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Natural hybridisation and phylogenetic position of *Saxifraga trabutiana* (Saxifragaceae) inferred from ISSR markers and ITS sequences

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Natural hybridisation between *Saxifraga trabutiana* and *Saxifraga granulata*, two species belonging to different series of sect. *Saxifraga*, is reported. The hybrid, *S.* × *sorianoi,* is endemic to the high mountains of Sierra de Filabres (Spain). The high morphological variation of *S. granulata* makes it difficult to differentiate all individual variants of this species from true hybrids found in sympatric populations. To test the hypothesis of the hybrid origin of *S.* × *sorianoi,* we have used parental-specific molecular markers, obtained by ISSR (Inter-Simple Sequence Repeat) analysis and restriction of ITS (Internal Transcribed Spacer) sequences. In agreement with that hypothesis, marker profiles showed strong additivity according to its parental species. On the other hand, the ITS sequence of *S. trabutiana* was analysed along with those from other species of sect. *Saxifraga* to determine its relative position within this complex group. *Saxifraga trabutiana* clustered with *S. erioblasta* and *S. rigoi*, all of them classified into series *Gemmiferae*. Conversely, other taxa of the same series such as *S. globulifera*, which is morphologically very similar to *S. trabutiana*, are scattered over different clusters. This is in line with what has been described for other plants of sect. *Saxifraga*. Discrepancies between molecular phylogeny and the relationships inferred from morphological data are discussed in a general context of reticulation within sect. *Saxifraga*.

Key words: conservation, molecular markers, natural hybridisation, reproductive barriers, *Saxifraga* × *sorianoi*, taxonomy

Introduction

Genus *Saxifraga* comprises more than 500 species spread over Europe, northern Africa, Asia, and the American continent (Webb & Gornall 1989). Hybridisation within this genus, even among distantly related species, is a well-documented phenomenon (Engler & Irmscher 1916,

Luizet 1931, Webb & Gornall 1989). However, few reports deal with the molecular characterisation of such hybrids. In a previous work, we have described the morphological characterisation of a putative hybrid between *S. trabutiana* and *S. granulata*, named *S.* × *sorianoi* (García-Maroto & Gómez-Mercado 2002). Although it shared traits with both progenitors, the hybrid is more similar to *S. granulata*. However, great morphological variation has been found within *S. granulata*. Engler and Irmscher (1916), for instance, described four subspecies of this plant, comprising four varieties and nine forms. Therefore, it was conceivable that the putative hybrid plants just represented extreme morphological variants within the *S. granulata* population.

Saxifraga trabutiana and *S. granulata* belong to sect. *Saxifraga*, an extremely heterogeneous group comprising more than 70 species mainly distributed across Europe and northern Africa (Webb & Gornall 1989). According to the most recent classifications (Webb & Gornall 1989, Vargas 1997), *S. granulata* is included in series *Saxifraga*. This series consists of plants characterised by presence of underground axillary buds (bulbils from which rooting and development of stems occurs). *Saxifraga granulata* shows a wide distribution in Eurasia and northwest Africa, and it is present in most of the Iberian Peninsula. On the other hand, *S. trabutiana* belongs to series *Gemmiferae*, which contains species with summer-dormant buds. It is distributed along the North of Africa (Morocco and Algeria) and Sierra de Filabres of Spain (Vargas 1997). Although it has been morphologically characterised (Engler & Irmscher 1916, Vargas 1997), phylogenetic relationships between *S. trabutiana* and other plants of sect. *Saxifraga* have not been investigated before. This is relevant since discrepancy between morphology and molecular phylogeny has been previously reported for plants of this section (Soltis *et al*. 1996, Vargas 2000). Moreover, *S. trabutiana* has been recently included in a number of red lists under the UICN category of vulnerable (Aizpuru *et al*. 2000, Blanca *et al*. 2002). This raises a considerable interest in the study of *S.* × *sorianoi* since an endangered status can be inferred for these plants.

