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A cytological and molecular study of the genera *Scorzonera* L. and *Podospermum* (L.) DC (Asteraceae)

Winfield Mark Owen[1], Giovanni D'Amato[2], Raffaele Ilio De Dominicis[2], Paola Salimbeni[3] and Gian Franco Tucci[3]

[1] SCRI, Invergowrie, Dundee, DD2 5DA, Scotland.
[2] Dip. Biologia Vegetale - Università "La Sapienza" – Piazzale A. Moro 5, 00185 Roma, Italy
[3] D.A.B.A.C. - Università della Tuscia – Dipartimento di Agrobiologia ed Agrochimica, Via S. Camillo de Lellis, 01100 Viterbo, Italy

Abstract — The genus *Scorzonera* L. s.l. encompasses enormous morphological variation and has created problems for taxonomists. *Podospermum* has been treated as either a section of a broadly defined *Scorzonera* or as an independent genus. In Italy, there are twelve *Scorzonera* species and three *Podospermum* species. The relationship between these species is not at all clear. To clarify these relationships, a survey, including thirteen of the fifteen taxa found in Italy, was carried out using: i) sequence analysis of ribosomal internal transcribed spacer (ITS) and external transcribed spacer (ETS) regions; ii) analysis of Amplified Fragment Length Polymorphisms (AFLPs); iii) chromosome counts. In addition, to broaden the scope of the work and allow taxonomically significant interpretation of the data to be made, sequences from a range of related species were obtained from the EMBL nucleotide sequence database. Our analyses clearly indicate that *Scorzonera* s.l. is a polyphyletic grouping. The three approaches used were consistent with each other although showing different levels of resolution. There was broad division, on the basis of chromosome number, either 2n = 12 or 14. This was supported by PCO analysis of AFLP fragments which indicate three groups. Finally, phylogenetic analysis of sequence data produced four to six highly supported clades that might appropriately be given independent taxonomic status.

Key words: AFLP, chromosomes, ETS, ITS, phylogeny, *Scorzonera,* taxonomy.

INTRODUCTION

The genus *Scorzonera* L. s.l. comprises about 160 species - these are widely spread in arid regions of Eurasia and Africa and exhibit great morphological variability. The taxonomic treatment of the genus has been highly contentious (Nazarova 1997; Mavrodiev *et al.* 2004), and regional Florae present differing treatments of the species contained therein. The contrasting treatments offered by Flora d'Italia (Pignatti 1982) and Flora Europaea (Charter 1976) are a case in point. Pignatti (1982), following the system initially proposed by de Candolle (1805), places some species of *Scorzonera* s.l. in the separate genus *Podospermum.* In Flora Europaea (Charter 1976), using a treatment based on the system proposed by Lipschitz (1935), *Podospermum* is not granted generic status, while the genus *Scorzonera* is divided into three sections: *Podospermum* (L.) DC Boiss, *Scorzonera* L. and *Lasiospora* Less. More recent treatments have recognised the polyphyletic nature of *Scorzonera* s.l. and split various taxa from it and granted them independent generic status (Nazarova 1990; 1997).

Beyond the broad issue of the treatment of these two genera, the relationship between the taxa is not clear. For instance, *S. purpurea* has at times been placed in the genus *Scorzonera* and at others in *Podospermum* (Mavrodiev *et al.* 2004). The taxon *S. rosea* is given generic status by Pignatti but considered to be a subspecies of *S. purpurea* in Flora Europaea (Charter 1976). On the basis of pappus length, Pignatti distinguishes *S. villosa* Scop. from *S. hirsuta* L.: in the former the pappus is 12 – 15 mm long, whilst in the second it is approximately 20 mm. In addition, *S. villosa* is said to produce achenes that may be either villous or glabrous - generally, however, this is considered of little taxonomic value. Flora Europaea places *S. villosa* in the section *Scorzonera* and describes it as having only glabrous achenes. *S. hir-*

Corresponding author: M.O. Winfield; email: Mark.Winfield@scri.ac.uk

suta, on the other hand, is placed in the section *Lasiospora* and is distinguished from the former taxa in having densely villous or lanate achenes and longer pappus-hairs in proportion to the length of the achene. There are also differences between the two florae with regard the collocation of *S. glastifolia* Willd. and *S. trachysperma* Guss. with respect to *S. hispanica* L.. On the basis of morphology and geographic distribution, PIGNATTI considers them to be three distinct species: *S. hispanica*, found in Friuli and Piemonte, has linear spatolate leaves, and a branched stem; *S. glastifolia*, found in Piemonte, Liguria and the Appennini, has strictly linear leaves and an unbranched stem; *S. trachysperma,* found in the south of Italy, has a basal rosette of leaves with the cauline leaves much reduced, and a simple unbranched stem. In Flora Europaea, however, only a single, highly morphologically variable species, *S. hispanica*, is described.

According to PIGNATTI (1982), there are twelve species of the genus *Scorzonera* and three species of the genus *Podospermum* in Italy. The genus *Scorzonera* in this classification contains perennial species that have entire leaves, and achenes that may or may not have a tubular base. Species of *Podospermum*, on the other hand, may be perennial, biennial or annual, and have pinnatisect leaves and achenes with a tubular base. The aim of this study was to provide evidence to clarify the relationship between the genera *Scorzonera* and *Podospermum,* and to give support to the positioning of the species found therein. We have used both cytological (chromosome counts) and molecular (ITS/ETS sequence data and AFLP analysis) approaches. Chromosome counts were made for 11 of the 15 Italian species. Sequencing of ribosomal ITS1, ITS2 and ETS (partial) regions was performed and phylogenetic trees constructed using Parsimony, Maximum Likelihood and Batesian Analysis. To obtain a broader sampling of the genome, AFLP analysis was also performed using three primer pairs. Sequences from ITS and ETS regions have been shown to be phylogenetically informative at the genus and species level (BALDWIN AND MARKOS 1998) as has the utility of shotgun, broad scale genome probing techniques such as Randomly Amplified Polymorphic DNA (RAPD) and AFLP analysis (JORGENSEN *et al.* 2003; VILATERSANA *et al.* 2005). Finally, to place the species studied in a correct taxonomic framework, sequences of a range of taxa closely related to those directly under study were obtained from the National Center for Biotechnology Information (NCBI) nucleotide sequence database and phylogenetic trees constructed.

