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Research Article

Withania somnifera: Diversity and phylogeny - An overview

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Abstract: The diversity and phylogenetic relationships within Solanaceae are interesting. *Withania,* despite being an important genus of the family, has not found proper attention for such studies and even species delimitation within the genus is debatable. Different authors at different points of times classified *Withania somnifera* into different morphological forms and split it into two different species. Similarly, there are various uncertainties about phylogenetic relationships below the family level in Solanaceae and *Withania*. In the present study, morphological and molecular data have been used to overview the diversity and phylogeny of Withania, particularly *W. somnifera*. The morphological studies, RAPD, ISSR, cytochrome P450 sequence, derived primers, SSR analogues, and ITS sequence data support that the species has a lot of polymorphism bifurcated into two well-demarcated varieties. However, there is no conclusive support for dividing the species into more than two forms or splitting the species into two different species, *rbc*L gene sequence data supports a close relationship of the genus with *Physalis* and associated genera.

Keywords: *Withania*; Solanaceae; polymorphism; phylogeny; PCR; sequence

Introduction

Withania Pauq. 1825 is an important genus of Solanaceae, a family well in harboring a tremendous amount of diversity (Yamanaka *et al.* 2003; Knapp, *et al.* 2004). The genus consists of 26 species, with *W. somnifera* (L.) Dunal. a widely distributed one, found in many parts of the world, including India. There is a lot of polymorphism at morphological (Atal and Schwarting, 1962) as well as chemical level in the species (Lavie *et al.* 1975). Atal and Schwarting (1962), following a survey population in the Indian region, concluded the existence of many distinct morphological forms of the species. A preliminary study by Nagi, *et al.* (2000) also indicated the existence of wide amplitude of polymorphism (53%) in the species.

Many authors have time and again raised certain varieties of *W. somnifera* (L.) Dunal to the species status (Kaul, 1957) and in certain instances some species of *Withania* have been transferred to the genus Physalis (Clarck, 1883). Although the genus has been established as early as 1825 by Pauquy but many species like *W. qaraitica* A. G. Mill. & Biagi and *W. sphaerocarpa* Hepper & Boulos have been described as lately as 1988. (Singh and Kumar, 1998) indicating that the genus being so

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important is poorly described. Species delimitation is still debatable within the genus (Singh and Kumar, 1998) as many species have been segregated on trivial characters. *W. obtusifolia* Tackh. and *W. kansuensis* Kuang & A. M. Lu from *W. somnifera* (L.) Dunal on the basis of leaf morphology. Even *W. coagulans* (Stocks) Dunal is confused with Physalis alkekengi L. (http./www.henriettsherbal.com/eclectic/kings/physalis.html). Tribal arrangements within the Solanoideae (to which *Withania* belongs) are not clear even after many taxonomic treatments of the family (D'Arcy *et al.* 2001). Olmstead *et al.* (1999) on the basis of preliminary survey argued separation of *Withania* along with *Tubocapsicum* Makino and *Aureliana* Sendtn. from tribe Capsiceae, and have placed them in tribe Physaleae on the basis of restriction pattern of chloroplast DNA. *W. somnifera* is a focal species of the genus because of its tremendous medicinal (Singh and Kumar, 1998 and Dutta *et al.* 2019) use and nearly cosmopolitan distribution (Singh and Kumar, 1998) so warranting taxonomic attention. The studies so far about its diversity and phylogeny are inadequate. They need rectification, which can be augmented by molecular phylogenetics. Further for better optimum and sustainable commercial exploitation of the taxon like *W. somnifera*, knowledge of its biodiversity and phylogenetic relationships is very important. Though morphological characters are still indispensable to the taxonomists, they are not alone adequate and need support from other branches, particularly molecular biology. Morphological data is plagued by constraints of phylogenetic inference and limited number of unambiguous characters available for analysis, so warrant using molecular markers to reveal genetic diversity that surpasses other types of markers in their utility (Scotland *et al.* 2003). In combination with explicit methods for phylogenetic analyses, molecular data has reshaped concepts of relationships at all taxonomic hierarchy levels (Qui *et al.* 1999). Molecular data has the advantage that it is much more informative and can be analysed more objectively and statistically than morphological data.

In the last few decades, the generation of sequence data has provided an opportunity to assess the relationship of various taxa in Solanaceae in phylogenetic terms. So such a treatment is also relevant in case of *W. somnifera*. Ribosomal genes, the spacers and chloroplast genes are found throughout the plant kingdom and are customarily used to resolve taxonomic queries at generic and above levels (Negrisolo *et al.* 2004; Verma *et al.* 2006). In the present study, molecular data and morphological data have been used to overview diversity and phylogeny of *W. somnifera*. PCR based primers along with sequence data of ribulose 1, 5-bisphosphate carboxylase/ mono-oxygenase large subunit (*rbc*L) and ITS regions flanking to the 5.8S rRNA gene have been used for this purpose. The study's main focus is to give an overview of diversity and phylogenetic trends of *W. somnifera*. Moreover, it is intended to clarify taxonomic position of so claimed *W. ashwagandha* (Kaul, 1957; Malik *et al.* 2018). Such study can also be used as a base for a holistic study of the whole genus of *Withania*.