In order to assess the hybrid origin of *Saxifraga* × *sorianoi*, we followed a combined molecular approach by using Inter-Simple Sequence Repeat (ISSR) markers and restriction polymorphism analysis of the Internal Transcribed Spacer (ITS). ISSR markers have been previously shown to be useful tools to address questions of hybridisation and introgression in natural populations, due to the relative simplicity and high resolution of the technique (Wolfe & Liston 1998). Studies related to conservation genetics have been conducted on *Saxifraga* using ISSR among other markers (Hollingsworth *et al*. 1998). Analysis of sequences from the nrDNA ITS region is a common tool in phylogenetic studies of the angiosperms (Baldwin 1992). In particular, comprehensive phylogenetic studies have been conducted in *Saxifraga*, based on ITS and other genes (Soltis *et al*. 1993, Soltis *et al*. 1996, Vargas *et al*. 1999, Conti *et al*. 1999, Vargas 2000). ITS sequences have also been used to investigate the origin of plant polyploid genomes. While in some instances additivity was observed (Van Houten *et al*. 1993, Baldwin *et al*. 1995, Wendel *et al*. 1995), in other cases homogenization of the ITS sequences in the direction of one of the progenitors is reported (Kim & Jansen 1994, Wendel *et al*. 1995, Brochmann *et al*. 1996). Utilization of ITS data was problematic in the case of *S. svalbardensis* since it could not discriminate between different hybridization hypotheses (Brochmann *et al*. 1998).

In our study, we have obtained initial evidences on the hybrid origin of *Saxifraga* × *sorianoi*, based on the mainly biparentally inherited ISSR markers. To reinforce our preliminary results, the ITS sequences for the putative parental species were determined. This allowed the development of specific markers generated by restriction of the ITS fragments from both parents, in order to investigate their presence in the putative hybrid. On the other hand, the comparative analysis of the ITS sequence from *S. trabutiana* allowed us to discuss its phylogenetic position and relationships with other species of sect. *Saxifraga*.

Material and methods

Plant materials and DNA extraction

Plant material was collected at different locations of Sierra de Filabres (Almería, Spain) during

the spring of years 2000 and 2001, and stored at –70 °C until processing. Individuals were sampled at distances of at least 10 m in each population. Pooled leaves corresponding to single plants were analysed. The hybridisation area, corresponding to the syntopic population of *Saxifraga granulata* and *S. trabutiana*, was located at the "Barranco del Negro" (30SWG4021) at about 1900 m of altitude. Ten putative hybrid plants, located at different patches, and thus probably representing single hybridisation events (F_1 plants), were collected. For *S. granulata*, 15 individuals were taken from three different populations (including 5 individuals in the hybridization area) located at least 5 km apart. In addition, eight individuals of the single *S. trabutiana* population situated close to the hybridisation area were analysed. The differentiation between the putative hybrid and parental species was made on morphological characteristics. Contrary to *S. trabutiana*, the putative hybrid displays well developed leaf blades, rosetted growth habit, and underground bulbils instead of summer-dormant buds. It is distinguished from *S. granulata* in that the leaves are flabellated, deeply emarginated and with fewer lobes. Voucher specimens (GDAC 44583) are deposited on the GDAC herbarium of the Universidad de Granada (Spain). Access to the photographic register of the voucher is also available at http://www.ugr.es/herbario.

Total DNA was extracted from frozen leaf tissues using the DNeasy-kit (QIAGEN) following the manufacturer's protocol.

