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SELECTION OF CHLOROPLAST DNA MARKERS FOR THE DEVELOPMENT OF DNA BARCODE AND RECONSTRUCTION OF PHYLOGENY OF *SENECIO ASIRENSIS* BOULOS AND J.R.I. WOOD

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Abstract

Senecio asirensis Boulos & J.R.I. Wood (family: Asteraceae) is an endemic plant of Kingdom of Saudi Arabia found at a few places. The medicinal value of this plant is due to the presence of a variety of complex secondary metabolites. For the development of DNA barcode for this species the chloroplast spacer sequences *rps16* and *psbA-trnH* were found more variable and effective. Among the various loci of molecular DNA markers, nrDNA-ITS was used for the evaluation of phylogenetic relationship with other *Senecio* species retrieved from the GenBank database. *Senecio asirensis* had a separate clade from all other *Senecio* species suggesting its endemism to Saudi Arabia. However, using BLAST search of ITS sequence of this species on NCBI a maximum (98%) similarity to the other *Senecio* species was found. We analysed the data using the methods viz., maximum parsimony (MP), Neighbor-joining (NJ) and maximum likelihood for the accuracy of the results and found congruence in phylogeny for this species. Thus, based on nr-ITS marker, *S. asirensis* clearly showed its phylogenetic relationship to the other *Senecio* species.

Introduction

Senecio is one of the largest genera, which is represented by many species. All *Senecio* species have variation in morphology and growth pattern containing annual, climber, peri-annual, succulent, semi-aquatic, stragglers and shrubs. *Senecio asirensis* is an endemic species of Saudi Arabia which is distributed at a few places such as Jabal Fayfa, Raida, Bal Lasmar, Jabal Samdah, Bal Jurshi and Tannouma (Chaudhary, 2000). Apart from *S. asirensis*, the other species of *Senecio* such as *Senecio bojeri*, *S. flavus*, *S. glaucus*, *S. hadiensis*, *S. haggariensis*, *S. lyratus*, *S. schimperi*, *S. sumarae*, and *S. vulgaris* are distributed in different geographical regions of Saudi Arabia. *Senecio asirensis* is a perennial, which may attain height up to100 cm. Some *Senecio* species are very poisonous due to the presence of pyrrolizidine alkaloids (Stegelmeier, 2011). A number of medicinally important compounds such as furanoeremophilanes have been isolated and characterized from these species (Ahmed *et al.,* 2004). Some species of *Senecio* such as *Senecio spartioides*, *S. douglasii* var. longilobus, *S. jacobaea* and *S. intergerrimus* var. exaltatus (Schaneberg *et al*., 2004) have hepatotoxic pyrrolizidine alkaloids with and without chromophores. A few *Senecio* species have gastro-protective effect (Lakshmanan *et al*., 2012) which have been used for the treatment of sexually transmitted infections (Wet *et al.,* 2012).

Senecio genus can hybridize inter-specifically frequently in its natural habitat (Kirk *et al.,* 2004) and the hybridized species generally have more advanced potential characters for yield and quality traits (Hercus & Hoffman, 1999; Fritz, 1999; Orians, 2000). However, natural hybridizations has been reported between *S. keniodendron* and *S. keniensis* (Beck, 1992), *S. vulgaris* and *S. squalidus* (Lowe & Abbott, 2000), *S. germanicus, S. hercynicus* and *S. ovatus* (Hodalova & Marhold, 1996; Hodalova, 2002), and between *S. vulgaris* and *S. vernalis* (Comes, 1994). Moreover, some species of *Senecio* such as *S. squalidus* (Abbott *et al*., 2000) and *S. cambrensis* (Harris & Ingram, 1992) have arisen from hybrid origins. Thus, hybridization seems to have a potential role in the evolution of *Senecio* species.

