GSC Biological and Pharmaceutical Sciences, 2018, 05(02), 017–025

Available online at GSC Online Press Directory

GSC Biological and Pharmaceutical Sciences

e-ISSN: 2581-3250, CODEN (USA): GBPSC2

Journal homepage: https://www.gsconlinepress.com/journals/gscbps

(RESEARCH ARTICLE)

Assessment of genetic diversity among different sugarcane genotypes using internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA)

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Publication history: Received on 29 September 2018; revised on 09 October 2018; accepted on 15 October 2018

Article DOI: https://doi.org/10.30574/gscbps.2018.5.2.0108

Abstract

Internal transcribed spacer or ITS region of nuclear ribosomal DNA (rDNA) has been used to evaluate genetic assortment and phylogenetic relationship in nine sugarcane genotypes including *Saccharum* species and another related genus as *Erianthus*, Narenga and hybrid. DNA was extracted from selected genotypes and ITS (ITS-1 and ITS-2) regions were amplified using specific primers. The sequence lengths ITS-1 showed 205- 207 bp, while ITS2 was ranged from 211- 218 bp. However, G+C content (%) 65.2% - 67% in ITS-1 and in ITS-2 68.4% - 99.7%. The sequence lengths of fragment and GC content of ITS-1 and ITS-2 regions showed variable. To evaluate the phylogenetic association of both the region of ITS (ITS-1 and ITS-2) neighbor-joining (NJ) method was employed. The cluster A of ITS-1 and cluster B for ITS-2 and cluster C combined between ITS1+ ITS2 sequences gave two distinct groups A and B. The group A represented the ITS1 sequences which showed two subgroups I and II. The A-I subgroup consisted of wild species of sugarcane; *Erianthus*, *Narenga* and *S. robustum,* whereas the A-II subgroup consisted of the *Saccharum* species and hybrid. The ITS2 sequences in the group B showed better correlation amongst each other. The sequences ITS-1 & ITS-2 combined and compared with some selected sequences from NCBI database using NJ method. The results have confirmed that ITS region can be used for evaluating the genetic assortment in *Saccharum* and its closely related genes.

Keywords: Genetic diversity; ITS; rDNA; PCR; Sugarcane

1. Introduction

Sugarcane (*Saccharum* spp. hybrids) is secure second rank an imperative crop and acting as significant role in agricultural and industrial economy [1], provides more than 70% sugar and more than 30% ethanol production in tropical and subtropical countries [2-5]. Currently, sugarcane is cultivated in 20.42 million ha producing 1,333.2 million tonnes with an average cane productivity of 65.20 tonnes/ha [6]. Sugarcane belongs to family *Poaceae* tribe Andropogoneae.

Present sugarcane cultivars are highly polyploidy in nature and often aneuploid, (2n=100-130) [7-8]. The *Saccharum* genus having six spp. viz., the noble cane *S. officinarum* L. two wild spp. Including *S. spontaneum* L. and *S. robustum* Brandes et Jeswiet ex Grassl, and three sub spp., *S. sinense* Roxb., *S. barberi* Jeswiet., and *S. edule* Hassk. And four other sugarcane genus including *Erianthus* Michx., *Narenga* Burkiee., *Miscanthus* Anderss., and *Sclerostachya*. For the evolutionary relationships among species in *Saccharum* complex, it is still unclear although researchers have attempted to disclose the phylogenetic relationships of *Saccharum* complex using molecular markers, plastid intergenic spacers, and ITS of nrDNA [9-10].

