Hybrid origin of the Oxford Ragwort, *Senecio squalidus* L: morphological and allozyme differences between *S. squalidus* and *S. rupestris* Waldst. and Kit.

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ABSTRACT

Morphometric and allozyme variation was surveyed in *Senecio rupestris*, a species native to central and southern Europe, and compared with that within the Oxford ragwort, *Senecio squalidus*, and two Sicilian species, *S. aethnensis* and *S. chrysanthemifolius* and their hybrid offspring. In addition, a limited survey of chloroplast DNA variation was conducted in *S. rupestris* to augment previous results for this species. Canonical Variate Analysis of 32 morphometric traits recorded on plants raised under the same conditions showed that *S. squalidus* is morphologically distinct from *S. rupestris*, and is similar to hybrid material between *S. aethnensis* and *S. chrysanthemifolius* collected from Mt Etna, Sicily. *Senecio squalidus* produces leaves that are more dissected than *S. rupestris*, and which have a more acute angle between the apex of the primary vein and the apices of the adjacent marginal tooth sinuses. A UPGMA analysis of genetic distances between populations based on allele frequencies at eight allozyme loci also distinguished *S. rupestris* from *S. squalidus. Senecio squalidus* contained two alleles for acid phosphatase, one of which (*Acp-2b*) was not present in *S. rupestris.* Allozyme and chloroplast DNA variation was geographically structured within *S. rupestris*, such as to indicate that refugia may have existed for this species in central Italy and the Balkans during the last ice-age, and that alpine populations are a product of post-glacial colonisation. The close similarity in morphology and allozyme composition of *S. squalidus* to hybrid material of *S. aethnensis* and *S. chrysanthemifolius*, suggests that it is a hybrid derivative of these two Sicilian species rather than a derivative of *S. rupestris*.

KEYWORDS: hybrid speciation, introduced plants, population genetics, *Senecio*, Asteraceae.

INTRODUCTION

In a previous paper (Abbott *et al.* 2000), we presented evidence supporting the proposal by Crisp (1972) that Oxford ragwort, *Senecio squalidus*, L. (Asteraceae) is a diploid hybrid species which originated from hybrid material between *S. aethnensis* Jan. ex DC. and *S. chrysanthemifolius* Poiret that was introduced to the British Isles from Mt Etna, Sicily. Morphometric analysis of material raised under glasshouse conditions showed that *S. squalidus* (2n=20) was phenotypically intermediate in leaf shape and capitulum size to that of *S. aethnensis* (2n=20) and *S. chrysanthemifolius* (2n=20), and closely resembled hybrid material between these two species. Allozyme analysis further showed that populations of *S. squalidus* were very similar genetically to Sicilian material with some populations clustering tightly with hybrid material and with *S. chrysanthemifolius*. Most notably, *S. squalidus* and hybrid material were polymorphic at the *Acp-2* locus and exhibited almost identical frequencies for two alleles (*a* and *b*) that distinguish pure populations of the two parent species.

Prior to the above analysis, Walters (1963) had emphasized that *S. rupestris* Waldst. and Kit. (2n=20), a species that occurs on mountains in central and southern Europe was morphologically very similar to *S. squalidus* in the British Isles. Because of this similarity, Alexander (1979) in a taxonomic revision of Mediterranean *Senecio*, section *Senecio*, subsumed *S. rupestris* under

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S. squalidus although considered British plants to be atypical of the taxon. This would suggest that *S. squalidus* and *S. rupestris* have a common origin. For the purposes of this paper, we treat as separate species *S. squalidus* found in the British Isles and *S. rupestris* from central and southern Europe, and examine how similar they are in morphology and in allozyme composition.

A survey of chloroplast (cp) DNA RFLP variation in *S. squalidus*, *S. aethnensis*, *S. chrysanthemifolius* and *S. rupestris* (Abbott *et al.* 1995) showed that both British *S. squalidus* and Sicilian *S. chrysanthemifolius* are monomorphic for the same cpDNA haplotype (B). In contrast, *S. aethnensis* from populations occurring between 1650 to 1890 m on Mt Etna is polymorphic for haplotypes A and B as is hybrid material. Only one accession of *S. rupestris* (from Abruzzi, central Italy) was found to possess haplotype B; other accessions of this species contained either haplotype A (from the Italian Alps, Romania, N Bulgaria, and introductions to Germany), or haplotype C (from S Bulgaria and Greece).

Here we compare morphometric and allozyme variation in *S. rupestris* with that within *S. squalidus*, *S. aethnensis*, *S. chrysanthemifolius*, and *S. aethnensis* × *S. chrysanthemifolius* hybrid material. The results for material other than *S. rupestris* were published previously (Abbott *et al*. 2000). Consequently, we repeat these here in summary form and solely for comparative purposes. In addition, we report the results of a further survey of chloroplast DNA variation in *S. rupestris*, which augments previous results for this species and provides an improved understanding of the geographical distribution of cpDNA haplotypes in this material.

MATERIALS AND METHODS

PLANT MATERIAL

Seed was collected separately from approximately 30 plants from each of 18 populations of *S. rupestris* from central and southern Europe (Table 1, Fig. 1). Material raised from seed was compared with material cultivated from seed from 9 populations of *S. squalidus* from the British Isles, three populations of each of *S. aethnensis*, *S. chrysanthemifolius*, and three of their hybrid swarms occurring on Mt Etna, Sicily (Fig. 1 and Abbott *et al.* 2000).

MORPHOMETRIC ANALYSIS

Five plants (one offspring per mother plant) from each of 13 populations of *S. rupestris* (nine populations from Italy, two from Bulgaria, and one each from Romania and Greece) (Table 1) were raised with 5 plants from each of two populations of *S. squalidus* (from Oxford and Edinburgh) and five plants of each of *S. aethnensis*, *S. chrysanthemifolius*, and also of hybrid material from Mt Etna (two plants from population 7, and three plants from population 8). The 90 plants were grown from seed to maturity as single individuals in pots of 13 cm diameter containing compost. Pots were fully randomized in a 15 × 6 block within a greenhouse. Details of growth conditions are given in Abbott *et al*. (2000). On the day of full anthesis of the apical capitulum, each plant was harvested and measured for 32 characters. Fifteen of the characters were descriptors of the capitulum, while 15 described leaf size and shape. The remaining two characters were plant height and inflorescence length. Details of measurement are given in James (1995). Data of variables that were not normally distributed were transformed into natural logarithms before subjecting them to one-way ANOVA using SPSS Base (version 7.5, SPSS Inc. 1997) to detect differences between population means. Canonical variate analysis (CVA) conducted using NTSYSpc (version 2.0, Rohlf 1998) was also conducted. This analysis derives linear canonical variates that maximize the separation among groups (populations) relative to variability within groups. For the purpose of CVA analysis *S. aethnensis* × *S. chrysanthemifolius* hybrid plants were combined into one group. After testing for a significant difference between groups by single classification multivariate analysis (MANOVA), the relationships among groups were displayed by plotting mean canonical scores of each group against each other for the first two canonical variates. CVA was then repeated ten times with data of one *S. squalidus* plant omitted, in turn, each time. After each analysis the plant omitted was assigned to a group using the respective group discriminant function as criterion.