MATERIALS AND METHODS

Study species - The taxa used in the study are those found in Italy: *P. canum* C.A. Meyer (syn. *S. cana*), *P. laciniatum* (L.) DC, (syn. *S. laciniata*), *S. aristata* Ramond, *S. austriaca* Willd., *S. callosa* Moris, *S. deliciosa* Guss., *S. glastifolia* Willd, *S. hirsuta* L., *S. hispanica* L., *S. humilis* L., *S. purpurea* L., *S. trachysperma* Guss., and *S. villosa* Scop. Two of the fifteen taxa reported to be present in Italy, *S. rosea* and *P. resedifolium*, were not found and so are not included in the study. The taxa *Centaurea aeolica* Guss. and *Silybum marianum* (L.) Gaertner, were also collected and their ITS and ETS sequences included to provide an outgroup for the molecular analysis (Table 1).

Table 1 — List of species examined their sites of collection and the accession numbers given to the sequences on submission to the EMBL nucleotide sequence database.

Species	Locality	Accession No.
P. canum (syn. *S. cana*)	Lucoli (L'Aquila)	AM117044
P. laciniatum (syn. *S. laciniata*)	San Quirico d'Orcia	AM117045
S. aristata	Abetone (Pistoia)	AM117046
S. austriaca	Monte Prinzera (Parma)	AM117047
S. callosa	Monte Limbara (Sassari)	AM117048
S. deliciosa	Menfi (Trapani)	AM117049
S. glastifolia	Tolfa (Rome)	AM117050
S. hirsuta	Lentella (Chieti)	AM117051
S. hispanica	Cultivated: Botanic Garden Rome	AM117052
S. humilis	Cultivated: Botanic Garden Rome	AM117053
S. purpurea	Forca Canapine (Perugia)	AM117054
S. trachysperma	Ferrandina (Matera)	AM117055
S. villosa	Matera	AM117056
Centaurea aeolica		AM117057
Silybum marianum		AM117058

Samples were collected from their natural habitat and are conserved both *in vivo* and as herbarium specimens in the Department of Botany (Dipartimento di Biologia Vegetale) of "La Sapienza" University, Rome.

Related sequences were obtained by performing a BLAST search of the NCBI nucleotide sequence database (see table 2 for accession number of these sequences). ETS sequences were not available for any of the species studied. Where possible, only those accessions with complete ITS1 and ITS2 sequences were used in the analysis. These included, where available, replicates of sequences extracted by us, plus a range of taxa that Flora Europaea gives as closely related to the taxa directly under study (table 2).

Table 2 — Species list (n=22) and accession number for the sequences obtained from the NCBI nucleotide sequence database. N.B. The first given name in the list below is that used in the NCBI database; where these are synonymous to the names used by us, the relevant synonym is presented in brackets.

Species	Accession No.
Koelpinia linearis	AJ633492
K. turanica	AJ633491
Lasiospora hirsuta (syn. *S. hirsuta*)	AY508197
Podospermum canum (syn. *S. cana*)	AJ633480
P. laciniatum (syn. *S. laciniata*)	AJ633475
S. angustifolia	AJ633488
Scorzonera aristata	AY508192
S. austriaca	AY508216
S. cretica	AJ633481
S. crispatula	AJ633486
S. doria	AJ633478
S. graminifolia	AJ633487
S. hirsuta	AJ633479
S. hispanica	AJ633472
S. humilis	AJ633476
S. purpurea	AJ633477
S. villosa	AJ633482
S. undulata	AJ633485
Takhtajaniantha pusilla (syn. *S. pusilla*)	AY508205
Tourneuxia variifolia	AY508202
Tragopogon dubius	AJ633503
T. minor	AJ633495

Karyotyping - Root tips were obtained from 11 of the 13 taxa studied - *P. laciniatum* was available only as an herbarium specimen; *S. callosa* did not grow well and good root tips could not be obtained. These were placed in 0.3% colchicine for 2 h at room temperature to accumulate dividing cells in metaphase. Root-tips were fixed in 3:1 ethanol:glacial acetic acid for a minimum of 2 h and stored at 4°C until used. For chromosome counts, root tips were hydrolysed in 1N HCl at 60° for 7-8 mins and then stained with Feulgen (HEITZ 1936).

DNA extraction - Extractions of total genomic DNA were made from single leaves of fresh material except in the case of *P. laciniatum* for which dried, herbarium material was used. The CTAB method of ROGER and BENDICH (1994) was used throughout.

Sequencing - Sequencing of the ITS and ETS regions was performed using a Perkin Elmer 2400 thermocycler under the following conditions: Taq polymerase kit (Invitrogen®, cat.10342-012) with a thermal profile of 95° for 3 min to denature the DNA, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 30 s. There was a final extension of 72°C for 7 min, and then the samples were held at 4°C. The primers used for the amplification of the ITS regions were those of WHITE *et al.* (1990). In the case of *S. callosa*, however, these primers were not effective, and amplification was obtained using primers anchored to 18S (5'-CGTAACAAGGTTTCCGTAGG-3') and 25S (5'-CAGCGGGTAGTCCCGCCTGA-3') regions (VENORA *et al.* 2000). The ETS region was amplified using primers U6 (5'-GTACGGTGCATGAGTGGT- 3') and U317 (5'-ATATGACTACTGGCAGGA-3') designed by TUCCI *et al.* (1994). The thermal profile used was: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 50°C for 30 s and 72°C for 1.5 min. An extension of 72°C for 7 min was added to the end of the programme.