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Universally in the entire organism gene coding is available for ribosomal DNA. The tremendous importance of the restriction fragment and sequence analysis of nuclear rDNA as a reliable approach for addressing the genomic relationships among cultivated species and its wild relatives has been well recognized [11-12]. Nucleotide sequences of ITS (1, 2) of 18S to26S nr DNA has proved to be helpful in taxonomic studies [13-14]. Then rDNA-ITS have been used in the evolutionary analysis of inter or intraspecific genetic variation and due to elevated rate of mutation it is also used in selection of parent in breeding program in different plants [15-17] and in sugarcane [10, 18]. The length of ITS regions and its sequences of rDNA repeats are considered to be quickly developing and hence may differ. Widespread PCR primers of ITS regions are developed from extremely conserved flanking regions, and comparatively, small size enables easy amplification of ITS region (600-700 bp) of rDNA repeats. To evaluate the ITS regions small quantity of DNA are necessary and it is easy to produce, quick and this whole thing makes the ITS region an exciting topic for phylogenetic and evolutionary investigations [13; 19] and additionally for biogeographic studies [20-23]. The present investigation was to study the genetic diversity and phylogenetic relationship on the basis of ITS regions (ITS1 and ITS2) in sugarcane genotypes.

2. Material and methods

2.1. Plant material

Fresh plant material (young leaves) of *Saccharum* genotypes and other related genus from the farm of Vasantdada Sugar Institute, Manjari (Bk), Pune and Sugarcane breeding Centre, Amboli, including 9 sugarcane clone namely Vellai (*Saccharum officinarum*); IJ-76-417(*Saccharum robustum*); Holes (*Saccharum spontaneum*); Dhaur Alig (*Saccharum barberi)*; IK-76-91 (*Erianthus arundinaceus*); *E. ciliaris*, *E. elegans*, *Narenga porphyrocoma* and CoC 671 sugarcane cultivar were selected for the experiment (Table. 1).

2.2. DNA isolation and PCR amplification of ITS regions

Collected leaves were frozen in liquid nitrogen and kept in -80°C for preservation. The total genomic DNA was isolated from each stored sample by using the CTAB method with little modification [24]. The quantity and quality of extracted DNA were evaluated by UV spectrophotometric method and agarose gel electrophoresis. The final working concentration of DNA was diluted at 10 ng/µL was used in PCR.

The PCR was carried out by using specific primers of ITS-1 (5'-GCT GCG TTC TTC ATC GAT GC- 3' and 5'-GGA AGT AAA AGT CGT AAC AAG G- 3') and ITS-2 (5'-GCA TCG ATG AAG AAC GCA GC-3' and 5'-TCC TTC CGC TTA TTG ATA TGC-3') regions [25]. These primers are used in equal ratio. Amplification of genomic DNA was carried out using thermal cycler (Applied BioSystems) and the reaction mixture volume of 50 µL consisting of sterilized glycerol (5 µL), 10×Taq buffer (10 µL),2 mM dNTPs (5 µL), 25 mM $MgCl_2$ (6 µL), 1 U Taq DNA Polymerase (0.4 µL), 10 µM ITS-1 and ITS-2 primers (20 µL) (Sigma, USA) and of 40 ng template DNA (4 µL)and programmed for 40 cycles were as follows; 1 min primer annealing at 48 °C, 45 s extension at 72 °C, and a final extension of 10 min at 72 °C. PCR products were resolved on 3% (w/v) agarose gel electrophoresis (AGE) with 1×TAE buffer, stained with EtBr and documented under UV trans-illuminator (UViTech).

2.3. Cloning and sequencing of ITS regions

The amplified PCR product was eluted and purified by using gene clean kit (Gene Clean II, Bio 101) by following manufacturer instructions. The isolated purified product was introduced in GMT vector (Promega) followed by transformed in E. coli (DH5α) and identified through blue/white screening as well as confirmed by PCR and sequencing of clones (minimum three) of every species were carried out in the sense and antisense direction.

2.4. Sequence and phylogenetic analysis

The sequences of ITS region obtained from the sugarcane varieties were subjected to sequencing and the raw sequences were edited and verified for vector sequence contamination followed by the sequence alignment and phylogeny analysis of final edited sequences. The sequence alignment was obtained using the ClustalW algorithm [26]. The BioEdit software provided the graphic view of the sequence alignment generated. The sequence alignment assignment provided a brief description of our ITS sequences and the database sequences in terms of sequence identity and gap percentage and E-value notes through the NCBI, BLAST analysis. The phylogeny software, MEGA6 [27-29] provided comparative analyses of our ITS sequences and that of the NCBI database ITS sequences. The phylogeny was generated using the MEGA6.0 software using Neighbor-Joining method (1000 bootstraps) [30].

