

Assessment of different marker systems for diversity analysis in monokaryotic lines of button mushroom strains using RAPDs, ISSRs, IRAPs and REMAPs

Mamta Gupta*, Shwet Kamal, Anupam Barh, Rakesh K Bairwa and VP Sharma

ICAR-Directorate of Mushroom Research, Solan – 173213 (HP), India

*Corresponding author; E-mail: mamta14biotech@gmail.com

ABSTRACT

Improvement of button mushroom is a specific area involving isolation of single spores and inter-mating of non-fertile spores to develop new cultivars. The breeding of *Agaricus bisporus* is complicated proposition because of its unusual secondary homothallic sexual behavior where majority of the basidia produce two spores, each containing two nuclei of opposite mating-type and only a few basidia are tri- or tetrasporic yielding homokaryotic/monokaryotic spores. There is no way to identify the later as *A. bisporus* mycelium unlike other basidiomycetes lacks clamp connections and is multinucleate. The only promising method to identify non-fertile isolates is the cumbersome fruiting trial. Because of their mobility and activity, transposons have proved to be valuable markers for genetic diversity and variability. Use of outward facing primers of retro-element insertion sites, the IRAP technique, (or between retro elements and simple sequence repeats, the ISSR method) has been useful to provide multi-locus anonymous markers. The SSAP, IRAP and REMAP methods are multiplex and are used to generate several anonymous marker bands. The TEs are known to have strains/species specific signatures, which can be used for identification of non-fertile isolates in *A. bisporus*. In the present study, a total of 1000 single spore isolates were developed from 11 different strains of button mushroom and were evaluated for their fertility. A total of 33 SSIs were identified as non-fruiting and were characterized using different molecular marker systems like RAPDs, ISSRs, IRAPs and REMAPs. The study aimed to identify specific markers linked with fertility of single spore isolates and also the markers will be used to construct a linkage map of the hybrids developed using these non fertile single spore isolates.

Keywords: *A. bisporus*, monokaryon, RAPD, ISSR, IRAPs

Button mushroom is the most commonly grown mushroom in India and about 80% of the total mushroom production is of button mushroom only (Sharma *et al.*, 2017). Still at present only a few improved strains are available in India. The germplasm available in the country of these mushrooms has also not been systematically collected. Considering the need of improved cultivars, it was felt necessary to collect conserve and characterize the germplasm from different parts and initiate efforts to select/develop improved cultivars/ hybrids. The first serious efforts on intermating isolates from different strains of button

mushroom was made by Dr. G. Fristche in Netherlands in late 70s, that leads to development of two cultivars, namely, U1 and U3 (Fristche, 1983). Presently, most of the varieties cultivated are the selections or very much similar to these two varieties (Sonnenberg *et al.*, 2011).

Life cycle of button mushroom is secondary homothallic and hence absence of clamp connections and possess multinucleate mycelium. There is limited ways to identify the non-fertile monokaryons. The most used method is the fruiting trials. Moreover, it

is practically impossible to screen all the single spore isolates for fertility through fruiting trials. Molecular markers like RAPDs, RFLPs, AFLPs, ISSRs, SSRs, SCAR, ESTs, microarrays, etc. permit study of any morphological, physiological or developmental process (through profiling and mapping) in which genetic variants exist with a minimum of priori information. The TEs (transposable elements) have strains/species specific signatures, which can be used for identification of non-fertile isolates in *Agaricus bisporus* while it can also be used for speedy species or strain identification for strain protection. The SSAP, IRAP and REMAP methods are multiplex and are used to generate several anonymous marker bands. REMAP markers are also applicable in several phylogenetic and biodiversity studies. These markers are now used in several studies to observe biodiversity and phylogenetic relations of the species and to investigate the retrotransposons.

The present study was carried out for molecular characterization of monokaryons identified through fruiting trials and subsequently develops DNA based markers to identify the monokaryons / non-fertile isolates without going for cumbersome fruiting trial.

MATERIALS AND METHODS

Germplasm used: A total of 1000 single spores were isolated from eleven strains, namely, S-11, U-3, A-4, A-6, A-16, A-2, A-15, A-94, S-465, S-130 and a wild strain WI-1. All the strains used in the study were obtained from culture collection of ICAR-Directorate of Mushroom Research, Solan, and ICAR-Indian Agriculture Research Institute, New Delhi.

