Reciprocal hybridization at different times between *Senecio flavus* and *Senecio glaucus* gave rise to two polyploid species in north Africa and south-west Asia

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Summary

• The analysis of hybrid plant taxa using molecular methods has considerably extended understanding of possible pathways of hybrid evolution.
• Here, we investigated the origin of the tetraploid *Senecio mohavensis* ssp. *breviflorus* and the hexaploid *Senecio hoggariensis* by sequencing of nuclear and chloroplast DNA, and by analysis of the distribution of taxon-specific amplified fragment length polymorphism (AFLP) fragments.
• Both taxa originated from hybridization between the diploid *Senecio flavus* and *Senecio glaucus*. Whereas *S. glaucus* was the female parent in the origin of *S. mohavensis* ssp. *breviflorus*, *S. flavus* was the female parent in the origin of *S. hoggariensis*.
• The distribution of AFLP fragments suggests that *S. hoggariensis* is an allohexaploid species with two diploid genomes of *S. glaucus* and one diploid genome of *S. flavus*. The high frequency of *S. flavus*-specific fragments in *S. mohavensis* ssp. *breviflorus* is explained either as the result of introgression between a primary hybrid and *S. flavus* or as the result of intergenomic recombination in a primary hybrid. These two alternative processes cannot easily be distinguished.

Key words: allopolyploidy, amplified fragment length polymorphism (AFLP), hybridization, intergenomic recombination, introgression, *Senecio*.

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Introduction

Interspecific hybridization plays an important role in plant and animal evolution (Arnold, 1997), and can result in new taxa via introgressive hybridization, homoploid hybrid speciation, and polyploid hybrid speciation (Abbott, 1992). Although this has long been known and accepted (Stebbins, 1950, 1959), much progress in understanding these different trajectories of hybrid evolution has been made in the recent past, mainly but not exclusively through the application of molecular methods (for a review, see Hegarty & Hiscock, 2005). The widespread occurrence of introgressive hybridization was recently reviewed by Rieseberg & Wendel (1993), and detailed insights have been gained into homoploid hybrid speciation (Rieseberg *et al.*, 1995, 2003; Rieseberg, 1997). In polyploid hybrid speciation, much progress has been made in understanding mechanisms and rates of polyploid formation (Ramsey & Schemske, 1998; Otto & Whitton, 2000) and in appreciating the recurrent origin of polyploid species formation (Soltis & Soltis, 1999). Recently, interest has focused on the early evolution of polyploids (Soltis & Soltis, 1995, 2000, 2004; Leitch & Bennett, 1997; Bayer, 1999; Wendel, 2000; Ramsey & Schemske, 2002; Hegarty *et al.*, 2005). The existence of intergenomic recombination was first suggested by the observation of substantial segregation in, for example, allopolyploid *Nicotiana* (Kostoff, 1938), *Gilia* hybrids (Grant, 1966a,b) and the recently evolved allohexaploid *Senecio cambrensis* Rosser (Ingram & Noltie, 1984). However, cytogenetic characterization of the chromosome complement of hybrid species such as *Nicotiana tabacum* (Kenton *et al.*, 1993) and studies of experimental allopolyploids, particularly

in *Brassica* (Song *et al.*, 1995; Lukens *et al.*, 2004; Pires *et al.*, 2004) and *Triticum-Aegilops* (Feldman *et al.*, 1997; Liu *et al.*, 1998; Levy & Feldman, 2004), were needed to demonstrate conclusively that substantial intergenomic recombination can take place, resulting in the loss of parental characters and the origin of new hybrid characters within only a few generations of hybrid offspring (but see Liu *et al.*, 2001; Ainouche *et al.*, 2004). The study of artificial allopolyploids in *Brassica* by Song *et al.* (1995) also suggested that the amount of genome evolution is proportional to the degree of relatedness of the parent species, with more genome change occurring in distant crosses, and that the genome of the paternal parent changes more than that of the maternal parent.

The hybrid origin of new taxa is well documented in certain groups of *Senecio*. In the British Isles, hybridization between the diploid ($2n = 20$) *Senecio squalidus* L., itself a hybrid between *Senecio aethnensis* Jan. ex DC and *Senecio chrysanthemifolius* Poiret (Crisp, 1972; Abbott *et al.*, 2000, 2002), and the tetraploid ($2n = 40$) *Senecio vulgaris* L. gave rise to the tetraploid *Senecio vulgaris* var. *hibernicus* Syme (Stace, 1977; Ingram *et al.*, 1980; Abbott *et al.*, 1992), the tetraploid *Senecio eboracensis* R.J. Abbott & A.J. Lowe (Irwin & Abbott, 1992; Lowe & Abbott, 2000, 2003; Abbott & Lowe, 2004), and, recurrently, the hexaploid ($2n = 60$) *Senecio cambrensis* (Rosser, 1955; Weir & Ingram, 1980; Ashton & Abbott, 1992; Harris & Ingram, 1992). Moreover, the hexaploid ($2n = 60$) Canary Islands endemic *Senecio teneriffae* Schultz Bip. (Kadereit, 1984) is probably a hybrid derivative of *S. vulgaris* and the diploid ($2n = 20$) *Senecio glaucus* L. (Lowe & Abbott, 1996). The species involved in these various hybridizations are all closely related to each other (Comes & Abbott, 2001).