3. Results

3.1. ITS-1 and ITS-2 sequences of nrDNA

All the amplified product from nine sugarcane are sequenced and have been submitted to nucleotide sequence database (EMBL) under the accession number LT220885 to LT220902.

3.2. PCR amplification of ITS-1 and ITS-2 regions

PCR amplification of the rDNA-ITS (ITS1 and ITS2) region produced one major band approximately 300 bp (ITS1) and 350 bp (ITS2) (Figure 1 A-B).

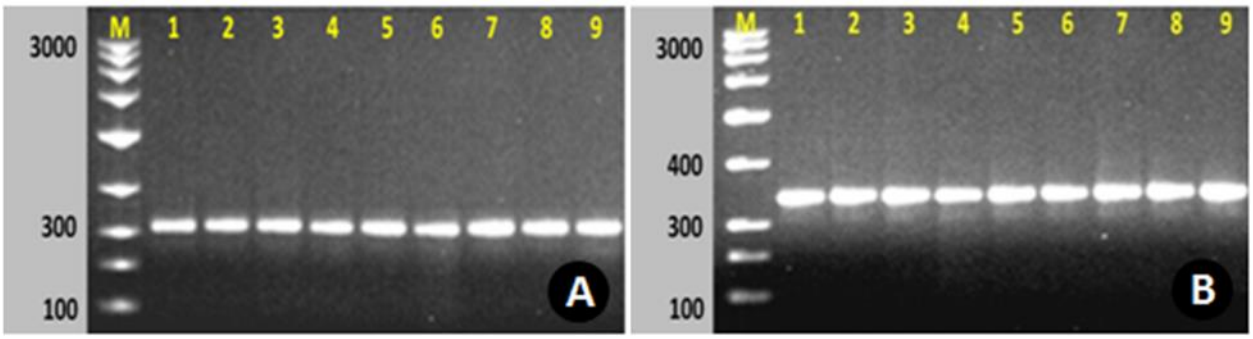

Figure 1 PCR amplification of ITS-1 and ITS-2 (M: DNA marker; 1–9 genotypes of sugarcane mentioned in Table 1)

3.3. Sequence length analysis of ITS-1 and ITS-2 and G+C content

All nine amplified ITS regions produce an inconsistent site, lengths, and percentage of GC content. The sequence lengths of ITS-1 ranged from 205 to 207 bp, while ITS-2 was ranged from 211 to 218 bp. However, the percentage of GC content is slightly lower in the ITS-1 region (65.2 to 67 %) compared to the ITS-2 region (68.4 to 99.7%) (Table 1).

Table 1 Length and G+C% content in the ITS1 and ITS2 regions of nine sugarcane genotypes used in this study

Sr. No.	Genotypes	Clone	Status	ITS1		ITS-2	
				Length	G+C%	Length	G+C%
1	So	Vellai	Wild	207	65.7	214	70.6
2	Sr	IJ-76-417	Wild	206	66.5	215	68.4
3	Ss	Holes	Wild	207	65.7	215	70.7
4	Sb	Dhaur Alig	Wild	207	66.2	215	70.7
5	Ea	IK-76-91	Wild	206	67.0	213	71.4
6	Ec	-	Wild	205	66.8	213	72.4
7	Ee	-	Wild	206	66.0	211	71.6
8	Np	-	Wild	207	65.2	218	99.7
9	Cv	CoC 671	Cultivated	207	66.7	215	70.7

Note: So- *Saccharum officinarum*; Sr- *S. robustum*; Sp- *S. spontaneum*; Sb- *S. barberi*; Ea- *Erianthus arundinaceus*; Ec- *E. ciliaris*; Ee- *E. elegans*; Np- *Narenga porphyrocoma*; Cv- Hybrid

