Journal of Spices and Aromatic Crops
Vol. 23 (2) : 233–242 (2014)
www.indianspicesociety.in/josac/index.php/josac

Indian Society for Spices

Evaluating the taxonomic status of *Solanum nigrum* L. using flow cytometry and DNA barcoding technique

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Received 22 April 2013; Revised 01 August 2013; Accepted 20 March 2014

Abstract

Solanum nigrum L. is a widely distributed species whose taxonomic status remains controversial. The ploidy determination of all the accessions were done based on stomatal, pollen and some morphological characters but morphometry could not provide solution to distinguish these species. In this study, ploidy status of all the accessions were done using flow cytometry and DNA barcoding technique was also applied to identify and distinguish 13 distinct germplasm collections. DNA of all 13 accessions was isolated and sequenced. The sequence was aligned using DNASTAR offline Software. The DNA sequence was subjected to BLAST for identifying at species level. The intra specific variation between the species was calculated using MEGA 5.0. The phylogenetic analysis indicated that among the 13 accessions, six were identified as *S. americanum* Mill, three were identified as *S. nigrum* Linn. and four were identified as *S. villosum* Mill.

Keywords: BLAST, DNA barcode, ploidy analysis, *Solanum americanum*, *S. nigrum*, *S. villosum*, taxonomic status

Introduction

Solanum is one of the most important and largest genera of the family *Solanaceae*, comprising of about 84 genera and 3000 species. *Solanum nigrum* L. is a complex consisting of few species, is commonly known as black nightshade and is the largest and the most variable species of the genus *Solanum.* The most complicated type of plant group is polyploidy complex in which species mostly possess common morphological features besides their closely similar genomes. *S. nigrum* L. complex is reported to have about 30 morphologically distinct taxa (Schilling & Andersen 1990) with basic chromosome x=12. This complicated polyploidy complex has led to much of the taxonomic confusion surrounding this species. For this reason, the species have been re-classified many times but no satisfactory revision of the whole section has yet been devised.

The situation has been further complicated by a number of authors, who have persistently treated different members of the section as belonging to one species, *S. nigrum* L. The medicinally important taxa belonging to *S. nigrum* Complex are *S. americanum* Mill., *S. nigrum* L. and *S. villosum* Mill. *Solanum scarbum* Mill., *S. chenopodioides* Lam., *S. retroflexum* Dunal.

which displays similar morphological features with few phenotypic variations. Natural hybridization is probably more widespread in this section and is supposed to be the reason for complexity. In any breeding program, it is important to determine the ploidy level since polyploidy has been an important process in the evolution of plants that can contribute to novel gene expression and divergence.

Recently taxonomists have started using a number of techniques to solve the taxonomic confusion which could not be resolved by morphological markers. One of these could be the use of DNA barcoding which is an effective, reliable and simple tool to resolve the confusion in morphological identification. It is a diagnostic technique for species identification, using a short, standardized DNA region (www.barcoding.si.edu). It is a technique in which species identification is performed by using DNA sequences from a small fragment of the genome, with the aim of contributing to a wide range of ecological and conservation studies in which traditional taxonomic identification is not practical. The objective of the present study was to distinguish and authenticate morphologically similar *Solanum* species from each other using this barcoding technique.

Materials and methods

Thirteen morphologically distinct accessions of *Solanum nigrum* L. complex were selected from germplasm collection maintained at Department of Medicinal and Aromatic Crops, Tamil Nadu Agricultural University, Coimbatore (Table 1.). The study was conducted during 2012–13. Observations on morphological characters were recorded in the form of multiscale scores. The scale was adopted as per the IPGRI descriptors to measure the various morphological parameters like plant growth habit, leaf characters, stem characters, flower characters, fruit characters and seed characters