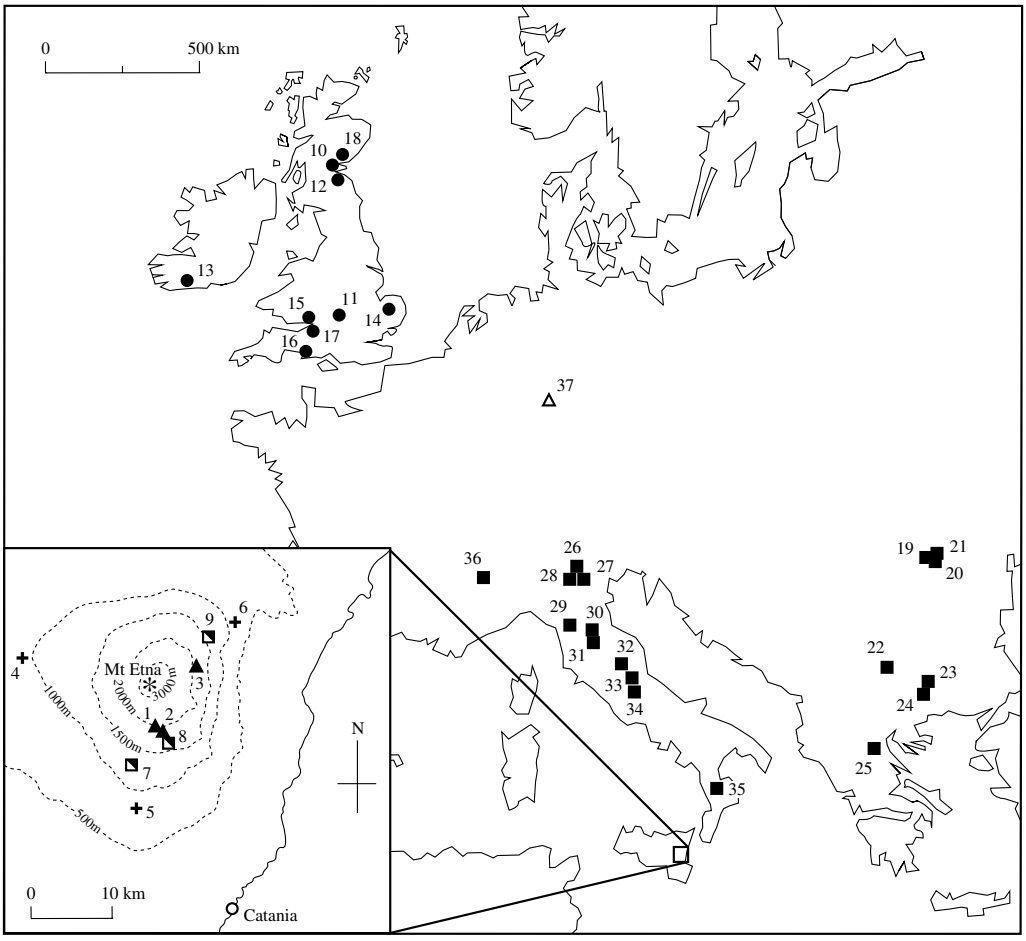

FIGURE 1. Locations of populations of *Senecio* surveyed. Main map: *S. squalidus* (●), *S. rupestris* (■). Map inset: *S. aethnensis* (▲), *S. chrysanthemifolius* (✚), and *S. aethnensis* × *S. chrysanthemifolius* (◩). Key to population numbers of *S. rupestris* is given in Table 1, and for other taxa in Abbott *et al.* (2000).

ALLOZYME ANALYSIS

Allozyme variation was assayed in all populations for six enzymes by means of starch gel electrophoresis procedures described previously (Abbott *et al.* 2000)). The enzymes assayed were acid phosphatase (*Acp*), aspartate aminotransferase (*Aat*), β–esterase (*β-Est*), glyceraldehyde-3-phosphate dehydrogenase (*G3pd*), phosphoglucoisomerase (*Pgi*), and phosphoglucomutase (*Pgm*).

The genetics of electrophoretic variants were interpreted as described by Abbott *et al.* (2000). Allozyme variation was estimated by computing the mean number of alleles per locus (*A*) (including monomorphic loci), mean percentage of polymorphic loci (*P*), and mean unbiased expected heterozygosities (*He*) using the BIOSYS-1 software (vers. 1.7; Swofford and Selander 1989). Hardy-Weinberg equilibrium was tested at each locus in each population and across loci in all populations using the GENEPOP software (vers. 3; Raymond and Rousset 1995) probability test option (Fisher's method). Estimates of genetic distance between all populations were calculated (after Nei 1972) and subjected to an unweighted pair group analysis (UPGMA) using BIOSYS-2 (Swofford and Selander, 1997).

