

Molecular confirmation of the hybrid origin of the critically endangered western Mediterranean endemic *Sonchus pustulatus* (Asteraceae: Sonchinae)

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Abstract The critically endangered composite *Sonchus pustulatus* Willk. despite being known from fewer than ten locations in southern Spain and northern Africa, has never been characterized in robust phylogenetic context. Here, we report molecular evidence that strongly supports a hybrid origin for *S. pustulatus*. Although parentage cannot be identified with certainty, analysis of DNA sequence variation from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) supports a phylogenetic placement of *S. pustulatus* close to other species in the poorly known section *Pustulati*, whereas examination of chloroplast DNA (cpDNA) places *S. pustulatus* most closely with species from the sections *Sonchus* and *Asperi*. This is one of several instances of topological non-concordance reported for the genus *Sonchus*. Monophyly of *S. pustulatus* in both gene genealogies supports the null hypothesis of a single origin, and the relatively large amount of nucleotide substitutions is indicative of an origin in the range of millions of years. A hypothesis of a northern African origin of *S. pustulatus* followed by dispersal to the Iberian Peninsula during the Messinian salinity crisis/later Quaternary glaciations is proposed on the basis of

biogeographic patterns and calibrated estimations of molecular evolution.

Keywords Critically endangered species · Internal transcribed spacer (ITS) · *matK* gene · Reticulate evolution · *Sonchus pustulatus*

Introduction

Hybridization and introgression have long been viewed as important in plant evolution and speciation (Knobloch 1972; Grant 1981; Ellstrand et al. 1996; Arnold 1997; Rieseberg et al. 2003; Mallet 2007). Hybridization involving chromosome doubling (i.e., allopolyploidization) is an especially well-established mode of speciation in plants (Levin 2002; Mallet 2007). By contrast, recombinational hybrid speciation, in which the genome remains diploid (i.e., homoploid hybrid speciation), can be harder to define and more difficult to detect. Increasingly robust methods of phylogenetic reconstruction are helping overcome the challenges in detecting and discerning allopolyploid and homoploid hybridization (e.g., Arnold 1997; Hegarty and Hiscock 2005). Several studies, in particular, have recently shown how homoploid hybridization can promote plant evolution and speciation (e.g., Comes and Abbot 2001; Arnold et al. 2003; Rieseberg et al. 2003).

Patterns of plant speciation in the Mediterranean Basin have been prominently shaped by repeated episodes of fragmentation, contraction and expansion of distribution brought on by dramatic geological events, such as the Betic crisis and Messinian salinity crisis, or severe shifts of climate occasioned by Quaternary glaciations (Thompson 2003; Veith et al. 2004; Ortiz et al. 2007). One important

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evolutionary consequence of these events was the extensive opportunities for hybridization between otherwise geographically isolated species (e.g., Gutiérrez Larena et al. 2002; Thompson 2003). A recently suspected case of homoploid hybridization in the Mediterranean Basin involves *Sonchus* sect. *Pustulati* (Kim et al. 2007). According to Boulos (1972, 1973), this section comprises four diploid suffrutescent perennials from the western Mediterranean Basin: *S. pustulatus* Willk., *S. fragilis* Ball, *S. briquetianus* Gandoger, and *S. masquindalii* Pau and Font Quer. The taxonomic status of *S. briquetianus* is somewhat questionable, and it is probably a coastal form of the highly polymorphic *S. tenerrimus* L. [José A. Mejías, unpublished data; also see electronic supplementary materials (ESM) Fig. S1]. A phylogenetic analysis of the subtribe Sonchinae (Asteraceae: Cichorieae) has yielded topological incongruence between nuclear and plastid trees with regard to this group (Kim et al. 2007). According to the internal transcribed spacer (ITS) phylogeny, section *Pustulati* (*S. briquetianus* not being sampled) is a strongly supported monophyletic group (100% bootstrap support and 15 synapomorphic substitutions) with *S. pustulatus* being sister to *S. fragilis*. The chloroplast DNA (cpDNA) phylogeny suggests drastically different relationships, especially regarding the phylogenetic position of *S. pustulatus* relative to the remaining two species of section *Pustulati*. The *matK* gene strict consensus tree suggest that *S. pustulatus* is embedded within a clade containing species in sections *Sonchus* L. and *Asperi* Boulos (i.e., *S. tenerrimus*, *S. bourgeaui*, *S. microcephalus*, *S. asper*, *S. oleraceus*, *S. hydrophilus*, and *S. kirkii*). This topological incongruence between biparentally (nuclear) and uniparentally (plastid) inherited genomes may indicate possible hybridization and chloroplast capture during the origin of *S. pustulatus* (see other examples in Rieseberg 1991; Soltis and Kuzoff 1995; Wendel et al. 1995; Soltis et al. 1996; Nieto Feliner et al. 2002; Albadalejo et al. 2005; Okuyama et al. 2005; McBreen and Lockhart 2006). Although the above evidence was indicative of a hybrid origin of *S. pustulatus*, it could only be preliminary, since it was based on just a few individuals from a single Moroccan population. It was likewise insufficient sampling that was used to distinguish whether *S. pustulatus* was hybrid-derived or if the pattern of incongruence could be accounted for by recent and ongoing gene flow from close populations of *S. fragilis*, a species that does not occur in Spain.

In this study, we sampled extensively for the phylogenetic analysis of *Sonchus* section *Pustulati* in order to investigate further the hybrid origin of *S. pustulatus*. We first wanted to test the bi-phyletic ancestry of *S. pustulatus* in the Iberian Peninsula and Morocco. We also wished to determine the identity of the putative parents of *S. pustulatus* and whether disjunct populations in the

Iberian Peninsula and northern Africa represented single or multiple origins.