Materials and Methods

For the morphological studied of *W. somnifera*, characters like habit, root, stem, leaf, flower, fruit and seed characters of the accessions collected from different regions of India (Table 1), were studied. Fresh leaf material was used for DNA extraction following the protocol of Ahmad *et al.* (2004). The DNA samples' quantity and quality were estimated by comparing band intensities on a 0.8% agarose gel and by spectrophotometric method. After thorough screening 6 RAPD primers (Table 2), 3 non-anchored ISSR primers (PL515, PL520 and

PL522), 8 pairs of cytochrome P450 sequence based primers and 3 pairs of SSR analogue primers (Table S1) were used. Cytochrome P450 sequence-based primers used were derived from cytochrome P450 sequences of plant cytochrome P450 family *CYP*78 (Larkin, 1994) and SSR primers used in this study were originally used by Miyao *et al.* (1996) for rice and subsequently Smelcerovic *et al.* (2006) for *Hypericum* species. The RAPD and ISSR primers were used to screen the collected 32 accessions for polymorphism. The cytochrome P450 sequence derived primers, SSR-analogue primers, those for amplifying ITS1-5.8S-ITS2 rDNA region, and AFLP primers were used to reveal genetic diversity using five selected accessions of *W. somnifera* and single available accession each of *W. frutescens* and *W. coagulans*. Amplification of rDNA region containing ITS and 5.8S was carried out by primers (Forward P119: 5′-TCCGTAGGTGGTGAACCTGCGG-3′ and reverse P120: 5′-TCCTCCGCTTATTGATATGC-3′) recommended by White *et al.* (1990). For *rbc*L gene amplification universal primers (Forward primer PL669: 5′-ATGTCACCACAAACAGAAAC-3′ and reverse primer: PL670: 5′TCCTTTTAGTAAAAGATTGGGCCGAG-3′; Savolainen *et al.* 2000) were used for amplifying and sequencing *rbc*L gene in the three species of *Withania*.

PCR amplification was performed in a volume of 20 µl containing: 2 µl 10X Taq DNA polymerase buffer, 2.25 mM of $MgCl_2$, 0.2 mM of dNTP mix, 12-20 pM of each primer (Bio Bsic Inc. Canada), 5-10 ng genomic DNA and 1 unit of *Taq* DNA polymerase (Bio Basic Inc. Canada). DNA amplification was performed in PCR thermocycler, Master Cycler Gradient (Eppendorf, Germany). After initial incubation for 3 minutes at 95°C, the samples for enzymatic amplification were subjected to PCR. The cycling programme in all cases ended with additional extension programme of 5 minutes at 72°C. After amplification, the reaction products were subjected to electrophoresis in 1.5% agarose gels in 1XTAE buffer at 5-8 V/cm stained with ethidium bromide. Gels were photographed under UV light with the help of a digital camera. A Gene Ruler™ DNA Ladder Mix (Fermentas Inc. USA) was used as the molecular standard. Cluster analysis (UPGMA) was used to generate a collective dendrogram for the two types of markers. Genetic diversity was estimated by Shannon diversity index (Lewontin, 1972).

The ITS1-5.8S-ITS2 amplicons were cloned prior to sequencing in pGEM®-T Easy vector (Promega Corp., USA). The gel purified PCR products of *rbc*L amplicons were directly sequenced following Savolainen *et al.* (2000) procedure. Additional internal primers (Forward: PL674: 5′-GCGTTGGAGAGATCGTTTCT-3′ and reverse PL675: 5′- TCGCATGTACCTGCAGTAGC-3′) of Savolainen *et al.* (2000) besides the PCR primers were used for sequencing *rbc*L sequences. New sequences' identity was checked using BLASTN programme (Altschul *et al.* 1997) available at the NCBI web server (www.ncbi.nlm.nih.gov). like Soltis *et al.* (1999) and Bell (2004) all sequences were readily aligned by eye with corresponding sequences of close relatives of the species in the public domain (http//www.ncbi.nlm.nih.gov). The end sequences which were difficult to align like Soltis *et al.* (1999) were excluded from the comparison. The sequences were also aligned for the phylogenetic tree and other features by ClustalW using the Lasergene software package (DNAStar Madison WI).

Sequences obtained from the public domain (www.ncbi.nlm.nih.gov) representing different tribes of Solanaceae and certain Convolvulaceae species (as outgroups) were used for generating *rbc*L sequence-based dendrogram. Phylogenetic tree for *rbc*L and ITS1-5.8S-ITS2 rDNA sequence data was generated following Saitou and Nei (1987) procedure.