F-statistics (Wright 1951) were estimated for *S. rupestris* as outlined by Weir (1990) using the computer program FSTAT (vers 1.2; Goudet 1995). Mean values of F, θ and f (Weir and Cockerham's, 1984) estimators of Wright's (1978) parameters of total inbreeding (*Fit*),

TABLE 1. LOCATIONS, SITE DESCRIPTIONS, COORDINATES, AND ALTITUDE OF *SENECIO RUPESTRIS* POPULATIONS

No.	Locality	Lat. (N)	Long. (E)	Alt. (m)	Collector (date)
19	Romania, Sinaia, by stream approx. 3 km W. of Sinaia	45·20	25·32	1400	RIM (12.08.92)
20	Romania, Sinaia, roadside in forest, approx. 2 km W. of Sinaia	45·20	25·33	1500	RIM (12.08.92)
21*†	Romania, Sinaia, on shingle by river, 2 km N. of Sinaia	45·21	25·33	1400	RIM (13.08.92)
22*†	Bulgaria, Sofia, Mt Vitosha, environs of hotels	42·21	23·16	1800	RIM (15.08.92)
23*†	Bulgaria, Pamporovo, near Smolyan, roadside	41·40	24·41	1550	RIM (18.08.92)
24	Bulgaria, near Smolyan, 10 km S. of Shiroka Laka, by stream	41·42	24·36	1500	RIM (19.08.92)
25*†	Greece, Mt Olympus, open disturbed habitat	40·04	21·25	2150	RIM (21.08.92)
26*†	Italy, Trentino, Molveno, environs of first chair lift station	46·09	10·59	1370	RJA & HPC (19.08.94)
27*†	Italy, Trentino, Lake Garda, Mt Baldo, environs of chair lift stn.	45·43	10·52	1850	RJA & HPC (18.08.94)
28*†	Italy, Trentino, Mt Tremalzo, Rif. Fco. Guella, disturbed ground	45·50	10·40	1580	RJA & HPC (19.08.94)
29*†	Italy, Tuscany, Abetone, carpark	44·11	10·40	1390	RJA & HPC (21.08.94)
30*†	Italy, Tuscany, Mt Falterona, roadside	43·52	11·42	1350	RJA & HPC (20.08.94)
31*†	Italy, Tuscany, Mt Secchieta, waste ground	43·43	11·37	1450	RJA & HPC (21.08.94)
32*†	Italy, Umbria , Mt Sibbelini, near Mt Prata above Pso. di Gualdo, environs of Rif. “La Baita”	42·52	13·12	1650	RJA & HPC (22.08.94)
33*†	Italy, Abruzzi, Gran Sasso d'Italia (southern range), by cable car stn. and roadside, calcareous rocks	42·27	13·34	2130	RJA & HPC (23.08.94)
34*†	Italy, Abruzzi, Mgna della Maiella, near La Maieletta, roadside exposed calcareous rock	42·09	14·07	2000	RJA & HPC (24.08.94)
35†	Italy, Calabria, La Sila Grande, Fago del Soldalo, environs of Rif. del Montanaro	39·15	16·35	1450	RJA & HPC (18.04 96)
36	France, la Vallee d'Aoste et la Savoire, Col du Petit Saint Bernard roadside, near La Thuile	45·40	06·52	2175	RJA & HPC (07.09.97)

* Populations included in survey of morphometric variation.
† Populations included in survey of cpDNA variation. Additional material examined was from Engadin (Switzerland), Pirin (Bulgaria) and Timfristos (Greece).
RJA (R. J. Abbott), HPC (H. P. Comes), RIM (R. I. Milne).
Details of locations of populations of *S. aethnensis* (1–3), *S. chrysanthemifolius* (4–6), *S. aethnensis* × *S. chrysanthemifolius* (7–9) and *S. squalidus* (10–18) are given in Abbott *et al*. (2000).

subdivision among populations (*Fst*), and inbreeding within populations (*Fis*)] were computed and their standard deviations were calculated by jackknifing over loci. To examine a possible genetic association between particular populations of *S. rupestris*, an analysis of hierarchical F_{XY} statistics (Wright 1978) was conducted using the BIOSYS-1 program. Here a two tier hierarchy was defined such that subscripts X and Y refer to populations within groups, and groups within the total.

CHLOROPLAST DNA ANALYSIS

Total genomic DNA for restriction analysis of cpDNA variation was extracted as described in Comes *et al.* (1997) from leaf tissues of individuals of *S. rupestris* from the populations indicated in Table 1. Methods for restriction enzyme digestion, fragment separation, DNA transfer, and hybridization were also as described previously (Comes *et al.* 1997). Filters were hybridized with cloned fragments of digoxigenin-labeled cpDNA from *Lactuca sativa* (Jansen and Palmer 1987).

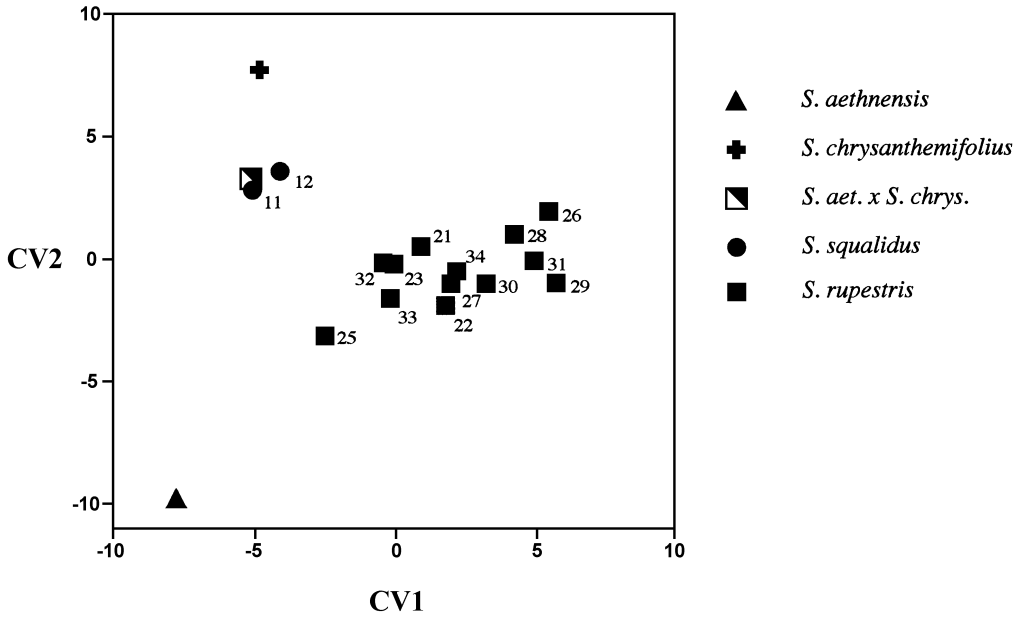

FIGURE 2. Plot of mean canonical scores for populations of *Senecio squalidus*, *S. rupestris*, *S. aethnensis*, *S. chrysanthemifolius*, and *S. aethnensis* × *S. chrysanthemifolius* on axes of canonical variates 1 and 2. Key to population numbers of *S. rupestris* is given in Table 1, and for other taxa see Fig. 1 and Abbott *et al.* (2000).

