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MOLECULAR IDENTIFICATION AND COMPARATIVE ANALYSIS OF NOVEL 18S *RIBOSOMAL RNA* GENOMIC SEQUENCES OF A WIDE RANGE OF MEDICINAL PLANTS

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Abstract

Ribosomal RNAs (rRNAs) are universally distributed and known for their functional equivalence among all the known organisms. Analysis of small-subunit rRNAs (16-18S rRNAs) can permit the accurate statistical measurement of a broad range of phylogenetic relationships due to highly conserved sequences. Therefore, we identified and partially sequenced novel isoforms of *18S rRNA* gene from 7 wild, medicinal plants (*Ferocactus glaucescens, Capparis decidua, Calatropis procera, Maytenus royleana, Prosopis Juliflora, Ficus carica and Mentha spicata*) and three cultivated plants (*Cyamopsis tetragonoloba, Eruca sativa* and *Solanum lycopersicum*). The genomic sequences of *18S rRNA* from all of these diverse plants were analyzed and confirmed by using bioinformatics tools and submitted to genebank. We used ClustalW for pairwise alignment of these novel sequences with other known *18S rRNA* sequences to find out their phylogenetic relationships. Our results have shown highly conserved nature of *18S rRNA* with variable regions might be indications of some historical signals. Secondary structure constrains of rRNA can affect their phylogenetic interpretations rarely. These novel *18S rRNA* sequences can also be used as internal controls for several types of molecular analysis after accurate validations of their consistent expression in the given plant species in future studies, as less is known about these housekeeping genes of wild plants.

Introduction

Recently several analyses together with molecular evidences have surprisingly improved our understanding of plant phylogenies (Shinwari & Shinwari, 2010). However, several evolutionary relationships within many major groups of land plants still remained unclear and can be further explored by phylogentic analysis of several interesting housekeeping genes (Shinwari *et al*., 2011). Housekeeping genes are integral part of cellular metabolism. We need to we find elsewhere for additional and novel characteristics to elaborate the existing picture of plant phylogeny. Ribosomal RNA (rRNA) has frequently been used for reconstruction of deep branches of plant evolutionary history. Small-subunits (16S, 18S) rRNA sequences were used in several attempts to infer the life history (Woese & George, 1977; Woese, 1987; Olsen & Woese, 1996; Woese, 1998). In past, the 18S rRNA sequence analysis was used to predict early eukaryotic diversifications (Bhattacharya & Medlin, 1995) and based on those predictions, the fungi were placed in a sister group to animals (Wainright *et al.*, 1993). Similarly, 18S rRNA sequences have been successfully used in reconstruction of eukaryotic phylogeny into many groups of plants including algae, bryophytes, gymnosperms and angiosperms (Buchheim & Chapman, 1991; Chaw *et al.*, 1993; Chaw *et al.*, 1995; Hedderson *et al.*, 1996; Chaw *et al.*, 1997; Soltis *et al.*, 1997; Chapman *et al.*, 1998; Hedderson *et al.*, 1998). Similarly several other studies showed morphological and physiological characterization of plants (Mumtaz *et al*., 2011). Molecular polymorphism and phylogenetic relationships has also been extensively studied (Akbar *et al*., 2011).

18S rRNA sequences have been used in several studies with main focus on the origin of land plants with their placement into distinct phylogenetic groups (Mishler *et al.*, 1994; Hedderson *et al.*, 1996; Hedderson *et al.*, 1998). All of these studies actually portray the unique patterns of land plant's relationships in many clades and usually cannot provide an accurate general outline of few plant phylogenies due to limited samplings of taxons (Hedderson *et al.*, 1996), which can leads to some disagreements on the relationships of major lineages. The larger sampling can improve the inferences of plant phylogenies based on 18S rRNA or related sequences. This seems a challenging job due to the limited number of known genome/gene sequences covering wide range of plants. Most of the available genomic information is unfortunately still limited to the model plants and few crops and not much is known about wild or some cultivated plants with more or less economic importance and medicinal values (Shinwari & Qaisar, 2011). The grouping of such sequences into functional groups based on their expression levels is very beneficial as it can provide a basic framework to direct further research for defining their particular roles in form of gene products in evolution.