3.4. Sequence alignment and phylogenetic analysis of ITS-1 and ITS-2

The sequence alignments of ITS-1 and ITS-2 generated by ClustalW showed the variable sites like T-A and C-G mismatch base insertion or base deletion and C-T and G-A transversion, as shown in Figure 2. The phylogenetic relationship of all nine sugarcane genotypes was carried out for ITS-1 and ITS-2 sequences using Neighbor-Joining method and combined nucleotide sequences data of ITS1 + ITS2 (Figure 3 A-C). Some of the sequences downloaded from NCBI database generated dendrogram for the comparison study with some sugarcane species and related genera as shown in (Figure 4 A-C).

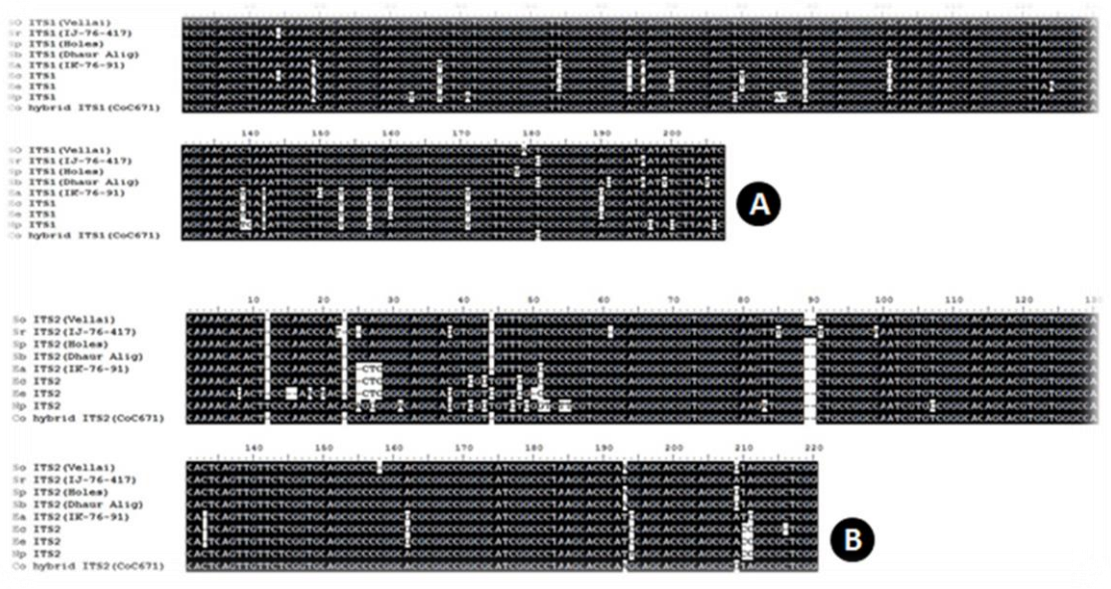

Figure 2 Sequence alignment among the ITS-1 (A) and ITS-2 (B) Code for the sugarcane genotypes as given in Table 1