Ploidy determination

Stomata

The sample for stomatal study was taken from the center portion of the physiological leaf. The sample leaves were cut into one cm^2 bits and boiled for two minutes in water and then transferred to 70% ethanol and kept for 24 h to remove chlorophyll. The sample was washed with water and kept over a clean slide containing glycerin with the upper surface of the lamina bit in contact with the slide and sealed with a cover slip and examined under microscope of 45X magnification. The number of stomata per microscopic field (0.152 mm^2) was counted atleast at 10 different fields. The mean was arrived and expressed as stomatal density per mm^2. The length and breath of the stomata were also measured by using ocular micrometer and it was expressed in microns. The size of the stomata was calculated by multiplying the length and breath and was expressed in μM^2.

Pollen

Pollen grains were collected from freshly collected anthers by gently tapping the anthers on glass slides containing a drop of glycerol. Then cover slips were placed over the pollens and slides were observed under a microscpoe with the aid of ocular and stage micrometers connected with a computer in ordinary light. Diameters of 50 pollen grains in each genotype were recorded and the data were analysed in Q 500 MC WIN software programme. The pollen viability was tested with acetocarmine glycerine stain. The number of normal and shrivelled pollen grains was recorded under different fields of microscope. Pollen grains which stained well and looked plump and normal were considered to be viable and the shrivelled and unstained ones as non viable and the mean value was expressed in per cent.

Number of flowers in each infloresence of 10 tagged plants of all accessions was counted and mean was tabulated. Diameter of all the flowers and berries was measured in 10 selected plants of all accessions and mean was tabulated. Number of seeds present in all berries of 10 selected plants of all accessions was counted and mean was tabulated.

Flow cytometry analysis

Young leaves of selected accessions were used

Table 1. Morphological characterization of Makoi (*Solanum nigrum* L.) genotypes

Genotypes	Growth habit	Stem characters				Leaf characters				Flower characters		Fruit	Seed
		Branching	Pubescence	Shape	Color	Shape	Pubescence	Margin	Color	Size	Color	color	color
TN *Sn* 08	5	5	5	1	1	2	5	3	2	2	1	1	2
TN *Sn* 10	7	7	7	2	5	1	7	2	4	3	1	1	2
TN *Sn* 12	5	5	5	1	1	2	5	3	2	2	1	1	2
TN *Sn* 19	5	5	0	1	1	2	3	1	3	1	1	1	2
TN *Sn* 23	5	5	5	1	1	2	5	3	2	2	1	1	2
TN *Sn* 30	5	7	7	2	5	1	7	3	4	3	1	2	1
TN *Sn* 32	5	5	7	2	5	2	7	3	4	3	1	2	1
TN *Sn* 38	5	5	5	1	1	2	5	3	2	2	1	1	2
TN *Sn* 44	3	3	7	2	5	2	7	2	4	3	1	3	1
TN *Sn* 47	5	7	7	2	5	2	7	3	3	3	7	3	1
TN *Sn*51	5	5	0	1	1	2	3	1	3	1	1	1	2
TN *Sn* 52	5	5	5	1	1	2	5	3	2	2	1	1	2
TN *Sn* 53	5	5	5	1	1	2	5	3	2	2	1	1	2

Plant growth habit 3- Prostrate; 5- Intermediate; 7- Erect
Stem branching 3- Sparse; 5- Intermediate; 7- Dense
Stem pubescence 0- Absent; 3- Sparse; 5- Intermediate; 7- Dense
Stem shape 1- Cylindrical; 2-Angled; 3-Flattened
Stem color 1- Green; 3- Light purple; 5- Purple; 7- Dark purple
Leaf shape 1- Deltoid; 2- Ovate; 3- Lanceolate
Leaf pubescence 0-Absent; 3- Sparse; 5- Intermediate; 7- Dense
Leaf margin 1-Entire; 2- Undulate; 3- Sinuate
Leaf color 2-Light green; 3- Green; 4- Dark green
Flower size 1-Small; 2- Medium; 3-Big
Corolla color 1- White; 3- Yellow; 7- White with purple stripes
Fruit color 1-Purplish black; 2- Red; 3- Orange
Seed color 1-Straw; 2-Brown; 3-Black