18S rRNA are housekeeping genes (HKGs) and are ubiquitously expressed in all tissues and cell types for the maintenance of the basic cellular functions in living cells. Furthermore, the expression of such genes is assumed to be comparatively constant or nearly constant during all the environmental or experimental conditions. Most commonly used plant housekeeping genes are β-actin (*ACT*), α-tubulin (*TUA*), ubiquitin (*UBQ*), glyceraldehde-3-phosphate dehydrogense (*GAPDH*), *18S* or *6S* ribosomal RNA and elongation factors (*EF*) etc., (Nicot *et al.*, 2005; Hu *et al.*, 2009; Garg *et al.*, 2010; Maroufi *et al.*, 2010).

Normalization of a target gene expression to the HKGs in several molecular expression analyses is required to minimize the variations in target gene quantifications irrespective of experimental conditions. But an accurate

selection of housekeeping genes with stable expression under given conditions is a prerequisite to achieve the above objective (Jain *et al.*, 2006; Majerowicz *et al.*, 2011; Borges *et al.*, 2012). Alternatively 2 or more housekeeping genes can also be used as internal controls for data normalization to minimize the experimental errors (Thellin *et al.*, 1999; Vandesompele *et al.*, 2002). Again, most of the internal control gene identifications and validation studies are still limited to model plants or crops. Recent studies have shown that different experimental conditions can affect the stable expression of some internal controls due to their involvement in multiple cell signalling/defense pathways (Vandesompele *et al.*, 2002: Sitwat *et al.,* 2012). It's really important to identify the novel homologues or orthologues of known housekeeping genes of non-model plant species to study their metabolic pathways and related variations according to their habitats or experimental conditions.

This paper attempts to identify, sequence and characterize novel homologues and orthologues of 18S rRNA genes from a diverse group of plants including seven wild, medicinal plants (*Ferocactus glaucescens, Capparis decidua, Calatropis procera, Maytenus royleana, Prosopis juliflora, Ficus carica and Mentha spicata*) and three cultivated plants (*Cyamopsis tetragonoloba, Eruca sativa* and *Solanum lycopersicum*) to describe their phylogenetic relationships based 18S rRNA sequences. As we need to explore wide range of housekeeping genes from non-model plants for their potential use as an internal control gene (reference gene). These novel, partial gene sequences of *18S rRNA,* isolated from above mentioned plants can also be used as internal controls for normalizations of several types of gene expression studies of these selected plants after accurate validations of their consistent expression in particular plant species under given experimental conditions in future.

Materials and Methods

Plant materials: Here, we used ten diverse plants for identifications and sequencing of different homologues of 18S rRNA gene as given below with their economical and medicinal values.

Cyamopsistetra gonaloba is commonly known as Guar or cluster beans, belongs to family *leguminasae*. It is a drought-tolerant crop, commonly grown in arid and semi-arid regions with annual rainfall of 200-600mm. Guar beans can be used as vegetables for human consumption, also grown for cattle feed and for green manure. It contains large endosperm comprises of significant amounts of galactomannan gum that forms a viscous gel in cold water. The gum obtained is the primary marketable product of the plant. Highly refined guar gum is used as a stabilizer for cheeses, stiffener in ice cream, and is a meat binder whereas the lower grade of guar gum is used in cloth and paper manufacturing industries (Undersander *et al.*, 1991).

Eruca sativa commonly called taramira belongs to the family *Brassicaceae*, and is used as spice and vegetable for human consumption. It is important for preparations of some traditional medicines and remedies (Flanders & Karim, 1985). It is also well known for the drought resistance and salt tolerance (Shannon & Grieve, 1999).

Calotropis procera belongs to family *Asclepidaceae* and is important for its medicinal properties. Its different parts have been reported to exhibit antioxidant, analgesic, and anti-inflammatory properties. It has bactericidal and vermicidal effects and can be used to treat leprosy and elephantiasis (Sing *et al.*, 2002). Its latex has been reported as a useful remedy for the coetaneous infections, leprosy, inflammation, eczema, and malarial and low grade fevers (Kumar & Basu, 1994). It is a drought-resistant and salt tolerant plant.

Capparis decidua is a member of family *Capparaceae*. Fruits (green berries) can be used as vegetables and have anti-diabetic action. The bark has been reported for the treatments of cough, inflammations and asthma. Roots are useful to cure fever and buds are good to cure boils. Leaves can be used as appetizers, and are helpful in cardiac problems. Shoots are commonly used for antifertility tonic. Root bark act as anthelmintic and purgative and wood coal is effective for muscular injuries. It is highly tolerant to persistent drought conditions and is known for its adaptations to the arid conditions.