Results

Withania somnifera is a shrub with stem brownish green or straw coloured. The stem height varies from 15-135 cm. The branching starts right from the base of the stem and leaves are simple and petiolate with margin entire. The root is a tap root system which divides into 2-5 smaller roots and occasionally there is only a single unbranched root. The root is brown to light reddish brown in colour and about 9-47 cm in length. The root crown is conical or knotty. The leaf apex is acute or in certain cases slightly obtuse. Both the surfaces of leaves are dull green with cordate or oblique bases. Leaves on vegetative shoots are alternate and large; those on floral branches are opposite, arranged somewhat laterally in pairs of one large and one small leaf with the arrangement going obscure towards the branch's apex. The inflorescence is axillary (or slightly extra-axillary) cyme with cluster of 5-25 inconspicuous pale green flowers. Pedicel is about 5 mm in length. The flowers are 9-12 mm (long) X 3-4 mm (diameter) in size. They are complete, actinomorphic, cyclic, gamosepalous, gamopetalous, hypogynous and pentamerous.

The size of sepals is 6-9 mm (long) X 1-5-3 mm (broad). Corolla is yellowish green, narrowly companulate, 7-8 X 2-2.5 mm in size, tomentose at throat and with ovate lobes. The petals are 5 (occasionally 4 or 6 in certain flowers), recurved, acute, pubescent and greenish yellow. Stamens are epipetalous and about 5-8 mm in length. Anthers are oblong with linear and slender filaments. Anthers are yellowish-white, ovoid and about 1 mm in diameter. Ovary is globose, 2-4 mm in diameter, with terminal filiform exerted style (3-5 mm long) and shortly bifid rounded stigma. The fruiting density varies from 2 to 10. Fruiting calyx becomes enlarged, inflated and entirely enveloping the fruit. It is membranous or leathery with 10 longitudinal ribs and an invaginated base. The fruit is a juicy globose soft berry. Seeds are numerous, compressed and discoid or reniform. Berry is shiny, globose, about 4-5 mm in diameter when mature and with a distinct brownish scar of the style at its apex. Each fruit remains enclosed inside a persistent calyx which becomes gradually bigger and thicker nearly closely inflated. Placentation is axile with seeds 1.8-2.0 mm long and about 1 mm thick. The morphological studies revealed two main morphotypes, morphotype I and morphotype II (Table 3). Morphotype I is one with shorter stems (height ≤ 30 cm), smaller leaves (≤ 2.2-2.8 X 4.5-6.0 cm), lean branching and fruit yellowish orange berries. The morphotype II is with tall stems (≥30 cm), bigger leaves (2.3-7.2X4.2-14.0 cm), upright branches and fruit as red berries. Accession AGB-030 of morphotype II appears to have certain intermediate characters (Table) and probably is an interconnecting link between the morphotypes. There are no other constant morphological differences between different accessions of the species. Morphotype I consists of two accessions viz. AGB-015 and AGB-025, while rest of the accessions constitute morphotype II.

In the present study a total of 108 clear, repeatable bands were scored in over all six RAPD (82) and three ISSR primers (26). Out of them 96 (73 and 23 in RAPD and in ISSR respectively) were polymorphic, accounting for overall polymorphism of 88.8% (Table 2). The number of bands per primer in RAPD varied from 9 (in S143), through 14 (in S11) to 22 (in S5). In non-anchored ISSR primers, the number bands varied from 7 (in PL520) to 11 (PL522). Among the RAPD primers S5 (Fig. 1 and Table 2) showed the highest Shannon diversity index (4.32) followed by S11 (4.13) with lowest by S136 (1.09). S11 and S157 showed the highest percentage of polymorphism (100%) while S136 again showed the least percentage polymorphism (72.7%). Among the non-anchored ISSR primers (Table 2) PL522 (Fig. 1) was highly polymorphic (H′ = 1.39, percentage polymorphism = 100%) and PL520 was the least informative (H′ = 0.60, percentage polymorphism = 71.4%). A dendrogram (Fig. 3) generated using pooled molecular data divided the 32 accessions, in which accession AGB-015 and AGB-025 align together, while the rest of the accessions form almost a single clade.

The average number of bands per primer in case of cytochrome P450 sequence derived primer was 8.87 with a range of 4 (PL454 & PL457) to 13 (PL465 & PL466) (Table 6). The primer pair PL454 and PL457 was least informative in the number of bands; percentage polymorphism and Shannon diversity index (Table 6). The highest Shannon diversity index was shown by the primer pair PL456 and PL466 and the highest percentage polymorphism by the primer pair PL458 and PL461. Among the SSR analogue primers, the pair PL563 and PL564 showed the highest Shannon diversity index and percentage polymorphism. In both types of primers 71 bands were scored in which 52 were polymorphic constituting, for overall polymorphism of 73% (Table 6). Overall average Shannon diversity index per primer for both was 1.86. The analysis revealed by their collective dendrogram (Fig 3b) supported aligning of the accessions into two main morphotypes. Further it showed that *W. somnifera* was closer to *W.* frutescence than to *W. coagulans*.