Single spore isolation: All the strains were cultivated on pasteurized compost using standard cultivation practice to collect the spore prints for single spore isolation. Single spores were isolated using serial dilution technique on the standardized media containing dextrose – 5.0 g; MgSO_4 – 0.25 g; KH_2PO_4 – 0.95 g; Succinic acid – 1.25g; Agar – 15.0 g; water 500 mL and pH- 5.0. *Agaricus* mycelium was placed on the lid to trigger the spore germination.

Cultivation of single spore isolates: The spawn of a total of 1000 isolated single spores was prepared

on wheat grains following standard practice and was cultivated on pasteurized compost in 10 kg bags. The yield of the each bag was taken up to four weeks and then average for each SSI was calculated. The SSIs remained non-fruiting in first flush were considered as non-fertile and were used for this analysis

DNA isolation: Genomic DNA was extracted from liquid nitrogen dried mycelium grown on malt extract broth medium (malt extract 10 g L^{-1} and dextrose 5 g L^{-1}) as per Punja and Sun (2001). The DNA concentration was estimated using a spectrophotometer taking optical density at 260 and 280nm. The final working concentration of DNA was standardized to 50 $\text{ng } \mu\text{L}^{-1}$

Genotyping of Non-fertile Single spore isolates: The SSIs and hybrids were genotyped along with their parents for diversity analysis and developing markers for confirmation of hybrids using RAPD, microsatellite (ISSRs) and retro-element based markers (IRAPs and ReMAPs). A total of 50 RAPD, 7 ISSR, 29 outward facing IRAP and 9 ReMAP primers were used.

Bio-informatic analysis: Data obtained by scoring the presence (1) or absence (0) of amplified fragments from the RAPD, ISSR, IRAP and ReMAP profiles were analyzed using NTSYS pc software 2.02 (Nei and Li, 1979). To validate the analysis, bootstrap analysis was performed using Winboot software keeping 1000 bootstrap comparisons.

RESULTS AND DISCUSSION

In the present study, a total of 1000 single spore isolates were isolated from 11 different strains of button mushroom and were evaluated for their fertility and yield. A total of 33 SSIs were identified as non-fruiting and were characterized using different molecular marker systems i.e. RAPDs, ISSRs, IRAPs and REMAPs. The profile generated through these markers was analyzed using NTSYS PC version 2.2. For molecular characterization 50 RAPD markers used and marker profile generated scored as presence and absence of bands. The analysis results of RAPD profiles indicated that the monokaryons have very limited genetic diversity (8%) and only a few markers could

Table 1. List of ISSR primers used in the study

Primer name	Sequence	Primer name	Sequence
P3	GAGAGAGAGAGAGAGAT	P31	CTCTCTCTCTCTCTCTRG
P8	CTCTCTCTCTCTCTCTAGA	P38	CTCCTCCTCCTCCTCCTC
P22	AGAGAGAGAGAGAGAGYC	P39	CAAGGCAAGGCAAGG
P30	GAGAGAGAGAGAGAGAC		