Prosopas juliflora belongs to family *Mimosaceae*. It is a leguminous, perennial phreatophyte. It grows in very hot and dry areas with high temperatures like 48°C with annual precipitation of 150-750mm (Darke, 1993; Geilfus, 1994). Its pods are one of the earliest known foods of ancient man. Pods are fermented to make wine. Leaves can be used as forage. Wood is used for floors, furniture, and many items. Toasted seeds can be added to coffee. The gum is used as an emulsifying agent. Gum is used in confectionary. Roots also contain 6–7% of tannin, which discourage the Rhizobia. It is used as a folk remedy for colds, catarrh, diarrhea, dysentery, eyes, inflammations, itching, measles, stomachaches, sore throats, and wounds (Duke & Wain, 1981). Aqueous and alcoholic extractions are remarkably antibacterial.

Maytenus royleana belongs to family *Celasteraceae*. It is highly drought tolerant plant and can survive in arid/semi-arid regions. Bark or leaves in powdered form is used for herbal therapy for the treatment of bone fractures (Rauf *et al.*, 2012).

Ficus carica is dicot and belongs to family *Moraceae.* It is a monoecious and deciduous tree or a large shrub. Common Fig plant is used as a laxative, expectorant, emollient and analgesic. It is usually used in preparations of laxative syrups in combinations with Senna and carminatives. The fruit can be used treatments of colds. Fresh figs can be used for treatment of boils and very small tumors. Its white milky juice extracted from the stems and leaves is used for removal of warts.

Mentha spicata (Spearmint) is a herbaceous, rhizomatous and perennial plant, belongs to family *Lamiaceae.* Its leaves produce an essential oil used for flavor in candies, gums, ice creams, drinks. It is also used commercially for preparations of hygiene products (toothpaste, mouth-washs, etc). It has been used in many continents as an alternative treatments due to its antiemetic, antispasmodic, antiseptic, carminative, diuretic, restorative, stimulant, stomachic and tonic. The medicinal herb tea made from the leaves is used in the treatment of fevers, bronchitis, chills, cramps, chronic gastritis, common cold,

diuretic, morning sickness, nasal congestions, halitosis, nausea, painful menstruation, and many minor problems.

Genomic DNA extraction: Genomic DNA was extracted from leaves of all the selected plants by CTAB (Cetyl Trimethyl Ammonium Bromide) method (Richards, 1997). Plant leaves (~0.3 g) were harvested, washed with 70% ethanol and homogenized in preheated (65°C) 2X CTAB buffer followed by incubation at 65°C for 45 minutes and centrifugation at 10,000 rpm for 10 minutes. The supernatant was then collected and transferred to new tubes. Equal volume of chloroform-isoamylalcohol (24:1) was added and mixed with the supernatant followed by centrifugation at 10,000 rpm for 10 minutes. Equal volume of chilled isopropanol and 1 M sodium acetate was added to the supernatant. The mixture was kept at -20°C for 30 minutes for DNA precipitation. Finally centrifuged at 12,000 rpm for 10 minutes and subsequent washings were done to remove impurities followed by air drying. The pellet was resuspended in 40µl of Tris EDTA buffer containing 10 µg/µl of RNase. The DNA samples were incubated at 37 °C for 30 minutes to remove RNA impurities and purified samples were stored at -20°C for further use.

DNA samples were quantified by using NanoDrop-1000 spectrophotometer (ND/-1000 V3.7.1, Thermoscientific) and the DNA samples were diluted to a final concentration of 200ng/µl for further molecular analysis. Similarly the DNA samples were also analyzed by loading samples on 1% agarose gel stained with ethidium bromide for gel electrophoresis.

Polymerase chain reaction (PCR): PCR was performed to amplify of *18s rRNA* gene from all of the above selected plants by using gene specific primers (Haq *et al*., 2010) and Promega's master mix (Cat. # M7502) according to manufacturer's instructions at following PCR conditions for amplification. First denaturation was done at 95^{o}C for 5 min, followed by 35 cycles denaturation for 45 sec at 94^{o}C, annealing at 55°C for 1 min followed by extension for 1 min at 72^{o}C. Final extension was done for 10 min at 72^{o}C. PCR products were checked on 1% agarose gel.