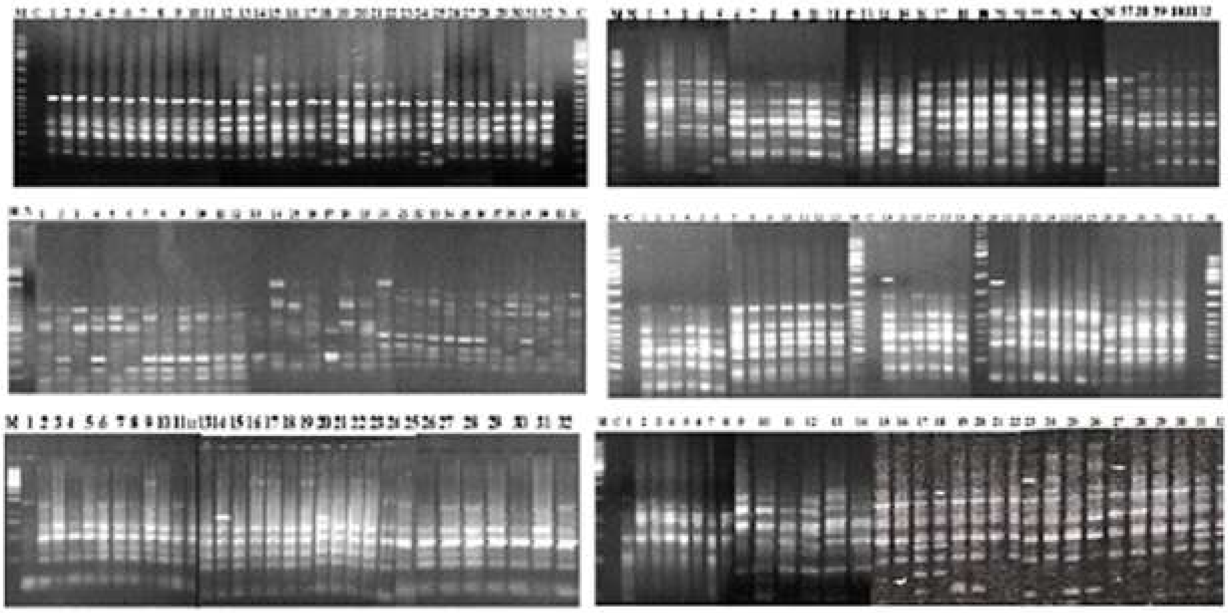
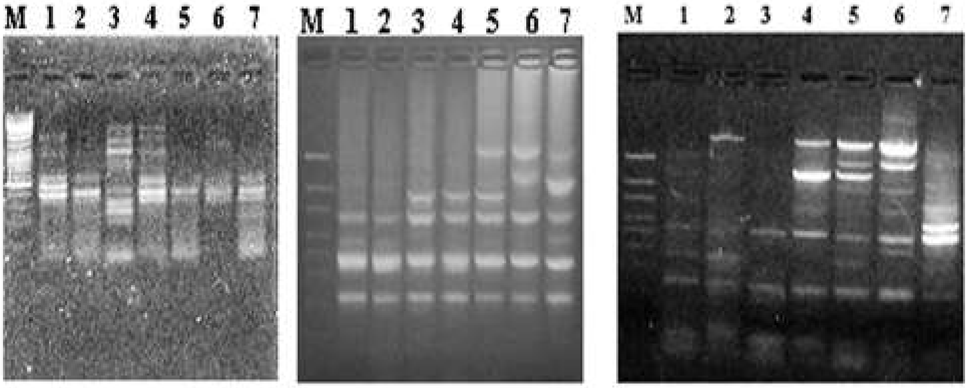


Figure 1. RAPD patterns of different accessions of *Withania somnifera* produced by the selected primers.

Lane M: 1 kb ladder (Gene RulerTM DNA Ladder Mix), lane C: Negative control, lane 1:AGB-001, lane 2: AGB-002, lane 3: AGB-003, lane 4: AGB-004, lane 5: AGB-005, lane 6: AGB-006, lane 7: AGB-007, lane 8: AGB-008, lane 9: AGB-009, lane 10: AGB-010, lane 11: AGB-011, lane 12: AGB-013, lane 13: AGB-014, lane 14: AGB-015, lane 15: AGB-016, lane 16: AGB-017, lane 17: AGB-019, lane 18: AGB-021, lane 19: AGB-022, lane 20: AGB-025, lane 21: AGB-026, lane 22: AGB-027, lane 23: AGB-028, lane 24: AGB-030, lane 25: AGB-031, lane 26: AGB-034, lane 27: AGB-037, lane 28: AGB-038, lane 29: AGB-042, lane 30: AGB-044, lane 31: AGB-049, lane 32: AGB-054.