Table 2. List of IRAP and ReMAP primers used in the study

Primer name	Sequence	Primer name	Sequence
IRAP Primers		IRAP Primers	
Sukkula	GATAGGGTCGCATCTTGGGCGTGAC	MarY1L	GTGCCCTGTGTGTTTTTCCT
Sukkula LTR	AACAGAAGTAGTGGCAGCTTGAGAG	MarY1R	AAGGGGTACTCCGCCTCTAA
IRAPCrocusSuk	AACAGAAGTAGTGGCAGCTTGAGAG	RTE-L	CACCGTTGCCTTTTCTGTCT
Nikita	CGCATTTGTTCAAGCCTAAACC	RTE-R	AGTAAACGAGGGTGGTCACG
IRAPCrocusNik	CAGTTTGTGATCAAGTCATAACC	CPLTR1/R	GTGCAAGCGACACACAAACT
LTR6149	ACTACATCAACCGCGTTTATT	CPLTR2/R	GCAGTGTTTCTACAGCGAAG
LTR6149_TEO	CTCGCTCGCCCACTACATCAACCGCGT TTATT	CPLTR1/F	CTCGCTTCTTCAGCTTGACC
LTR6150	ATGTCTATGTATCCACACATGTA	CPLTR1/R	GTGCAAGCGACACACAAACT
ShortLTR6150	CCATGTCTATGTATCCACACATGTA	CPLTR2/R	GCAGTGTTTCTACAGCGAAG
ReverseTy1	CCYTGNAYYAANGCNGT	Microsatellite Primers	
Reverse TY2	TRGTARAGRAGNTGRAT	MS1	GGCGGCGGCGGCGGCGGCGGCT
IRAPCrocus 5'LTR	AACAGAAGTAGTGGCAGCTTGAGAG	MS2	ATGATGATGATGATGATGATGG
5'LTR1	TTGCCTCTAGGGCATATTTCCAACA	MS4	CACACACACACACACACACAG
3'LTR	TGTTTCCCATGCGACGTTCCCAACA	Ps 48	GAGGTGGGGAAAAATATGGG ACGAAC
marY1-LTR-L	GAAGGAAATGAGGTGGGGAA	PI281	CTTCACATATACTGGGCATCA GCAAGGG
marY1-LTR-R	TTTTTAGGTTATATTTGATTTTGTG GTCTT	PDGSL313-1	CGATGTATGTGGCTGTGCCAGT ACCAT
LTR1L	AAAGTACGCAGCCCCTATCA	PDGSL719-2	TGGGCCGCCCTTGATGGCTCA TATT
LTR1R	GAAGTGGCGGAACCAATAGA	UBC807	AGAGAGAGAGAGAGAGT
LTR2L	TATAGCCAACCCTCCACAGC	UBC818	CACACACACACACACAG
LTR2R	AACTGGACAAGGCAACAAGG		

RAPD amplifications were performed as per protocol given by Singh *et al.* (2004) while ISSR amplifications were performed following Nazrul and Yin Bing (2011). IRAPs and ReMAP amplifications were performed following Teo *et al.* (2005).

be identified (Fig.1). The tree obtained from RAPD data classified the dendrogram in 2 groups. Group one contains 4 non- fertile (NF) spores and remaining NF spores falls under second group. Total 7 ISSR markers used to characterize non-fertile spores. ISSR markers have shown higher genetic diversity (56%) in monokaryotic isolates (Fig 2). The phylogenetic tree results from ISSR data leads to 2 major groups of non-fertile spores. Further second group is subdivided in many small groups. It was interesting to observe that the retro element based marker i.e. IRAPs and REMAPs have clearly delineated the fertile isolates from non-fertile isolates in larger population also (Fig. 3 and 4) and showed 22% genetic diversity in non-fertile monokaryons. The study showed that ISSR marker system worked very well in diversity analysis in monokaryons of button mushroom but the IRAP and ReMAP markers have an advantage over ISSR that it can be used for diversity analysis as well as for segregation of non-fertile isolates from the fertile ones.

In mushrooms, RAPDs have been used in assessment of intraspecific and interspecific genetic diversity, screening of homokaryotic breeding lines and confirmation of hybridization amongst compatible homokaryons (Khush *et al.*, 1992; Sunagawa *et al.*, 1995). Horgen and Anderson (1989) used RFLP marker based system for confirmation of intraspecific hybrids of *A. bisporus*. In last two decades different DNA based markers have been exploited for various types of genetic studies of edible mushrooms. Castle *et al.* (1987) used RFLP markers for diversity analysis of *A. brunnescens*. Moore *et al.* (2001) used RAPD technique to differentiate cultivars of the button mushroom. Ma and Luo (2002) used ITS-RFLP in *Pleurotus* genus for genotype identification. Chillali *et al.* (1998) analyzed ITS and IGS regions for evaluation of molecular diversity in the fungus *Armillaria*. ISSR markers were used by Zhang *et al.* (2007) in *Lentinula edodes* for strain identification and by Guan *et al.* (2008) in *A. bisporus* for differentiation of strain. Ghorbani Faal *et al.* (2009) succeeded partly to differentiate closely related hybrid strains of white button mushroom using AFLP technique and also obtained some unique bands for a few strains. ISSR marker system is very useful technique for most systematic and ecological

evaluations due to variable nature of microsatellite regions, minimum requirements and easy application as well as the reasonable cost (Reddy *et al.*, 2002).