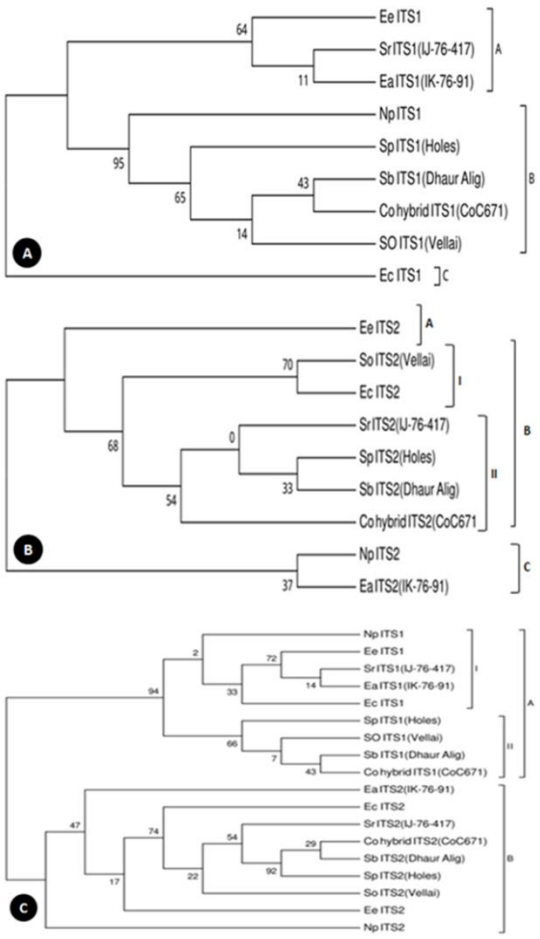

Figure 3 Phylogenetic tree of sugarcane genotypes based on ITS-1 (A), ITS-2 (B) and combined ITS-1+ITS-2 (C) sequences

The phylogeny tree generated between the ITS-1 sequences revealed 3 groups; A, B and C. The A group comprised of the *Erianthus* species, the *Saccharum* series were clustered in group B and the C group was left only with the *Erianthus*

cilliaris which showed a correlation with that of group A (Figure 3A). The phylogeny tree of ITS2 sequences showed better correlation amongst each other (Figure 3B). The phylogeny between combined ITS1 + ITS2 sequences gave two distinct groups A and B. The group A represented the ITS1 sequences which showed two subgroups I and II. The A-I subgroup consisted of wild species of sugarcane; *Erianthus*, *Narenga* and *S. robustum,* whereas the A-II subgroup consisted of the *Saccharum* species. The ITS2 sequences in the group B showed better correlation amongst each other (Figure 3C).

The Phylogeny of ITS (ITS-1, ITS-2, and ITS-1 + ITS-2) sequences both our sequences and other research group submitted sequences taken from NCBI database were used to study the phylogenetic relationship between sugarcane genotypes with some related species consider as out-group. The dendrogram clustered into 4 groups of (A, B, C, and D). The group A clustered the *Erianthus* species with the *S. robustum* and *Saccharum arundinaceus.* The group B consisted of *Narenga, S. officinarum, S. spontaneum, S. officinarum* hybrid and *S. barberi* which showed better clustering with *Zea perennis*, *Saccharum fallax, S. robustum*, *S. barberi* and *S. spontaneum*; whereas the group C formed a different group with *Miscanthus* and *Zea mays*. The group D showed the separate *Saccharum fulvum* which relates to the group A (Figure 4A). The phylogeny ITS-2 sequences with their NCBI homology were clustered into 4 groups which clearly reflects that group An *Erianthus* showed association with the *S. barberi* and *S. robustum* ITS-2, group B shows major cluster of our ITS-2 sequences with that of, *Saccharum fulvum*, *S. robustum* and *Saccharum arundinaceus.* The group C showed only *Narenga porphyrocoma* (ITS-2) to show a relation with Zea mays, S*. spontaneum* and *Narenga porphyrocoma* (ITS-2, AF345233), whereas the group D was separated with *Miscanthus* and *Narenga fallax* from the other groups (Figure 4B).

The phylogeny generated between the ITS-1 + ITS-2 sequences and their NCBI homologies revealed that ITS1 sequences shown a fair distribution among the homologs whereas the ITS-2 sequences have shown to form a cluster among themselves with *Narenga porphyrocoma* and *Erianthus* forming separate groups (Figure 4C).