Sequencing of partial 18s rRNA gene: Sequencing PCR products were purified by using Axygen prep kit (Catalog No AP-PCR-250) according to the manufacturer's instructions. Sequencing was performed by using Beckman CEQ 8800 sequencer. Sequencing PCR reaction mixture was made by adding RRv3.1 master mix as recommended by suppliers. Sequencing PCR was done by denaturing the template at 95°C for 1min, followed by 30 cycles of denaturation at 95°C, annealing at 55°C (18srRNA) for 30 seconds each, and extension at 72°C for 4min, followed by final extension at 72°C for 10min.

Analysis of sequences: The sequences were initially analyzed by using BioEdit software. To confirm the identified partial *18S rRNA* gene sequences, we first used BLAST with "somewhat similar sequences (blastn)" options to find out the similarities of these genes with other known plant genes. Then several other plant genes with high similarity were downloaded from NCBI with their accession numbers and were used for alignment by using BioEdit software followed by construction of heuristic parsimony phylogenetic trees for evolutionary analysis.

Results and Discussion

Ten novel partial sequences of *18S rRNA* gene were isolated and characterized from a group of seven diverse wild plants (*Ferocactus glaucescens, Capparis decidua, Calatropis procera, Maytenus royleana, Prosopis Juliflora, Ficus carica and Mentha spicata*) and three cultivated plants (*Cyamopsis tetragonoloba, Eruca sativa* and *Solanum lycopersicum*). For identification and charctrizations of *18S rRNA* genes we isolated the genomic DNA from the leaves of all of the above plants by using CTAB method (Richards, 1997). Good quality genomic DNA is one of the prerequisite for PCR and other PCR based technologies. The quality and quantity of extracted DNA was further analyzed by NanoDrop (ND/-1000 V3.7.1) and agarose gel electrophoresis, which showed the presence of high molecular weight DNA with minimum degradations in each case. These genomic DNAs of all the selected plants were used as template for polymerase chain reaction (PCR) to amplify *18SrRNA* gene individually by using gene specific primers. Approximately 200 to 290bp products were amplified from each selected plant species as shown in Fig. 1 and absence of amplified product of *18SrRNA* gene in case of non-template control was indicative of gene specific amplifications in PCR in each case as expected. We sequenced this entire product individually and the *18S rRNA* partial gene sequences of *F.glaucescens, S.lycopersicum, C.decidua, C.procera, C. tetragonoloba, E. sativa, M.royleana, P.juliflora, F.carica* and *M.spicata* were submitted to genebank (Genebank accession numbers JX444499-JX444508 respectively) after initial analysis by BLAST, which showed high degree of similarities with previously known homologues of plant *18S rRNA* genes downloaded from NCBI database. Our results confirmed that all the sequenced products are unique and novel sequences of *18S rRNA* gene isolated from non-model plant species.

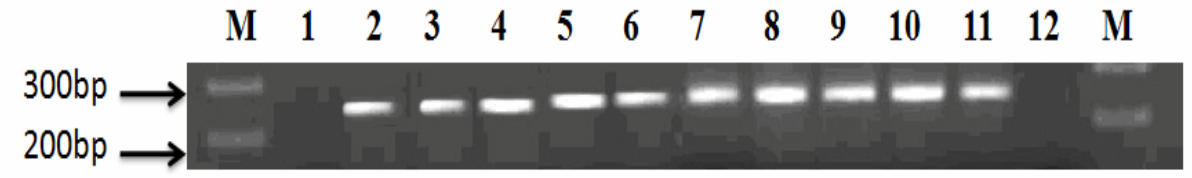

Fig. 1. Amplification of partial sequences of *18S rRNA* gene from a diverse group of plants including *Ferocactus glaucescens, Capparis decidua, Calatropis procera, Maytenus royleana, Prosopis Juliflora, Ficus carica, Mentha spicata, Cyamopsis tetragonoloba, Eruca sativa* and *Solanum lycopersicum*. Gene specific primers of *18S rRNA* were used to amplify genomic DNA extracted from all of the above selected plants (Lane 2 to 11 respectively). PCR products were run on 1% agarose gel along with 100bp DNA ladder. No amplification was seen in the non-template controls (Lanes 1 and 12).

