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Short Communications

Intraspecific variation in the internal transcribed spacer (ITS) regions of rDNA in *Withania somnifera* (Linn.) Dunal

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Intraspecific variation in ITS regions of the rDNA among the five wild and five cultivated genotypes of *Withania somnifera*, were evaluated at nucleotide sequence level using restriction fragment length polymorphism (RFLP). The entire internal transcribed spacer (ITS1-5.8S-ITS2) region was first amplified by PCR and then cleaved with four different restriction enzymes (EcoRV, Hinf I, Afa I & Hae III). Restriction endonuclease digests, types, and sequence length composition of ITS 1 and ITS 2 of nuclear ribosomal DNA provided discrete differences between the cultivated and wild genotypes. A 710 bp single amplified product was obtained in all the five wild genotypes whereas, two ITS bands named as ITS type A and B of 709 bp and 552 bp, respectively were obtained in the five cultivated genotypes. A single deletion at 672 position was noted in ITS type A of cultivated genotypes. There was no restriction site in 552 bp ITS band for all the four restriction enzymes used. The variation of ITS at amplification as well as digestion level is in conformity with morphological and phytochemical differences in *W. somnifera* genotypes.

Keywords: *Withania somnifera*, RFLP, internal transcribed spacer (ITS), intraspecific variation

Withania somnifera also known as Ashwagandha or Asgandh is considered a wonder herb with diverse therapeutic values in the Ayurvedic and indigenous medical systems. Ashwagandha roots are compared with ginseng roots for their restorative properties and have been given the name 'Indian ginseng'. The plant is reported to possess adaptogenic, antiinflammatory, immunosuppressive, antioxidant, immunomodulatory and anticancerous properties[1-5]. In India, the plant grows widely in drier areas in the subtropical and semi-temperate regions from plains up to a height of 1700 m. Commercial cultivation of *W. somnifera* in India is carried out in ~ 5000 ha land in Manasa (Madhya Pradesh) and in some parts of Rajasthan, Andhra Pradesh and Uttar Pradesh[6,7]. The estimated annual production of Ashwagandha roots in India is about 2000 tons. An extreme degree of variability exists in *W. somnifera* with respect to growth habit, chemical profiling and morphological characteristics of plants in different parts of India and in other countries[8,9]. The cultivated morphotypes are reported to be distinct from the wild ones[9-12]. In Arabic and Persian literature, both cultivated and wild plants are separately described, former referred to as *Kaknaj-i-bostani* and the latter as *Kaknaj-i-jaballi*[10]. Further, Negi *et al*[13] grouped the Indian germplasm into Nagori and Kashmiri groups based on AFLP data.

Internal transcribed spacer (ITS) regions of the nuclear rDNA genes, ITS1 and ITS2, have proved one of the most informative regions of variable DNA for phylogenetic analysis at the level of species relationships within genera[14,15]. The ITS regions are non-coding DNA that are transcribed to RNA, but spliced out during ribosome assembly. Being non-coding, they accumulate DNA mutations much more rapidly than the 5.8S rDNA gene. Ribosomal genes, although present in high copy number, are usually homogeneous in DNA sequence within individuals through the process of concerted evolution and so are effectively equivalent to the study of variation of a single gene locus[16].

However, in some cases, concerted evolution is incomplete and multiple nuclear rDNA sequence types co-exist in the genome[17]. The nuclear genomic ITS1, 5.8S and ITS2 regions of rDNA are contiguous and, being ~700 bases in total length, can be readily amplified by PCR as a single unit and studied together. Significant levels of genotypic and phenotypic variability, more especially the wild and cultivated morphotypes, do exist in the species[9-12]. The present study was aimed to confirm the classification into cultivated and wild genotypes based on ITS region of rDNA in *W. somnifera*.

The seeds of the wild and cultivated genotypes (Fig. 1) were collected from Bikaner (Rajasthan) and

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Fig. 1—Genotypes of *W. somnifera:* a. Representative cultivated genotype with yellow berries & b. Representative wild genotype with red berries.