Sequence editing and alignment - Control of sequence data was performed in DNAMAN. In all cases, forward and reverse strands were aligned to ensure that sequence data was correct. Where any doubt existed about the quality of the sequence, the sample was re-sequenced. The ITS (sequence including 5.8) and ETS sequences were exported to a single text file and concatenated. Alignment was performed in Clustal X using default gap opening and extension penalties. A small amount of manual editing of the resulting alignment was performed (c. 20/1084) to adjust for perceived misalignment around gaps.

Phylogenetic analysis - The final alignment file was imported into PAUP. To determine the most appropriate model for maximum likelihood analysis the programme MODELTEST (POSADA and CRANDALL 1998) was used. Within the PAUP block, *Centaurea aeolica* and *Silybum marianum*

were defined as outgroup species. Although the whole of the concatenated sequence was input to PAUP (facilitating alignment), the 5.8 sequence was excluded from the phylogenetic analysis. Maximum likelihood was performed using a heuristic search; 2000 bootstrap replications were performed and a 50% majority rule tree obtained. Bayesian analysis was performed with 1.5 million generations, sampling every 1000 generations and a burn-in of 1500. A 50% majority rule tree with clade credibility values was obtained.

AFLP analysis - Selective primers were 5' end labelled with either 6-Fam or Hex (TAGN, Newcastle) and PCR was performed in a Tetrad thermal cycler (MJ Research) programmed for 12 cycles of 30s at 94°C, 30s at 65°C (reducing 0.7°C per cycle), 1 min at 72°C, followed by 23 cycles of 30s at 94°C, 30s at 56°C and 1 min at 72°C. One microlitre of PCR was mixed with 0.04 µl of Genescan ROX 500 internal lane size standard (Applied Biosystems) and 6.96 µl of HiDi formamide (Applied Biosystems). AFLP analysis was carried out using an ABI 3730 Genetic Analyser using Genemapper version 3.7 (Applied Biosystems).

For the analysis of AFLP markers, the binary matrix was subjected to Principal Coordinate Analysis (PCO) using Genstat 7 (PAYNE *et al.* 1993). The first five coordinates were recorded; the first three were plotted (Fig. 12).

RESULTS

Karyotypes - Chromosome counts of 11 taxa were made (counts not made from *P. laciniatum* and *S. callosa*). The two taxa *S. hirsuta* and *S. villosa* possessed 12 chromosomes, and both had a large chromosome with a satellited short arm. All other species studied were 2n = 14 (Figs 1-11).

AFLP analysis - The total number of fragments produced from the three primer pairs was 177. All of these were polymorphic, and many were specific to an individual taxon. PCO analysis based on the AFLP data matrix produced three distinct clusters (Fig. 12). The first principal coordinate (13.9% of variation explained) separated the three taxa *S. callosa, S. hirsuta* and *S. villosa* (cluster A) from all others. The second and third coordinates (11.1% and 10.4% of variation explained, respectively) separated *S. glastifolia, S. hispanica* and *S. trachysperma* (cluster B) from the remaining taxa (cluster C). The only taxon that didn't cluster with other taxa was *S. austriaca* that lay at an intermediate position between clusters B and C (Fig. 12).

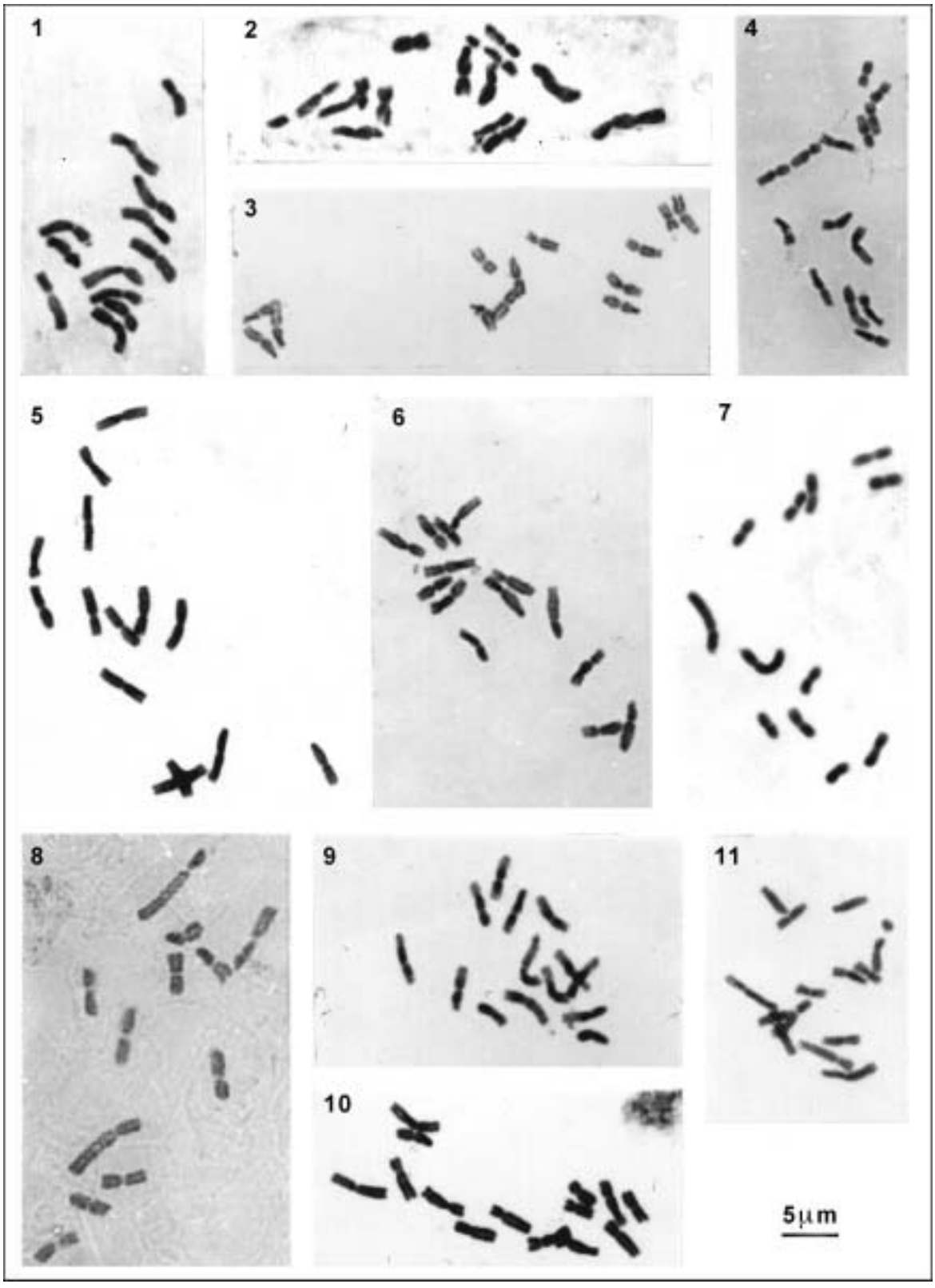
Sequence analysis - The length of the concatenated alignment (ETS, ITS1, rDNA 5.8S subunit and ITS2) was 1084 base pairs (bp); sequence lengths ranged from 1021 (*S. aristata*) to 1048 (*S. hirsuta*). The length of the aligned sequences and the range for each is shown in Table 3.