To find out the similarities and conserved patterns of newly isolated sequences of *18S rRNA* genes from above mentioned plants, we aligned these novel partial sequences together with already known *18SrRNA* genes of other plant species as shown in Fig. 2. Our data suggests that this particular segment of ~200-290 bp of *18S rRNA* is highly conserved among these selected plant species. Most of these sequences had less variable sequences as compared to few sequences with high degree of variability. Interestingly, we could not see any major differences or unique conserved regions characteristic of monocots or dicots.

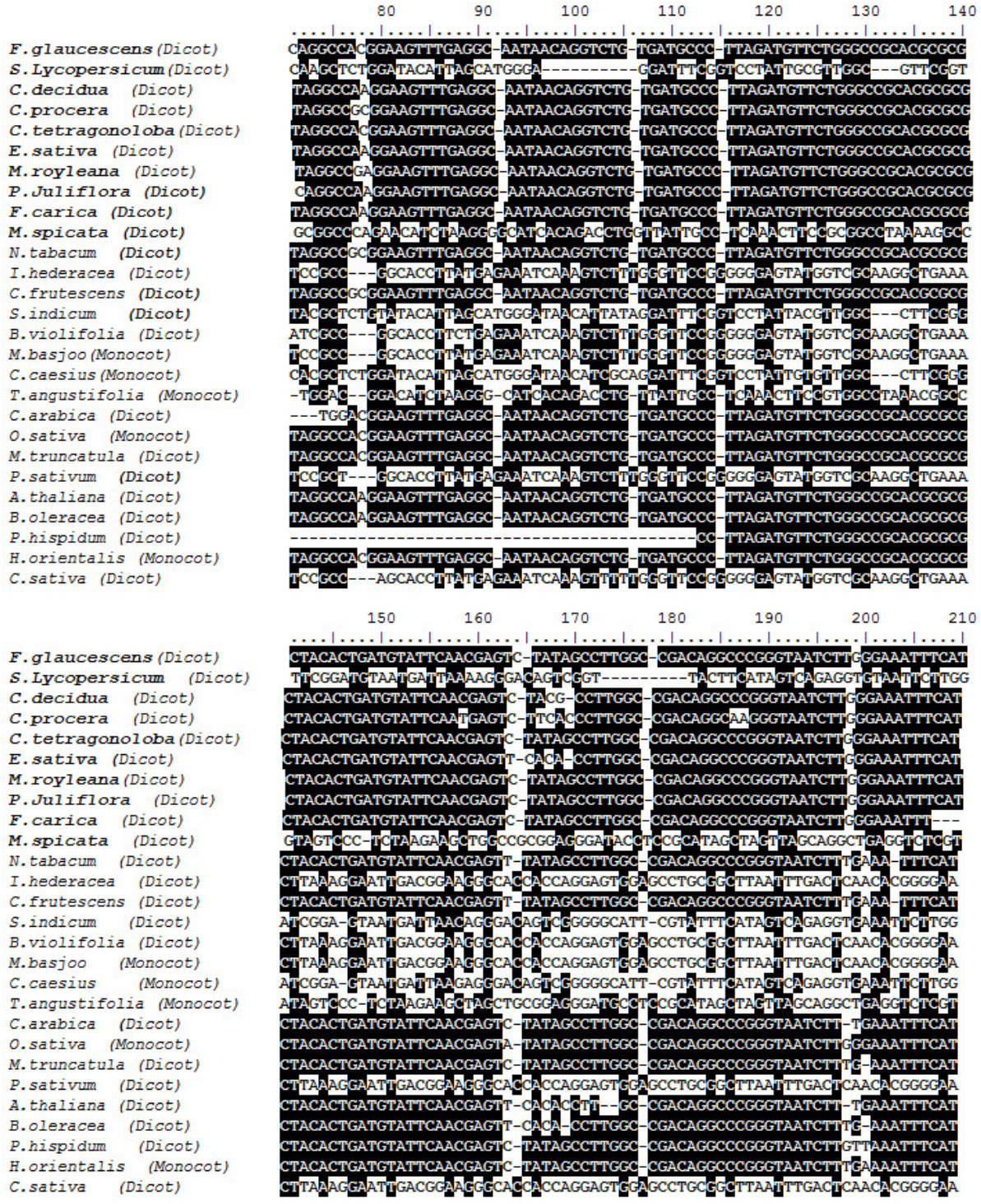

Fig. 2. Alignment of novel genomic DNA sequences of partial *18S rRNA* gene isolated from *Ferocactus glaucescens, Capparis decidua, Calatropis procera, Maytenus royleana, Prosopis Juliflora, Ficus carica, Mentha spicata, Cyamopsis tetragonoloba, Eruca sativa* and *Solanum lycopersicum* with genomic DNA sequences of*18S rRNA* genes of previously known plant *18S rRNA* genes.