ITS analysis

The flanking primers used for PCR amplification of the ITS region, ITS-UP (5´-AGTCGTAACAAGGTTTCCGTAGGT-3´) and ITS-DN (5´-CCTCCGCTTATTGATATGCTTAAACTC-3´), were specifically designed by us from the 18S-26S rDNA region of Saxifragaceae species and purchased from SIGMA-GENOSYS. Reaction mixtures contained 10 ng of total DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 mM of the flanking primers and 0.6 units of Taq DNA polymerase (GIBCO BRL), in a final volume of 25 μl. The PCR was performed in a 2400 Perkin Elmer Thermocycler using the following program. A denaturation step of 2 min at 94 °C, followed by 38 cycles of 15 sec at 94 °C, 45 sec at 55 °C and 90 sec at 72 °C, and a final elongation cycle of 5 min at 72 °C. PCR products were analysed in a 1.3% agarose gel and bands extracted using the QIAEX II Agarose Gel Extraction kit (QIAGEN), following instructions from the manufacturer. These products were directly sequenced in both strands using the primers ITS-UP and ITS-DN, in an automated ABI Prism 377 sequencer.

For the restriction analysis of the ITS fragments, five samples from each of the hybrid and putative parental species were used. About 100 ng of the ITS fragments generated as described before were digested with the restriction enzymes *Xho*I and *Ava*I (SIGMA), according to the manufacturer's instructions. Both enzymes are six-cutters with recognition sites CTCGAG for *Xho*I and CPyCGPuG for *Ava*I. These enzymes were chosen since different restriction patterns are predicted from the ITS sequences of *Saxifraga granulata* and *S. trabutiana*. The resulting fragments were separated on 1.2% agarose gels followed by staining with ethidium bromide.

ISSR analysis

Ten different primers were initially assayed in ISSR analysis, of which seven produced suitable banding patterns. The oligonucleotides used, IS-B: $(CA)_6AC$; IS-C: $(CA)_6GT$; IS-E: $(CA)_6GG$; IS-F: $(GAA)_6$; IS-I: $(GA)_6GG$; IS-J: $(GA)_6CC$; IS-G: $VMV(GT)_7$ were purchased from SIGMA-GENOSYS. The PCR reaction mixtures contained 10 ng of DNA, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of the primer and 0.6 units of Taq DNA polymerase (GIBCO BRL), in a final volume of 25 μl. PCR reaction consisted of a denaturation step of 2 min at 94 °C, followed by 40 cycles of 15 sec at 94 °C, 45 sec at 44–45 °C (for primers IS-B,-C,-E, -I, and -J) or 55 °C (primer IS-G) and elongation for 1 min and 30 sec at 72 °C, followed by an extension of 8 min at 72 °C. Amplification products were run in 1.3% agarose gels and visualised by staining with ethidium bromide. Only clear, well-resolved amplification products were scored and reproduced in at least two experiments. Species specific markers were selected based on their

presence in all individuals of one of the putative parents and absence in at least 90% of individuals of the other one.

Phylogenetic analysis

The ITS sequences obtained in this work for *Saxifraga granulata* (acc. no. AF482692) and *S. trabutiana* (acc. no. AF482693) were aligned with those of 42 species of sect. *Saxifraga* selected from GenBank, including that of *S. granulata* obtained from Italy (accession numbers were given in Vargas *et al*. 1999, 2000) plus *Saxifraga spathularis* (sect. *Cymbalaria*). The alignment was performed using the program ClustalX v.1.8 (Thompson *et al*. 1994) with minor manual modifications. Fitch parsimony analysis was carried out using the PHYLIP v.3.6 (Phylogeny Inference Package) software (Felsenstein 2001). Bootstrap searches were performed after 100 replicates on the contig file, and the resulting file was fed to the PROTPARS protein parsimony utility under the multiple data set option. The analysis was performed with randomized input of sequences, equal weighting of all characters and of transitions/transversions. *Saxifraga spathularis* was used as the outgroup based on previous data (Webb & Gornall 1989, Vargas 2000). The consensus tree was generated by the CONSENSE program with the majority rule option, and bootstrap values were assigned to each node.