The standard part of the genome known as 'barcode' can be used for the identification of the plant species. Various chloroplast and nuclear DNA markers have been used for the development of DNA barcode (Jeanson *et al*., 2011; Hollingsworth *et al*., 2011; Dong *et al*., 2012). Molecular markers are more reliable than the morphological and biochemical markers (Kumar *et al*., 2009). *Senecio* species such as *S. bombaynsis*, *S. belgaumensis, S. dalzilli* and *S. edgeworthii* were characterized using RAPD markers (Sumangala *et al*., 2011). Although a variety of molecular markers have been used for the study of plant phylogeny, nuclear ribosomal internal transcribed spacer (nr-ITS) sequence is the most widely used marker as it is more reproducible under wide variable conditions. This region has more insertions/deletions which are more informative for the study of phylogenetic relationship among plant species (Baldwin *et al.*, 1995). ITS sequences (mainly ITS 2) of nuclear rRNA genes have been used for the study of phylogenetic relationships at species level (Shneer, 2009; Chen *et al.,* 2010). However, phylogenetic tree based upon a single locus must be interpreted with caution as nuclear gene has a tendency of hybridization and there is a strong evidence of monophyly from sequences of both nuclear and chloroplast genes (Barber *et al*., 2002). These regions are highly used in phylogenetic studies due to its high discriminatory power and technical ease of amplification (Kress *et al*., 2005). It also provides highest informative characters for the identification and authentication of plant species at inter- and intra-species levels. Due to its high copy number in the genome, it is easily amplified from the total crude genomic DNA extracted from the old and/or dried plant samples. This region also has hyper-variability and poor alignability across most taxonomic groups. It is the most frequently used locus followed by the *trnH–psbA*, *rbcL*, and *matK* loci. This locus has been used for the authentication of

those plant species which have similar morphology to the other species (Li *et al*., 2011; Rana *et al*., 2012). However, in some cases ITS regions were unable to differentiate at inter-species level (Luo & Yang, 2008; Wu *et al*., 2005). The segment ITS2 of the ITS region has been used as a DNA barcode for the identification of plant species (Kress *et al*., 2005). Since, *S. asirensis* is an endemic plant to Saudi Arabia and its phylogenetic study has not been carried out until now, so various loci and chloroplast genomes were screened for development of DNA barcode and nrDNA-ITS employed for the study of its phylogeny.

Material and Methods

Taxon sampling: *Senecio asirensis* was collected from the Abha region of Saudi Arabia. It was identified based on morphological characteristics by a well-versed taxonomist at King Saud University, Saudi Arabia (Fig. 1).

Fig. 1. Photograph of *Senecio asirensis* collected from the Abha region of Saudi Arabia.

DNA extraction and sequence data generation: The genomic DNA was extracted from the leaves (dried in silica gel) using modified CTAB method (Khan *et al*., 2007). The purified genomic DNA was used in PCR for the amplification of nr-ITS locus using universal primers ITS1/ITS4, respectively. The PCR bead (GE healthcare, Spain) was employed for the amplification of the nr-ITS locus. The single reaction consisted of 20µl of deionized sterile water, 25ng DNA per reaction volume and 10 pM/µl of each forward and reverse primer. After mixing the all PCR components, the reaction was set up in Techne, thermal-cycler. The first denaturation was carried out at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 49°C for 1 min and extension at 72°C for 1 min with a final extension step of 72°C for 5 min.

PCR product purification and sequencing: The PCR products were purified according to the manufacturer instructions (SolGent PCR purification Kit) prior to sequencing. The amplified PCR product was directly sequenced at Macrogen Inc., South Korea using the dye terminator chemistry.

Sequence alignment and phylogenetic analyses: The ITS sequences of nrDNA of *Senecio asirensis* were subjected to BLAST on http://blast.ncbi.nlm.nih.gov/Blast.cgi to confirm our sequences from related genera available on the GenBank database. The related ITS sequences of *Senecio* species (53) were retrieved from the GenBank database. *Paraixeris denticulata* and *Pilosella rhodopea* were used as an out group for the phylogenetic reconstruction. All sequences were edited and assembled using BIOEDIT v.

7.0.9.0 (Hall, 1999). Some sequences were edited manually with adjustments as needed. All characters were treated as equally weighted and unordered, and the gaps as missing data. The branch support was evaluated using 1000 bootstrap (BS) replicates with random sequence addition, equal weighting and TBR branch swapping, holding one tree at each replicate. The ITS sequence (size: 645 bp) generated from *S. asirensis* in the present study has been submitted to the GenBank (accession No: KC751413).