for ploidy analysis. The samples were packed in zipped polyethene cover and sent to Directorate of Medicinal and Aromatic Plants, Anand, Gujarat for analysis. The procedure explained by Galbraith *et al.* (1983) was followed for ploidy analysis. The leaf material was chopped with a one-sided sharp razor blade into a petri dish containing extraction buffer prepared according to the method of Galbraith *et al.* (1983). After few hours the buffer containing cell constituents was passed sequentially through nylon filters of 50 µm and 20 µm mesh size to separate nuclei from the cell debris. The buffer with nuclei was then centrifuged at high speed (800 rpm for 5 min). Then the supernatant was discarded, and the pellet was resuspended in 100 µL of a propidium iodide (PI) staining solution at a concentration of 100 µg mL^{-1}. Diploid (2x) cultivated type of *Solanum nigrum* L. was used as an internal reference standard. The ploidy level was determined by measuring the size of the nuclear genome in the form of histograms.

Confirmation of genetic diversity using DNA barcoding

DNA extraction, PCR amplification and DNA sequencing

DNA was isolated from the fresh leaf samples of all 13 distinct accessions by using the modified CTAB method (Khanuja *et al.* 1999). Isolated DNA was used as the template for Polymerase Chain Reaction. The total volume of 20 µL PCR mixture contained 1 µL of 50 ng of DNA template, 2 µL of 10X Taq Buffer (Fermentas®), 2 µL of 25 mM $MgCl_2$ (Fermentas®), 2 µL of 2 mM dNTPs (Fermentas®), 0.5 µL of each Forward and Reverse primers (10 pM) and 0.1 µL of 5 U Taq DNA Polymerase (Fermentas®). The reaction was carried out in a thermal cycler (Eppendorf, Germany). The forward and reverse primer sequences of internal transcribed spacer (*ITS*) region used in the present study are F- 5′ GGA AGG AGG AGT CGT AAC AAG G 3′ (Modified from White *et al*. 1990); R- 5′ TCC TCC GCT TAT TGA TAT GC 3′ (White *et al*. 1990). The PCR product was separated in 1% agarose gel and documented (Alpha Digidoc, USA). Sequencing of PCR product was outsourced to Chromous Biotech, Bengaluru, India.

Sequencing alignment and phylogenetic analysis

The chromatographic traces of the forward and reverse sequences of *ITS* region were assembled and edited using the DNASTAR offline software (http://www.dnastar.com/). The contiguous alignments were removed and the sequences of 13 *Solanum nigrum* species were aligned using Clustal W algorithm as implemented in the BioEdit tool (Hall 1999). The individual *ITS* region was subjected to pairwise alignment with the reference sequence EF108406.1 obtained from the GenBank, which resulted in the split up of *ITS* region into *ITS1*, 5.8S rRNA and *ITS2*. The DNA sequences were subjected to BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) for identification at species level. The aligned sequences were imported into the Molecular Evolutionary Genetic Analysis (MEGA 5) tool (Tamura *et al*. 2011). The phylogenetic analysis was carried out using the Neighbour Joining method by applying the test of phylogeny as bootstrap with 1000 replicates. The sequences were submitted to the GenBank, NCBI, USA.

Results and discussion

The family *Solanaceae* to which the genus *Solanum* belongs is a cosmopolitan family. *Solanum nigrum* L. is one of the largest and most valuable species groups of this genus. Though this species group is often referred to as the *S. nigrum* complex, the section is composed of a large number of morphologically distinct taxa, which show greatest diversity. These *Solanum* species display varying amounts of phenotypic variation, particularly in their vegetative features such as plant habit, leaf size, leaf shape, stem shape and form. It is, therefore, often difficult to define the limits within such features. Many species exhibit considerable genetic variations, both vegetatively and florally. These variations may occur in different populations of the same species or in different infraspecific categories of a species. Sometimes the characters may be genetically plastic. The leaf margin may vary from entire to dentate and berry colors have wide range of variations from green, purple to black, yellow, orange to red. Stems showed variation in purple

pigmentation, presence of ridges and thorns. Flowers showed variation in size and some groups were reported to have purple striping on the petals.