The ETS sequences were slightly more variable than the ITS sequences (mean distance between taxa was 0.2078, 0.1871 and 0.1745 for the ETS, ITS1 and ITS2 regions, respectively).

The concatenated sequence (5.8 was excluded from the phylogenetic analysis) had 521 variable characters of which 371 were parsimony informative. Heuristic searches found two most parsimonious trees, which had the same topology apart from the relative positions of the three taxa *S. deliciosa S. glastifolia* and *S. hispanica* (tree not shown). Nested likelihood ratio tests performed in MODELTEST indicated that the best model of nucleotide substitution was TrN+G (base frequencies: A = 0.2348; C = 0.2208; G = 0.2337; T = 0.3107; gamma shape distribution of $\alpha = 0.9549$). These parameters were used in a heuristic search for a maximum likelihood tree and for Baysian analysis (Fig. 13). The ML and Baysian trees were consistent with each other and with that produced by Parsimony. That is, there were four distinct clades all with greater than 97% support. For convenience of description, these clades have been given the name of one of the taxa contained therein: Hirsuta, Hispanica, Aristata and Po-

Table 3 — Range of sequence lengths and aligned sequence length for the 12 ingroup taxa sequenced by our group. The base frequencies, again only for the ingroup taxa, for the four regions is also shown

	Length (aligned)	Range	A	C	G	T
ETS	425	387 - 407	0.194	0.208	0.256	0.341
ITS1	265	253 – 256	0.253	0.257	0.273	0.217
5.8	163	160 - 162				
ITS2	231	217 - 226	0.176	0.264	0.278	0.283
Concatenated	1084	1017 - 1051	0.217	0.241	0.266	0.276



Figs 1-11 — Metaphase plates of 11 out of 13 species examined. Fig. 1. *S. Purpurea*, Fig. 2. *S. deliciosa,* Fig. 3. *S. hispanica,* Fig. 4. *S. trachysperma,* Fig. 5. *S. humilis,* Fig. 6. *S. aristata,* Fig. 7. *S. hirsuta,* Fig. 8. *S. villosa,* Fig. 9. *S. glastifolia,* Fig. 10. *S. austriaca,* Fig. 11. *P. canum.* (*P. laciniatum* and *S. callosa* are not shown because of the difficulty of obtaining clear metaphase spreads for these two species).