Results and Discussion

Senecio asirensis is an endemic plant of Saudi Arabia with considerable medicinal value. A few species of *Senecio* are found in the Kingdom. This genus has many species worldwide and a large number is found in Turkey which is widely used in treating various human diseases (Lotfi *et al.,* 2010). We amplified various loci of the chloroplast genome for the development of a DNA barcode and for the evaluation of phylogenetic relationship of *S. asirensis* with those of *Senecio* species whose sequences are available in the GenBank databases (NCBI) (http://www.ncbi.nlm.nih.gov/). The amplified sequences viz., *rps-16*, *rpoB, rpoC1* and *psbA-trnH* from *S. asirensis* were subjected to BLAST at GenBank database for sequence homology and confirmation of the amplified locus. The *rpoB* and *rpoC1* showed 99% similarity to the other species of the same genus, therefore, further application of these loci for the development of DNA barcode is less applicable for *S. asirensis*. The *rps16* and *psbA-trnH* loci showed 95% and 96% similarity to the other genera of the same family, respectively. Thus, among the chloroplast DNA markers, *rps16* and *psbA-trnH* both can be used for the development of DNA barcode as both spacer sequences showed more variations. Plastid regions with raw sequence differences 2% were grouped as the most variable segments, and can be used for DNA barcoding when normalized for length of 300 to 800 bp (Lucas *et al.,* 2012). However, due to the less availability of these chloroplast loci for the *Senecio* genus at the GenBank database, the reconstruction of phylogeny was not possible to draw. Therefore, more successful and highly used marker, nrDNA-ITS was used for the reconstruction of phylogeny of *S. asirensis* with those taxa whose sequences were easily retrieved from the GenBank database. The amplified region nr-ITS (ITS1-5.8s-ITS2) from *S. asirensis* showed 98% similarity to the same genus using BLAST at the GenBank database. This locus has been used in many plant species for the evaluation of phylogenetic relationships due to its high discriminatory power and being easily amplifiable. Such phylogenetic studies reveal the origin and evolution of taxa in their natural habitat. The identification keys based on morphological features of the plant still hold pivotal position in taxonomy (Heinrich, 2008). However, morphological (Carlsward *et al*., 1997) anatomical (Stern *et al*., 1994), biochemical (Korejo *et al*., 2010) and phytochemical (Anon., 1988; Li *et al*., 2010) markers have some limitations because of their less reliability. The 2 species of *Senecio* viz., *S. scandens* and *S.vulgaris* were authenticated using UPLC–DAD/ESI-MS based on pyrrolizidine alkaloid (Yang *et al*., 2011) as it is commonly found in *Senecio* species and also has been reported in other genera of the same family (Stegelmeier, 2011).

The sequence based DNA markers have potential reproducibility under wide variable conditions and have been used in many studies. They provide more information as compared to other markers. Therefore, ITS locus was used to infer the phylogenetic relationship of *S. asirensis* which is endemic to Saudi Arabia. The nr-ITS locus of *S. asirensis* was amplified using universal primers and its size was found 645bp (ITS1-5.8s-ITS2). This sequence was subjected to BLAST on NCBI to confirm our genus and its similarity to the other species of the same genus. For the determination of exact size of this species, sequence alignment was performed among the different species of *Senecio* which showed a maximum identity using the ClustalX version 1.81 (Thompson *et al*., 1997). The residue of each sequence was cleaned using Genedoc software **(**Nicholas & Nicholas, 1997**)** and erroneous sequences were deleted from the final analysis. The correct sequences were processed for further analysis. The phylogenetic tree was reconstructed among the various species of *Senecio* using MEGA 5 (Tamura *et al*., 2011). There were a total of 574 positions in the final dataset. *Senecio asirensis* clustered in a separate clade in the phylogenetic tree (Fig. 2a, 2b & 2c) when three methods viz., maximum likelihood (Jones *et al*., 1992), Neighbor-joining (NJ) (Saitou & Nei, 1987) and maximum parsimony (MP) (Eck *et al*., 1966) were employed for the reproducibility and thus congruence in phylogeny was found.