The morphological characterization of all the 13 genotypes were done based on IPGRI descriptors and are tabulated in Table 1. Based on the multiscale scoring a dendrogram was constructed by using NTSYS programme. A cluster diagram was obtained from the morphological descriptors at a coefficient of 0.62 (Fig. 1). The genotypes TN *Sn* 08, 12, 23, 38, 52 and 53 were grouped in one cluster. Another cluster comprised of TN *Sn* 19 and 51. TN *Sn* 30, 32, 44, 47 with red colored berries were grouped in another cluster. TN*Sn* 10 was unique in its morphological character and does not cluster with any of the genotypes.

The species belonging to this group constitutes a polyploidy series with diploid (2n=2x=24), tetraploid (2n=4x=48) and hexaploid (2n=6x=72). To evaluate the ploidy nature of the genotypes the stomatal, pollen, flower and fruit characters were measured (Table 2). Both pollen diameter, stomatal size, number of flowers inflorescence^{-1} and diameter of flower and berries tended to increase with ploidy level while number of stomata unit area^{-1} and number of seeds berry^{-1} decreased with increase in ploidy level. Flow cytometry results confirmed the ploidy status of the genotypes based on the histograms (Fig. 2). TN *Sn* 08, 12,

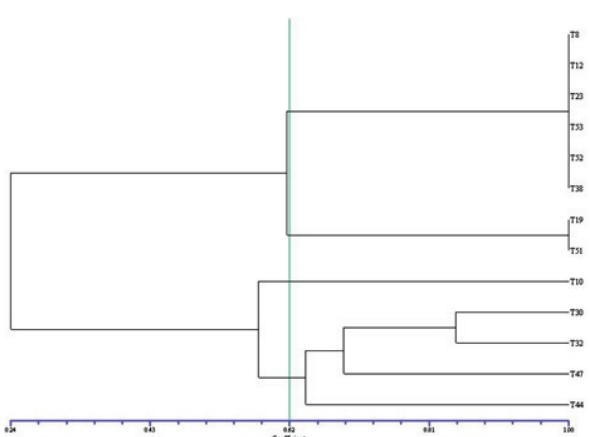
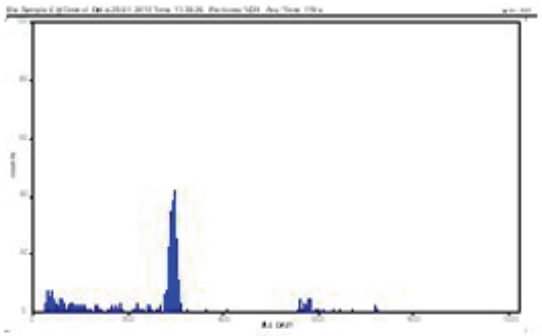


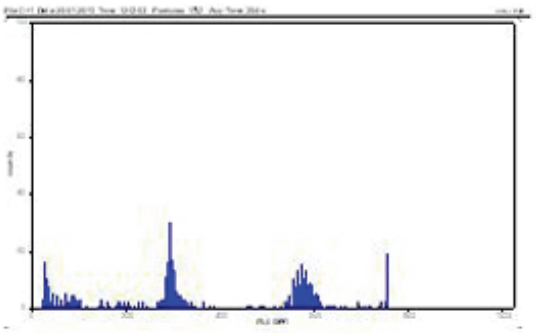
Fig. 1. Dendrogram based on morophological scoring of genotypes using UPGMA cluster analysis