A much more distant cross was apparently involved in the origin of two tetraploid ($2n = 40$) subspecies of *Senecio mohavensis* A. Gray, the North American ssp. *mohavensis* and the south-west Asian ssp. *breviflorus* (Kadereit) M. Coleman. Subspecies *breviflorus*, originally described as *Senecio flavus* (Decne.) Schultz Bip. ssp. *breviflorus* Kadereit (Kadereit, 1984), from south-west Asia, has been shown to be a tetraploid hybrid derivative of diploid *S. glaucus* and diploid *S. flavus* through a combination of chloroplast DNA (cpDNA), internal transcribed spacer (ITS), random amplified polymorphic DNA (RAPD), isozyme and chromosomal evidence (Liston *et al.*, 1989; Liston & Kadereit, 1995; Coleman *et al.*, 2001, 2003; Comes & Abbott, 2001). *Senecio glaucus* is widely distributed over north Africa and south-west Asia, and *S. flavus* grows in north and south-west Africa (Alexander, 1979; Kadereit, 1984; Coleman *et al.*, 2001). Chloroplast DNA analysis provides strong evidence that in this cross *S. glaucus* was the female parent (Comes & Abbott, 2001), and homogenization of ITS also took place towards *S. glaucus* (Comes & Abbott, 2001; Coleman *et al.*, 2003). In the study by Coleman *et al.* (2003), *S. flavus* was found to be more closely related to other genera of Asteraceae-Senecioneae than to *S. glaucus*. This clearly demonstrates that the two species are only very distantly related. Indeed, a broad survey of ITS variation in Asteraceae-Senecioneae suggested that *S. flavus* may not even be part of a re-defined *Senecio* (P. B. Pelser *et al.*, Department of Botany, Miami University, Oxford, OH, USA, personal communication). These molecular data confirm earlier experimental work. Thus, Alexander (1975, 1979) did not succeed in hybridizing *S. flavus* with *S. glaucus*, *Senecio leucanthemifolius* Poiret, *Senecio aegyptius* L. or *Senecio hoggariensis* Batt. & Trab., and of the three hybrid individuals between *S. flavus* and *S. squalidus*, a close relative of *S. glaucus*, obtained by Kadereit (1983) two were completely pollen-sterile and one had a very low percentage (3.5%) of stainable pollen grains.

Considering the differences in chromosome number among *S. glaucus*, *S. flavus* ($2n = 20$) and *S. mohavensis* ($2n = 40$) in combination with the very close morphological similarity between *S. flavus* and *S. mohavensis*, it remains unclear by which mechanism *S. mohavensis* originated. Coleman *et al.* (2001) considered the possibilities of an origin either via autopolyploidy (of *S. flavus*) followed by introgression with *S. glaucus* or via allopolyploidy. Here we investigate the systematic distribution of species-specific amplified fragment length polymorphism (AFLP) fragments in *S. mohavensis*, its putative parental taxa (*S. flavus* and *S. glaucus*), and several close relatives (*S. aethnensis*, *Senecio vernalis* Waldst. & Kit. and *S. vulgaris*) to further investigate this problem. In a similar way, a qualitative analysis of AFLP fragment variation recently was used to interpret hybrid evolution in *Achillea* by Guo *et al.* (2005). We will discuss the possibility that the genomic constitution of *S. mohavensis*, as revealed by AFLP fragment distribution, may have arisen through allopolyploid intergenomic recombination, but will show that this unusual mechanism of allopolyploid hybrid speciation may be difficult to distinguish from introgression. We also include another species of *Senecio* in this study, the hexaploid ($2n = 60$) *S. hoggariensis* from north Africa and south-west Asia (Hoggar Mountains, Tibesti Mountains, south-east Egypt and Sinai; Alexander, 1979). This species has morphological similarities with both *S. glaucus* and *S. flavus* and thus may be yet another hybrid derivative of these two species. In addition to AFLP variation, we analysed sequences of both nuclear ribosomal DNA (ITS) and three cpDNA gene regions (the *trn*T-L intergenic spacer, the *trn*L intron and the 3′ *trn*K intron) in *S. hoggariensis* and its close relatives. As the mode of inheritance of cpDNA in *Senecio* is strictly maternal (Harris & Ingram, 1992), examination of relationships with both nuclear and cytoplasmic markers not only provides a more accurate picture of the phylogenetic relationships of this species, but also allows identification of the maternal and paternal parents.

Materials and Methods

Plant material

Plant material was taken from specimens grown in the experimental glasshouse of Mainz Botanic Garden, if available,

and stored at −80°C. Alternatively, herbarium material was used. Details about the origin of plant material used for AFLP and/or DNA sequence analysis are given in Table 1.

DNA extraction

Plant material was ground in liquid nitrogen. DNA was extracted using the DNeasy™ plant minikit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA concentration was measured spectrophotometrically with a GeneQuant RNA/DNA calculator (Pharmacia, Cambridge, UK), or estimated visually in ethidium bromide-stained agarose gels. Extracted DNA was stored at −20°C.