The separate clading of *S. asirensis* in phylogenetic tree showed its endemism to Saudi Arabia, which was also confirmed by some unique morphological markers as discussed above. Thus, due to easy amplification and more discriminatory power of nr-ITS, the phylogenetic study of several taxa has been performed such as *Cymbidium* (Sharma *et al*., 2012), *Eriobotrya* (Zhao *et al.,* 2011), *Paphiopedilum armeniacum*, *Paphiopedilum micranthum*, *Paphiopedilum delenatii* and their hybrids (Sun *et al.,* 2011), and *Gavilea* (Chemisquy & Morrone, 2012). However, a single gene is less reproducible for reconstruction of phylogenetic tree as well as development of DNA barcode, therefore it is better to sequence more genes for the accuracy of the results (Sandersson & Driskell 2003). The more sequences coming from different genes provide more information than a tree constructed from a single gene. Therefore, a combined study should be performed using the other markers such as morphological, anatomical, phytochemical, chloroplast loci and nuclear DNA markers. Furthermore, we are collecting the populations of *S. asirensis* from different places of Saudi Arabia for the assessment of genetic diversity and the development of the DNA barcode based on these chloroplast loci and nrDNA-ITS, so that its DNA based identification could be easily done. Since many species of *Senecio* have toxic compounds, so non-toxic ones can be identified and authenticated based on these DNA barcodes, if the taxa have similar morphology.

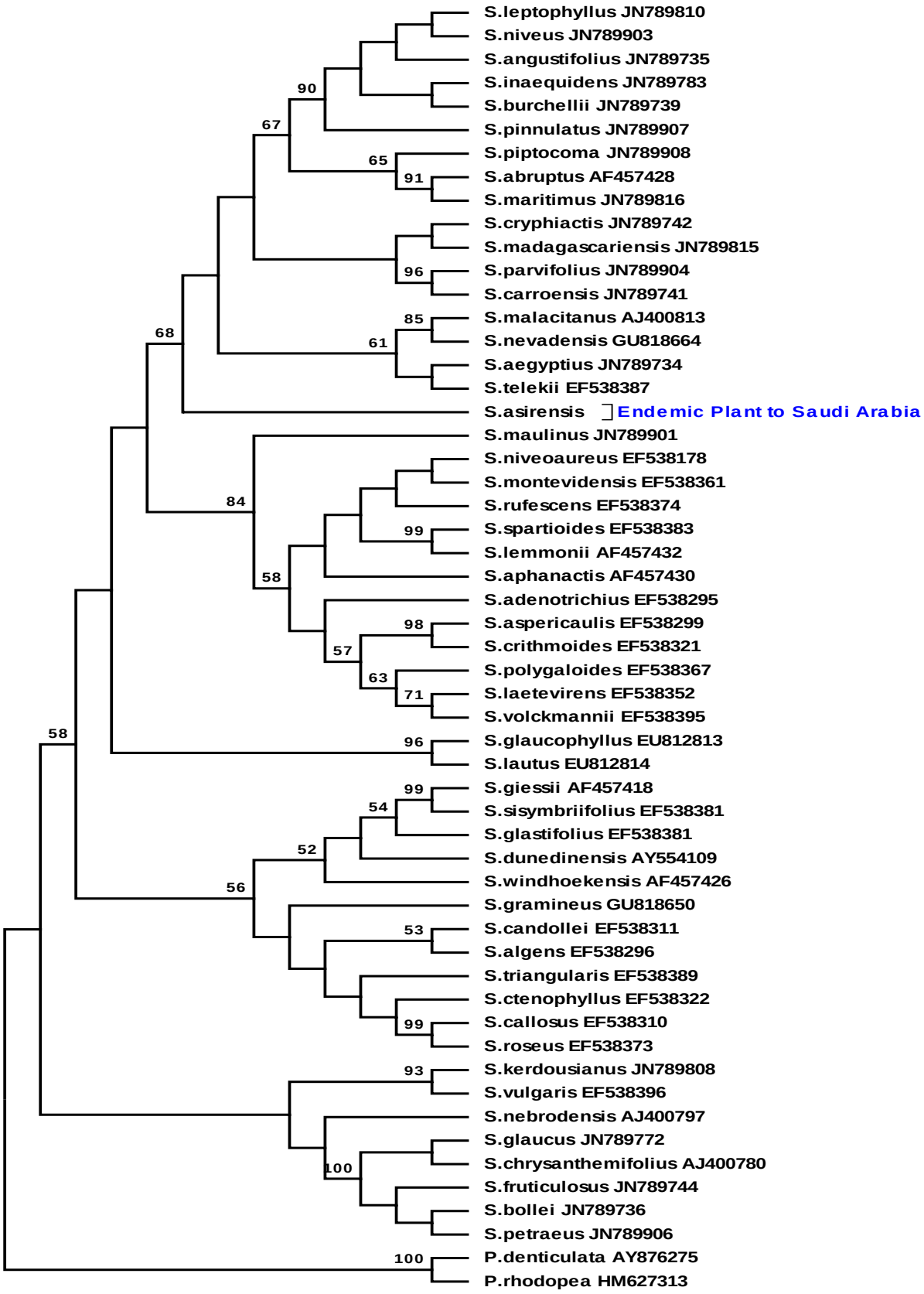

Fig. 2a. Phylogram based on nr-ITS locus among the various species of *Senecio* including *Senecio asirensis* using the maximum likelihood method, Bootstrap value >50% were shown on branches.