Table 2. Stomatal, pollen, flower and fruit characters of *Solanum nigrum* L. complex

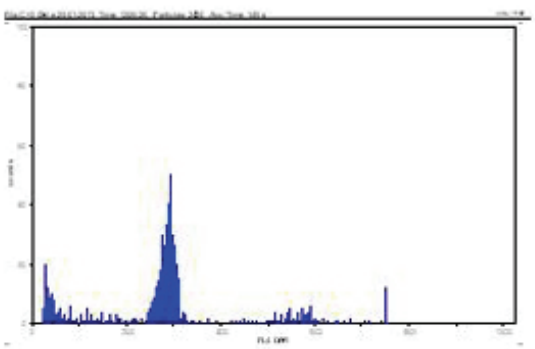
Genotypes	No. of stomata unit area^{-1}	Stomatal length (µ)	Stomatal breath (µ)	Pollen diameter (µ)	Pollen fertility (%)	No. of flowers inflorescence^{-1}	Flower diameter (cm)	Berry diameter (cm)	No. of seeds berry^{-1}
TN *Sn* 08	15.22	20.97	13.82	23.49	92.14	3	0.53	0.62	37.83
TN *Sn* 10	9.11	25.03	16.70	27.06	98.56	8	0.96	0.92	31.33
TN *Sn* 12	15.56	20.84	13.90	23.46	93.04	3	0.55	0.65	37.83
TN *Sn* 19	16.67	20.13	13.54	23.05	92.47	3	0.49	0.58	37.83
TN *Sn* 23	15.11	20.89	13.92	23.47	93.25	3	0.56	0.64	37.67
TN *Sn* 30	11.22	23.91	14.98	26.90	96.23	5	0.69	0.72	34.83
TN *Sn* 32	11.33	23.99	15.08	26.91	95.14	5	0.70	0.76	34.83
TN *Sn* 38	14.67	21.03	13.94	23.56	92.89	3	0.55	0.63	38.50
TN *Sn* 44	10.67	24.18	15.31	26.95	95.47	5	0.71	0.78	33.17
TN *Sn* 47	11.44	24.12	15.06	26.87	96.04	5	0.70	0.71	33.17
TN *Sn* 51	16.11	20.16	13.28	23.03	92.45	3	0.50	0.58	38.17
TN *Sn* 52	15.56	20.93	13.93	23.50	93.74	3	0.55	0.65	37.00
TN *Sn* 53	15.76	21.01	13.90	23.52	93.56	3	0.54	0.62	37.67