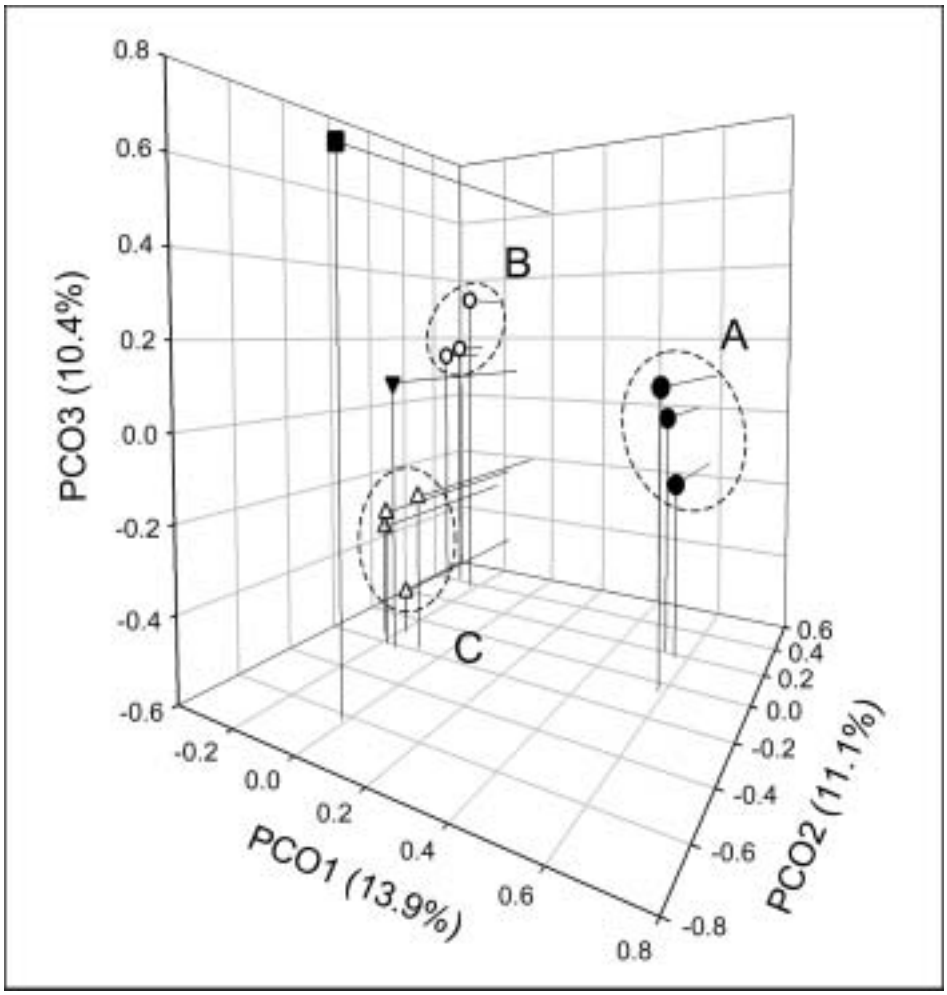


Fig. 12 — PCO plot (first three components) based on the AFLP data. The in-group species fall into three clusters: A) *S. callosa, S. hirsuta* and *S. villosa*; B) *S. glastifolia, S. hispanica* and *S. trachysperma*; C) *S. humilis,, S. aristata, S. purpurea* and *P. canum*. *S. austriaca* (black triangle) falls between clusters B and C. The out-group taxon, *Centaurea aeolica* (black square) lies distant from all the in-group taxa.

dospermum (Fig. 13): the Hirsuta clade contains *S. callosa*, *S. hirsuta* and *S. villosa*; the Hispanica clade contains *S. trachysperma*, *S. deliciosa*, *S. glastifolia* and *S. hispanica*; the Aristata clade contains *S. aristata* and *S. humilis*; and the Podospermum clade contains *S. cana*, *S. laciniata* and *S. pupurea* (an alternative interpretation would be that *S. pupurea* is sister to the *Podospermum* clade). *S. austriaca* was not well resolved in either tree: in the ML tree it formed a polytomy with the Hispanica clade and Aristata/Podospermum clades; in the Bayesian tree, it lay poorly supported (clade credibility value of 68), with the Hispanica clade.

The 5.8 sequence (not used in the ML and Baysian analyses) was 160 bp long in all taxa except *S. trachysperma* in which it was 162 bp. Although, as one would have expected *a priori*, this was the most highly conserved region of the sequence, there was a small number of base substitutions between the species. These were consistent with the phylogeny produced form the rest of the sequence and so are unlikely to be the result of sequencing error: i.e., at position 4 the two *P. podospermum* species have a T while all other taxa possess C; at position 124 the taxa in the Hirsuta clade have a G while all other taxa have A; at 147 the members of the Hispanica clade and *S. purpurea* have T and all others have C. The variation in the length of the ITS2 sequence was also consistent with the taxonomic relationships; that is, the three taxa in the Hirsuta clade (Fig. 13) all possessed an ITS2 region of 226 bp. In all other taxa the ITS2 region was shorter than this.

The length of the multiple alignment containing both the Italian samples (n = 13; out-group taxa not included) and those obtained from the