Materials and methods

Study system and sampling

Currently, the genus *Sonchus* comprises 54 species and is divided into three subgenera (Boulos 1972). Subgenus *Dendrosonchus* (19 species) consists of woody plants endemic to Macaronesia, and subgenus *Origosonchus* (14 species, herbaceous perennials) occurs exclusively in Africa. Subgenus *Sonchus* comprises 21 species (annuals, biennials, and perennials) and includes several cosmopolitan weedy species. Boulos (1972, 1973) designated *S. pustulatus* as the type species of section *Pustulati*. This section comprises four diploid, suffrutescent chamaephytes, 10–35 cm in height, with pinnatisect leaves and achenes with thickened margins, narrowly endemic to low–mid altitude (up to 700 m above sea level) calcareous and other basic rocky cliffs in the western Mediterranean Basin: *S. pustulatus* Willk., *S. fragilis* Ball, *S. briquetianus* Gandoger, and *S. masquindalii* Pau and Font Quer (Boulos 1973; Fennane and Ibn Tattou 1988; Mejías 1988; Bañares et al. 2004). Chromosome numbers are identical, i.e. $n = 9$ and $2n = 18$ (Stebbins et al. 1953; Roux and Boulos 1972; Talavera et al. 1984; Mejías 1988; Mejías and Andrés 2004; José A. Mejías, unpublished data). *Sonchus pustulatus* is the most widely distributed member of sect. *Pustulati*, with populations in both southern Spain and northern Morocco and Algeria (ESM, Fig. S1). Recently published surveys of the Iberian populations found only three sites (Barranco del Caballar, Faro de San Telmo, and Playa de la Parra, peripheral to the city of Almería), approximately 100 individuals (Bañares et al. 2004; Mota et al. 2005). A careful survey has yielded one more population and ca. 900 individuals as a whole (J. A. Mejías, unpublished data).

Previous phylogenetic study of the subtribe Sonchinae (Kim et al. 2007) was with limited sampling in section *Pustulati*. Only one Moroccan population from Oued Laou valley (i.e., Chefchaouen, Talembote) of *S. pustulatus* was sampled, and only two individuals from a single population of both *S. fragilis* and *S. masquindalii* were included. In this study, additional seeds from new accessions of *S. pustulatus* were field collected and germinated in greenhouses at the Universidad de Sevilla. Representatives of *S. pustulatus* from Spain included five individuals from three sub-populations located several hundred meters apart at Barranco del Caballar (Almería). Two individuals, Spain-3 and Spain-4, are siblings. Six additional individuals from Morocco [two from Talembote (Chefchaouen),

and four siblings from Es-Sebt-de-Saïd] and two additional individuals of *S. fragilis* and *S. masguindalii* were also included in the analysis.

DNA extraction and sequencing

Methods of DNA isolation/sequencing followed those of Lee et al. (2005) and Kim et al. (2007). Total genomic DNA was isolated from fresh, dried, or herbarium leaf tissue using DNeasy Plant Mini kits (QIAGEN, Valencia, CA, USA). Polymerase chain reaction (PCR) products were purified using a QiaQuick PCR purification kit (QIAGEN) and direct sequencing of PCR products was performed with an ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). PCR and DNA sequencing primers were the same as those described by Lee et al. (2005) and Kim et al. (2007). Extension products were purified and separated on an ABI377 automated sequencing machine (Applied Biosystems).

Individuals of *S. pustulatus* from Morocco had been shown to be highly polymorphic in a previous study (two polymorphic sites in ITS1 and 11 in ITS2) (Kim et al. 2007). One of those individuals was subsequently cloned, and eight clones were sequenced so that the different ITS repeat types could be identified. An individual from Almería which showed no polymorphisms was also cloned, and four cloned sequences were generated as further confirmation of direct sequencing results. All cloning was performed with the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, USA). Base calling and sequence editing were performed with Sequencher 4.7 (Gene Codes, Ann Arbor, MI, USA).

Phylogenetic analysis

Aetheorhiza bulbosa was used as an outgroup for the ITS and *matK* data, based on the previous phylogenetic study (Kim et al. 2007). Ingroup taxa included representative species of *Sonchus* sections *Sonchus*, *Asperi*, *Maritimi*, and *Arvenses*, as well as newly sequenced additional individuals of section *Pustulati*. The sampling of subgenus *Sonchus* includes all but two species (*S. macrocarpus*, an Egyptian endemic, and *S. malaianus*, an Indonesian endemic), and the ITS and *matK* data matrices contain the same species, with exceptions in two species (*S. mauritanicus* and *S. wightianus* not represented in the *matK* data). Phylogenetic analyses using Fitch parsimony were performed with PAUP* (version 4.0; Swofford 2001), using the heuristic search option (100 random additions) with tree-bisection–reconnection (TBR) branch swapping and the multiple parsimony (MULPARS) option on. Insertions and deletions (indels) were treated as missing data. Support

for groups was examined by 1,000 bootstrap replicates (Felsenstein 1985) using the heuristic search option from the 100 random additions sequence with TBR branch swapping. In addition, decay analysis (Bremer 1988) was performed to assess the robustness of the monophyletic groups. Pairwise sequence divergence was calculated, using the Kimura 2-parameter method (Kimura 1980) and a neighbor-joining (Saito and Nei 1987) tree was constructed using PAUP*. Each data set was also analyzed, using likelihood methods to determine the stability of the parsimony results with an explicit model-based approach (Felsenstein 1981). Optimal models of molecular evolution were chosen, using the likelihood ratio test (Goldman 1993; Whelan and Goldman 1999) implemented in ModelTest 3.7 (Posada and Crandall 1998). Model parameters were then imported into PAUP*, and a heuristic search (asis sequence addition, TBR branch swapping, and MULPARS option on) was executed. Maximum likelihood (ML) bootstrap analyses with 100 replicates were conducted, using the same parameter values obtained from the ModelTest and heuristic options.

Results

ITS phylogeny

Lengths of ITS1 and ITS2 are within the range of other *Sonchus* species previously reported (Kim et al. 1996a, b, 2004, 2007; Lee et al. 2005). A total of nine individuals of *S. pustulatus* from Morocco have identical ITS1 and ITS2 sequences with unusually high polymorphic sites (i.e., two and 11 in ITS1 and ITS2, respectively). Thus, we cloned one individual from Chefchaouen, Morocco-1, and sequenced eight clones (ESM Appendix S1). The cloning results revealed three different ITS repeat types (ribotype I, clone-1,2,5,6,7,8; ribotype II, clone-3; ribotype III, clone-4), and all the polymorphic sites by direct sequencing were confirmed by additivity of base pairs among the three clone sequences. All five individuals of the Almería population had identical ITS sequences. Cloned sequences of one individual, Spain-1, were also identical to the five individual ITS sequences, further confirming direct sequencing results. Four individuals of *S. fragilis* had identical ITS sequences, with no polymorphisms. Four individuals of *S. masguindalii* from Al Hoceïma had identical ITS sequences and differed from one accession cultivated at the Botanical Garden La Orotava (Canary Island, Spain; originally from Morocco) by four base pair changes and a one base pair deletion in the ITS2 region.