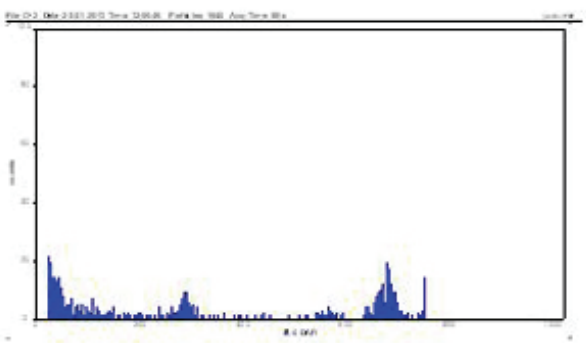
TN *Sn* 19 (control) Diploid



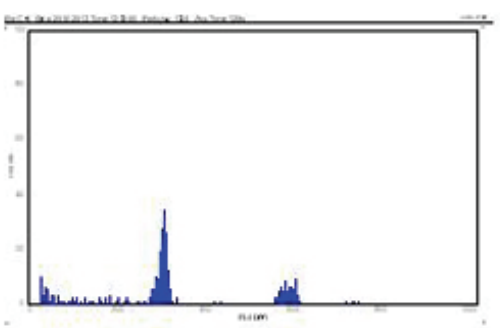
TN *Sn* 08 –Diploid



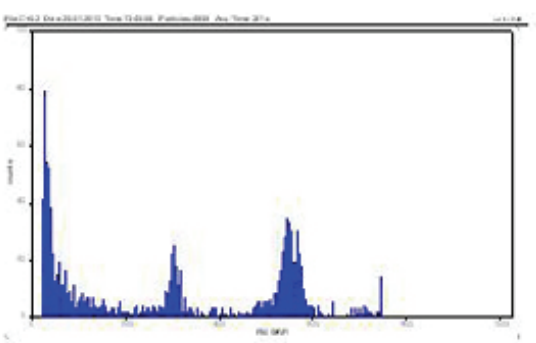
TN *Sn* 10 –Hexaploid



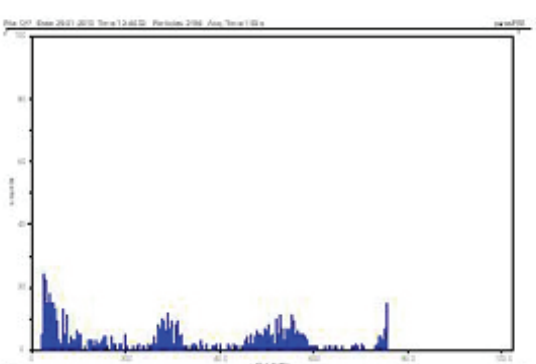
TN *Sn* 12 –Diploid



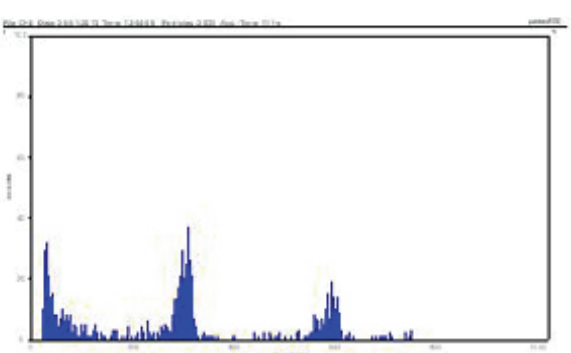
TN *Sn* 23 –Diploid



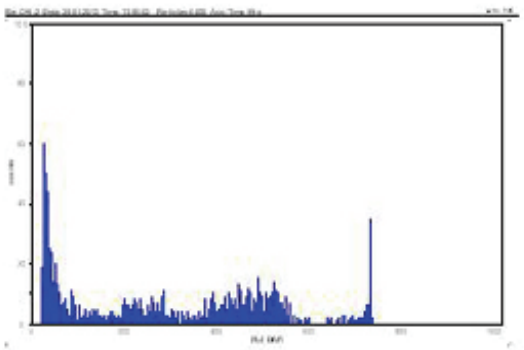
TN *Sn* 30 –Tetraploid



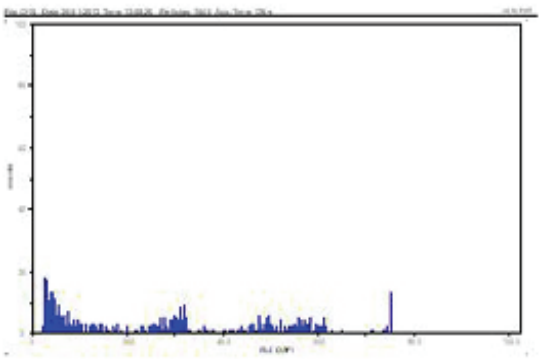
TN *Sn* 32 –Tetraploid



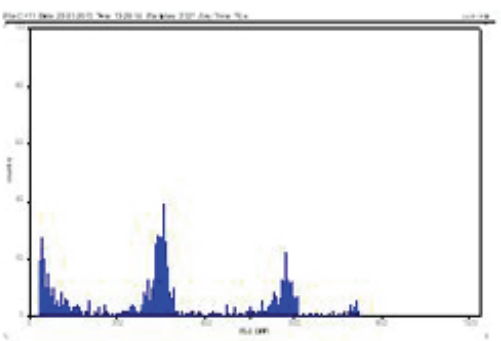
TN *Sn* 38 –Diploid



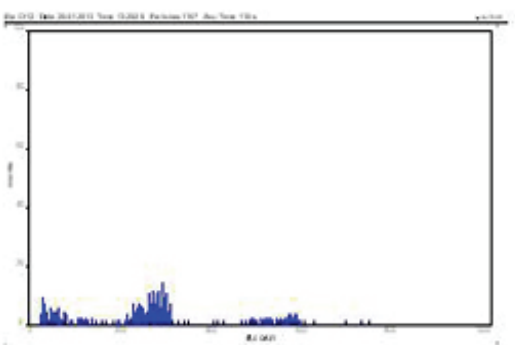
TN *Sn* 44 –Tetraploid



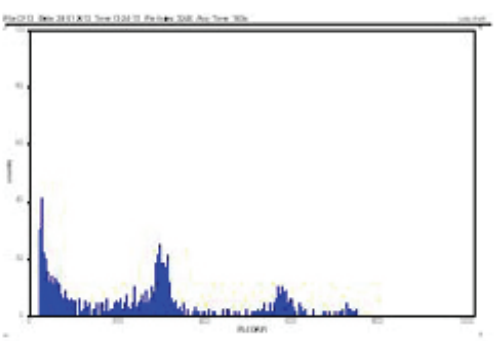
TN *Sn* 47 –Tetraploid



TN *Sn* 51 –Diploid



TN *Sn* 52 –Diploid



TN *Sn* 53 –Diploid

Fig. 2. Ploidy confirmation of genotypes using flow cytometric histograms

19, 23, 38, 51, 52 and 53 were diploids. While TN *Sn* 30, 32, 44, 47 were tetraploids and TN *Sn* 10 was a hexaploid species.

Confirmation of genetic diversity using DNA barcoding

S. nigrum, *S. americanum* and *S. villosum* are three medicinally important species of genus *Solanum* (Jennifer & James 1997). In the past their taxonomic status remained highly controversial. Clarke (1885) did not separate them and considered all the three species as *S. nigrum*. Hawkes & Edmond (1972) gave the rank of subspecies to *S. villosum* of *S. luteum,* however, they considered *S. americanum* as a separate species. Baytop (1978) considered *S. villosum* as sub species of *S. nigrum*. Nasir (1985) considered *S. nigrum* as species with two varieties (*nigrum* and *villosum*). Morphologically these species are very much similar.

The correct identification and interrelationship of the members of the *S. nigrum* complex have