Hybridized probes were visualized using a digoxygenin nonradioactive labeling and detection kit (Boehringer Mannheim). The aim was to determine whether individuals possessed cpDNA haplotype A, B or C. This was achieved using a single probe-enzyme combination, *Lactuca sativa* probe pLsC 6/*Cla*I, which identifies a restriction site loss that distinguishes haplotype B from A, and also a 0·33–0·35 kb deletion which distinguishes haplotype C from A (Abbott *et al.* 1995).

RESULTS

MORPHOMETRIC ANALYSIS

The first three canonical variates derived from canonical variate analysis (CVA) accounted for 34·35, 25·44 and 11·51% of the total variance respectively. A MANOVA showed that differences between group means were highly significant (p<0·001, according to Wilk's Lambda, Pillai's trace, and Hotelling-Lawley trace tests of significance). The plot (Fig. 2) of mean canonical scores of groups for canonical variate 1 (CV1) against variate 2 (CV2) clearly separated the two Sicilian taxa from one another along CV2 and less so along CV1. The two British populations of *S. squalidus* and the group of hybrid plants from Mt Etna clustered with each other, and were positioned between the two Sicilian taxa but more towards *S. chrysanthemifolius*. These five groups were clearly separated from *S. rupestris* populations by CV1 and CV2. An examination of population means for each character (Table 2) shows that *S. chrysanthemifolius* possesses highly dissected leaves in contrast to *S. aethnensis* which produces almost entire leaves. It is also evident that *S. squalidus* produces leaves that are more dissected than those of *S. rupestris*, and which have a more acute angle between the apex of the primary vein and the apices of the adjacent marginal tooth sinuses. The latter character had high weightings on both CV1 and CV2 (covariance was 1·91 and -2·82 respectively).

The assignment of *S. squalidus* individuals to groups after omitting each one, in turn, from a CVA, showed that two such plants were assigned to the *S. aethnensis* × *S. chrysanthemifolius* hybrid group (one each from Edinburgh and Oxford), six were assigned to the Oxford *S. squalidus*

TABLE 2. CHARACTER MEANS OF *SENECIO* POPULATIONS

	Population																				
	S. aet.	*S. chr.*	*S. a* × *S. c*		*S. sq.*	*S. sq.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*S. ru.*	*P*
	3	4	7	8	11	12	21	22	23	25	26	27	28	29	30	31	32	33	34		
Height mm	606	703	585	695	593	483	387	443	397	412	375	384	404	392	414	410	375	391	329	***	
Inflorescence length mm	28·8	21·7	21·6	27·8	31·3	24·4	22·8	24·2	35·7	33·3	19·4	27·0	22·2	26·5	31·6	23·5	40·9	33·2	23·1	*	
Capitulum length mm	11·5	9·9	10·4	10·5	10·8	10·7	11·1	10·8	10·6	10·9	10·3	11·1	10·6	10·5	10·9	10·5	11·0	10·1	10·7	ns	
Pedicel length mm	17·3	11·8	11·1	17·3	20·5	13·8	11·8	13·5	25·1	22·3	7·6	15·8	11·5	16·1	20·7	13·0	30·0	22·5	12·4	**	
No. of pedicel bracts	3·0	3·4	2·0	5·3	3·2	2·4	3·4	2·8	3·8	2·8	2·8	3·4	2·4	2·2	2·8	2·8	2·0	2·0	1·8	ns	
Capitulum apical width mm	5·6	4·2	4·9	5·5	5·7	4·9	5·0	4·9	4·3	5·4	4·7	5·0	5·0	5·0	4·7	4·8	5·0	5·1	5·1	***	
Capitulum basal width mm	5·8	4·7	5·8	5·3	5·9	5·7	5·0	5·1	4·8	5·0	4·5	4·8	4·7	4·7	4·6	4·9	4·6	5·0	4·6	***	
No. of phyllary bracts	21·8	21·2	21·0	21·0	22·8	21·6	23·0	22·4	20·8	20·8	22·6	23·8	21·6	22·2	21·6	21·8	21·4	21·2	20·4	ns	
No. of calyculus bracts	7·6	5·0	9·0	6·7	8·2	9·0	8·8	6·0	8·4	8·2	6·6	8·8	6·8	10·0	8·4	8·0	7·6	6·8	7·0	ns	
Calyculus bract length mm	3·1	2·3	2·9	3·5	3·0	2·8	2·7	2·4	3·0	2·8	3·4	3·1	3·7	3·4	3·2	3·1	3·0	3·3	3·0	***	
No. of florets per capitulum	116	97	106	119	136	106	116	124	109	125	98	106	106	94	96	101	83	90	88	***	
Ray floret length mm	13·2	9·5	9·4	10·3	13·0	12·5	13·9	11·2	12·0	13·1	12·0	14·0	12·0	12·1	13·0	12·9	11·7	12·5	11·8	***	
Ray floret width mm	3·7	2·6	3·1	3·6	3·7	3·6	3·4	3·0	2·8	3·5	2·9	3·0	2·8	3·0	2·8	2·9	2·9	3·5	2·8	***	
No. of disc florets	105	84	93	106	123	93	102	112	97	113	85	92	92	82	83	88	71	78	76	***	
Disc floret length mm	8·0	6·8	6·9	7·3	7·2	7·0	7·2	7·0	7·0	7·2	7·3	7·3	6·9	7·1	7·2	7·0	7·2	7·5	6·8	ns	
Disc floret corolla length mm	2·8	2·4	2·5	2·8	2·5	2·6	2·5	2·3	2·4	2·5	2·4	2·4	2·3	2·4	2·5	2·3	2·6	2·8	2·5	***	
Disc floret corolla width mm	0·99	0·9	0·89	0·94	0·92	0·87	0·84	0·74	0·66	0·8	0·94	1·11	0·87	0·97	0·83	0·87	1·02	0·96	0·87	***	
Longest leaf length mm	133	128	138	144	177	152	154	143	137	116	168	144	190	185	163	180	121	133	116	***	
Midleaf length mm	107	108	116	124	125	126	123	108	118	101	136	116	147	154	132	142	103	111	99	***	
Midleaf lobe number	3·4	12·4	13·0	11·0	11·6	12·4	8·4	8·8	9·0	7·4	9·0	10·6	9·0	10·2	10·0	9·4	9·8	9·2	8·6	***	
Midleaf maximum left width mm	18·7	42·1	42·7	37·5	38·7	41·7	30·4	28·4	27·3	23·9	37·3	25·9	38·5	36·7	33·2	36·6	30·6	29·6	28·5	***	
Midleaf maximum right width mm	19·4	46·0	48·0	37·7	45·1	42·9	30·4	20·8	24·2	22·5	36·4	24·5	40·4	36·0	32·9	35·6	28·4	28·7	27·3	***	
Midleaf basal length left mm	61·7	51·9	46·5	72·0	64·9	72·6	72·9	66·1	61·5	55·8	56·5	55·3	71·7	83·5	53·7	76·0	52·1	62·0	55·4	ns	
Midleaf basal length right mm	65·9	44·8	82·0	64·0	71·9	71·6	60·8	75·6	69·7	58·2	64·9	63·2	86·3	76·3	64·7	74·1	52·7	60·8	62·6	*	
Midleaf apical angle A † (deg)	109·0	62·7	69·0	98·3	97·4	83·6	104·4	115·0	109·8	117·2	116·8	123·2	104·8	120·2	108·8	98·6	102·8	108·0	108·8	***	
Midleaf apical angle B †† (deg)	41·4	58·6	74·5	96·0	104·2	95·0	79·8	94·4	88·2	85·0	87·8	93·0	81·2	95·2	87·6	89·6	85·2	84·6	102·2	***	
Midleaf 2ndary vein angle ‡ (deg)	20·4	66·6	61·0	44·0	40·2	53·8	46·8	35·0	31·8	46·2	48·6	36·8	45·2	40·6	47·0	40·8	52·8	55·4	43·2	***	
Square root of midleaf area mm	52	44	49	54	56	51	53	50	54	42	67	52	73	80	66	75	45	53	48	***	
Midleaf perimeter mm	328	1062	1240	797	1186	1240	734	626	662	528	964	724	1012	928	850	856	606	656	670	***	
Square root midleaf area/length mm	0·49	0·40	0·42	0·44	0·44	0·40	0·44	0·45	0·46	0·42	0·50	0·45	0·50	0·52	0·51	0·52	0·43	0·48	0·49	***	
Midleaf perimeter/length mm	3·1	9·8	10·7	6·4	9·4	9·9	6·1	5·7	5·6	5·3	7·2	6·2	6·9	6·0	6·4	6·1	5·9	5·9	6·7	***	
Midleaf dissection ‡‡	6·2	24·1	25·4	14·6	21·1	24·8	13·9	12·5	12·4	12·6	14·5	13·9	14·0	11·7	12·8	11·9	13·8	12·3	13·7	***	