Results

Inter-Simple Sequence Repeat (ISSR) analysis

Ten ISSR primers were tested in a preliminary search for species-specific markers. Seven of these primers screened yielded useful PCR patterns and were selected for further analysis. A total of 60 clear and reliable bands were scored, of which 12 were exclusive to *Saxifraga trabutiana*, and 4 to *S. granulata* (Table 1). Additive profiles of parental specific bands were observed in most individuals of the putative hybrid population (Table 1 and Fig. 1). Thirteen of these markers were present in all hybrid plants tested. Lower frequencies (0.9) in the hybrid population were obtained for the other three specific markers (SGR-B1, STR-E1, SGR-E2). Since ISSR markers are inherited in a dominant way, a possible explanation for this is that for some of the hybrid plants the parent was heterozygous for the concerned loci. Alternatively, as we did not sample all plants in parental populations, it might be that some of the chosen markers were not in fact species-specific, being absent from some parents.

Table 1. Relative frequencies of the ISSR bands in 8, 15 and 10 individuals, respectively, of *S. trabutiana* (STR), *S. granulata* (SGR) and hybrid populations.

Primer	Marker	Size (bp)	*S. trabutiana*	*S. granulata*	Hybrid population
IS-B	STR-B1	575	1	0	1
	STR-B2	820	1	0	1
	SGR-B1	870	0	0.93	0.90
IS-C	STR-C1	540	1	0	1
	STR-C2	825	1	0	1
	STR-C3	1225	1	0	1
IS-E	STR-E1	550	1	0.07	0.90
	STR-E2	775	1	0	1
	SGR-E2	615	0	1	0.90
IS-G	STR-G1	675	1	0	1
	STR-G2	1260	1	0.13	1
	SGR-G1	1025	0	1	1
IS-I	STR-I1	455	1	0	1
	STR-I2	500	1	0.13	1
	STR-I3	1480	1	0	1
IS-J	SGR-J1	1200	0	1	1

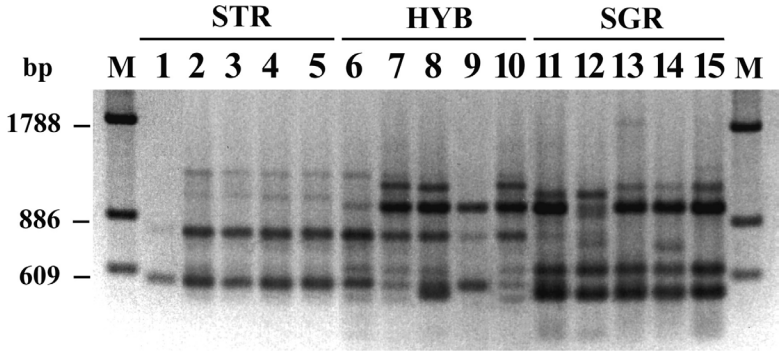

Fig. 1. Photograph of an agarose gel showing ISSR bands of the putative hybrid *Saxifraga* × *sorianoi* and its putative parental species *S. trabutiana* and *S. granulata*. The analysis includes five individuals each of *S. trabutiana* (STR, lines 1–5), *S.* × *sorianoi* (HYB, lines 6–10) and *S. granulata* (SGR, lines 11–15). The PCR was performed using primer IS-E as described in Material and Methods. The molecular weight markers, between 1788 and 609 bp, are indicated as M.

ITS sequence analysis from *Saxifraga trabutiana* and *S. granulata*

The ITS sequences, comprising the ITS-1, 5.8S and ITS-2 units, from *Saxifraga granulata* and *S. trabutiana* were determined and aligned with those available for 42 species belonging to sect. *Saxifraga*. The aligned sequence matrix generated a total of 700 characters, 278 of which corresponded to variable sites and 126 to parsimony-informative sites. Pairwise comparisons of the ITS sequences using the Kimura 2-parameter model of sequence evolution yielded a 1.8% and 1.9% of divergence between *S. trabutiana* and its closest relatives *S. rigoi* and *S. erioblasta*, respectively. The divergence value between *S. trabutiana* and other species also classified into series *Gemmiferae* (*S. globulifera*, *S. reuteriana* and *S. conifera*) ranges between 5.4% and 5.6%.