AFLP procedure

Nineteen individuals representing eight taxa were included in the AFLP analysis (Table 1). Three of the *S. hoggariensis* individuals were offspring of the same parent (Ehrendorfer 20b; see Table 1). All reactions and polymerase chain reaction (PCR) conditions followed the procedure of Vos *et al.* (1995) with modifications and chemicals as described in Kropf *et al.* (2003), except that 150 ng of DNA was used for the restriction–ligation step. The restricted DNA was simultaneously ligated to adaptors (*Eco*RI: 5′-CTCGTAGACTGCGTACC-3′/5′-AATTGGTACGCAGTC-3′; *Mse*I: 5′-GACGATGAGTCC-TGAG-3′/5′-TACTCAGGACTCAT-3′) at 23°C for 14 h. A preselective amplification was carried out using the primers E+1 (5′-GACTGCGTACCAATTCA-3′) and M+1 (5′-GAT-GAGTCCTGAGTAAC-3′). For the selective amplifications, the three primer combinations E37/M61 (E+1 + CG/M+1 + TG), E39/M57 (E+1 + GA/M+1 + GG) and E45/M54 (E+1 + TG/M+1 + CT) were used.

Products resulting from selective amplification were separated on a 6% polyacrylamide gel with an internal size standard (ROX 500; Applied Biosystems, Weiterstadt, Germany). The gel was run for approx. 4 h on an ABI Prism 377™ automated sequencer (Applied Biosystems) and analysed with GENESCAN software (version 3.1; Applied Biosystems). Fragments in the range from 75 to 500 bp were scored automatically as present (1) or absent (0) with the GENOTYPER analysis software (version 2.1; Applied Biosystems) and manually corrected after visual inspection of the electrophoretograms. Ambiguous peaks were scored as missing data.

ITS sequence analysis

The entire ITS region, comprising ITS-1, 5.8S and ITS-2, was sequenced in one accession of *S. hoggariensis* (Table 1), using primers 18S and 28S of Muir & Schlötterer (1999). Amplifications were performed in 25-μl volumes containing 1.0 μl of 50 mM $MgCl_2$, 2.5 μl of 10 × BioTherm PCR buffer (GeneCraft, Münster, Germany), 0.25 μl of 20 mM dNTP, 1.0 μl of each primer at 25 pmol μl^{-1}, 0.75 U BioTherm Taq polymerase (GeneCraft), and 0.4 μl of genomic DNA (*c.* 5–40 ng). PCR conditions consisted of 60 s at 94°C followed by 35 cycles of 18 s at 94°C, 30 s at 55°C and 60 s at 72°C, and a post-treatment of 78 s at 55°C and 8 min at 72°C. The ITS fragments were then cloned using the TOPO TA™ Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Eight colonies were picked for sequencing in order to check for intra-individual variation. The ITS region was cycle-sequenced with the BigDye® Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems) using the same primers as for the amplification. PCR conditions were 10 s at 96°C, followed by 4 min at 55°C repeated 30 times. The three different ITS sequences generated in this study have been deposited in GenBank (see Table 1 for accession numbers).

Chloroplast DNA sequence analysis

The *trn*T-L intergenic spacer, the *trn*L intron, and the 3′ *trn*K intron were sequenced for eight taxa. In some cases different accessions of the same taxon were used for different cpDNA regions (Table 1). The *trn*T-L intergenic spacer and the *trn*L intron were amplified with primers a, b, c and d from Taberlet *et al.* (1991). The 3′ *trn*K intron was amplified with primers 1023F and 1559R from Pelser *et al.* (2002). PCR conditions for all three cpDNA regions followed Pelser *et al.* (2002). Purified PCR products of the *trn*L and 3′ *trn*K introns were cycle-sequenced in one direction using primers d and 1559R, respectively, while those of the *trn*T-L spacer were sequenced in both directions using the same primers as in the amplification. The sequencing reactions followed the same protocol as for the ITS region. The 24 different cpDNA sequences generated in this study have been deposited in GenBank (see Table 1 for accession numbers).

Data analyses

AFLPs The AFLP data matrix was inspected for taxon-specific fragments that were recorded for all taxa. AFLP fragments found in only one taxon were regarded as taxon-specific (or private) even when they were not fixed in that taxon. When determining taxon-specific fragments in *S. flavus* and *S. glaucus*, fragments shared between these two species and *S. mohavensis* were considered private to the former two species because they are known to be the parents of *S. mohavensis*. The proportion of taxon-specific fragments of potential parental taxa present in *S. hoggariensis*, *S. mohavensis* ssp. *mohavensis* or *S. mohavensis* ssp. *breviflorus*, respectively, was then used to infer the mode of participation of parental taxa in the origin of these suspected hybrids. We assume that AFLP fragments shared among taxa are homologous but realize that this is not necessarily the case (Bussell *et al.*, 2005; Koopman, 2005; see also Discussion).

Sequence data All DNA sequences (ITS and cpDNA) were edited and automatically aligned using the program