In a work by Guan *et al.* (2008), 12 strains of *A. bisporus* collected from different provinces in China tested by six ISSR primers. Although all the strains were successfully differentiated, the results showed a high similarity coefficient between the strains, implying that they might originate from a single maternal strain, U1. Later Malekzadeh (2011) assessed similarity of 18 *A. bisporus* genotypes, including four cultivars, 13 hybrid strains and their single spore progenies along with an indigenous wild strain, using 20 ISSR primers. Out of 20 primers, 10 proved to be discriminative in *A. bisporus*. Current knowledge of repetitive elements in *A. bisporus* remains sporadic. Barroso *et al.* (2000) identified the first microsatellite sequence in *A. bisporus* while Foulongne-Oriol *et al.* (2009) developed marker set of 33 SSRs for molecular studies. Sonnenberg *et al.* (1999) identified Abr1, a transposable element of class II, and Sonnenberg (2000) and Kerrigan *et al.* (2004) described diverse retrotransposons. *A. bisporus* exhibits a particular signature in tandem repeats patterns, TEs distribution, and telomeric sequences (Foulongne-Oriol *et al.*, 2013). Retro elements have been identified in a wide range of eukaryotes and, in some cases, used as DNA markers to examine phylogeny, to create mutants and recombinants, and to recognize mutated genes (Bennetzen, 2000, Bingham, and Zachar, 1989, Boeke, 1989, Flavell and Smith, 1992, Hirochika, 1997).

Retrotransposon microsatellite amplified polymorphism (REMAP) technique uses an outward-facing LTR primer and an SSR specific primer to amplify the DNA region between a long terminal repeat (LTR) of the retrotransposon and simple sequence repeat (SSR) (Kalendar *et al.*, 1999; Kalendar and Schulman, 2006). The gypsy-type retro-element marY1 was first identified in *Tricholoma matsutake* (Murata and Yamada, 2000) and later in other basidiomycetes species (Murata and Miyazaki, 2001; Murata *et al.*, 2001, 2005) and The REMAP markers have demonstrated their feasibility in the fingerprinting of strains of various mushroom species (Le *et al.*, 2008). So RAPD markers are not efficient