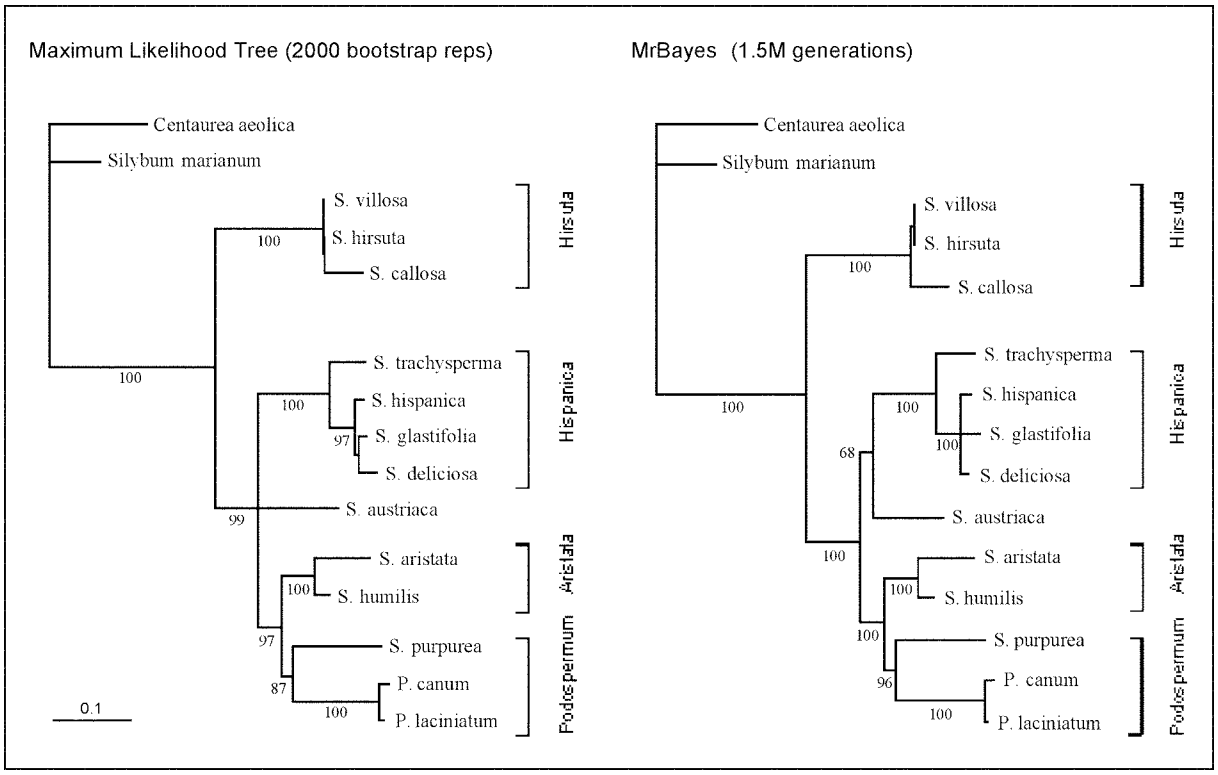


Fig. 13 — ML and Baysian trees (50% consensus trees) derived from the alignment of the sequences of the 13 species collected for this study. Bootstrap values on the ML tree are based on 2000 replicate; the clade credibility values are based on 1.5M iterations. Trees rooted with *Centaurea aeolica* and *Silybum marianum*.

NCBI database (n = 22) was 668bp: 322bp were constant, and of the 346 variable sites, 276 were informative. Both Parsimony and Maximum Likelihood analyses were performed on this combined data set. In both analyses, replicate samples either fell together with (*P. canum, S. austriaca, S. aristata, S. humilis, S. hispanica* and *S. purpurea*), or very close to (*P. laciniatum, S. villosa*), their sister sequence. The three sequences of *S. hirsuta* (N.B. *Lasiospora hirsuta* – accession number AY508197 – is a synonym of *S. hirsuta*) did not all fall together and were somewhat interspersed with those of other taxa. However, they did all fall in the same clade (Figs. 14 and 15). The sequences of the other samples of *Scorzonera* obtained from the NCBI database were principally collocated with the taxa to which one would have expected from their treatment in Flora Europaea (CHARTER 1976). That is, *S. cretica* and *S. doria* fell in the Hirsuta clade with *S. hirsuta* and *S. villosa*; *S. crispatula* fell into the Hispanica being similar to *S. hispanica*; *S. graminifolia* fell out with the *Podospermum* species (Figs. 14 and 15). *S. undulata*, on the other hand, that one might have expected to fall out with *Podospermum* or *S. purpurea*, was almost indistinguishable from the two *S. hispanica* samples and fell into the Hispanica clade.

The ML (Fig. 14) and parsimony (Fig. 15) trees were very similar in their general topology and the clades identified on the basis of the analysis including only the Italian samples (Fig. 13) were again evident. The Hisrsuta clade was, as in figure 13, very distinct from all other groups. *Scorzonera s.l.* is clearly not a single taxonomic entity. Taxa belonging to the genera *Tourneuxia* (monotypic), *Tragopogon* and *Koelpinia* split the group: on the parsimony tree (Fig. 15) all three lie between the Hirsuta clade and the other "*Scorzonera*" clades, while on the ML tree (Fig. 14) *Koelpinia* lies sister to the Aristata and Podospermum clades. *S. austriaca* fell out as distinct taxon closely related and sister to the monotypic taxon *Takhtajaniantha.*

The only significant difference between the trees with respect to the taxa of interest (i.e., those belonging to *Scorzonera* s.l.) was the collocation of *S. purpurea*. On the ML tree (Fig. 14) it fell out with *S. angustifolia* as a sister clade to that containing *aristata* and *S. humilis*. On the parsimony tree (Fig. 15) it formed a sister to that of the Podospermum clade.