often been a puzzle to the evolutionary biologists (Rao *et al.* 1977). DNA barcoding community has proposed several molecular markers for identification of plant species. Among the proposed barcode candidates, nuclear *ITS* region usually exhibits high levels of variation, including indel polymorphism (Graham *et al.* 2000) and serves as an efficient candidate for species identification, provides relative ease of sequencing and alignment (Kress *et al.* 2005; Hollingsworth *et al*. 2011; Taberlet 2007; Baldwin *et al.* 1995). In the present study, the interrelationship between the 13 *Solanum nigrum* L. complex collected from different geographical location was analyzed using the barcode candidate *ITS*. The multiple sequence alignment of the *Solanum* sp. displayed distinct nucleotide variation between the three important species *S. americanum* Miller, *S. villosum* Miller, *S. nigrum* L. (Table 3). The evolutionary analysis conducted in the MEGA5 tool involved 685 codon positions in the final data set. All the positions containing gaps and missing data were eliminated. The *ITS* region exhibited 97.5% overall constant sites and 2.5% variable sites among which 2.3% constituted parsimony informative sites. The pairwise comparison of *S. americanum*, *S. nigrum* and *S. villosum* showed 98.5% identity. The overall average pairwise distance was estimated to be 0.01. Most of the parsimony informative characters were shared between *S. nigrum* and *S. americanum*. Blast analysis also confirmed the closeness between, *S. nigrum* and *S. americanum*. The best nucleotide substitution model predicted using the MEGA5 tool was attributed to be Tamura-3- parameter (T92) model. The phylogenetic analysis performed using Neighbour joining statistical method produced three distinct clades, wherein the three species *S. americanum* Miller, *S. nigrum* L., *S. villosum* Miller were grouped under clade I, II and III, respectively (Fig 3).