P = level of significant difference. *<0·05; **<0·01; ***< 0·001

† angle between the apex of the primary vein and the apices of the adjacent marginal tooth sinuses.

†† angle between the apex of the primary vein and the apices of the adjacent secondary veins.

‡ angle between the midlobe secondary vein and the primary vein.

‡‡ perimeter of the midleaf divided by the square root of its area.

TABLE 3. ALLELE FREQUENCIES AT POLYMORPHIC LOCI IN *SENECIO* TAXA AFTER POOLING DATA OVER POPULATIONS. (N = SAMPLE SIZE)

Locus/allele		Taxon *S. aethnensis*	*S. chrysanth.*	*S. aet.* × *S. chrys.*	*S. squalidus*	*S. rupestris*
Acp-2	N	(62)	(95)	(76)	(209)	(483)
a		0·645	----	0·204	0·170	1·000
b		0·355	1·000	0·796	0·830	-----
Aat-3	N	(62)	(94)	(76)	(214)	(483)
a		-----	-----	-----	-----	-----
b		-----	-----	-----	-----	0·003
c		-----	-----	-----	-----	0·001
d		0·911	0·925	0·960	0·327	0·970
e		0·089	0·075	0·040	0·673	0·025
f		-----	-----	-----	-----	0·001
β-Est-3	N	(52)	(87)	(82)	(215)	(496)
a		-----	-----	-----	-----	0·001
b		-----	-----	-----	-----	0·009
c		-----	-----	-----	-----	0·040
d		-----	-----	-----	-----	0·065
e		0·673	0·632	0·591	-----	0·531
f		0·327	0·368	0·409	1·000	0·352
g		-----	-----	-----	-----	0·002
Pgi-2	N	(61)	(88)	(84)	(211)	(478)
a		-----	-----	-----	-----	0·027
b		0·107	-----	-----	-----	0·418
c		0·893	1·000	1·000	1·000	0·555
Pgm-2	N	(59)	(85)	(81)	(237)	(495)
a		-----	0·023	-----	-----	0·022
b		0·881	0·906	0·920	0·996	0·974
c		0·119	0·071	0·080	0·004	0·004
G3pd-1	N	(63)	(89)	(84)	(197)	(480)
a		1·000	1·000	1·000	1·000	0·996
b		-----	-----	-----	-----	0·004

group (two Edinburgh and four Oxford plants), one was assigned to the Edinburgh *S. squalidus* group (an Edinburgh plant), and the remaining plant (from Edinburgh) was assigned to the *S. chrysanthemifolius* group. None of the *S. squalidus* plants was assigned to any of the *S. rupestris* groups, emphasising that these two species are distinguished morphometrically.

ALLOZYME ANALYSIS

Three of the eight loci investigated (*Acp-2*, *Pgi-1* and *Pgm-1*) were monomorphic in all populations of *S. rupestris* surveyed. At the *Acp-2* locus, all populations of *S. rupestris* were monomorphic for the allele *Acp-2a* (Table 3). In contrast, all populations of *S. aethnensis*, plus the *S. aethnensis* × *S. chrysanthemifolius* hybrid populations investigated, were polymorphic for the two *Acp-2* alleles (*a* and *b*) as were five of the nine populations of *S. squalidus*. The remaining four populations of *S. squalidus* were monomorphic for the *Acp-2b* allele as were the three populations of *S. chrysanthemifolius* surveyed.

Allele frequencies in *S. rupestris* were similar to those in the Sicilian taxa at the *Aat-3*, *β-Est-3*, *Pgm-2* and *G3pd-1* loci, except that *S. rupestris* normally possessed one or more rare alleles in addition to the common alleles at these loci (Table 3). There was a contrast between *S. squalidus* and the other *Senecio* taxa, in that it was monomorphic at the *β-Est-3* locus, and contained the *Aat-3e* allele at a much higher frequency.

TABLE 4. MEAN GENETIC DISTANCES AMONG POPULATIONS WITHIN AND BETWEEN *SENECIO* SPECIES (STANDARD ERRORS ARE IN BRACKETS).