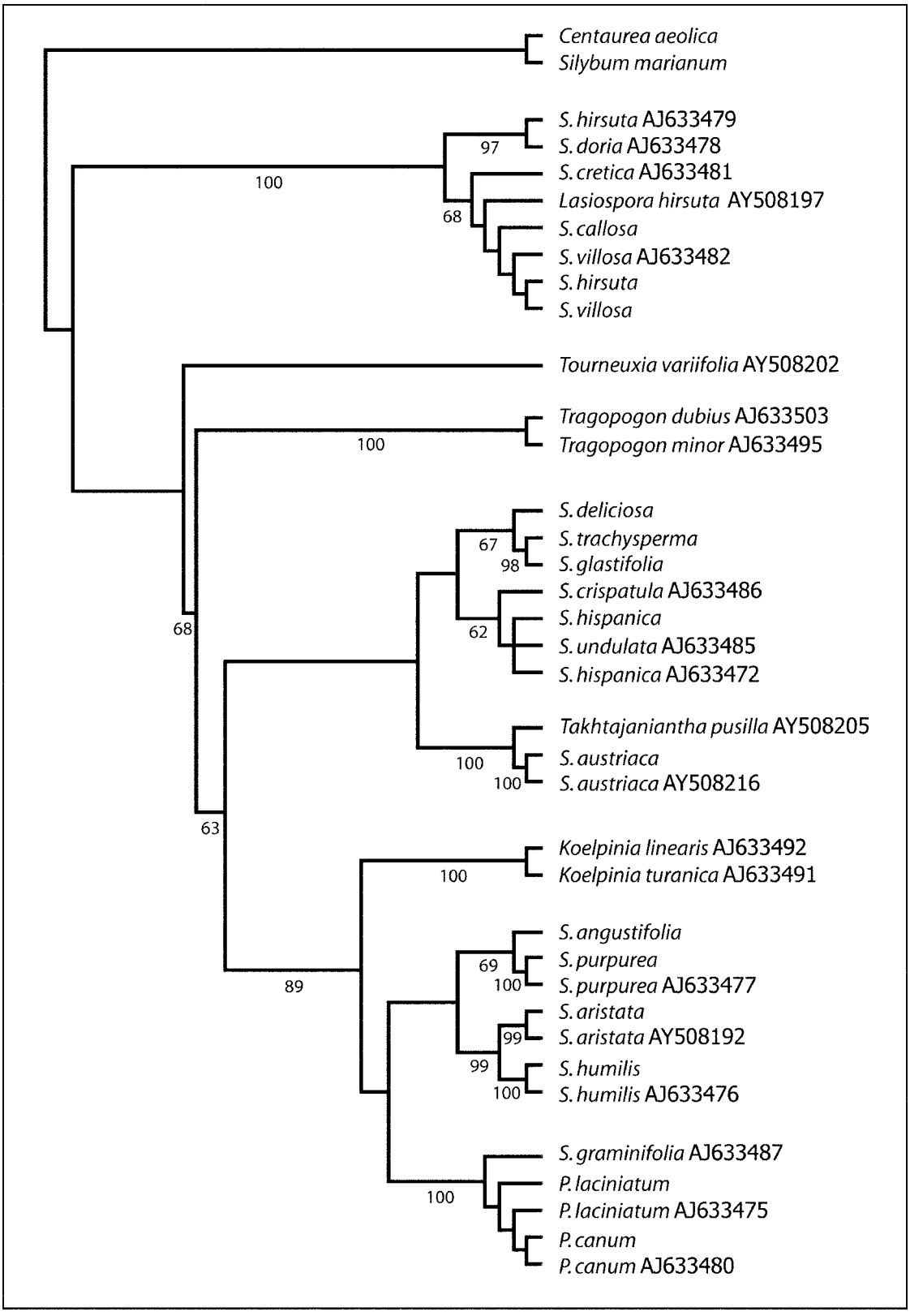


Fig. 14 — Cladogram (ML) derived from the alignment of the sequences from the combined data set; i.e., our sequences (13 in-group plus 2 out-group species) and the 22 sequences obtained from the NCBL database (accession numbers of the latter are shown on the cladogram).

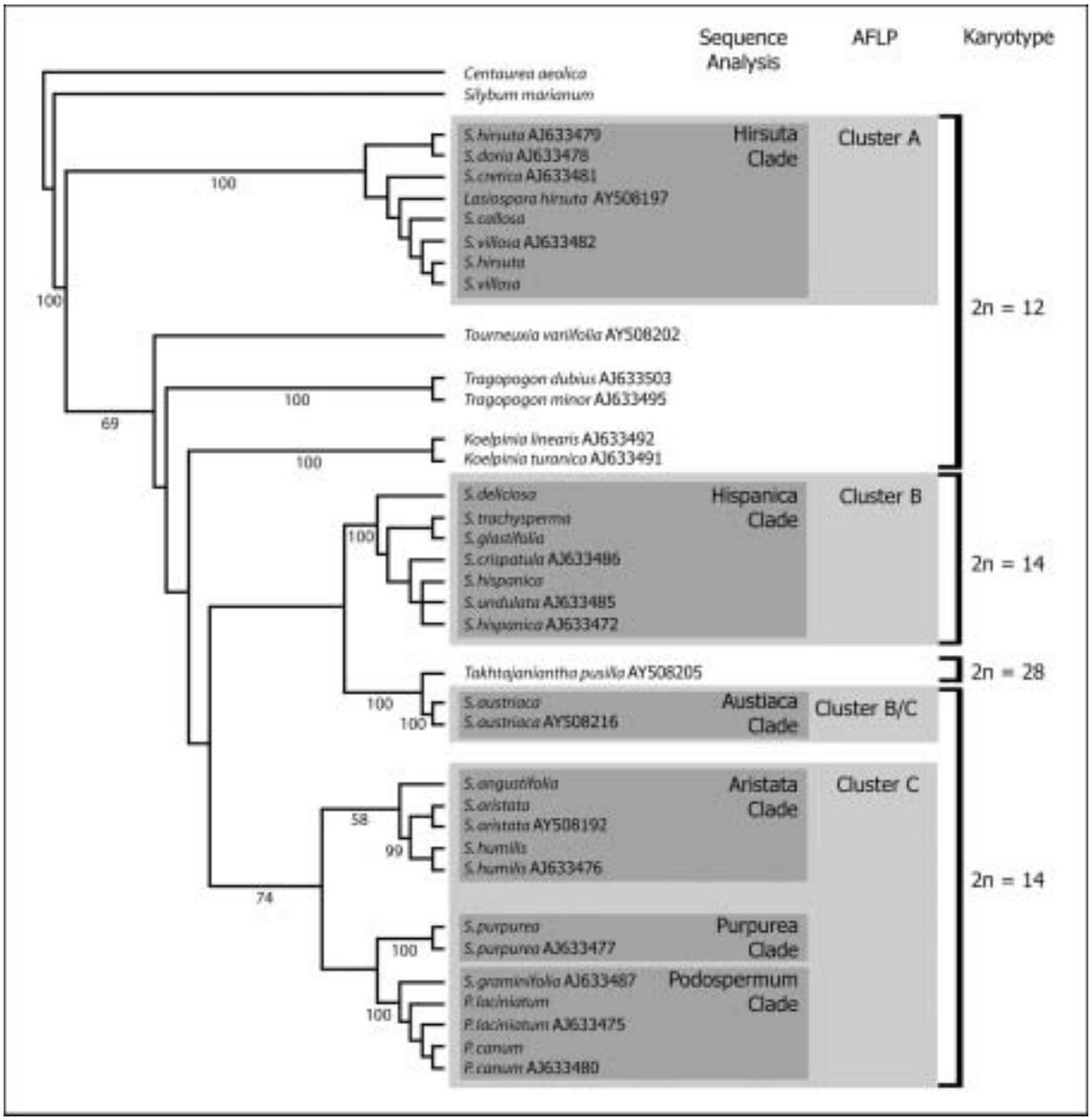


Fig. 15 — Cladogram (Parsimony) derived from the alignment of the sequences from the combined data set; i.e., our sequences (13 in-group plus 2 out-group species) and the 22 sequences obtained from the NCBL database (accession numbers of the latter are shown on the cladogram). The three groups evidenced in the PCO analysis of AFLP data are highlighted. Chromosome numbers are also shown: in the case of *Koelpinia linearis, K. turanica, Takhtajaniantha pusilla, Tourneuxia variofolia, Tragopogon. dubius* and *T. minor* chromosome numbers are reported from NAZAROVA (1997) and MAVRODIEV *et al.* (2004). Bootstrap values are based on 1000 replicates.