Neemunch and Manasa (Madhya Pradesh), respectively. The seeds were germinated in earthen pots and raised under uniform conditions at experimental field of Indian Institute of Integrative Medicine, Jammu. Total genomic DNA was extracted from young fresh leaves of all the genotypes using the CTAB method[18]. The DNA was quantified using DNA Quant Flurometer (Amersham Pharmacia Biotech Limited, UK). Double stranded DNA of the complete ITS region (ITS1-5.8S-ITS2) was amplified using the primers ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′). Amplifications were carried out in 25 µL reaction volume with 1X reaction buffer, 0.8 m*M* $MgCl_2$, 0.2 m*M* dNTP mixture (Amersham Pharmacia Biotech Limited, UK), 0.5U/ 25 µL Taq DNA polymerase (Bangalore Genei Pvt Ltd, India), 2.5 µL of each primer and 50 ng of total DNA. Amplification was performed in a thermal cycler (BIORAD) programmed for an initial denaturation at 94°C for 4 min, then 94°C for 1 min, 50°C for 1 min, 72°C for 2 min for 40 cycles with a 7 min final extension at 72°C. The amplified ITS region of rDNA was cloned in pGEM-T easy vector system (Promega) as described by Goel *et al*[19]. The positive clones were sequenced via sequencing centre, Delhi University South Campus, New Delhi. For ITS sequence data, alignments were done with CLUSTAL X program[20] using default setting with a fixed gap penalty of 6.66, and DNA transition weight of 0.5 in the multiple alignment parameter option.

The amplified ITS region was restricted with four restriction enzymes to obtain ITS-restriction fragment length polymorphism (ITS-RFLP). The 12.25 µL reaction mix in 1.5 mL Eppendorf tubes contained 10 µL of amplified product, 1.2 µL of enzyme buffer and 10 U of the restriction enzyme (New England Biolabs). After gentle mixing the mixture was incubated overnight at 37°C. The enzymes used were EcoRV, Hinf I, Afa I and Hae III, as per the specifications of the manufacturers. The digested products were fractionated on 1.5% agarose gel containing ethidium bromide (0.05 µg/mL), in 0.5X TBE buffer. Lambda DNA digested with HindIII (Promega) and 100 bp ladder (Bangalore Genei) were loaded alongside the digested products to serve as size markers. After agarose gel electrophoresis, the gel was photographed in UV light (LKB Pharmacia, USA).

A 710 bp single amplified product was obtained in all the wild genotypes for the entire rDNA ITS (ITS1-5.8S-ITS2) region, whereas, two ITS bands of 709 bp and 552 bp, named as ITS type A and B, respectively, were amplified unambiguously in all the cultivated genotypes (Fig. 2a). The smaller ITS band of cultivated genotypes shows a high degree of sequence heterogeneity from larger band of the same variety and that of the wild genotypes as shown in sequence alignment matrix (sequence alignment not shown). The amplified product(s) was separately digested with the restriction enzymes (EcoRV, Hinf I, Afa I and Hae III) and it was very interesting to note that there was no restriction site for any of the enzymes tested in 552 bp ITS band of cultivated genotypes. The Fig. 2b shows the restriction pattern generated with ITS+EcoRV combination. In wild genotypes, two bands of 400 bp and 260 bp were distinct (the remaining restriction fragment(s) of ~ 50 bp is not visible on the agarose gel) while in cultivated genotypes, in addition to bands of 400 bp and 260 bp, one undigested band of ~ 552 bp (ITS type B) was evident. Hence, it is clear beyond doubt that the polymorphism observed at digestion level was because of undigested 552 bp ITS band of cultivated genotypes.

The ITS sequence ranged from 552 bp (ITS type B) in cultivated accessions to 710 bp in wild accessions. The ITS type A in cultivated accessions was 709 bp long and differed from ITS sequence of wild accessions by 1 bp indel at 672 position (Fig. 3). The ITS type B in cultivated accessions differed from ITS type A and 710 bp ITS sequence of wild genotypes by 171 mutations comprising 151 substitutions and 20 indels ranging from 1-29 bp (Fig. 3). Thus, it can