A total of 485 characters for 50 accessions were used for phylogenetic analyses. Of the 485 characters, 299 characters (62%) were constant, 44 variable characters (9.1%)

were parsimony uninformative, and 142 characters (29%) were parsimony informative between outgroup and ingroup. The heuristic search found a total of 24 equally parsimonious trees with a tree length (TL) of 305, a consistency index (CI) of 0.7607 (excluding uninformative characters, 0.7192), and a retention index (RI) of 0.9333. The strict consensus tree is shown in Fig. 1. The neighbor-joining (NJ) tree (not shown) was identical to the strict consensus tree, except for the position of *S. palustris*. Model test selected “general time reversible plus gamma” (“GTR + G”) as the best-fit model based on the Akaike information criterion (AIC), for the ITS data, and the ML analysis found five trees, which were identical except in the position of the direct sequencing results of Moroccan *S. pustulatus* populations. The hierarchical likelihood ratio tests (hLRTs) (TrN + G) also recovered two of the five identical trees as the AIC. All three different optimality criteria strongly suggested that the section *Pustulati*, which includes three species, is monophyletic (100% bootstrap and four decay value support) and that each species is

monophyletic (98%, 100%, and 76% bootstrap support for *S. masguindalii*, *S. fragilis*, and *S. pustulatus*, respectively) (Fig. 1). It also suggested that *S. fragilis* is most closely related to *S. pustulatus* and that *S. masguindalii* is sister to the two species.

matK phylogeny

A total of ten *matK* gene sequences were generated for *S. pustulatus*: all individuals from Morocco, Morocco-1, -2, -3, -5, -6, -7, and -8, had identical *matK* gene and flanking sequences. Three individuals from Almería also had identical *matK* gene sequences and differed from the Moroccan populations by a one base pair change in the *trnK* 3' intron. Based on the previous phylogenetic analysis of subtribe Sonchinae, it became quite clear that *S. pustulatus* is distantly related to the other two species in the same section *Pustulati*: *S. fragilis* and *S. masguindalii* are part of a highly unresolved polytomy of two subgenera *Sonchus* L. and *Origosonchus* Boulos, *Aetheorrhiza*, and several Pacific

Fig. 1 Strict consensus tree of 216 equally parsimonious trees based on ITS sequences of nuclear ribosomal DNA (nrDNA) (TL = 153, CI = 0.8627, and RI = 0.9742). Numbers above and below branches represent decay values and bootstrap supports, respectively. Dark arrow indicates the clade to which *S. pustulatus* belongs and gray arrow indicates the monophyly of *S. pustulatus*. *S. Sonchus*, [§] section *Asperi*, [¶] section *Arvenses*

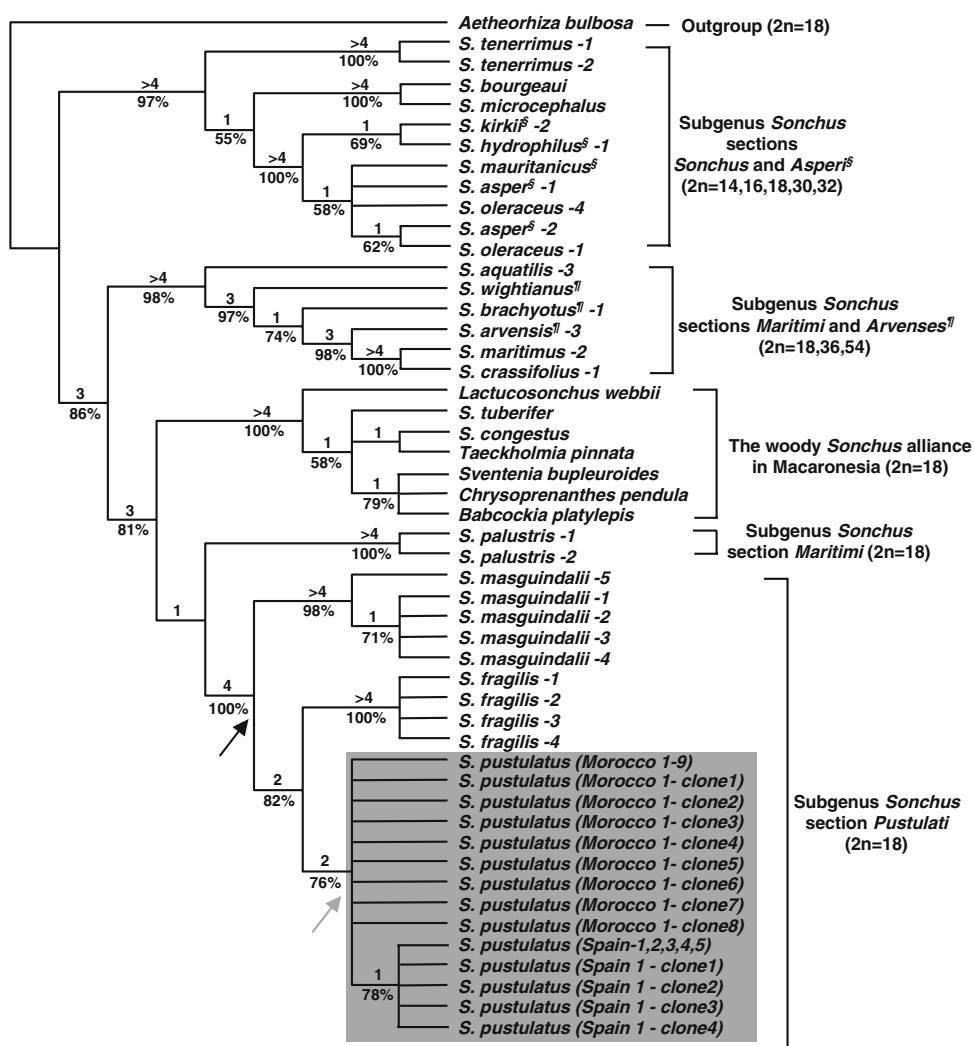
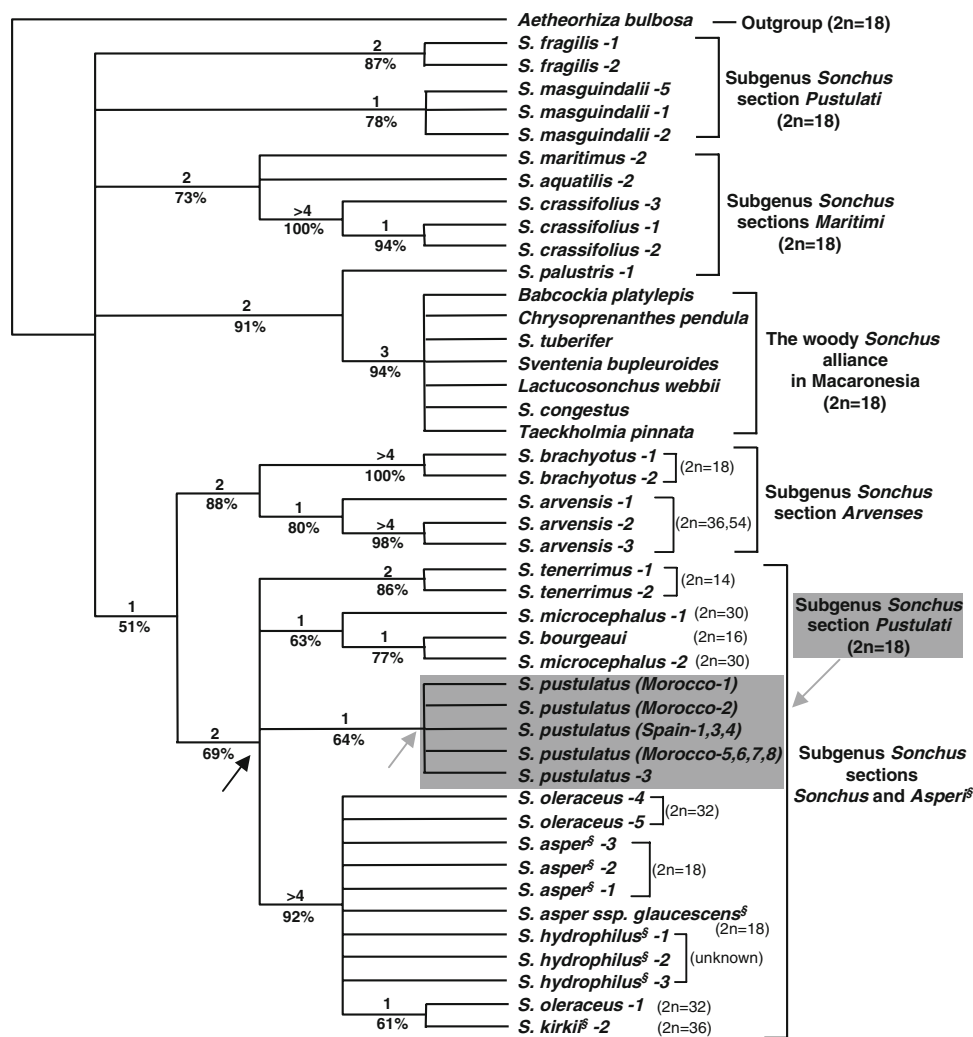