The rDNA region of ITS1-5.8S-ITS2 flanked by partial sequence of 18S and 28S rDNA was about 700 bp. The sequence yielded ITS1+5.8S+ITS2 rDNA region of 673-686 bp in *W. somnifera* and 682 in *W. frutescens* and 685 in *W. coagulans*. For the accessions AGB-002, AGB-009, AGB-015, AGB-025 and AGB-030 of *W. somnifera,* the accession numbers obtained were DQ455564, DQ455560, DQ455562, DQ455563 and DQ455565 respectively. While for *W.*

frutescens it was no. DQ455561 and for *W. coagulans* it is DQ343619). The ITS length was 282-293 in *W. somnifera* and 291 both in *W frutescens* and *W coagulans.* Similarly, 5.8S rRNA was 164 in all three species. The G+C % of ITS1 in *W somnifera* was in the range of 64.8-68.1, while that in *W. frutescens* and *W coagulans* it was 66.3 and 67.6, respectively. The G+C % of 5.8S rDNA in *W. somnifera* was 227 and 230 in *W frutescens* and *W coagulans,* respectively. The G+C % for ITS2 varied from 60.2-62.1 in *W. somnifera* and was 57.7 in *W. frutescens* and 59.5 in *W. coagulans.*



Figures 2. Banding pattern by SSR analogue primers in selected accessions of *W. somnifera* M:1 kb DNA marker. 1: AGB-002, 2: AGB-009, 3: AGB-015, 4: AGB-025, 5: AGB-030, 6: *W. frutescens* and 7: *W. coagulans*

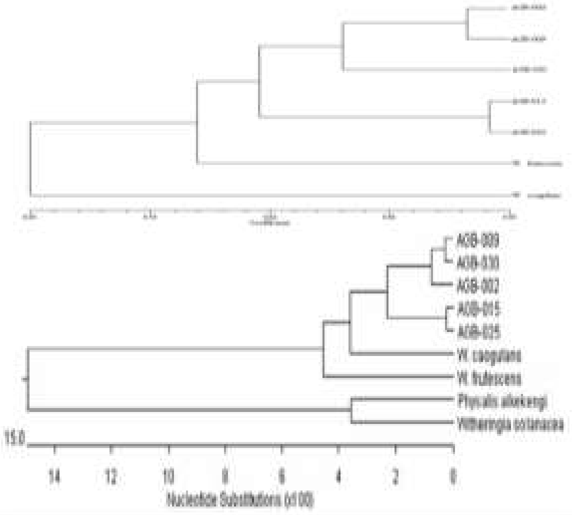


Figure 3. A) Dendrogram showing relationships between some selected accessions of *W. somnifera* (representing two morphotypes), *W. frutescens* and *W. coagulans* based on combined data of cytochrome P450 sequence based and SSR analogue primers. B) Phylogenetic tree generated proportional to sequence divergence based on ITS1-5.8S-ITS2 rDNA sequences.

The ITS1 and ITS2 length is within the range (see review Verma *et al.* 2006). The percent identity and divergence within the samples sequenced were also within range as expected within and between species of a genus (Ahmad *et al.* 2006). The phylogenetic tree (Fig. 8) proportional to sequence divergence plotted showed all *W. somnifera* group accessions with further bifurcation into two subgroups like the RAPD and ISSR primers.

Using the universal primers of Savolainen *et al.* (2000) the sequencing results showed the total length of amplified *rbc*L fragment about 1450 bp as also reported in other angiosperms (Savolainen *et al.* 2000). All the sequences were submitted to the Genbank with following acquired accession numbers (DQ353862, DQ368401 and DQ368402). The *rbc*L sequence data showed that *Withania* is closer to *Physalis* than to *Capsicum.*

Table 1. List of accessions of *Withania somnifera* used in the present study and their original site of collection.

S. No.	Accession	The original location of the collection site
1	AGB-001	Jammu
2	AGB-002	Jammu
3	AGB-003	Jammu
4	AGB-004	Jammu
5	AGB-005	Punjab
6	AGB-006	Punjab

7	AGB-007	Punjab
8	AGB-008	Haryana
9	AGB-009	Punjab
10	AGB-010	Himachal Pradesh
11	AGB-011	Himachal Pradesh
12	AGB-013	Himachal Pradesh
13	AGB-014	Himachal Pradesh
14	AGB-015	Utter Pradesh
15	AGB-016	Haryana
16	AGB-017	Utter Pradesh
17	AGB-019	Delhi
18	AGB-021	Utter Pradesh
19	AGB-022	Utter Pradesh
20	AGB-025	Madhya Pradesh
21	AGB-026	Madhya Pradesh
22	AGB-027	Madhya Pradesh
23	AGB-028	Madhya Pradesh
24	AGB-030	Madhya Pradesh
25	AGB-031	Gujarat
26	AGB-034	Gujarat
27	AGB-037	Gujarat
28	AGB-038	Rajasthan
29	AGB-042	Rajasthan
30	AGB-044	Rajasthan
31	AGB-049	Rajasthan
32	AGB-054	Rajasthan

