), H1 (KJ774270, KJ774196, KJ774343); **Sgen4**, H1 (KJ774271, KJ774197, KJ774344), H1 (KJ774272, KJ774198, KJ774345), H1 (KJ774273, KJ774199, KJ774346); **Sgen5**, H1 (KJ774274, KJ774200, KJ774347); Sgen6, —; **Sger1**, H5 (KJ774276, KJ774202, KJ774349), H5 (KJ774277, KJ774203, KJ774350), H13 (KJ774275, KJ774201, KJ774348), H13 (KJ774278, KJ774204, KJ774351), H13 (KJ774279, KJ774205, KJ774352), H13 (KJ774280, KJ774206, KJ774353), H13 (KJ774281, KJ774207, KJ774354); **Sger2**, H5 (KJ774283, KJ774208, KJ774356), H13 (KJ774282, KJ774209, KJ774355), H13 (KJ774284, KJ774210, KJ774404); **Sger3**, H12 (KJ774285, KJ774212, KJ774358), H13 (KJ774286, KJ774211, KJ774357); **Sger4**, H1 (KJ774287, KJ774213, KJ774359); **Sger5**, H13 (KJ774288, KJ774214, KJ774360), H13 (KJ774289, KJ774215, KJ774361); **Sger6**, H5 (KJ774290, KJ774216, KJ774362); **Sger7**, H1 (KJ774292, KJ774217, KJ774363), H5 (KJ774291, KJ774218, KJ774364) H5 (KJ774293, KJ774219, KJ774365); **Sint1**, H14 (KJ774294, KJ774220, KJ774366), H14 (KJ774295, KJ774221, KJ774367); **Sint2**, H11 (KJ774296, KJ774222, KJ774368), H11 (KJ774297, KJ774223, KJ774369); Sint3, —; Sint4, —; **Smos1**, H5 (KJ774298, KJ774224, KJ774405); **Smos2**, H5 (KJ774299, KJ774225, KJ774370); Smos3, —; **Smos4**, H14 (KJ774300, KJ774226, KJ774371); **Spen1**, H13 (KJ774301, KJ774227, KJ774372); **Spen2**, H13 (KJ774302, KJ774228, KJ774373); **Spen3**, H5 (KJ774303, KJ774229, KJ774374); **Spra1**, H15 (KJ774304, KJ774230, KJ774375); Spub1, —; **Spub2**, H4 (KJ774305, KJ774231, KJ774376), H4 (KJ774306, KJ774232, KJ774377), H4 (KJ774307233, KJ774, KJ774378), H4 (KJ774308, KJ774234, KJ774379), H4 (KJ774309, KJ774235, KJ774380), H4 (KJ774311, KJ774237, KJ774382), H13 (KJ774310, KJ774236, KJ774381); **Spub3**, H13 (KJ774312, KJ774238, KJ774383); Spub4, —; **Svay1**, H6 (KJ774313, KJ774239, KJ774406), H6 (KJ774314, KJ774240, KJ774384), H6 (KJ774315, KJ774241, KJ774385), H6 (KJ774316, KJ774242, KJ774386); **Svay2**, H2 (KJ774317, KJ774243, KJ774387), H6 (KJ774318, KJ774244, KJ774388), H6 (KJ774319, KJ774245, KJ774389); **Svay3**, H3 (KJ774321, KJ774247, KJ774391), H6 (KJ774320, KJ774246, KJ774390), H6 (KJ774322, KJ774248, KJ774392); **Svay4**, H6 (KJ774323, KJ774249, KJ774393), H7 (KJ774324, KJ774250, KJ774394); Svay5, —; Sbub1, —; **Scad1**, H1 (KJ774327, KJ774253, KJ774397), H6 (KJ774325, KJ774251, KJ774395), H6 (KJ774326, KJ774252, KJ774396), H6 (KJ774328, KJ774254, KJ774398); **Scad2**, H1 (KJ774329, KJ774255, KJ774399); **Scad4**, H1 (KJ774330, KJ774256, KJ774400), H1 (KJ774331, KJ774257, KJ774401); Scad5, —; **Sjea1**, H5 (KJ774332, KJ774258, KJ774402).

GenBank accession numbers for ITS sequences published in previous works used in this study.

S. adscendens L., EF028688; ***S. aquatica*** Lapeyr., AF261161; ***S. arachnoidea*** Sternb., AF261162; ***S. aizoides*** L., AF504547; ***S. babiana*** T. E. Díaz & Fern. Prieto, AJ233879; ***S. camposii*** Boiss. & Reut., AF261170; ***S. canaliculata*** Boiss. & Reut., AF261170; ***S. cebennensis*** Rouy & E. G. Camus, AF261169; ***S. cespitosa*** L., AF261170; ***S. conifera*** Coss. & Durieu, AJ233865; ***S. cuneata*** Willd., AJ233881; ***S. erioblasta*** Boiss. & Reut., AJ233866; ***S. exarata*** Vill., AJ233861; ***S. fragosoi*** Sennen, AJ233867; ***S. globulifera*** Desf., AJ233874; ***S. hariotii*** Luiz. & Soulié, AF261181; ***S. hartii*** D. A. Webb, AF261189; ***S. intricata***, AJ133030; ***S. latepetiolata*** Willk., AF261183; ***S. losae*** Sennen, AJ133027; ***S. maderensis***, AJ233882; ***S. moncayensis*** D. A. Webb, AJ133028; ***S. nevadensis*** Boiss., AF261185; ***S. pentadactylis***, AJ133031, AY354307, AY354308; ***S. portosanctana***, AJ233883; ***S. pubescens*** subsp. ***iratiana*** (F. W. Schultz) Engl. & Irmsch., AF261187, AF261188; ***S. reuteriana*** Boiss., AJ233874; ***S. rigoi*** Porta, AJ233878; ***S. rosacea*** Moench, AF261190; ***S. spathularis*** Brot., KC749988; ***S. trabutiana*** Engl. & Irmsch., AF482693; ***S. tridactylites*** L., EF028687; ***S. trifurcata*** Schrad., AJ233885.

Supplementary Material

The following supplementary material is available online for this article at http://onlinelibrary.wiley.com/doi/10.1111/jse.12105/suppinfo:

Table S1. Compilation of chromosome counts and their references of taxa from *Saxifraga* subsect. *Triplinervium* with available DNA content assessment.