A total of 621 most-parsimonious trees were found. The majority-rule consensus tree using *Saxifraga spathularis* as the outgroup is shown in Fig. 2. CI and RI indexes were 0.71 and 0.72, respectively, considering both informative and uninformative sites. The basal position of *S. conifera* and a basal polytomy including the rest of clades of sect. *Saxifraga* is in agreement with previous results (Vargas 2000). Our sequence from the parental species *S. granulata* is grouped together with that previously reported for an Italian specimen of *S. granulata* (acc. no. AJ233860), and *S. graeca*, *S. cespitosa*, *S. rosacea* and *S. hortii* (91% bootstrap). On the other hand, *S. trabutiana* is clustered together with *S. erioblasta* and *S. rigoi*, all of them belonging to series Gemmiferae (93% bootstrap support). Grouping of the same three species in a distinct clade is also obtained using other methods of analysis such as neighbor-joining and minimum evolution (not shown). However, other taxa of the series *Gemmiferae* such as *S. globulifera*, which is morphologically similar to *S. trabutiana*, *S. conifera*, *S. rigoi* and *S. fragosoi*, are placed in different clades (Fig. 2).

Restriction polymorphism analysis of the ITS sequences

To further elucidate the hybrid origin of the *Saxifraga* × *sorianoi* specimens, we took advantage of the fact that the ITS sequences of the putative parental species were available, to deduce species specific patterns generated by digestion of the ITS fragments from different specimens with appropriate restriction enzymes (Fig. 3A). As expected from their sequences, the *Xho*I enzyme digests the ITS fragment of *S. granulata* generating two fragments of about 700 bp and 90 bp, of which the smaller fragment is not visible on the gel (Fig. 3B: lines 9–13), while the *S. trabutiana* ITS is not cut by the enzyme (Fig. 3B: lines 1–4). The presence of two bands (Fig. 3B: lines