Discussion

Diversity within Solanaceae is not any unusual feature (Knapp *et al.* 2004) so also within *Withania*. Morphological (Atal and Schwarting, 1962) and chemical variations (Lavie *et al.* 1975) within *W. somnifera* can be envisaged by its wide geographical distribution. Our studies support the view that *W. somnifera* harbours large diversity at morphological (Kaul, `1957 and Atal and Scharting, 1962) and DNA (Negi *et al.* 2000) level; however, we could not get support for its segregation into more than two forms as done by Atal and Shwarting (1962). There is no difference into the root structure of different accessions except that the plants with less stem height have shorter roots. The forms of Atal and Shwarting (1962) seem probably result of edapho-climatic factors, as roots tend to be longer and unbranched in sandy soils (form II of Atal and Schwarting, 1962) and in dry habitat conditions (form III of Atal and Schwarting, 1962), which may be reason of unbranched roots in these forms. However, form I of Atal and Shwarting (1962) is similar to morphotype I of our study. AFLP studies of Negi *et al.* (2000) also supported existence of two well-distinguished varieties of the species (Nagori and Kashmiri). Though they have not given morphological attributes of the varieties but by Kashmiri variety they mean the accessions found in Jammu & Kashmir, which belong to morphotype II. Latoo *et al.* (2006) attribute large diversity of the species to a peculiar reproductive mechanism and protogyny in the species.

Randomly amplified polymorphic DNA (RAPD) is the simplest type of technique for developing molecular markers (Welsh and McClelland, 1991). The collective effect of short sequence primers and low annealing temperature allows amplification of more than one band making the assay useful for efficient screening of polymorphism between individuals (Scott *et al.* 1993). RAPD being a fast-screening technique (Sobral and Honeycutt, 1993), needing no prior sequence knowledge is an attractive marker type and is better for resolving genetic diversity at intra-specific level (Scott *et al.* 1993). Like RAPDs, non-anchored ISSR markers quick and easy to handle but they seem to have the reproducibility of SSR markers because of the longer length of their primers. They are based on the premise that SSR loci are evenly dispersed throughout eukaryotic genome (Li and Ge, 2001). The chance of hitting two SSRs with a common motif, oriented on opposite DNA strands within amplifiable distance of one another is high enough that a single-primer amplification should yield a high degree of polymorphic bands (Reddy *et al.* 2002). However, various workers allege of non-reproducibility about both these marker types, yet if laboratory conditions are monitored carefully both RAPD (Ahmad *et al.* 2006) and ISSR (Bornet and Branchard, 2001) can give consistent results. Inter simple sequence repeat

(ISSR) sequences besides detecting polymorphism, can be a new approach to studying SSR distribution and their frequency (Bornet *et al.* 2002). Non-anchored ISSR primer amplification in cauliflower (Bornet *et al.* 2002) showed that most of the internal regions of ISSR had homologies with known sequences, mainly with genes coding for proteins implicating their significance. Bornet *et al.* (2002) also found that these displays long and numerous homologies with *Arbidiopsis thaliana* genome. The overall mean Shannon-Weaver diversity (H′) index with the RAPD and ISSR was 2.13, indicating better resolution 7of genetic diversity. Song *et al.* (2000) also observed similar results in *Lansium domesticum* with RAPD markers.

Cytochrome P450 multigene superfamily being widely distributed in genome (Shalk *et al.* 1999) plays important roles in primary and secondary metabolism (Ohkawa *et al.* 1998). *Arabidiopdsis thaliana* (relatively simple with less secondary metabolites) genome has shown that about 0.9% of genes could be categorized as cytochrome P450 genes (Riechmann *et al.* 2001). It is here to emphasize that cytochrome P450 form bulk of genes in plant species. Information from multi-gene families like cytochrome P450, could be used as a benchmark in functional genomic analysis and for diverse genetic studies applications (Somerville and Somerville, 1999) particularly of plant genomes (Yamanaka *et al.* 2003). Relatively small number of primers is needed for amplifying such genes and could be useful genetic markers in cases where good sequence information is not available (Yamanaka *et al.* 2003). Unlike the study of Yamanaka *et al.* (2003), in the present case, the primers were derived from maize cytochrome P450 gene sequences (Larkin, 1994), not from a phylogenetically distant species (like the human cytochrome P450 genes as in the study of Yamanaka *et al.* 2003). In the present study, eight pairs (consisting of four forward and six reverse) of primers provided sufficient polymorphism in a highly reproducible manner. Like Yamanaka *et al.* (2003) every primer combination was employed to amplify fragments in selected accessions. However, unlike them only forward-reverse combination was employed as in forward-forward or reverse-reverse combination (as in the study of Yamanaka *et al.* 2003), there are chances of getting more cytochrome P450 analogues rather than cytochrome P450s themselves. Even using only few accessions as representatives, such a high amount of polymorphism means that such primers can provide accession, variety, species, genus and family specific fragments as suggested by Yamanaka *et al.* (2003). The length of the amplified fragments was mainly below 1 kb. This is also the case of study of Yamanaka *et al.* (2003).