Table 1 Origin of plant material

Taxon	Collectors and/or collection no. (herbarium)	Origin	Type of material	GenBank accession numbers			
				3′ *trn*K intron	*trn*L intron	*trn*T-L intergenic spacer	ITS region
Senecio flavus	H. Merxmüller & W. Giess 28206 (M)	Namibia: Hanchabfontein Farm	F	DQ208168	DQ208176	–	–
S. flavus	D. Podlech 33668 (M)	Algeria: Wilaya Bechar	F	–	–	DQ208184	–
S. flavus	D. Podlech 40663 (RSA)	Morocco: Wadi Noun	F	–	–	–	–
S. mohavensis ssp. *breviflorus*	Danin *et al*. 5–1940 (HUJ)	Egypt: Sinai	F	–	–	–	–
S. mohavensis ssp. *breviflorus*	Liston & Boaz 255–1 (HUJ)	Israel: Dead Sea	F	–	DQ208178	–	–
S. mohavensis ssp. *breviflorus*	K. Naylor 279 (E)	Saudi Arabia: Jabarl	H	–	–	–	–
S. mohavensis ssp. *breviflorus*	R. Western 574 (E)	UAR: Abu Dhabi Emirate	F	DQ208170	–	DQ208186	–
S. mohavensis ssp. *mohavensis*	Engard & Hodgson 2121 (DES)	USA: Arizona, La Paz County	F	DQ208171	DQ208179	DQ208187	–
S. mohavensis ssp. *mohavensis*	Elias 10190 (RSA)	USA: Arizona, Maricopa County	F	–	–	–	–
S. aethnensis	NHM Paris 88–167	Cultivation	F	DQ208172	DQ208180	DQ208188	–
S. glaucus ssp. *glaucus*	R. J. Abbott & H. P. Comes (STA)	Israel: Judean Coast, Ashdod.	F	DQ208166	DQ208174	DQ208182	–
S. hoggariensis	F. Ehrendorfer 20b (MZA)	Algeria: Hoggar-Massif, descent from Assekrem	F	DQ208167	DQ208175	DQ208183	DQ208189–DQ208191
S. hoggariensis	D. Podlech 33320 (M)	Algeria: Hoggar-Massif, Wilaya Tamanrasset	H	–	–	–	–
S. vernalis	H. P. Comes 3	Germany: Ingelheim/Rhein	D	DQ208165	DQ208173	DQ208181	–
S. vernalis	H. P. Comes 7	Germany: Ingelheim/Rhein	D	–	–	–	–
S. vulgaris	H. P. Comes 8	Germany: Mainz	D	–	DQ208177	–	–
S. vulgaris	H. P. Comes 9	Germany: Mainz	D	DQ208169	–	DQ208185	–

All accessions were used in the amplified fragment length polymorphism (AFLP) analysis, with subsets used for sequencing of the 3′ *trn*K intron, *trn*L intron, and/or *trn*T-L intergenic spacer (ITS) regions of the chloroplast genome. In addition, three different sequences of the entire ITS region (ITS-1, 5.8S and ITS-2) of nuclear ribosomal DNA were retrieved from the single individual of *S. hoggariensis* after cloning (see text).

NHM, Natural History Museum. Type of material: F, fresh; H, herbarium specimen; D, silica-dried.

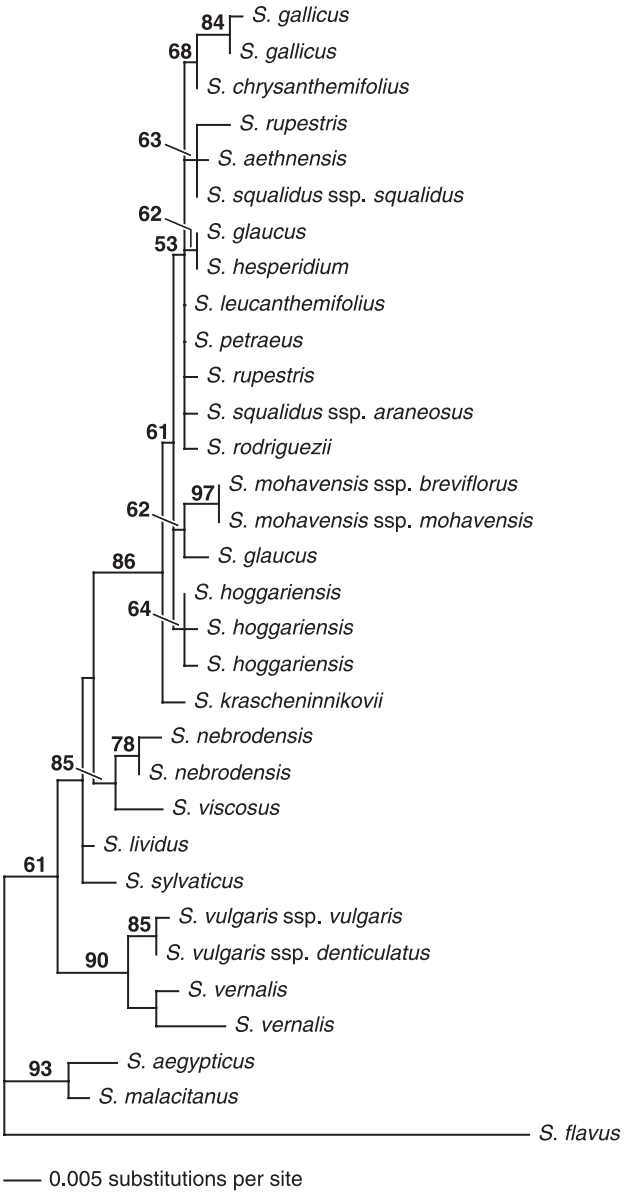

Fig. 1 Maximum-likelihood internal transcribed spacer (ITS) tree rooted with *Senecio flavus*. Bootstrap support values are shown above branches. Note that the three different ITS sequences of *Senecio hoggariensis* were retrieved from a single individual after cloning (see text and Table 1).