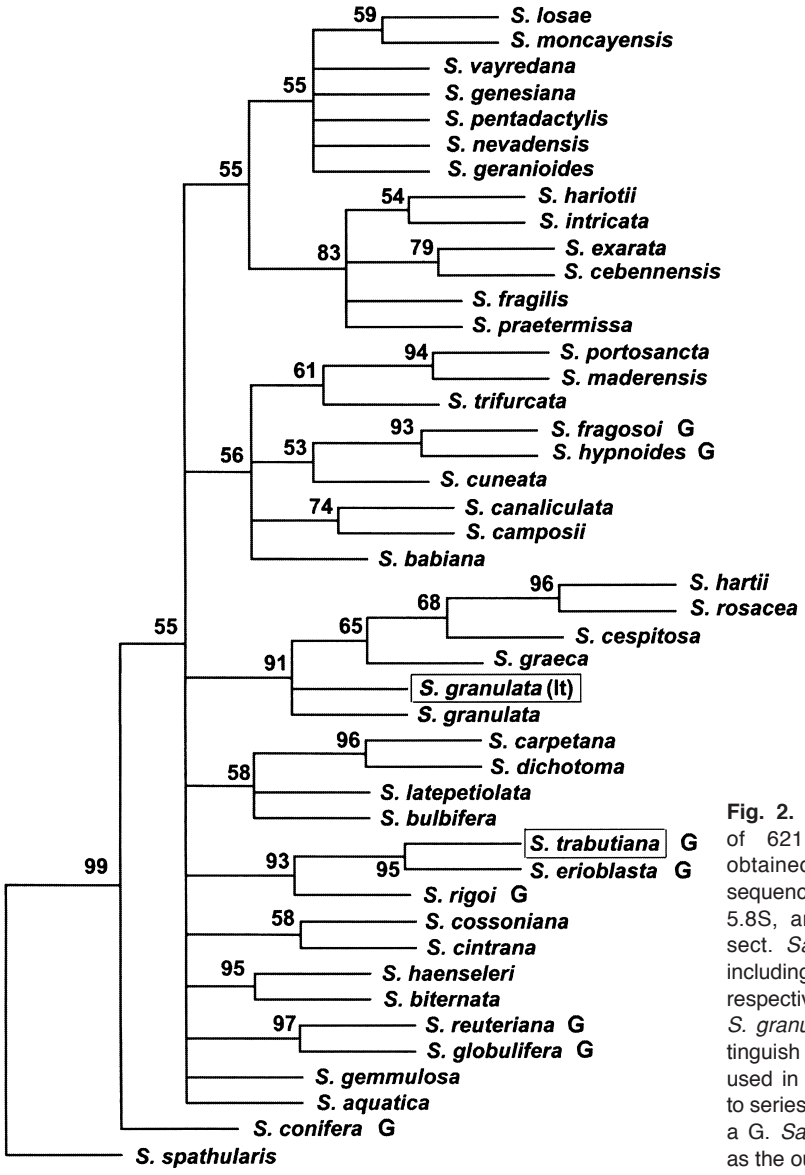

Fig. 2. Majority-rule consensus tree of 621 most-parsimonious trees obtained by parsimony analysis of sequences of the ITS region (ITS-1, 5.8S, and ITS-2) of 45 species of sect. *Saxifraga*. CI and RI values, including all sites, are 0.71 and 0.72, respectively. The Italian accession of *S. granulata* is marked by (It) to distinguish it from the Spanish population used in our work. Species belonging to series *Gemmiferae* are marked with a G. *Saxifraga spathularis* was used as the outgroup.

5–8), corresponding to the digested and the non-digested ITS fragments of *S.* × *sorianoi*, is compatible with its hybrid origin. A similar result was obtained when the DNA was digested with *Ava*I. In this case, useful species-specific bands of 335 and 230 bp for *S. trabutiana* (Fig. 3C: lines 2–6), and a 700-bp band resulting from the cut at the single *Ava*I site in the case of *S. granulata* are obtained (Fig. 3C: lines 12–16). An additive pattern is also obtained for the putative hybrid plants (Fig. 3C: lines 7–10).

Discussion

ITS phylogenetic analysis of *Saxifraga trabutiana*

Saxifraga trabutiana was described from northern Africa (Morocco and Algeria) and SE Spain (Engler & Irmscher 1916, Vargas 1997). In a recent revision of the Iberian species of *Saxifraga* (Vargas 1997), it has been classified within series *Gemmiferae* of sect. *Saxifraga*, a group of