Study of Smelcerovic *et al.* (2006) has revealed that SSR primers designed for one species can be used as markers in another phylogenetically distant species. It is unnecessary that such primers may amplify SSR regions in given such distant taxa; however, it is more important that the banding pattern generated is constant for a given sample at a relatively higher temperature. The longer primers (with both forward and reverse) used with stringent PCR conditions gave banding pattern more relevant than that by RAPD and ISSR.

AGB-015 and AGB-025 from morphotype I and AGB-002, AGB-009 and AGB-030 from morphotype II were selected as representative accessions for the present study. Out of the sixty-four screened primer pairs, sixteen pairs provided sufficient polymorphism (Fig. 18, Table 15 and 16). The highest number of bands was observed in case of primer pair *Eco*RI$_{ACA}$-*Mse*I$_{CAT}$ (183), while the pair *Eco*RI$_{ACG}$-*Mse*I$_{CTG}$ showed the lowest number of bands (43) and the lowest number of polymorphic bands (21). *Eco*RI$_{AGC}$- *Mse*I$_{CTT}$ showed the highest numbers of polymorphic bands (138) and the highest percentage polymorphism (81.18%). The lowest percentage polymorphism (43.04%) was shown by the primer pair *Eco*RI$_{AAC}$- *Mse*I$_{CTC}$. The AFLP data collectively showed AGB-015 and AGB-025 are closer than rest of the accessions.

The nuclear ribosomal DNA (rDNA), particularly internal transcribed spacer (ITS) region has become an important nuclear locus for systematic molecular investigations of angiosperms (Jobes and Thien, 1997). The high copy number of the nrDNA repeat facilitates the region's amplification and sequencing (Baldwin *et al.* 1995). The sequence of two internal transcribed spacers has proven useful for resolving relationships within and among closely related plant genera (Soltis and Kuzoff, 1993) and species (Jobes and Thien, 1997). High copy number of ITS, their concerted evolution, rate of evolution (Baldwin *et al.* 1995) and being phylogenetically interpretable with enough length make them ideal for phylogenetic analysis, particularly at the infra-generic level (Bayer *et al.* 1996 and Ahmad *et al.* 2006) and promotes the accurate reconstruction of species relationships from sequencing. The 5.8S rDNA locus can serve as a critical alignment-free anchor point for sequence comparisons (Hershkovitz and Lewis, 1996). In contrast to other components of nrDNA, ITS sequences evolve relatively quickly (Small *et al.* 1998) both with respect to indel accumulation and nucleotide substitutions accounting for most of the variations in nrDNA region (Ahmad *et al.* 2006 and see review Verma *et al.* 2005). ITS region can be aligned across families (Liu and Schardl, 1994 and Hershkovitz and Zimmer, 1996). The length of ITS1+5.8S+ITS2 rDNA region in the present study (Table 6) and its individual components are within the expected range (Baldwin *et al.* 1995, Chase, 2003, Ahmad *et al.* 2006 and see review Verma *et al.* 2005). The range of inter-specific and intra-specific sequence variation and length in ITS regions (Table 7 and Fig. 8) is also within expected limits (see review Verma *et al.* 2005).

AFLP enables to estimate diversity at multiple loci within DNA, even in species with low genetic base (Campbell *et al.* 2003 and Després *et al.* 2003) and without any obligate sequence knowledge (Ridout and Donini, 1999 and Hodkinson *et al.* 2000) and with high reproducibility (Money *et al.* 1996). Since its introduction, AFLP has become prime choice of polymorphism detection and has helped resolve intra and inter-generic polymorphism in many plant species (Powell *et al.* 1996 and Milbourne *et al.* 1997 and Bahulikar *et al.* 2004). In view of its high reproducibility and reliability (Money *et al.* 1996), AFLP was also used during the present investigation to estimate diversity at multiple loci distributed throughout the whole genome (Mackill *et al.* 1996 and Ridout and Donini, 1999) and without any prior sequence requirement (Ridout and Donini, 1999 and Hodkinson *et al.* 2000). Using 16 AFLP primer combinations, a total of 1813 bands were observed in *W. somnifera* during the present study and of these 999 bands were polymorphic, accounting for polymorphism of 55.1% (Table 16 and 17). The highest number (183) of bands was observed in case of primer pair *Eco*RI$_{ACA}$- *Mse*I$_{CAT}$, while the pair *Eco*RI$_{ACG}$- *Mse*I$_{CTG}$ yielded not only the lowest number (43) of bands and also the lowest number (21) of polymorphic bands. *Eco*RI$_{AGC}$- *Mse*I$_{CTT}$ gave the high number of polymorphic bands (138) and highest percentage polymorphism (81.18%) also, while the lowest percentage polymorphism (43.04%) was obtained when the primer pair *Eco*RI$_{AAC}$-*Mse*I$_{CTC}$ was used. Average number of bands per primer in the present study was 113.3 and it is in tune with the range observed in other species as well (Shan *et al.* 1999; Cardoso *et al.* 2000; Ubi *et al.* 2000; Wong *et al.* 2001; Coulibaly *et al.* 2002; Hodkinson *et al.* 2002b; Kim *et al.* 2002; Oleszczuk *et al.* 2002; Zerega *et al.* 2002; Campbell *et al.* 2003 and Brouat *et al.* 2004). The AFLP data, like the collective data of RAPD and ISSR, also support bifurcation of the species into two morphotypes (Fig. 19). Such Infraspecific grouping based on AFLP within *Musa acuminata* has also been reported by Wong *et al.* (2001). Furthermore, reasonable correspondence between AFLP and RAPD data, as recorded during the present study, has also been observed in *Bemisia tabaci* (Cervera *et al.* 2000), *Mystus nemurus* (Chong *et al.* 2000) and *Vitis vinefera* (Dalbo *et al.* 2001).