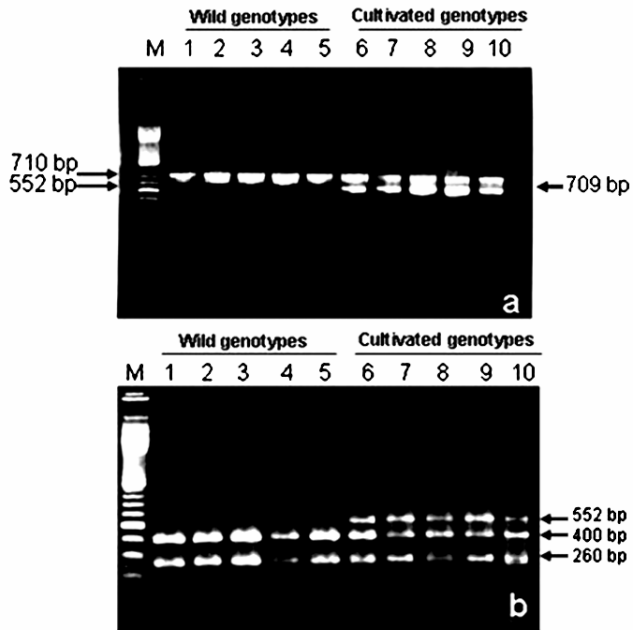

Fig. 2—a. Amplified rDNA ITS region with ITS1 and ITS4 primers of wild (Lanes 1-5) and cultivated (6-10) genotypes, M represents DNA marker; & b. Restriction fragment size patterns of the amplified ITS1-5.8S-ITS2 region using Eco RV.

be concluded that there are two situations: ITS type A only in wild genotypes and ITS type A with one indel and ITS type B in the case of cultivated genotypes.

Based on the results of the present study, a possible scenario appears to be that some relatively ancestral populations of *W. somnifera* acquired genetic changes that at a certain stage isolated it from the remaining population of the species. The period of isolation was sufficient to allow them to develop their distinct morphologies. In addition, since morphological characters are strongly affected by different selection pressures, for instance, selection of loci in response to cultivation practices in cultivated genotypes, the new evolutionary unit (cultivated genotypes) became very different in morphology.

The repeat gene family, like ITS in the present study, in a polyploidy can undergo three possible evolutionary fates – maintenance of repeat types, generation of new repeat types, and loss of repeat types via concerted evolution[21]. Our results suggested that wild genotypes exhibit a single ITS sequence type, presumably due to interlocus concerted evolution, whereas cultivated genotypes contain more than one repeat type, possibly reflecting the parental ITS sequences. Similar results have been reported in *Bromus*[22]. In *Bromus* allopolyploids, *B. hordeaceus* and *B. lanceolatus* and *B. secalinus* exhibited a single and more than one repeat type, respectively.

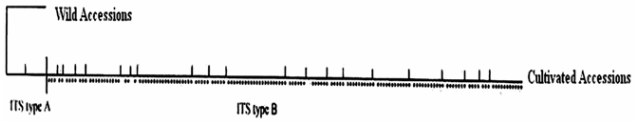

Fig. 3—Substitution and indel (insertion/deletion) events in ITS sequence of 10 genotypes. Solid circles represent single point mutations. Short vertical bars indicate indels.

In case of young hybridogenous taxa two ITS copies are present[23], which is the most probable situation in the present study and this possibility has also been described in the genus *Rosa* as a stable situation and has been defined as "nonconcerted" evolution[24]. In some cases these paralogues are old and might have lost their function and turned into pseudogenes[25].

Kaul[10] reported that the cultivated plants are different from the wild ones not only in their therapeutic properties but in all morphological characters like roots, stems, leaves, flowers, pollen grains, mature fruits, seeds and the enlarged calyx and suggested the name *Withania ashwagandha* for cultivated plants and *W. somnifera* for wild ones (Figs 1a & b). Negi *et al*[13] also confirmed their distinction based on AFLP. Our ITS data are in close agreement with the results of morphological and AFLP analyses. In earlier times also, genera were divided based on morphology, as is the case of genus *Hippophae* that was divided into two sections based on different fruit morphology[26]. In present study, analysis of the ITS regions provided evidence that wild and cultivated genotypes are not different only on morphological, chemical or AFLP basis but are also different based on ITS region of rDNA. Nuclear rDNA may be useful as an approximate molecular clock for diversification and phylogenetic analysis among species and populations within a species in variety of plants[27]. The present study supports the results of Kaul[10] and Negi[13] that wild and cultivated genotypes of *W. somnifera* are quite distinct. Thus, it could be concluded that RFLP may be useful in screening more individuals and populations before any conclusion is drawn in phylogeny.

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