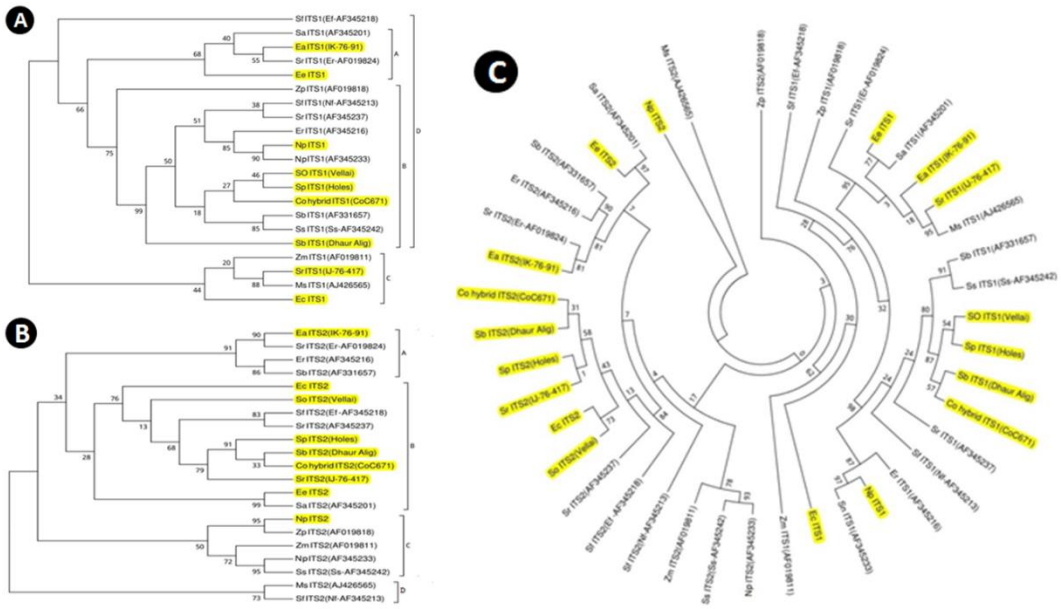

Figure 4 Phylogenetic analysis of sugarcane ITS-1(A), ITS-2 (B) and ITS-1 + ITS-2 (C) sequences with some of the sequences downloaded from NCBI database

[The initials representing the sugarcane ITS1 sequences: So-*Saccharum officinarum* (vellai); Sr: *Saccharum robustum* (IK-79-417); Sp: *Saccharum spontaneum* (Holes); Sb: *Saccharum barberi* (Dhaur Alig); Ea: *Erianthus arundinaceus* (IK-76-91); Ec: *Erianthus* ciliaris; Ee: *Erianthus* elegans; Np: *Narenga*; Co: *Saccharum officinarum* hybrid (CoC671); Er: *Erianthus rockii (*AF345216); Sa: *Saccharum arundinaceus* (AF345201); Sb: *Saccharum barberi* (AF331657); Nf: *Narenga fallax* (AF345213); Sf: *Saccharum fulvum* (AF345218); Er: *Erianthus ravennae* (AF019824); Sr: *Saccharum robustum*(AF345237); Ss: *Saccharum sinense* (AF345242); Ms: *Miscanthus sinensis* (AJ426565); Zp: *Zea perennis* (AF019818); Zm: *Zea may* (AF019811); Np: *Narenga porphyrocoma* (AF345233)].

4. Discussion

ITS is the region of 18s-26s nrDNAis consisted of two spacer of ITS-1 and ITS-2 sequences have additional informative sites and it has been extensively utilized to investigate the intra-specific dissimilarity and inter-specific association of plants [13, 15-17, 31-34]. To measure the degree of relatedness in between species, variation in DNA sequences is utilized. Differences in nucleotide sequences can be used to definite the degree of relatedness between spp. [35]. The

extremely conserved character of 18s and 26s rRNA genes allows alleviate the primer construction and PCR amplification [36], and many investigations have revealed the ITS region to be adequately unpredictable, as a result, it is helpful in providing information to compare taxa at the molecular level. The nrDNA-ITS seem to be a very helpful source of data for considering phylogenetic association within the *Saccharum*. Internal transcribed spacer regions generally explain the advanced rate of variation that is necessary for extrapolating phylogenies of closely allied species and populations in a known species [36-38]. Very recently [18] studied ITS-nrDNA to identify the genetic divergence and genetic variability of thirty sugarcane genotypes (*Saccharum officinarum* L.) for exploitation of the potential parent source in sugarcane improvement through breeding. Previously Internal transcribed spacer or nrDNA-ITS region were utilized to identify the intergeneric hybrids of *Saccharum* spp. × *E. fulvus*) [40] and evaluation of phylogenetic as well as the evolution of six *Sorghum* spp. [41].