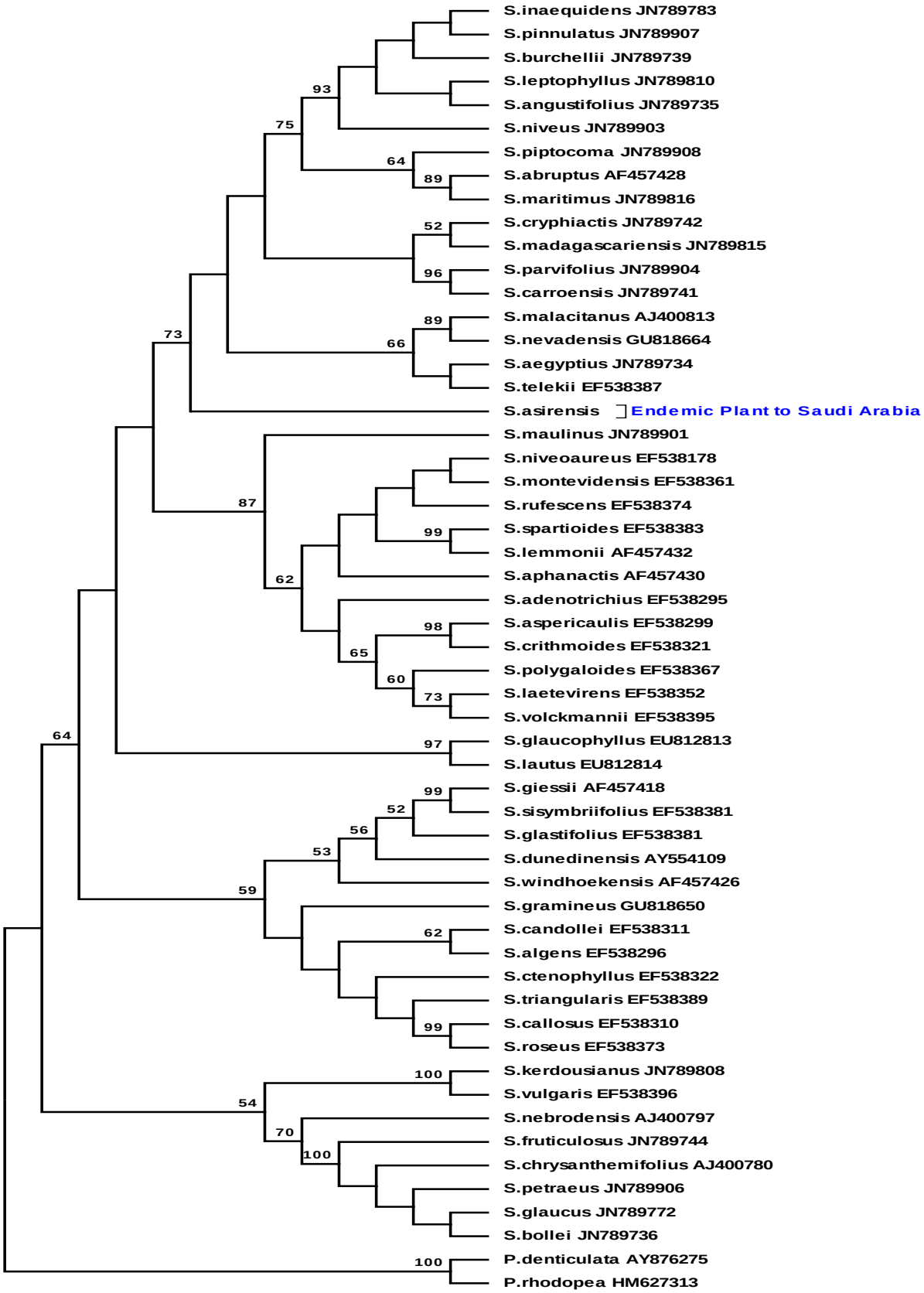

Fig. 2b. Phylogram based on nr-ITS locus among the various species of *Senecio* including *Senecio asirensis* using the Neighbor-joining (NJ) method, Bootstrap value >50% were shown on branches.