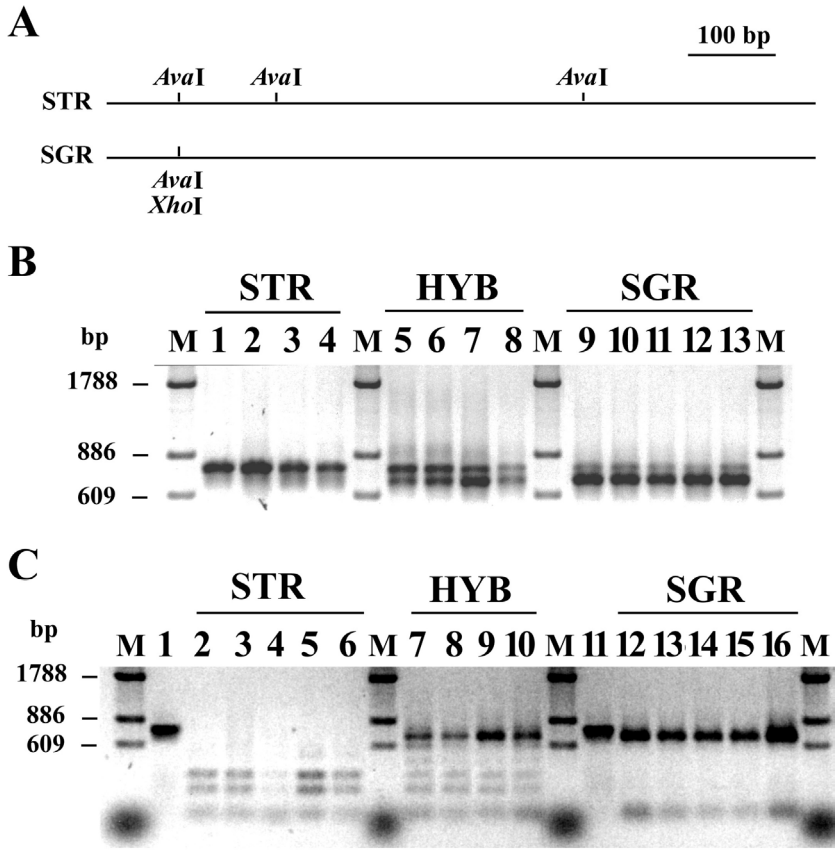

Fig. 3. Restriction polymorphism analysis of the ITS fragments from the putative hybrid *Saxifraga* × *sorianoi* and its parental species *S. trabutiana* and *S. granulata*. — **A**: Map of the restriction sites on ITS sequences from *S. trabutiana* (STR) and *S. granulata* (SGR) for the restriction enzymes *Xho*I and *Ava*I (panels **A** and **B**). — **B**: Agarose gel of the *Xho*I restriction analysis: ITS fragments of four individuals of *S. trabutiana* (STR, lines 1–4) and *S.* × *sorianoi* (HYB, lines 5–8), and five individuals of *S. granulata* (SGR, lines 9–13) were digested with the *Xho*I enzyme and the resulting fragments separated by electrophoresis. Molecular weight markers on line M. — **C**: Agarose gel of the *Ava*I restriction analysis: ITS fragments of five individuals of *S. trabutiana* (STR, lines 2–6) and *S. granulata* (SGR, lines 12–16), and four individuals of *S.* × *sorianoi* (HYB, lines 7–10) were digested with the *Ava*I enzyme and the resulting fragments were analysed, as above. Note the undigested ITS fragments for *S. trabutiana* (line 1) and *S. granulata* (line 11). Molecular weight markers on lane M.

plants mainly characterised by the absence of underground bulbils and the presence of axillary rosette buds (summer-dormant buds). However, a molecular analysis of the relationship between *S. trabutiana* and other *Saxifraga* species was not available. The phylogenetic analysis of the ITS sequences indicates a close relationship of *S. trabutiana* to *S. erioblasta* and *S. rigoi*, two taxa belonging to the same series. However, other plants of series *Gemmiferae* like *S. globulifera* or *S. fragosoi*, are placed in separate clusters (Fig. 2). Morphology of *S. trabutiana* is close to that of *S. globulifera*, sharing also some traits from *S. fragosoi*. While basal leaves in *S. trabutiana* and *S. globulifera* are flabellate to cuneiform, divided and 3–5 lobed (generally with 3 narrow lobes), in *S. erioblasta* they are spathulate and entire (rarely denticulate). In addition, summer-dormant buds are pedunculate and about 3–8 mm long in *S. trabutiana* and *S. globulifera*, while they are sessile and smaller in *S. erioblasta*. Conversely, summer-dormant buds in *S. trabutiana* are acute as those of *S. fragosoi*, while in *S. globulifera* their shape is blunt. This