Fig. 2 Strict consensus tree of 51 equally parsimonious trees based on *matK* gene and flanking region sequences of cpDNA (TL = 134, CI = 0.9552, and RI = 0.9721). Numbers above and below branches are decay values (up to four) and bootstrap supports, respectively. *Dark arrow* indicates the clade to which *S. pustulatus* belongs and *gray arrows* indicate the monophyly of *S. pustulatus*. *S. Sonchus*, [§] section *Asperi*



Islands endemics, while *S. pustulatus* belongs to a well-supported clade (clade III; Kim et al. 2007) containing two sections of subgenus *Sonchus*, *Sonchus* and *Asperi*. On average, pairwise sequence divergence between *S. pustulatus* and two other species was 0.28%, whereas, between *S. pustulatus* and several closely related species in clade III, it was 0.18%. When only *S. pustulatus* and *S. tenerrimus* were considered, the pairwise sequence divergence was, on average, 0.13%. Additional individuals from Morocco and Almería from this study further confirmed the previous results (see later “Results”). *Sonchus pustulatus* shares one five base pair tandem direct repeat (TATAA) insertion with *S. bourgeaui* in the *trnK* 5' intron.

A total of 2,882 aligned characters were used for phylogenetic analysis. Of these 2,882 characters, 2,757 characters (95.6%) were constant, 67 variable characters (2.3%) were parsimony uninformative, and 58 characters (2.0%) were parsimony informative between outgroup and ingroup. Although more characters were sampled than for nuclear DNA (2,882 vs 485), the number of parsimony

informative characters of cpDNA was much less than those of nuclear DNA (2.0 vs 29%). Maximum parsimony (MP) analysis found 51 equally parsimonious trees with a TL of 134, a CI of 0.9552, and an RI of 0.9721 (Fig. 2). An NJ tree (not shown) was almost identical to the MP trees and suggests that *S. tenerrimus* is most closely related to *S. pustulatus* (however, bootstrap support is less than 50%). The ML analysis based on AIC (TIM + I) found one tree (not shown) and is almost identical to the MP strict consensus tree. The *matK* phylogeny further confirmed the previous results and suggested that *S. pustulatus* is most closely related to the species in subgenus *Sonchus* sections *Sonchus* and *Asperi*, especially *S. tenerrimus*, *S. microcephalus*, and *S. bourgeaui* (Fig. 2). Two other species in the same section *Pustulati*, *S. fragilis* and *S. masguindalii*, are part of a highly unresolved polytomy of section *Maritimi* and the woody *Sonchus* alliance in the Macaronesian islands. The MP analysis using indels as fifth characters suggested that *S. bourgeaui* is sister to *S. pustulatus*, and this is entirely supported by a total of five

base pair tandem direct insertions shared by the two species.