In the current investigation, several variable sites were observed, with excellent reproducibility by repeated sequencing can be used as specific DNA fingerprinting sites. Similar kind of results was reported in sugarcane [18]. The sequence of rnDNA-ITS has been used in the genetic relationship analysis of the spp. belong to genus *Saccharum* and another related genus as *Erianthus*, *Narenga*, and hybrid**.** The result is obtained in the present study have shown that GC percentage is lower in ITS1 compare to ITS2 and also sequence length is longer in ITS-2 region than in ITS-1. A similar result was reported by [18,42], but in the present investigation, the variation sites found higher in ITS-2 region than ITS-1 as is observed in other plants [16, 43]. The possible reason behind that the spp. of genus *Saccharum* are highly complex polyploidy in nature which leads to the divergent evolutionary process of *Saccharum* as compared to other crops. Therefore, ITS approach can be used for the assessment of the genetic diversity in sugarcane**.**

The phylogenetic analysis of ITS-1 sequences revealed the *Saccharum* series were clustered in one group and *Erianthus cilliaris* was left in the separate group which showed a correlation with *Saccharum.* The phylogenetic analysis of ITS-2 has shown better correlation amongst species compares to ITS-1. In ITS-2 *Narenga porphyrocoma* and *Erianthus arundinaceus* formed one group with 37% bootstrap values (Fig. 3B). Phylogenetic analysis between the sugarcane ITS-1, ITS-2 and combines sequences and their respective NCBI database showed the ITS-1 sequences revealed a fair distribution among the groups whereas ITS-2 sequences have shown to form a cluster among *Narenga porphyrocoma* and *Erianthus* forming separate groups. Our results are also correlated with phylogenetic tree generated in the investigation of the thirty genotypes of sugarcane [18] obtained that *S. sense*and *S. barberi* species formed a single cluster, and they were situated between *S. spontaneum* and *S. officinarum*. This study supported the hypothesis that *S. sinense* and *S. barberi* were coming from interspecific hybrid species of *S. spontaneum*and *S. officinarum* [44]. According to classification by Irvine [45], derived that S. *spontaneum* and S. *officinarum* in two species similar observations in some extent observed in our study it supported by [18]. This conclusion coincided with RAPD, ISSR and SSR markers [46-47]. These results are supported by other marker systems as r-DNA spacers [48-49].

5. Conclusion

The present study shows that *Saccharum* and *Narenga* are closely related group whereas the *Erianthus* showed significant divergence. These outcomes are supported by other marker systems as r-DNA spacers. The information generated from sequence and its length from ITS region may be a helpful parameter for the evaluation of genetic diversity and phylogenetic studies in sugarcane species.

Compliance with ethical standards

Acknowledgments

Authors are gratefully acknowledged the Director-General, VSI, Pune for their invariable support, encouragement and providing infrastructure facilities throughout the research study and also thankful to Breeding Department, VSI for providing plant material.

Disclosure of conflict of interest

The authors declare that there are no conflicts of interest.
Study conception and Design: JFF, RMD, and AST
Acquisition of Data: JFF and AST
Analysis and interpretation of data: JFF, AST, and RMD
Drafting of the manuscript: JFF and AST
Critical revision: RMD

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How to cite this article

Jomhe FF, Thorat AS and Devarumath RM. (2018). Assessment of genetic diversity among different sugarcane genotypes using internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). GSC Biological and Pharmaceutical Sciences, 5(2), 17-25.