	S. aet.	*S. chrys.*	*S. squal.*	*S. rupestris*
S. aethnensis	**0·0127** (0·0019)			
S. chrysanthemifolius	**0·0726** (0·0070)	**0·0063** (0·0029)		
S. squalidus	**0·1772** (0·0095)	**0·1080** (0·0108)	**0·0367** (0·0054)	
S. rupestris	**0·0763** (0·0047)	**0·2207** (0·0064)	**0·3058** (0·0092)	**0·0673** (0·0045)

UPGMA analysis of Nei's genetic distances between populations based on allele frequencies at all eight loci separated populations into two main clusters (Fig. 3). One cluster comprised all populations of *S. rupestris*, the three populations of *S. aethnensis* and one of the hybrid populations (population 9). The other cluster contained all populations of *S. squalidus* plus the three populations of *S. chrysanthemifolius* and two hybrid populations. Within this second cluster, two sub-groups were evident, one of which contained seven *S. squalidus* populations, while the other consisted of the *S. chrysanthemifolius* and hybrid populations, and two *S. squalidus* populations. All of the main branches of the dendrogram had low bootstrap support (<50%), which is expected when differences between taxa are based on only one or a few loci. It is clear in the present example that variation at the *Acp-2* locus is largely responsible for distinguishing taxa.

A comparison of mean genetic distances among populations within and between taxa (Table 4) shows that genetic distance between *S. aethnensis* and *S. chrysanthemifolius* is low, although considerably greater than that between populations within either of these two taxa. A similar genetic distance was evident between *S. aethensis* and *S. rupestris*, while that between *S. rupestris* and *S. chrysanthemifolius* was approximately three times greater. The greatest mean genetic distance recorded was between *S. squalidus* and *S. rupestris*. This was considerably greater than that between *S. squalidus* and either Sicilian taxon.

Levels of diversity varied across populations of *S. rupestris* (Tables 5 and 6). Most diversity was present in populations from central Italy (Umbria/Abruzzi) in contrast to populations from the southern Alps which contained very little diversity (Table 5). Only in very few populations was the coefficient of inbreeding (*f*) high and deviations from Hardy-Weinberg equilibrium significant. Thus in most populations random mating was indicated.

In *S. rupestris*, population subdivision (θ) was significant while the mean coefficient of inbreeding within populations was not (Table 7). The very high *f* value recorded at *G3pd-1* was probably due to the extremely low level of polymorphism at this locus (Table 3). Hierarchical analysis of *F* statistics of these populations (Table 8) indicates that within *S. rupestris* most populations from the Balkans (19–21, 23–25) group with populations from central Italy (32–34), while most populations from the southern Alps (26, 28) cluster with populations from the Tuscany region of Italy (29–31) and with one population from the Balkans (22). However, the amount of genetic variation partitioned between these two groups (F_{XY} = 0·142) was less than that between populations within the groups (F_{XY} = 0·183).

CHLOROPLAST DNA ANALYSIS

Senecio rupestris surveyed for cpDNA variation was sampled from the central (Engadin) and southern Alps, Tuscany, central Italy, southern Italy and the Balkans (Romania, Bulgaria and Greece). The results (Table 9) confirmed previous findings (Abbott *et al.* 1995) that haplotype A is present in *S. rupestris* from the central and southern Alps, while haplotype B occurs in material from central Italy. Material from Tuscany and Calabria (not surveyed previously) possessed only haplotype A, indicating that haplotype B might be restricted in Italy to populations occurring centrally (Umbria and Abruzzi). However, haplotype B was also found in *S. rupestris* from two sites in the Balkans (Pirin, Bulgaria; and Timfristos, Greece), along with haplotype A in material from Romania and north Bulgaria and haplotype C in plants from south Bulgaria and Greece.