Discussion

Reticulate evolution of *S. pustulatus*

Topological incongruence between biparentally (nuclear) and uniparentally (chloroplast) inherited genomes has often been considered evidence of chloroplast capture via interspecific hybridization, sometimes followed by backcrossing (see other examples in Rieseberg 1991; Wendel et al. 1995; Nieto Feliner et al. 2002; Fuertes Aguilar and Nieto Feliner 2003; Doyle et al. 2004; Albadalejo et al. 2005; Okuyama 2005). However, topological incongruence is also an expected pattern in the process of lineage sorting of ancestral haplotypes. The deep evolutionary divergence encompassed by the seven substitutions (four non-homoplasious and three homoplasious) that differentiate cpDNA clades *S. fragilis*/*S. masguindalii* from *S. pustulatus* strongly favors a hybridization-based hypothesis. It is unlikely that convergent substitutions or lineage sorting of ancestral polymorphisms could account for the incongruent and distantly related positions of *S. pustulatus*. Our evidence suggests that *S. pustulatus* is a one-time, homoploid hybrid between *S. fragilis*, as a likely paternal contributor, and a still unknown maternal donor from the root lineage of sects. *Sonchus* and *Asperi*.

The designation of *S. fragilis* as the most probable paternal parent is not only based on DNA sequence data but is supported by independent evidence from biosystematics, geography, morphology and cytology. No statistically significant differences in fertility levels have been detected between inter-specific and intra-specific crosses from *S. pustulatus* and *S. fragilis* (J.A. Mejías, unpublished data). These two species also resemble each other morphologically, are sometimes sympatric, and share highly similar karyotypes.

According to the analysis of plastid DNA, the haplotypes of *S. pustulatus* are most similar to those of *S. tenerrimus*, a highly polymorphic species with some coastal forms morphologically quite similar to *S. pustulatus*. This suggests that *S. tenerrimus* is the most likely maternal donor. Cytological evidence, however, contradicts this interpretation (Fig. 2). *Sonchus tenerrimus* ($n = 7$, $2n = 14$) and other closely related taxa, such as *S. bourgeaui* ($n = 8$, $2n = 16$) and *S. microcephalus* ($n = 15$, $2n = 30$), have chromosome numbers that are different from those of *S. pustulatus* ($n = 9$, $2n = 18$; Mejías 1988; Mejías and Andrés 2004). Furthermore, several closely related species in other strongly supported clades (Fig. 2), i.e., *S. oleraceus* ($n = 16$, $2n = 32$),

S. hydrophilus (unknown, but autotetraploid was suggested; Boulos 1973), and *S. kirkii* ($2n = 36$), *S. asper* ($n = 9$, $2n = 18$) are tetraploids or amphidiploid and/or are characterized by highly divergent morphology. Therefore, it is highly unlikely that any extant species has contributed as a maternal donor in the hybrid origin of *S. pustulatus*. One plausible explanation is that a now extinct common ancestor with an equivalent chromosome number of $2n = 18$ ($n = 9$) leading to the lineage of sections *Sonchus* and *Asperi* was most likely the maternal donor for *S. pustulatus*.

Moroccan origin

Western Mediterranean biogeography has been shaped greatly since the Pliocene by Neogene tectonic dynamics and repeated climatic oscillations. These events produced ephemeral land bridges between northern Africa and Europe during the Betic crisis (15–16 Mya) and the Messinian salinity crisis (6.5–5.5 Mya) (for details and geologic maps see De Jong 1998; Veith et al. 2004; Barrón and Peyrot 2006) and lowered sea levels to approximately 120–150 m during glacial maximum (Ortiz et al. 2007), allowing plant populations to migrate temporarily between the two continents. As a consequence, some plant species found refugia in northern Africa during unfavorable periods, which has been shown in a phylogeographic study of *Hypochoeris salzmanniana* (Ortiz et al. 2007), a close relative of *Sonchus*. Since African populations of *S. pustulatus* are (1) phylogenetically ancestral to those in Iberia; (2) more widely distributed; (3) genetically more variable, and (4) close to populations of *S. fragilis* in Morocco, i.e., Tetouan, it is probable that *S. pustulatus* originated in Morocco.

The evolutionary origin and subsequent trans-Mediterranean dispersal of *S. pustulatus* can be roughly estimated using available information on rates of diversification in ITS sequences. Several different ITS substitution rates have been calculated for angiosperms and closely related genera in the Asteraceae: $3.94 \pm 0.10 \times 10^{-9}$ /year (*Dendroseris*, Sang et al. 1994); 3.62×10^{-9} /year (Cucurbitaceae, Jobst et al. 1998); 35×10^{-10} /year (herbaceous perennial *Astragalus*, Wojciechowski et al. 1999); and 30×10^{-10} /year (the Hawaiian silversword alliance, Baldwin and Sanderson 1998). The average of these is 0.725%/million years. This average would suggest a split between *S. pustulatus*/*S. fragilis* and *S. masguindalii* around 6.8 Mya (average pairwise sequence divergence of 4.933%), 5.81 Mya for *S. pustulatus* and *S. fragilis* (4.213%), and 3 Mya for populations of *S. pustulatus* in Almería and Morocco (2.22%). These dates roughly correspond to the Messinian salinity crises (5.5 Mya) (Pou 1988; Yokoyama et al. 2000; Collina-Girard 2001; Cheddadi et al. 2005).

[Similar divergence times were also estimated, based on the Bayesian method using Multidivtime (Kim S.-C. et al. unpublished data).] It is plausible that the origin and subsequent trans-Mediterranean dispersal of *S. pustulatus* were, perhaps, triggered by this dramatic geological event in the Mediterranean region and the onset of the first glaciation cycle, respectively (Thompson 2003).

Concluding remarks and future directions

Although the role of polyploidization in the evolution of the genus *Sonchus* is suggested to be important, based on the recurrence of polyploid species (ca. 30% of subgenus *Sonchus* are either tetraploids or hexaploids), little in contrast can be said about the potential impact of diploid hybrid speciation. This study represents the first molecular documentation of diploid hybrid speciation in the genus *Sonchus*, notably in subgenus *Sonchus*. Several other cases are suspected, based on current phylogenetic patterns. Another system worthy of study is represented by the two subgenera, *Dendrosonchus* and *Origosonchus*, both strictly diploids and endemic to the Macaronesian Islands and Africa, respectively.