Our study showed that some species exhibit intraspecific polymorphism that distinguish them from closely related species. The present study also confirmed the utility of the barcode candidate *ITS* in distinguishing plant species distributed across different geographical locations. Recently, nr *ITS* region and *ITS2*, one of the sub-components of the *ITS* region, have been suggested as core barcode candidates that identify interspecific and intraspecific variation

Table 3. List of *Solanum nigrum* L. complex species subjected to DNA barcode analysis

Accession number	Ploidy (flow cytometry)	Identified as	GenBank accession number
TN*Sn* 08	Diploid	*Solanum americanum* Miller.	KC540784
TN*Sn* 10	Hexaploid	*Solanum nigrum* L	KC540785
TN*Sn* 12	Diploid	*Solanum americanum* Miller.	KC540786
TN*Sn* 19	Diploid	*Solanum nigrum* L	KC540796
TN*Sn* 23	Diploid	*Solanum americanum* Miller.	KC540787
TN*Sn* 30	Tetraploid	*Solanum villosum* Miller.	KC540788
TN*Sn* 32	Tetraploid	*Solanum villosum* Miller.	KC540789
TN*Sn* 38	Diploid	*Solanum americanum* Miller.	KC540790
TN*Sn* 44	Tetraploid	*Solanum villosum* Miller.	KC540795
TN*Sn* 47	Tetraploid	*Solanum villosum* Miller.	KC540791
TN*Sn* 51	Diploid	*Solanum nigrum* L.	KC540792
TN*Sn* 52	Diploid	*Solanum americanum* Miller.	KC540793
TN*Sn* 53	Diploid	*Solanum americanum* Miller.	KC540794

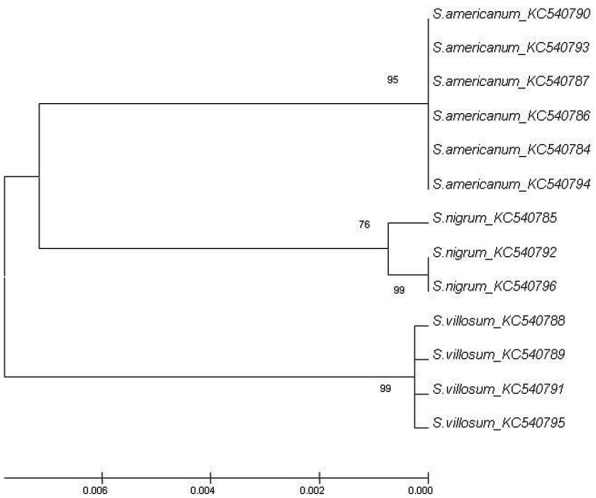


Fig. 3. Phylogenetic analysis of the *ITS* sequences of the *Solanum nigrum* L. complex using MEGA5 software (Phylogenetic tree was constructed using the Neighbour Joining method using *ITS* region for the 13 species of *Solanum nigrum* L. complex. The bootstrap support values are shown at the node of the branches).

within a group of taxa (Kress *et al*. 2005; Chiou *et al.* 2007; Chen *et al.* 2010). Our study revealed that DNA barcoding could be proposed as a conventional diversity identification tool to ensure the traceability of the morphologically distinct yet non-differentiable accessions. This could provide a DNA based model for documentation and conservation of genetic diversity of the complex group of taxa.

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