Although the nrDNA sequence data tend to separate the two morphotypes into two subgroups, the divergence level is still less than expected for two different species (Table 7 and Fig. 8). So, there is no conclusive support to the notion that morphotype I (AGB-015 and AGB-025) was described as Ahwagandha by Kaul (1957) as a distinct species.

Studies of various researchers (Heiser, 1960, Olmstead and Palmer, 1992, Olmstead and Sweare, 1994 and Fay *et al.* 1998) have revealed interesting phylogenetic pattern in Solanaceae. Phylogenetic studies of Solanaceae are interesting from the point that the basal clades contain species of annual habit with strongly zygomorphic flowers exactly opposite to the general trend in the rest of angiosperms (Stebbins, 1974). A capsular fruit occurs in most of the family tree's basal clades and berries are derived in the family (Knapp, 2002). In view of Stebbins and Knapp (2002) observations, Withania is an advanced genus because being with actinomorphic flowers, non-herbaceous habit, and berry as a fruit. Morphological (Axelius, 1996, D' Arcy *et al.* 2001 and Whitson and Manos, 2005) and other features (Olmstead *et al.* 1999) show phylogenetic proximity of *Withania* with other physaloid genera (a group of genera e.g., *Chamaesarcha*, *Leucophysalis*, *Physaliastrum*, *Margaranthus*, *Witheringia* and *Withania* etc., in the subfamily Solanoideae centred around the genus *Physalis*) rather than *Capsicum*. Olmstead *et al.* (1999) have also argued for separation of *Withania tubocapsicum* and *Aureliana* from tribe Capsiceae and have placed them in tribe Physaleae. Both *Withania* and other physaloid genera characteristically share nodal inflorescence and have longitudinally dehiscent anthers.

Chloroplast DNA data promises to provide an important new tool for reconstructing plant phylogenies (Clegg, 1993) and has helped resolve some long-standing problems in phylogenetics. Reasons for this focus on a single organelle that by itself accounts for less than 0.1% of the genetic complement of plants are, having a conservative rate of nucleotide substitution (Clegg and Curtis, 1984) and its slow rates of molecular evolution suitable for the study of phylogenetic relationships at or beyond the family level. It seems less likely to be plagued by problems of among-site and among lineage rate heterogeneity (Naylor and Brown, 1997, 1998). The chloroplast genome of *Nicotiana tabacum* was the first to be completely sequenced one of any flowering plant (Shinozaki, *et al.* 1986) and is being used as a reference genome, particularly within the family Solanaceae. Some chloroplast genes, including *rbc*L and *atp*B (Hoot and Douglas, 1998 and Soltis *et al.* 1998) have been predominantly used for such phylogenetic studies. Among all *rbc*L being ubiquitous in plant and coding for large subunit of an important protein ribulose 1-5, bisphosphate carboxylase / mono-oxygenase is more suitable for such study (Savolainen *et al*. 2000).

For *rbc*L the size of amplicon revealed by agarose gel electrophoresis is about 1450 bp which is within range as reported in other studies also (Savolainen *et al.* 2000). Comparing *rbc*L data from major representatives of Solanaceae family with that of *Withania* (Fig. 9) it is evident that *Withania* is closer to *Physalis* than to *Capsicum*, an evidence in support of view held by (Olmstead *et al.* 1992; Axelius, 1996; Olmstead *et al*. 1999; D' Arcy *et al*. 2001; Whitson and Manos 2005). Thus, from the present study, it can be concluded that *Withania* *somnifera* has a large amount of morphological as well as DNA level polymorphism and that there is no clear support for that the morphotype I (or *W. ashwagandha* called by Kaul, 1957) is any different species. It seems a distinct variety or a subspecies rather a species. Our data confirm the inclusion of genus *Withania* within subfamily Solanoideae. Further, the studies showed as in many taxonomic treatments its close kinship with genus *Physalis*. However, the data clearly depict that the genus is well distinct from the later on. The data seems to support the view of common ancestry for the clades of Solanoideae and Nicotianoideae (Fig. 9), which share 12 as basic chromosome number. However, like *rbc*L data we could not develop the phylogenetic tree from ITS1-5.8S-ITS2 data with other related species as many such published sequences are absent from public domain of sequences (http://www.ncbi.nlm.gov).

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