SEQUENCHER™ 4.1 (Gene Codes Co., Ann Arbor, MI, USA), and adjusted visually where needed. The ITS data of *S. hoggariensis* were analysed together with 29 sequences of 21 *Senecio* species obtained from GenBank [accession numbers AJ400777, AJ400779–AJ400786, AJ400789, AJ400794–AJ400801, AJ400803–AJ400809, AJ400812, AJ400813 (Comes & Abbott, 2001), AF457436 and AF457437 (Coleman *et al.*, 2003)] using *S. flavus* as the outgroup (see Fig. 1). Phylogenetic relationships were estimated using the maximum-likelihood criterion implemented in PAUP* v4.0b10 (Swofford, 2002). Using the program MODELTEST 3.5 (Posada & Crandall, 1998), the model of sequence evolution selected was SYM+G (Zharkikh, 1994) with gamma shape parameter $\alpha = 0.1544$ based on the Akaike information criterion. A heuristic search was then performed in PAUP* using the parameter settings obtained from MODELTEST, 500 random addition sequence replicates, tree bisection reconnection (TBR) branch swapping, STEEPEST DESCENT off, and MULTREES option on. Support for nodes was estimated by 100 bootstrap replicates (Felsenstein, 1985) using the model parameters obtained for the original data, 10 replications of random sequence addition, and TBR branch swapping. The aligned cpDNA sequence matrix (with all three regions combined) was analysed in PAUP* using maximum parsimony and the exhaustive search strategy.

Results

AFLPs

The three AFLP primer combinations used (E37/M61, E39/M57 and E45/M54) generated a total of 562 fragments across the 19 accessions and eight taxa analysed, with 156, 207 and 199 fragments per combination, respectively. Across the entire data set, 560 fragments were polymorphic, of which 125 were unique to individual accessions. Table 2 shows the total number of taxon-specific fragments for each taxon, and the proportion of those fragments found in *S. hoggariensis* and *S. mohavensis* subspp. *mohavensis* and *breviflorus*, respectively. A high percentage (65%) of species-specific fragments from *S. glaucus* were found in *S. hoggariensis*, compared with 22% from *S. flavus*, 22% from *S. aethnensis*, 19% from *S. vernalis* and 8% from *S. vulgaris*. In the case of *S. mohavensis* ssp. *breviflorus*, the majority of taxon-specific fragments were derived from *S. flavus* (41%), followed by those from *S. glaucus* (17%). The other species provided no major contribution (Table 2). Similar results were obtained for *S. mohavensis* ssp. *mohavensis*, with 39% from *S. flavus*, 19% from *S. glaucus* and very little contribution from the other species (Table 2).

ITS

The sequencing of eight clones of the entire ITS region (including 5.8S) from a single individual of *S. hoggariensis* yielded three sequences, differing from each other in one, two and three nucleotide positions, respectively. The complete alignment, including the three ITS sequences obtained from *S. hoggariensis*, resulted in 651 nucleotide positions, of which 555 were constant and 44 parsimony informative. The maximum-likelihood analysis of the ITS data showed that *S. hoggariensis* falls into a clade with *S. mohavensis*, *S. glaucus*, and several other diploid taxa such as *S. squalidus* (Fig. 1). Bootstrap support is high for this '*S. glaucus*/*S. mohavensis* clade', while relationships within the clade are largely unresolved. More importantly in the present context, the species of this

Table 2 Comparison of numbers and percentages of taxon-specific fragments among *Senecio hoggariensis*, *Senecio mohavensis* ssp. *breviflorus*, *Senecio mohavensis* ssp. *mohavensis*, and potential ancestors

Taxon	Taxon-specific fragments	Taxon-specific fragments in *S. hoggariensis*	Taxon-specific fragments in *S. mohavensis* ssp. *breviflorus*	Taxon-specific fragments in *S. mohavensis* ssp. *mohavensis*
S. hoggariensis	33	–	–	–
S. mohavensis ssp. *breviflorus*	17	1 (5%)	–	–
S. mohavensis ssp. *mohavensis*	17	0 (0%)	–	–
S. flavus	100	22 (22%)	41 (41%)	39 (39%)
S. glaucus ssp. *glaucus*	48	31 (65%)	8 (17%)	9 (19%)
S. aethnensis	32	7 (22%)	0 (0%)	2 (6%)
S. vernalis	37	7 (19%)	2 (5%)	1 (3%)
S. vulgaris	45	4 (8%)	2 (4%)	2 (4%)

Table 3 Distribution of nucleotide substitutions and indels in the *trn*T-L intergenic spacer, 3′ *trn*K intron, and *trn*L intron among *Senecio hoggariensis*, *Senecio mohavensis* ssp. *breviflorus*, *Senecio mohavensis* ssp. *mohavensis*, and potential ancestors

	*trn*T-L intergenic spacer				3′ *trn*K intron			*trn*L intron
	Position				Position			Position
Taxon	25	51	330	502	71	104–109	412	396
S. hoggariensis	C	C	T	T	T	+	T	A
S. mohavensis ssp. *breviflorus*	C	C	T	A	G	–	C	G
S. mohavensis ssp. *mohavensis*	C	C	T	A	G	–	C	G
S. flavus	T	A	T	T	G	+	C	G
S. glaucus ssp. *glaucus*	C	C	C	A	G	–	C	G
S. aethnensis	C	C	C	A	G	–	C	G
S. vernalis	C	C	T	A	G	–	C	G
S. vulgaris	C	C	C	A	G	–	C	G

clade are clearly more closely related to each other than to *S. flavus*.