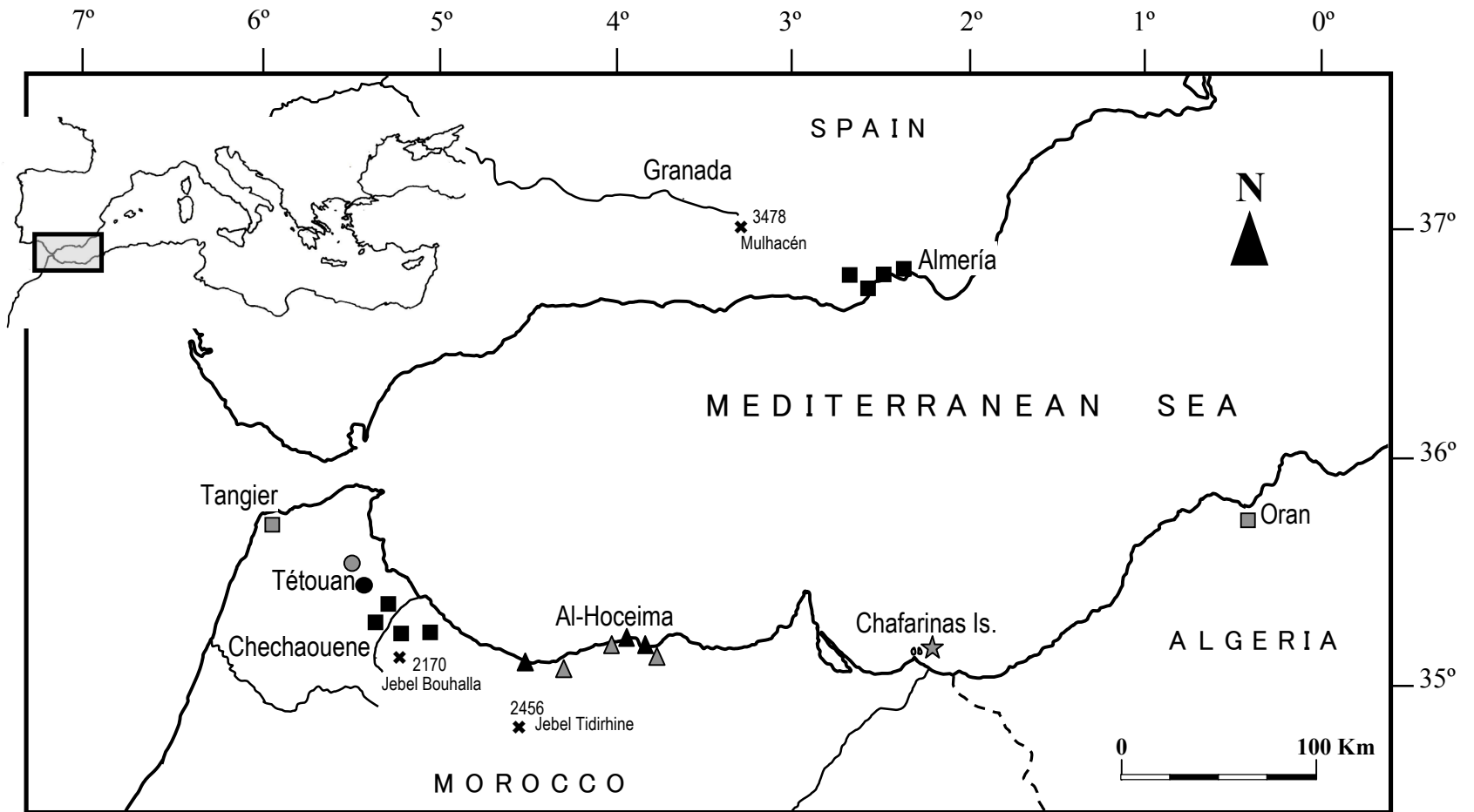
The presence of only four populations with ca. 900 individuals (J. A. Mejías, unpublished data) shows that *S. pustulatus* is a critically endangered species in the Iberian Peninsula. These populations are, unfortunately, in the vicinity of growing urban zones, and they are exposed to anthropogenic threats such as nitrification (Bañares et al. 2004). We encourage that the undertaking of detailed population genetic and demographic study be mandated for the persistence of this species in the Iberian Peninsula, and that similar studies also be required for populations in Morocco, where *S. pustulatus* most likely evolved.

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References

- Albadalejo RG, Fuertes Aguilar J, Aparicio A, Nieto Feliner G (2005) Contrasting nuclear-plastidial phylogenetic patterns in the recently diverged Iberian *Phlomis crinita* and *P. lychnitis* lineages (Lamiaceae). *Taxon* 54:987–997
- Arnold ML (1997) Natural hybridization and evolution. Oxford University Press, Oxford, 215 pp
- Arnold MA, Bouck AC, Cornman RS (2003) Verne Grant and Louisiana irises: is there anything new under the sun? *New Phytol* 161:143–149
- Baldwin BG, Sanderson MJ (1998) Age and rate of diversification of the Hawaiian silversword alliance (Compositae). *Proc Natl Acad Sci U S A* 95:9402–9406
- Bañares Á, Blanca G, Gúmes J, Moreno JC, Oritz S (eds) (2004) Atlas y Libro Rojo de la Flora Vascular Amenazada de España: Taxones Prioritarios. Dirección General de Conservación de la Naturaleza. Madrid, Spain, 1072 pp
- Barrón E, Peyrot D (2006) La vegetación forestal en el Terciario. In: Carrión JS, Fernández S, Fuentes N (eds) Paleoambientes y cambio climático. Fundación Séneca—Agencia de Ciencia y Tecnología de la Región de Murcia, Murcia, Spain, pp 56–76
- Boulos L (1972) Révision systématique du genre *Sonchus* L. s.l. I. Introduction et classification. *Bot Not* 125:287–305
- Boulos L (1973) Révision systématique du genre *Sonchus* L. s.l. IV. Sous-genre 1. *Sonchus*. *Bot Not* 126:155–196
- Bremer K (1988) The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* 42:795–803
- Cheddadi R, De Beaulieu J-L, Jouzel J, Andrieu-Ponel V, Laurent J-M, Reille M, Raynaud MD, Bar-Hen A (2005) Similarity of vegetation dynamics during interglacial periods. *Proc Natl Acad Sci U S A* 102:13939–13943
- Collina-Girard J (2001) L'Atlantide devant le détroit de Gibraltar? Mythe et géologie. *Earth Planet Sci* 333:233–240
- Comes HP, Abbott RJ (2001) Molecular phylogeography, reticulation, and lineage sorting in Mediterranean *Senecio* (Asteraceae). *Evolution* 55:1943–1962
- De Jong H (1998) In search of historical biogeographic patterns in the western Mediterranean terrestrial fauna. *Biol J Linn Soc* 65:99–164
- Doyle JJ, Doyle JL, Rauscher JT, Brown AHD (2004) Diploid and polyploid reticulate evolution throughout the history of the perennial soybeans (*Glycine* subgenus *Glycine*). *New Phytol* 161:121–132
- Ellstrand NC, Whittikus R, Rieseberg LH (1996) Distribution of spontaneous plant hybrids. *Proc Natl Acad Sci U S A* 93:5090–5093
- Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 17:368–376
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791
- Fennane M, Ibn Tattou M (1998) Catalogue des plantes vasculaires rares, menacées ou endémiques du Maroc. *Bocconea* 8:5–243
- Fuertes Aguilar J, Nieto Feliner G (2003) Additive polymorphisms and reticulation in an ITS phylogeny of thrifts (*Armeria*, Plumbaginaceae). *Mol Phylogenet Evol* 28:430–447
- Goldman N (1993) Statistical tests of models of DNA substitution. *J Mol Evol* 36:182–198
- Grant V (1981) Plant speciation, 2nd edn. Columbia University Press, New York, 563 pp
- Gutiérrez Larena B, Fuertes Aguilar J, Nieto Feliner G (2002) Glacial-induced altitudinal migrations in *Armeria* (Plumbaginaceae) inferred from patterns of chloroplast DNA haplotype sharing. *Mol Ecol* 11:1965–1974
- Hegarty MJ, Hiscock SJ (2005) Hybrid speciation in plants: new insights from molecular studies. *New Phytol* 165:411–423
- Jobst J, King K, Hemleben V (1998) Molecular evolution of the internal transcribed spacers (ITS1 and ITS2) and phylogenetic relationships among species of the family Cucurbitaceae. *Mol Phylogenet Evol* 9:204–219
- Kim S.-C, Crawford DJ, Jansen RK (1996a) Phylogenetic relationships among the genera of the subtribe Sonchinae (Asteraceae): evidence from ITS sequences. *Syst Bot* 21:417–432