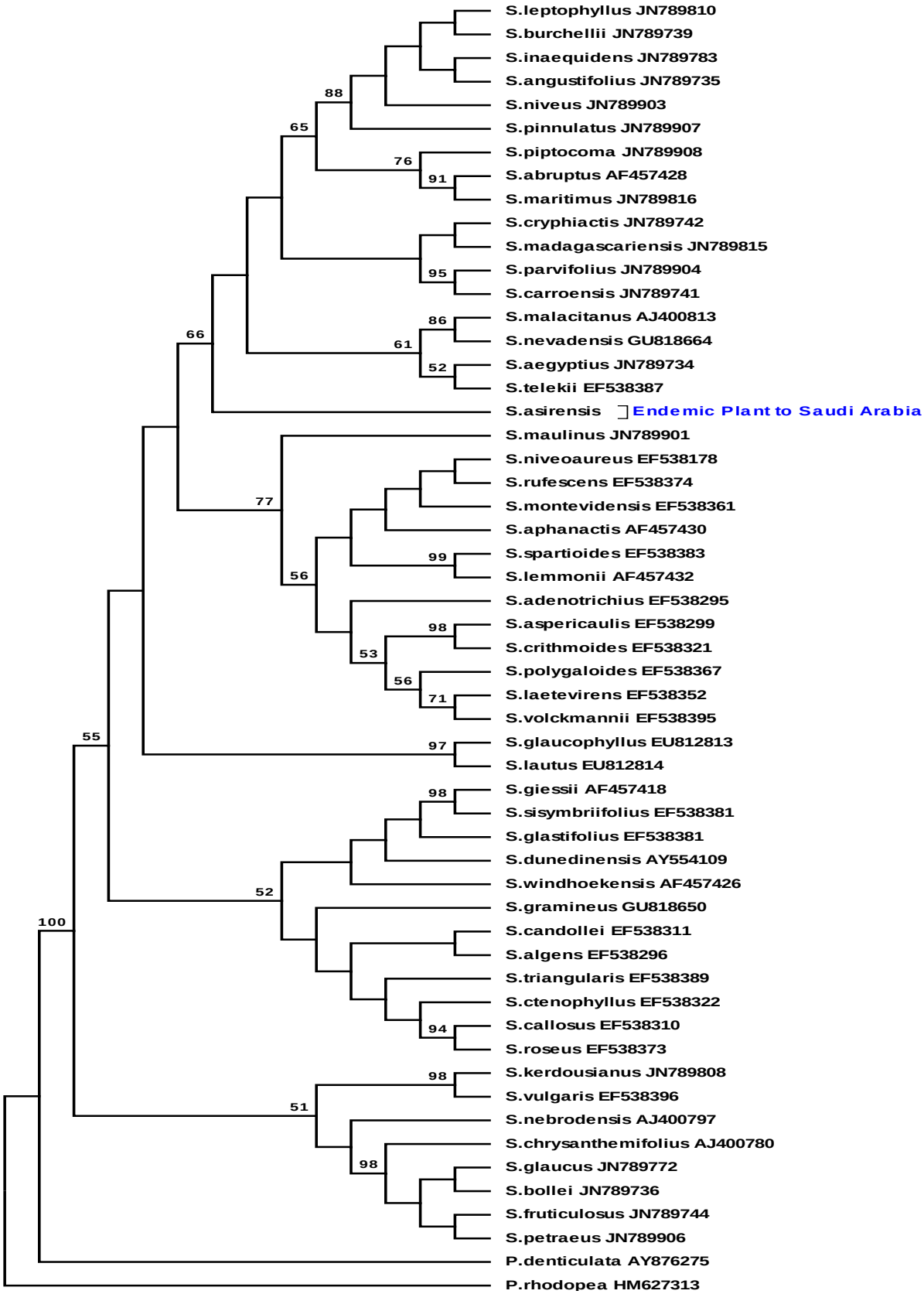

Fig. 2c. Phylogram based on nr-ITS locus among the various species of *Senecio* including *Senecio asirensis* using the maximum parsimony (MP) method. Bootstrap value >50% were shown on branches.

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