The numbers of differences in nucleotide positions among *S. glaucus*, *S. mohavensis* and *S. hoggariensis* are roughly equal. The ITS sequence of the *S. glaucus* (ssp. *glaucus*) accession from Israel (AJ400786) differs from the three *S. hoggariensis* sequences in four, five and six nucleotide positions, respectively, and in five positions from those of *S. mohavensis*. The two subspecies of *S. mohavensis* have the same ITS sequence. The Moroccan accession of *S. glaucus* [ssp. *coronopifolius* (Maire) Alexander; AJ400785] differs in three, four and five positions from *S. hoggariensis*, and in six positions from *S. mohavensis*. Finally, the ITS sequence of *S. mohavensis* differs in five, six and seven nucleotides from the three ITS sequences of *S. hoggariensis*, respectively.

Chloroplast DNA

The aligned sequences of the 3′ *trn*K intron, *trn*T-L spacer and *trn*L intron had lengths of 520, 502 and 464 bp, respectively, and contained seven substitutions and a 6-bp indel (Table 3). One short and highly variable region of the *trn*T-L spacer could not be used for parsimony analysis because of complicated patterns in length variation of a poly-adenine stretch. This region was thus excluded from the alignment. Of the variable positions, one substitution (*trn*T-L spacer: position 502) and the indel were shared by *S. hoggariensis* and *S. flavus*, and one substitution (*trn*T-L spacer: position 330) was common to *S. vulgaris*, *S. aethnensis* and *S. glaucus* (Table 3). Of the remaining substitutions, three were unique to *S. hoggariensis* and two to *S. flavus*. The maximum-parsimony analysis grouped *S. flavus* and *S. hoggariensis* as sister species with 85% bootstrap support (not shown).

Discussion

In the present study we employed sequence data for nuclear ribosomal (ITS) and chloroplast DNA as well as AFLPs to investigate the origin of the tetraploid *S. mohavensis* ssp. *breviflorus* and the hexaploid *S. hoggariensis*. We note that objections may be raised to the use of AFLPs in such a context, because the homology between co-migrating fragments may

be difficult to establish (Guo *et al.*, 2005). In fact, the amount of nonhomology between such fragments might be high at the sequence level, especially for distantly related taxa (Mechanda *et al.*, 2004). However, while this source of error has the potential to produce inaccurate genetic similarity measures, namely phylogenetic reconstructions (e.g. Robinson & Harris, 1999), the major interest here is in the *relative* genomic contributions from putative parental taxa, in terms of taxon-specific fragments, to assumed hybrid derivatives. Hence, even if a certain percentage of, for example, the *S. glaucus*-specific fragments found in, for example, *S. hoggariensis* should be nonhomologous, such percentages are likely to vary little across all potential parent/hybrid pairs, and thus should not affect our overall proportions of genomic contributions (see Table 2).

Furthermore, it must be emphasized that the AFLP data obtained are limited in extent; in particular, our absolute values related to 'taxon-specific fragments' are considered as approximations because only one or a few accessions from each taxon have been analysed. Accordingly, 'taxon-specific fragments' are no more than 'accession-specific fragments'. That said, all conclusions to be drawn from the analysis of these data would only be strengthened by more detailed intra(sub)specific surveys of AFLP variation. However, for *S. hoggariensis*, any wider sampling, at least in the Hoggar and Tibesti Mountains, is presently not possible for political reasons.

S. mohavensis ssp. *breviflorus*

Our qualitative analysis of AFLP fragment distribution among *S. mohavensis* ssp. *breviflorus* and its putative parents *S. glaucus* and *S. flavus* both confirms and extends existing knowledge. The analysis of cpDNA (Liston & Kadereit, 1995; Comes & Abbott, 2001), ITS (Comes & Abbott, 2001; Coleman *et al.*, 2003), RAPD (Comes & Abbott, 2001), chromosomal (Coleman *et al.*, 2001) and morphological variation (Kadereit, 1984; Coleman *et al.*, 2001) previously demonstrated that *S. mohavensis* ssp. *breviflorus* originated as a polyploid hybrid taxon from a cross between *S. glaucus* and *S. flavus*, that in this cross *S. glaucus* was the female parent, and that the homogenization of the strongly divergent ITS sequences of the two parent species was towards *S. glaucus*. The major sources of evidence for the participation of *S. flavus* in the origin of *S. mohavensis* ssp. *breviflorus*, originally described as *S. flavus* ssp. *breviflorus*, consisted of RAPD variation, where *S. mohavensis* ssp. *breviflorus* grouped with *S. flavus* rather than with *S. glaucus* (Comes & Abbott, 2001), flower morphology, where *S. mohavenis* ssp. *breviflorus* was found to differ from *S. flavus* mainly in the presence of shortly ligulate capitula with epappose ray floret achenes (Kadereit, 1984), leaf shape (Coleman *et al.*, 2001) and geographical distribution (Kadereit, 1984; Coleman *et al.*, 2001).