DISCUSSION

All the data gathered in the course of this study - cytogenetic, AFLP and sequence - point to the fact that the taxonomic entity *Scorzonera* s.l. is a polyphyletic grouping. On the basis of chromosome number, one could propose a division into two broad clades; one containing species with 2n = 12 and a second with 2n = 14 (Fig. 15). This division has been commented on by others (DIAZ DE LA GUARDIA and BLANCA 1985; 1987; NAZAROVA 1997). Analysis of AFLPs, is consistent with the chromosome data but indicates a further division of *Scorzonera* s.l. into at least three distinct groups. That is, cluster A, identified from the analysis of AFLPs, contains the 2n = 12 species, while the 2n

= 14 species may be divide into two distinct groups. The sequence data adds another level of resolution; they are consistent with the data from chromosome counts and the AFLP analysis but suggest an interpretation that would require the splitting of *Scorzonera* s.l. into several distinct groups each of which should perhaps be recognized as an independent genus.

The Hirsuta clade (cluster A in the PCO analysis) comprises the species *S. hirsuta* (syn. *Lasiospora hirsuta*), *S. villosa* and *S. callosa.* It is distinctly different from other clades both karyotypically (2n = 12) and molecularly. Indeed, members of this clade would appear to have more in common with non-Scorzonera taxa such as *Tourneuxia* and *Tragopogon* than with the other *Scorzonera* s.l. species. These data concord with those of MAVRODIEV *et al*. (2004), and support their suggestion that the section *Lasiospora* should be raised to generic status. This classification would unite *S. villosa* and *S. hirsuta* that in Flora Europaea, are placed in separate sections.

The Hispanica clade (cluster B in the PCO analysis) is also quite distinct being separated from the Aristata and Podospermum clades by members of the genus *Koelpinia* on the ML tree (Fig. 14). It contains *S. hispanica* and the closely related taxa *S. glastifolia, S. deliciosa* and *S. trachysperma*. The two taxa that lie closest to the Hispanica clade are *S. austriaca* and *Takhtajaniantha pusilla. S. austriaca* -not well resolved in the analysis containing only the Italian species - falls sister to *Takhtajaniantha pusilla* as in MAVRODIEV *et al.* (2004). Given that *Takhtajaniantha* is a distinct monotypic genus with a polyploid chromosome constitution (2n = 28), this collocation of *S. austriaca* suggests that it might also warrant being raised to the status of genus.

On the basis of sequence data, cluster C (from AFLP analysis) contains species that fall into two or three distinct clades: Aristata, Purpurea and Podospermum. The Aristata and Podospermum clade seem well supported. This positioning of *Podospermum* species is in agreement with the treatment by PIGNATTI (1982) rather than that of Flora Europaea in which *Podospermum* is considered a section within the genus *Scorzonera*. In a previous study (MAVRODIEV *et al.* 2004), the collocation of *S. aristata* was not resolved; indeed, it was thought to be somewhat distinct from other taxa of *Scorzonera* s.l. However, results here strongly support the collocation of *S. aristata* and *S. humilis* in a distinct clade that is sister to that of *Podospermum.* The placement of *S. purpurea*, on the other hand, proved to be ambiguous: on the ML tree it lay sister to the Aristata clade (Fig. 14) while on the parsimony tree it was sister to the Podospermum (Fig. 15) clade. However, it possesses a quite distinct karyotpye (NAZAROVA 1997; D'AMATO 2000), and is morphologically distinct from both *Scorzonera* and *Podospermum* (CHARTER 1976; MAVRODIEV *et al.* 2004). It may require independent treatment.

Unfortunately, our data did not help resolve some of the smaller issues about interspecies relationships. For instance, our sequence data suggested that *S. hirsuta* and *S. villosa* are almost identical - there were no differences in the ITS sequence and only an 8 bp insertion and a 1 bp substitution between the ETS regions. However, sequences for *S. hirsuta* taken from the public database were somewhat different from ours (and from each other) indicating a greater degree of difference between the two taxa. This was also suggested by AFLP data, the samples sharing only 31% of bands. Re-sampling and sequencing of a number of individuals would be required to resolve this question. The question of *S. hispanica* and its "subspecies" also remained unresolved: S. glastifolia has been considered a subspecies of *S. hispanica*. In the study of only the Italian sample, *S. glastifolia* and *S. delicosa* formed an unresolved group with *S. hispanica* (Fig 13). In the broader study, however, they fell away from *S. hispanica*. *S. trachysperma*, on the other hand, was distinct from *S. hispanica* in all analyses and would appear to warrant the independent taxonomic treatment given by PIGNATTI (1982).

In conclusion, then, our results clearly add to the debate about the taxonomic treatment of *Scorzonera* s.l. With the addition of the ETS region, and the added information from AFLP analysis, providing a broader sampling of the genome, our data gives support to the emerging conception of the phylogeny of *Scorzonera* and *Podospermum*. It is clear that it is not a monophyletic grouping and that current taxonomic treatment does not reflect phylogenetic relationships. At least three groups warrant being raised to the status of genus. *Podospermum* forms a distinctive clade and, as suggested by PIGNATTI (1982), should be considered a distinct genus. In addition one might formerly recognise clades for *S. hispanica* and related taxa, *S. aristata/S. humilis* and *S. austriaca*. *S. purpurea* would also appear to be distinct but its precise collocation is unclear.

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