incongruence between phylogenies inferred from molecular data and classifications based on morphological characters has been already pointed out for plants of sect. *Saxifraga* (Soltis *et al*. 1996, Vargas 2000). Both ancient and recent hybridisation events have been proposed (Vargas 2000) as the most likely explanation for the fact that morphological traits used for classification in sect. *Saxifraga* cannot be related to single clades of the ITS tree. Hybridization provides an efficient mechanism of obtaining new combinations of morphological traits. In the case of *S.* × *sorianoi* a new leaf morphology, as compared to that of parental species, is combined with other characteristics such as the presence of underground bulbils that defines series *Saxifraga*. Moreover, hybridization, even among distantly related plants of genus *Saxifraga*, is quite frequent (Webb & Gornall 1989, Vargas 1997), although fertility of F_1 plants is generally very low (Vargas & Nieto Feliner 1996)

Characterisation of the *Saxifraga* × *sorianoi* hybrid

Intermediate banding patterns from ISSR markers and ITS restriction polymorphism strongly support the occurrence of natural hybridisation between *Saxifraga trabutiana* and *S. granulata*. Strong additivity of molecular marker profiles in the hybrid, as compared with those from its progenitors, was found. This, together with the fact that seeds of *S.* × *sorianoi* are not viable, indicate that individuals found in natural populations likely represent the F_1 generation. The hybrid, previously referred to by the binomial *S.* × *sorianoi* (García-Maroto & Gómez-Mercado 2002) is the result of crossings between individuals of *S. trabutiana* and *S. granulata*, two species with overlapping distributions and similar habitats in "Sierra de los Filabres" (SE Spain). In that particular mountain range, *S. granulata* is widespread and occurs at different altitudes. Some individuals sporadically colonise siliceous crevices and meet the locally restricted population of *S. trabutiana* on grassy slopes above 1800 m. Regarding morphology, *S.* × *sorianoi* shows intermediate characteristics, in terms of morphology and hair covering, compared to those of the parental species. Nevertheless, vegetative traits such as the presence of underground bulbils, basal leaves with a well-developed blade and rosette growth habit, most resemble *S. granulata*. The inflorescence structure and flower morphology, on the other hand, are more similar to those of *S. trabutiana*. Hybrid populations consist of small patches of less than ten individuals, probably derived vegetatively from the same plant and often growing between the parents. Limited propagation seems to be due to the fact that hybrid plants do not produce viable seeds (García-Maroto & Gómez Mercado 2002). This observation is in agreement with data obtained by experimental hybridisation among species of sect. *Saxifraga*, where minimal seed-set was obtained for F_1 plants (Vargas & Nieto Feliner 1996). *Saxifraga* × *sorianoi* seems to fit the same breeding system and reproductive isolation, which is only manifested in the viability of the second generation. Hybridisation in mixed populations has also been studied in other sections of *Saxifraga*: *S.* × *kochii* (*S. oppositifolia* × *S. biflora*) (Gugerli 1997) and *S.* × *hansmannii* (*S. aizoides* × *S. mutata*) (Holderegger 1998). For these plants a relatively high and viable seed set is naturally obtained giving rise to at least few seedlings. Consequently, since sexual reproduction is usually accepted as a prerequisite for long-term survival in challenging environments, evolutionary potential to become distinct species has been recognised for these plants. In the case of the sexually isolated *S.* × *sorianoi*, the main propagation mechanism seems to be by means of their vegetative underground axillary buds. However, *S.* × *sorianoi* does not necessarily represent an evolutionary dead-end. Although we have not observed natural seed-set it may occur that few viable seeds are obtained in particularly optimal seasons as it has been suggested for *S. svaldbardensis* (Brochmann & Hapnes 2001). Since hybrid clones can be vegetatively maintained for many years it is possible that suitable gametes are occasionally generated which result in more fertile plants. In any case, the current status of these populations is highly vulnerable due to the reduced number of individuals and limited propagation of the hybrid plants. Consequently, it would be of great importance to implement a conservation program for this rare taxon.

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