The presence of species-specific AFLP fragments of both *S. glaucus* and *S. flavus* in *S. mohavensis* ssp. *breviflorus* (as well as in ssp. *mohavensis*; see Table 2) confirms the participation of these two species in the formation of *S. mohavensis*, and the higher proportion of *S. flavus* (41%) than *S. glaucus* (17%) fragments in *S. mohavensis* ssp. *breviflorus* is in complete accordance with RAPD and phenotypic variation. Our analysis of the among-species distribution of species-specific AFLP fragments clearly shows that *S. mohavensis* is not a simple allotetraploid species, and an explanation for the differential proportions of *S. glaucus* and *S. flavus* fragments is required.

Coleman *et al.* (2001) suggested either (1) a combination of autopolyploidy and introgressive hybridization, or (2) allopolyploidy as possible pathways for the origin of *S. mohavensis*. Although considering the presence of duplicated isozyme loci in *S. mohavensis* ssp. *breviflorus* in relation to *S. flavus* (Liston *et al.*, 1989) as evidence in favour of allopolyploidy, Coleman *et al.* (2001) concluded that the molecular data available are insufficient to allow an unequivocal decision to be made between these two pathways. Three additional pathways can be considered. It is possible that (3) introgression took place between an allopolyploid hybrid and *S. flavus*, (4) a homoploid *S. glaucus* × *S. flavus* segregant was stabilized through autopolyploidization, or (5) intergenomic recombination in an allopolyploid *S. glaucus* × *S. flavus* hybrid resulted in fast genomic rearrangements. The last possibility was also briefly discussed by Coleman *et al.* (2001).

Of these five possibilities, i.e. (1) autopolyploidy combined with introgression either before or after polyploidization, (2) simple allopolyploidy, (3) allopolyploidy plus introgression, (4) homoploid segregation and autopolyploidization, and (5) allopolyploidy plus intergenomic recombination, alternatives (1), (3) and (4) require the possibly high sterility barriers between *S. glaucus* and *S. flavus* (Alexander, 1975, 1979; Kadereit, 1983) and/or ploidy barriers to be overcome, and alternative (2) appears the least likely. As clearly pointed out by Coleman *et al.* (2001), more or less simple additivity of parental characters would be expected in the case of simple allopolyploidy. This, indeed, has been observed in other AFLP analyses of other hybrids or hybrid taxa (O'Hanlon *et al.*, 1999; Hedrén *et al.*, 2001; Hodkinson *et al.*, 2002) where, by and large, hybrids were intermediate between their parents. Considering these arguments, *S. mohavensis* appears to have originated by a combination of auto- or allopolyploidy and introgression, where introgression is defined as the 'permanent incorporation of genes from one set of differentiated populations into another' (Rieseberg & Wendel, 1993), by homoploid segregation and autopolyploidization, or by allopolyploidy and intergenomic recombination, whereby these last two processes are assumed to involve the homoploid or polyploid hybrid only. The difficulty of distinguishing these processes has previously been pointed out by, for example, Stebbins (1950) and Heiser (1973). When it is considered that 'from a mechanistic standpoint, hybridization and introgression may be viewed as a type of recombination' (Rieseberg & Wendel, 1993), it becomes obvious that these two possible

processes differ only marginally. In both cases, recombination among chromosomes of two different parent species (or other 'differentiated populations') takes place, and differences are found only in the crossing scheme. Whereas in the case of introgression backcrosses to parental species or populations are involved (although not as the only type of cross possible; Rieseberg & Wendel, 1993; Rieseberg, 1997), in the case of intergenomic recombination the parental species or populations do not participate in the further evolution of the homoploid or polyploid hybrids. Accordingly, polyploidization/introgression vs polyploidization/intergenomic recombination, in whichever temporal sequence, should not be considered fundamentally different or mutually exclusive pathways of polyploid or homoploid hybrid evolution. This also suggests that distinction of these two processes may prove difficult. In the light of the possibly high sterility of diploid hybrids between *S. glaucus* and *S. flavus*, we consider allopolyploidy plus intergenomic recombination the most likely mechanism for the origin of *S. mohavensis* ssp. *breviflorus*.

S. hoggariensis

Apart from its taxonomic treatment by Alexander (1979), *S. hoggariensis* has not been subject to further analysis. The species is similar to *S. glaucus* in leaf morphology and overall habit, and to *S. mohavensis* in having very narrow capitula (also found in *S. flavus*) with short purple ligules. In combination with its chromosome number of $2n = 60$ (Alexander, 1979; HPC & JWK, unpublished data), this raised the possibility that the diploid *S. glaucus* and the tetraploid *S. mohavensis* may have been involved in the origin of the hexaploid *S. hoggariensis*. This hypothesis, however, is not supported by our molecular data. Instead, our data clearly show that *S. hoggariensis*, like *S. mohavensis*, originated from a cross between *S. glaucus* and *S. flavus*, here with the latter species as the maternal parent.