- Kim S-C, Crawford DJ, Francisco-Ortega J, Santos-Guerra A (1996b) A common origin for woody *Sonchus* and five related genera in the Macaronesian Islands: molecular evidence for extensive radiation. *Proc Natl Acad Sci U S A* 93:7743–7748
- Kim S-C, Lu CT, Lepschi BJ (2004) Phylogenetic positions of *Actites megalocarpa* and *Sonchus hydrophilus* (Sonchinae: Asteraceae) based on ITS and chloroplast non-coding DNA sequences. *Aust Syst Bot* 17:73–81
- Kim S-C, Lee C, Mejías JA (2007) Phylogenetic analysis of chloroplast DNA *matK* gene and ITS of nrDNA sequences reveals polyphyly of the genus *Sonchus* and new relationships among the subtribe Sonchinae (Asteraceae: Cichorieae). *Mol Phylogenet Evol* 44:578–597
- Kimura M (1980) Simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120
- Knobloch IW (1972) Intergeneric hybridization in flowering plants. *Taxon* 21:97–103
- Lee C, Kim S-C, Lundy K, Santos-Guerra A (2005) Chloroplast DNA phylogeny of the woody *Sonchus* alliance (Asteraceae: Sonchinae) in the Macaronesian Islands. *Am J Bot* 92:2072–2085
- Levin DA (2002) The role of chromosomal change in plant evolution. Oxford University Press, Oxford, 230 pp
- Mallet J (2007) Hybrid speciation. *Nature* 446:279–283
- McBreen K, Lockhart PJ (2006) Reconstructing reticulate evolutionary histories of plants. *Trends Plant Sci* 11:398–404
- Mejías JA (1988) Diferencias cariológicas y morfológicas entre *Sonchus pustulatus* Willk. y *Sonchus tenerrimus* L. *Lagascalía* 15 [Suppl]:345–354
- Mejías JA, Andrés C (2004) Karyological studies in Iberian *Sonchus* (Asteraceae: Lactuceae): *S. oleraceus*, *S. microcephalus* and *S. asper* and a general discussion. *Folia Geobot* 39:275–291
- Mota JF, Cueto M, Pérez-García FJ, Garrido JA, Martínez-Hernández F, Medina-Cazorla JM, Sola AJ, Schwarzer H (2005) Contribución al conocimiento de la flora de Andalucía: citas novedosas de la provincia de Almería, el elemento estenócoro. *Acta Bot Malacitan* 30:227–231
- Nieto Feliner G, Fuertes Aguilar J, Roselló JA (2002) Reticulation or divergence: the origin of a rare serpentine endemic assessed with chloroplast, nuclear and RAPD markers. *Plant Syst Evol* 231:19–38
- Okuyama Y, Fujii N, Wakabayashi M, Kawakita A, Ito M, Watanabe M, Murakami N, Kato M (2005) Nonuniform concerted evolution and chloroplast capture: heterogeneity of observed introgression patterns in three molecular data partition phylogenies of Asian *Mitella* (Saxifragaceae). *Mol Biol Evol* 22:285–296
- Ortiz A, Tremetsberger K, Talavera S, Stuessy TF, García-Castaño JL (2007) Population structure of *Hypochaeris salzmanniana* DC. (Asteraceae), an endemic species to the Atlantic coast on both sides of the Strait of Gibraltar, in relation to Quaternary sea level changes. *Mol Ecol* 16:541–552
- Posada D, Crandall K (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–818
- Pou A (1988) La Erosión. Ministerio de Obras Públicas y Urbanismo. Madrid, 121 pp
- Rieseberg LH (1991) Homoploid reticulate evolution in *Helianthus* (Asteraceae): evidence from ribosomal genes. *Am J Bot* 78:1218–1237
- Rieseberg LH, Raymond O, Rosenthal DM, Lai Z, Livingstone K, Nakazato T, Durphy JL, Schwarzbach AE, Donovan LA, Lexer C (2003) Major ecological transitions in wild sunflowers facilitated by hybridization. *Science* 301:1211–1216
- Roux J, Boulos L (1972) Révision systématique du genre *Sonchus* L. s.l. II. Étude caryologique. *Bot Not* 125:306–309
- Saito N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Sang T, Crawford DJ, Kim S-C, Stuessy TF (1994) Radiation of the endemic genus *Dendroseris* (Asteraceae) on the Juan Fernandez Islands: evidence from sequences of the ITS regions of nuclear ribosomal DNA. *Am J Bot* 81:1494–1501
- Soltis DE, Kuzoff RK (1995) Discordance between nuclear and chloroplast phylogenies in the *Heuchera* group (Saxifragaceae). *Evolution* 49:727–742
- Soltis DE, Johnson LA, Looney C (1996) Discordance between ITS and chloroplast topologies in the *Boykinia* group (Saxifragaceae). *Syst Bot* 21:169–185
- Stebbins GL, Jenkins JA, Walters MS (1953) Chromosomes and phylogeny in the Compositae, tribe Cichorieae. *Univ Calif Publ Bot* 26:401–430
- Swofford DL (2001) PAUP*: phylogenetic analysis using parsimony (*and other methods) version 4.0. Sinauer, Sunderland
- Talavera S, Devesa JA, Galiano EF (1984) Notas cariosistemáticas sobre plantas norteafricanas. I. Compositae. *Candollea* 39:271–280
- Thompson JD (2003) Plant evolution in the Mediterranean. Oxford University Press, Oxford, 293 pp
- Veith M, Mayer C, Samraoui B, Barroso DD, Bogaerts S (2004) From Europe to Africa and vice versa: evidence for intercontinental dispersal in ribbed salamanders (Genus *Pleurodeles*). *J Biogeogr* 31:159–171
- Wendel JF, Schnabel A, Seelanan T (1995) An unusual ribosomal DNA sequence from *Gossypium gossypoides* reveals ancient, cryptic, intergenomic introgression. *Mol Phylogenet Evol* 4:298–313
- Whelan S, Goldman N (1999) Distributions of statistics used for the comparison of models of sequence evolution in phylogenetics. *Mol Biol Evol* 16:1292–1299
- Wojciechowski MF, Sanderson MJ, Fu FM (1999) Evidence on the monophyly of *Astragalus* (Fabaceae) and its major subgroups based on nuclear ribosomal DNA ITS and chloroplast DNA *trnL* intron data. *Syst Bot* 24:409–437
- Yokoyama Y, Lambeck K, de Deckker P, Johnston P, Fifield LK (2000) Timing of the Last Glacial Maximum from observed sea-level minima. *Nature* 406:713–716