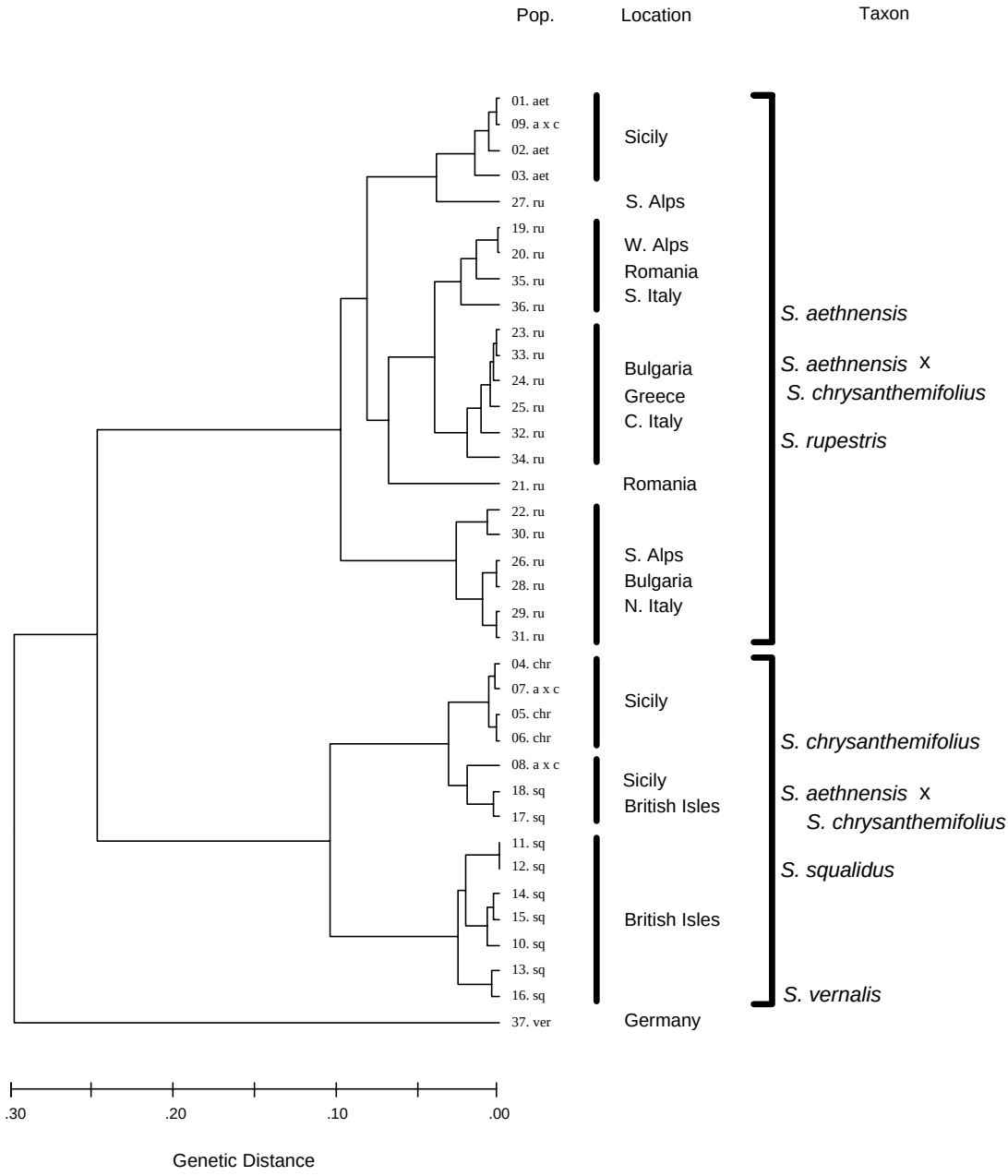

FIGURE 3. UPGMA tree constructed from genetic distances (after Nei 1972) between *Senecio* populations. Key to population numbers of *S. rupestris* is given in Table 1, and for other taxa see Fig. 1 and Abbott *et al.* (2000).

TABLE 5. ALLOZYME VARIABILITY ESTIMATES AND WEIR & COCKERHAM'S (1984) COEFFICIENT OF INBREEDING (*F=FIS*) IN POPULATIONS OF *S. RUPESTRIS.* TESTS OF HARDY-WEINBERG EQUILIBRIUM ACROSS LOCI WITHIN SAMPLES (FISHER'S METHOD) ARE INDICATED

Population	Sample size†	*A*†	*P*	*He*†	*f*††
19	19·9 (0·1)	1·4 (0·3)	25·0	0·096 (0·063)	-0·277
20	26·9 (0·1)	1·3 (0·2)	25·0	0·092 (0·060)	0·336*
21	17·0 (0·0)	1·5 (0·3)	37·5	0·157 (0·078)	0·257
22	27·3 (0·2)	1·8 (0·3)	50·0	0·161 (0·072)	0·130
23	24·1 (0·1)	1·4 (0·2)	37·5	0·130 (0·081)	0·097
24	29·9 (0·1)	1·8 (0·3)	50·0	0·141 (0·082)	0·191
25	30·0 (0·0)	1·5 (0·3)	37·5	0·128 (0·079)	0·323*
26	31·8 (0·5)	1·3 (0·2)	25·0	0·015 (0·010)	0·497
27	29·4 (0·2)	1·0 (0·0)	0·0	0·000	-------
28	28·1 (0·4)	1·3 (0·2)	25·0	0·040 (0·035)	-0·161
29	27·9 (0·6)	1·4 (0·3)	25·0	0·089 (0·062)	-0·069
30	26·0 (1·5)	1·4 (0·2)	37·5	0·082 (0·058)	0·021
31	27·9 (0·4)	1·4 (0·2)	37·5	0·110 (0·061)	0·010
32	31·4 (0·4)	1·5 (0·3)	25·0	0·156 (0·102)	-0·077
33	32·8 (0·3)	1·8 (0·3)	50·0	0·162 (0·088)	-0·010
34	30·0 (0·0)	2·0 (0·6)	37·5	0·178 (0·110)	0·063
35	12·0 (0·0)	1·1 (0·1)	12·5	0·010 (0·010)	0·000
36	34·0 (0·0)	1·4 (0·3)	25·0	0·076 (0·065)	0·086**

† Standard errors in parentheses
†† Hardy-Weinberg equilibrium rejected at * $P < 0{\cdot}05$ and ** $P < 0{\cdot}01$
A: mean no. of alleles per locus
P: percentage of polymorphic loci
He: mean expected heterozygosity

DISCUSSION

Morphometric analysis of material raised under the same greenhouse conditions showed that *S. rupestris* is morphologically distinct from *S. squalidus*. As previously reported by Abbott *et al*. (2000), *S. squalidus* closely resembles material sampled from the hybrid zone on Mt Etna, and possesses a phenotype intermediate to that of the two Sicilian taxa, although tending more towards that of *S. chrysanthemifolius* than *S. aethnensis*. The current analysis shows that *S. squalidus* produces leaves that are more dissected than those of *S. rupestris*, and which have a more acute angle between the apex of the primary vein and the apices of the adjacent marginal tooth sinuses.

The survey of allozyme variation confirmed the close similarity of *S. squalidus* to most *S. aethnensis* × *S. chrysanthemifolius* hybrid material and also to *S. chrysanthemifolius*. These were placed in a separate cluster from *S. rupestris*, *S. aethnensis* and one of the hybrid populations (9), in a UPGMA tree constructed from Nei's genetic distances between populations. The greatest mean genetic distance recorded between taxa was evident for *S. squalidus* and *S. rupestris* (0·306). This difference was considerably greater than that recorded between *S. squalidus* and *S. aethnensis* (0·177), and between *S. squalidus* and *S. chrysanthemifolius* (0·108).

S. rupestris populations from different parts of Europe differed greatly in level of allozyme diversity. Populations from central Italy and the Balkans contained relatively high levels of allozyme diversity based on mean number of alleles per locus and unbiased expected heterozygosity. In contrast, populations from the Alps contained very low levels or no diversity at all (Table 3), while populations from Tuscany contained intermediate levels of diversity. The relative high levels of allozyme diversity in central Italian and Balkan populations is of interest as except at high altitude these regions remained unglaciated during the last Ice Age (Hewitt, 1996)

TABLE 6. MEANS OF ALLOZYME VARIABILITY ESTIMATES (*A*, *P* AND *HE*) IN SAMPLED *SENECIO*

Species	Populations	*A*	*P*	*He*
S. aethnensis	1–3	1·50	50·0	0·174
S. chrysanthemifolius	4–6	1·50	37·5	0·098
S. aet. × *S. chrys.*	7–9	1·50	50·0	0·117
S. squalidus	10–18	1·23	20·8	0·063
S. rupestris - All	19–36	1·46	31·2	0·101
Balkans	19–25	1·53	37·5	0·129
S Alps	26–28	1·20	16·7	0·018
Tuscany	29–31	1·40	33·3	0·094
Umbria/Abruzzi	32–34	1·77	37·5	0·165