To find out the similarities and conserved patterns among newly isolated partial sequences of *18S rRNA* genes, we aligned these novel partial sequences together with already known *18SrRNA* genes of other plant species as shown in Fig. 2. Our data suggests that this particular segment of ~200-290 bp of *18S rRNA* is highly conserved among these selected plant species. Most of these sequences have less variability as compared to other sequences. Interestingly, we could not see any major differences or unique conserved regions characteristic of monocots or dicots.

Similaly, phylogenetic trees were also constructed by using all of above *18SrRNA* partial sequences as shown in Fig. 3. Our results showed high degree of conservation among this part of *18SrRNA* gene of all selceted plants. Analyses of 18S rRNA patterns and evolution in angiosperms fully or partially supported the clades showing transitions and transversions in the previous studies. Our data showed that these selected plants fall into three major clades. One major clade comprises of 14 plant species including *E.sativa, B.oleracea, A.thaliana, O.sativa, C.decidua, H.orientalis* etc. as shown in Fig. 3.

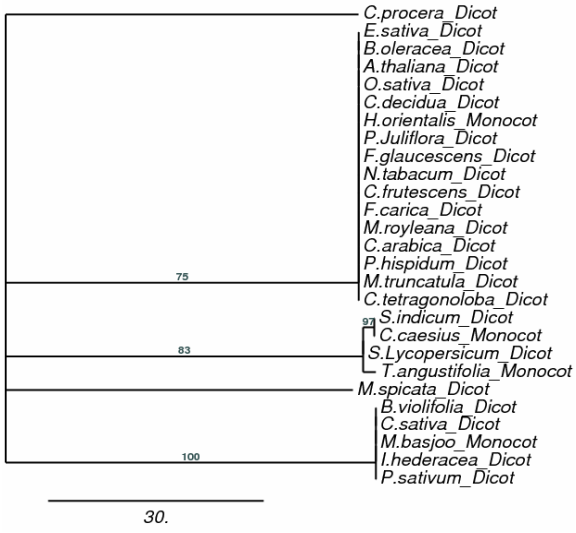

Fig. 3. Phylogenetic relationships of partial *18S rRNA* gene isolated from diverse group of plants including, *Ferocactus glaucescens, Capparis decidua, Calatropis procera, Maytenus royleana, Prosopis Juliflora, Ficus carica, Mentha spicata, Cyamopsis tetragonoloba, Eruca sativa* and *Solanum lycopersicum,* with already known sequences of *18S rRNA* genes by using Paup. The evolutionary history was inferred using Neighbor-Joining method. The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test, are shown on the top of the branches.

The second clade, grouped together four of the selected plant species (*S. indicum, C. caesius, S. lycopersecum, T. angustifolia*). Boot strap values are indicative of percentage of confidence on that particular branch. Third clade, a sister to the *M. spicata* consists of the remaining plants except *C. procera*, which is a separate branch in the tree. Unusual placement of any single species or clades may rarely occur by one or few divergent plant species that either contain errors in sequences or have no close relatives. These relationships inferred from these above analysis are not well supported as previously measured by bootstrap analysis. Although the general trend of the tree is pretty consistent with the known land plant phylogenies(Crane, 1985; Kenrick & Crane, 1997). Although more elaborated sampling is still required for such type of plant phylogenetic studies based on *18S rRNA* sequences, as this analysis does not clearly resolve the basal relationships of these plants due to high degree of conservations among these sequences.

Conclusively, our data suggests high levels of conservations among this particular region of these newly isolated partial sequences of 18S rRNA genes of this selected group of plants. This conserved region of 18S rRNA gene may be too invariant to display the sufficient phylogenetic signals to find relationships among these plants. Furthermore, limited number of variations in this conserved region may be constrained and the signal is masked by homoplasy due to multiple substitutions on these sites. Thus, analysis of partial 18S rRNA sequences alone for few taxons will likely yield trees that not well resolved and supported. However, these sequences, in combinations with other may improve the resolution and internal support (Soltis *et al*., 1998).

We also proposed to test these novel 18S rRNA genes for their potential use in the target gene expression studies for the normalizations of expression data after appropriate validations in future.

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