S2. Additional samples of species in section *Pustulati* used in the present study including the locality, voucher and herbarium numbers, and GenBank accession numbers. SEV = Herbarium Universidad de Sevilla. RNG = Herbarium the University of Reading. Accession numbers with asterisks were reported previously in Kim et al. (2007)

Taxon; Geographic Origin and Voucher; Accession Nos.: ITS1, ITS2; *matK*.

Sonchus pustulatus Willk.; Spain-1, Almería, Barranco del Caballar (SEV217577); EU526685, EU526712; EU529715. Spain-1, Clone 1; EU526690, EU526717; -. Spain-1, Clone 2; EU526691, EU526718; -. Spain-1, Clone 3; EU526692, EU526719; -. Spain-1, Clone 4; EU526693, EU526720; -. Spain-2, Almería, Barranco del Caballar; EU526685, EU526713; -. Spain-3, Almería, Barranco del Caballar; EU6687, EU526714; EU529716. Spain-4, Almería, Barranco del Caballar; EU526688, EU526715; EU529717. Spain-5, Almería, Barranco del Caballar; EU526689, EU526716; -. Morocco-1, Chefchaouen, Talembote, Oued Laou (SEV215730); DQ507920*, DQ507970*; DQ507988*. Morocco-1, clone1; EU526677, EU526704; -. Morocco-1, clone2; EU526678, EU526705; -. Morocco-1, clone3; EU526679, EU526706; -. Morocco-1, clone4; EU526680, EU526707; -. Morocco-1, clone5; EU526681, EU526708; -. Morocco-1, clone6; EU526682, EU526709; -. Morocco-1, clone7; EU526683, EU526710; -. Morocco-1, clone8; EU526684, EU526711; -. Morocco-2, Chefchaouen, Talembote, Oued Laou (SEV215731); DQ507921*, DQ507971*; DQ507989*. Morocco-3, Chefchaouen, road to Oued Laou from Chefchaouen, Taguesout (M. Ait Laflich et al. RNG P0003353); DQ507922*, DQ507972*; DQ507990*. Morocco-4, Chefchaouen, Talembote, Oued

Laou; EU526671, EU526698; -. Morocco-5, Chefchaouen, Talembote, Oued Laou; EU526672, EU526699; EU529711. Morocco-6, Rif Occidental, Entre Oued Laou y Talembote, Es-Sebt-de-Saïd (SEV217395); EU526673, EU526700; EU529712. Morocco-7, Rif Occidental, Entre Oued Laou y Talembote, Es-Sebt-de-Saïd (SEV217396); EU526674, EU526701; EU529713. Morocco-8, Rif Occidental, Entre Oued Laou y Talembote, Es-Sebt-de-Saïd (SEV217397); EU526675, EU526702; 529714. Morocco-9, Rif Occidental, Entre Oued Laou y Talembote, Es-Sebt-de-Saïd (J. A. Mejías); EU526676, EU526703; -.

S. fragilis Ball; Morocco-1, Morocco, Tétouan, Mts Gorgues (SEV217382); DQ507926*, DQ507976*; DQ507991*. Morocco-2, Tétouan, Mts Gorgues (SEV217383); DQ507927*, DQ507977*; DQ507992*. Morocco-3, Tétouan, Mts Gorgues; EU526694, EU526721; -. Morocco-4, Tétouan, Mts Gorgues; EU526695, EU526722; -.

S. masguindalii Fau & Font Quer; Morocco-1, Nador, Al Hoceïma (SEV217378); DQ507924*, DQ507974*; DQ507999*. Morocco-2, Nador, Al Hoceïma (SEV217379); DQ507925*, DQ507975*; DQ50800*. Morocco-3, Nador, Al Hoceïma-3 (SEV217380); EU526696, EU526723; -. Morocco-4, Nador, Al Hoceïma-4 (SEV217381); EU526697, EU526724; -. Morocco-5, Botanic Garden La Orotava, Tenerife, Canary Islands, Spain (A. Santos-Guerra 124-99); DQ507923*, DQ507973*; DQ507924*.