Chloroplast DNA variation showed that *S. hoggariensis* is most similar to *S. flavus*, sharing an indel in the 3′ *trn*K intron sequence and one substitution in the *trn*T-L spacer sequence (Table 3). This clearly suggests that *S. flavus* was the female parent. At the same time, *S. hoggariensis* differs from *S. flavus* at five positions, three of which are unique. The analysis of ITS sequence variation showed that *S. hoggariensis* falls into a clade with *S. glaucus* and *S. mohavensis*, and that this clade is far removed from *S. flavus* (Fig. 1). The ITS sequences of *S. hoggariensis* and *S. glaucus* differ in three to six nucleotide positions. Finally, our qualitative analysis of AFLP fragments (Table 2) showed that *S. hoggariensis* contains taxon-specific fragments from *S. glaucus*, *S. flavus* and *S. mohavensis* ssp. *breviflorus*, but also from the other four species included in the analysis. The proportional representation of species-specific fragments in *S. hoggariensis*, however, is very different. Whereas 65% (31 of 48) of the *S. glaucus*-specific fragments and 22% (22 of 100) of the *S. flavus*-specific fragments could be detected in *S. hoggariensis*, only 5% (one of 17) of ssp. *breviflorus*-specific fragments were detected in this species. These findings suggest that ssp. *breviflorus* was probably not involved in the origin of *S. hoggariensis*.

Considering all three molecular data sets together, we can speculate that *S. hoggariensis* originated in one of two ways: (i) in an initial step, *S. flavus* as female parent and *S. glaucus* as male parent gave rise to an allotetraploid hybrid (now extinct or unknown) that in turn became the female parent in another cross with *S. glaucus*, resulting in the formation of the allohexaploid *S. hoggariensis*; (ii) an initial triploid hybrid could have formed as a result of fertilization of a reduced egg of *S. flavus* by an unreduced pollen grain of *S. glaucus*, followed by chromosome doubling. In either instance, homogenization of ITS repeats probably occurred towards *S. glaucus* at an unknown stage during polyploid formation. While there is no evidence allowing us to decide between the above scenarios, both are favoured here because, in contrast to other possible pathways, they predict that the proportional representation of parental AFLP fragments in the allohexaploid *S. hoggariensis* should approximate 66% of fragments from *S. glaucus* (four haploid chromosome sets) and 33% of fragments from *S. flavus* (two haploid chromosome sets). These proportions conform remarkably well to those observed, with *S. hoggariensis* containing 65% of fragments from *S. glaucus* and 22% of fragments from *S. flavus* (Table 2). Moreover, these proportions close to expectation imply that no major genomic rearrangements, as discussed above as a possibility for the origin of *S. mohavensis* ssp. *breviflorus*, appear to have taken place in *S. hoggariensis*. However, we cannot exclude the possibility that the divergent homoeologous subgenomes of the latter species have not undergone significant epigenetic changes since their reunion, such as gene (in)activation through alterations in cytosine methylation (see Levy & Feldman, 2004), and this might be further explored in the future using methylation-sensitive AFLP (MSAP; Reyna-Lopez *et al.*, 1997; Ainouche *et al.*, 2004).

Although species-specific fragments of *S. aethnensis* (seven of 32; 22%), *S. vernalis* (seven of 37; 19%) and *S. vulgaris* (four of 45; 8%) were found in *S. hoggariensis*, we do not assume that these species were involved in the origin of *S. hoggariensis*. Instead, we prefer to postulate that either these fragments are nonhomologous or that they are plesiomorphic and the result of incomplete lineage sorting. It is also conceivable that not all of them are truly species-specific. Wider sampling of *S. glaucus* might have detected at least some of these fragments.

The relative ages of *S. mohavensis* ssp. *breviflorus* and *S. hoggariensis*

Our data seem to indicate that *S. hoggariensis* is of somewhat greater age than *S. mohavensis* ssp. *breviflorus*. In the chloroplast sequences, *S. hoggariensis* differs from *S. flavus* as its maternal parent in five nucleotide positions, three of which are unique to *S. hoggariensis*. In contrast, *S. mohavensis* differs from *S. glaucus*

(Israel) as its maternal parent in only one position, which is not unique. In the ITS sequences the difference in nucleotides between *S. glaucus* (Morocco/ Israel) and *S. mohavensis* to that between *S. glaucus* and *S. hoggariensis* is roughly equal (see also Fig. 1). In the AFLP data (Table 2), *S. hoggariensis* contains 33 species-specific fragments in comparison to 17 specific fragments in *S. mohavensis* ssp. *breviflorus*. The number of specific fragments found in *S. hoggariensis* is comparable to the numbers found in *S. glaucus* (35), *S. aethnensis* (29), *S. vernalis* (35), and *S. vulgaris* (40).

The split between a *S. vernalis*/*S. vulgaris* clade and a *S. glaucus*/*S. mohavensis* clade (see also Fig. 1) was estimated to have taken place 3.25 ± 0.96 Ma, the *S. glaucus*/*S. mohavensis* clade was estimated to have diversified 1.02 ± 0.55 Ma, and *S. mohavensis* was estimated to have originated less than 1 Ma (not exactly dated) by Coleman *et al.* (2003). Comparing the above approximations for species-specific fragments under the assumption of at least roughly clock-like accumulation of mutations in AFLPs (as demonstrated for other dominant fingerprint markers; Espinasa & Borowsky, 1998), and considering the date of origin of *S. glaucus*, we here postulate that *S. hoggariensis* originated very soon after the formation of the *S. glaucus* lineage around 1 Ma. According to Frenzel *et al.* (1992), xerophilous vegetation covered substantially larger areas around the Saharan high mountains during the last glacial maximum than it does today. Assuming that this also was the case during earlier Quaternary glacials, the highly disjunct distribution of *S. hoggariensis* in the Tibesti and Hoggar Mountains and the Sinai peninsula still must have involved substantial long-distance dispersal, but over shorter distances than those separating the three disjunct distribution areas of this species today.

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