A = average number of alleles per locus; *P* = percentage of polymorphic loci; *He* = expected heterozygosity

TABLE 7. WEIR & COCKERHAM'S (1984) ESTIMATES OF WRIGHT'S *F*-STATISTICS CALCULATED SEPARATELY FOR EACH LOCUS FOR ALL POPULATIONS OF *SENECIO RUPESTRIS*

Locus	*F*	θ	*f*
Aat-3	0·116 (0·094)	0·034 (0·017)	0·084 (0·096)
β-Est-3	0·540 (0·072)	0·453 (0·076)	0·159 (0·062)
Pgi-2	0·253 (0·105)	0·272 (0·094)	-0·026 (0·057)
Pgm-2	0·495 (0·207)	0·193 (0·087)	0·356 (0·172)
G3pd-1	0·972 (0·472)	0·065 (0·031)	0·938 (0·456)
Mean (Std. Dev.)	0·415 (0·162)	0·368 (0·111)	0·061 (0·101)
95% confidence interval	0·218–0·540	0·059–0·445	-0·013–0·271

Means and standard deviations were obtained by jackknifing over loci. Confidence intervals were obtained by bootstrapping over loci. Locus specific standard deviations were obtained by jackknifing over populations.
$F = Fit$ (within total), $\theta = Fst$ (among populations), $f = Fis$ (within populations)

TABLE 8. HIERARCHICAL *F*-STATISTICS FOR POPULATIONS OF *SENECIO RUPESTRIS* COMBINED ACROSS LOCI

Comparison x	y	Variance component	Fxy
Population	Group	0·1940	0·183
Group	Total	0·1757	0·142
Population	Total	0·3697	0·299

Groups: A (Balkans: 19–21, 23–25; central Italy: 32–34, populations)
B (S. Alps: 26–28; Tuscany: 29–31; and Balkan: 22, populations)
N.B. Populations 35 (La Sila Grande) and 36 (La Thuile) were excluded from analysis

and could have served as refugia for *S. rupestris* during this period. Populations of species that currently occur in areas that were refugia during previous glacial periods often contain high levels of genetic diversity relative to populations that occur in formerly glaciated regions (Comes & Kadereit 1998; Hewitt 2000). The low level of diversity within alpine populations of *S. rupestris* might stem, therefore, from the fact that these populations occupy formerly glaciated regions and are derived from colonists that sampled only a limited amount of genetic variation present in a refugial, source population.

TABLE 9. DISTRIBUTION OF CHLOROPLAST DNA HAPLOTYPES A, B AND C IN *SENECIO* MATERIAL

Taxon	No. of populations	Haplotype A	B	C
S. aethnensis	3	(2)	(3)	--
S. chrysanthemifolius	7	--	(11)	--
S. aet. × *S. chrys.*	4	(2)	(6)	--
S. squalidus	21	--	(22)	--
S. rupestris	22	22 (7)	6 (1)	18 (12)
Balkans	9	8 (6)	2	18 (12)
S. Alps	5	9 (3)	--	--
Tuscany	3	3	--	--
C. Italy (Umbria/Abruzzi)	4	--	4 (1)	--
S. Italy (Calabria)	1	2	--	--

Numbers of each haplotype not in brackets are results from present study combined with those of Abbott *et al*. (1995); numbers in brackets are from Abbott *et al*. (1995).
N.B. Indviduals haplotyped in present study were: Balkans (Romania - Sinaia 1A; Bulgaria - Mt Vitosha 1A, Pirin 1B, Pamporovo 1C; Greece - Mt Olympus 1 C, Timfristos 1B, Mistras 4C); C and S Alps (Engadin 1A, Mt Baldo 3 A, Mt Tremalzo 1A, Molveno 1A); Tuscany (Abetone 1 A, Mt Falterona 1A, Mt Secchieta 1A); C. Italy (Mt Sibbelini 1B, Gran Sasso 1B, Mt Maiella 1B); Calabria (La Sila Grande 2A).

Of further interest is the close genetic link between populations from central Italy (populations 32–34) and populations from the southern Balkans (23–25) indicated by hierarchical *F* statistics analysis (Table 8). This link might have arisen and/or been maintained by gene flow between these regions during previous glacial periods when a north-Adriatic land mass is believed to have connected the Balkans to central Italy (Witte,1965). Although central Italian and southern Balkan populations investigated for allozyme variation differed for cpDNA haplotype, cpDNA analysis revealed that the B haplotype in central Italian material is also present in other material examined from the southern Balkans (from Pirin, Bulgaria, and Timfristos, Greece). Hence, cpDNA evidence agrees, in part, with allozyme evidence in suggesting a genetic link between populations from these two regions.

Also indicated by the hierarchical *F*-statistics was a close genetic affinity between two of the southern alpine populations (26, 28), three populations from Tuscany (29–31) and the population from Mt Vitosha in north Bulgaria (22). All material from these populations also contained cpDNA haplotype A. This close affinity might reflect the evolutionary history of these populations as affected by events occurring during and following the glaciations of the Pleistocene; however, it is not possible to differentiate between the possibility of whether the two alpine populations might be derived from Bulgarian or Tuscan source populations, if indeed either of these two groups served as ancestral, source material.

This study has shown that British material of *S. squalidus* can be distinguished from *S. rupestris* by its morphology (leaf shape) and allozyme make-up. It resembles more closely hybrid material of *S. aethnensis* and *S. chrysanthemifolius*, indicating that it is a hybrid derivative of these two species rather than a derivative of *S. rupestris*. Nonetheless, *S. squalidus* closely resembles *S. rupestris* in overall morphology and consequently the two taxa are difficult to distinguish. This led Alexander (1979) to place *S. rupestris* into synonymy under *S. squalidus*. However, the current study shows that the two taxa are separate entities from an evolutionary perspective.

A final point of interest concerns the introduction of *S. squalidus* to the British Isles. Druce (1927) proposed that *S. squalidus* was introduced to the Oxford Botanic Garden from Mt Etna in the latter part of the 17th century. However, Harris (2002) has questioned this assertion following a detailed analysis of relevant herbarium material and the historical literature. According to Harris (2002), plant material morphologically similar to *S. squalidus*, was first grown in Britain in the Duchess of Beaufort's Garden at Badminton, England, in the early part of the 18th century, from seed supplied from Sicily by Cupani. This material was most likely the source of *S. squalidus* later grown in Oxford, which subsequently spread to many parts of the British Isles during the 19th and 20th centuries.

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