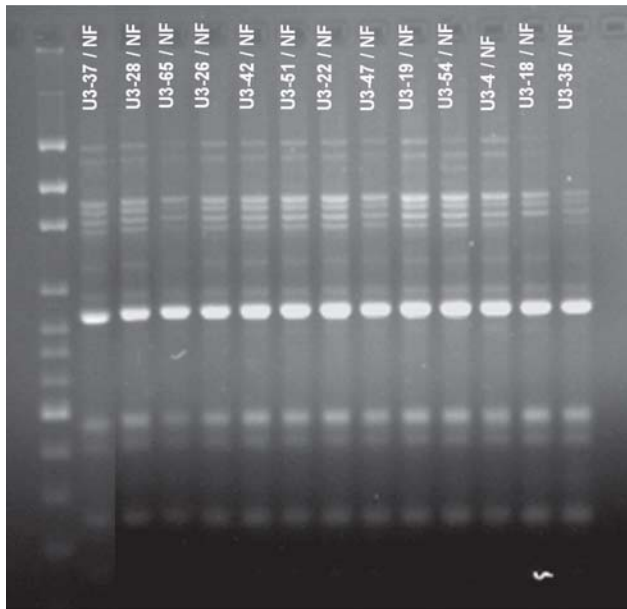


Fig. 1. RAPD profile of non-fertile isolates

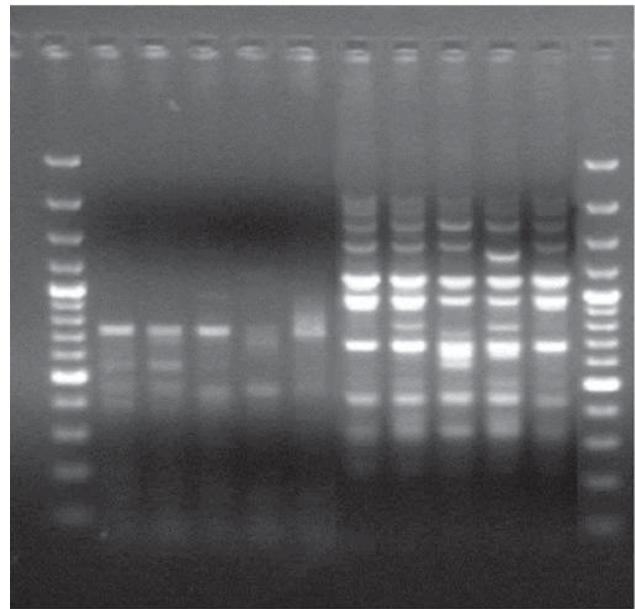


Fig. 2. ISSR profile of non-fertile isolates using P-3 and P-8 primers

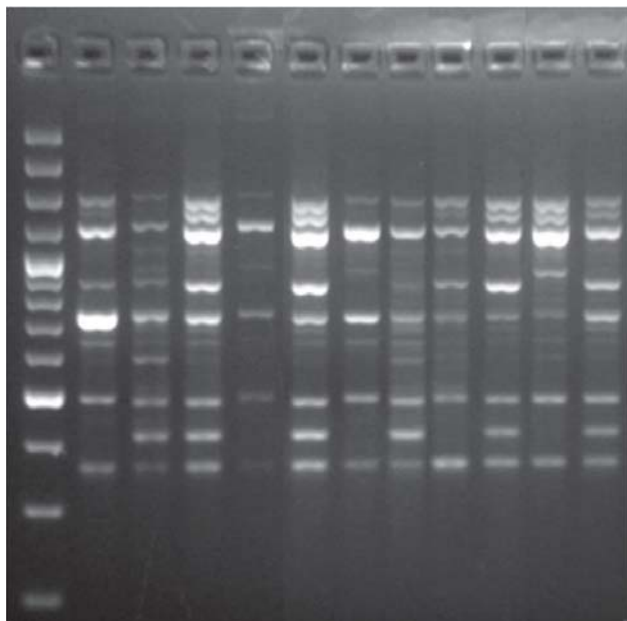


Fig. 3. IRAP Profile of non-fertile isolate

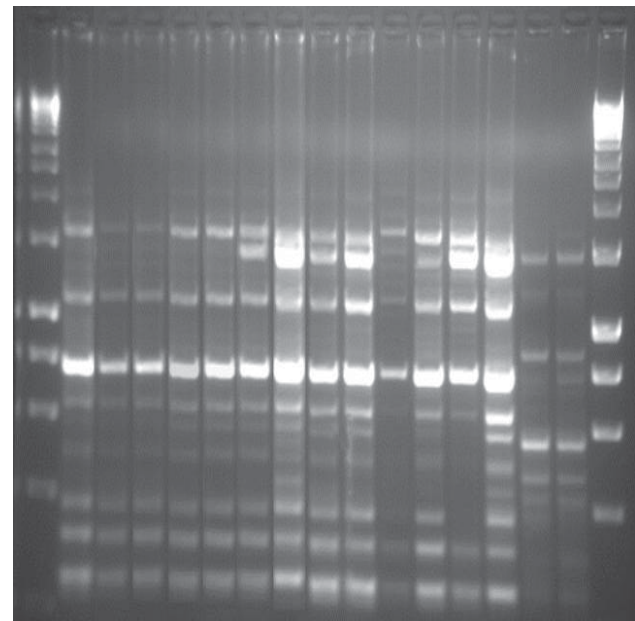


Fig. 4. REMAP Profile of non-fertile isolates

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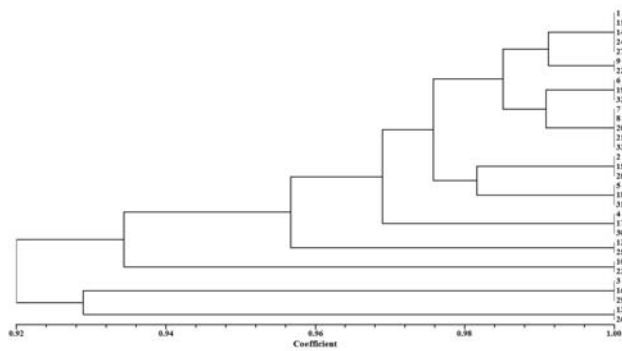


Fig. 5. Phylogenetic tree of RAPD data made by NTSys ver 2.2 showing low diversity in the non-fertile SSIs

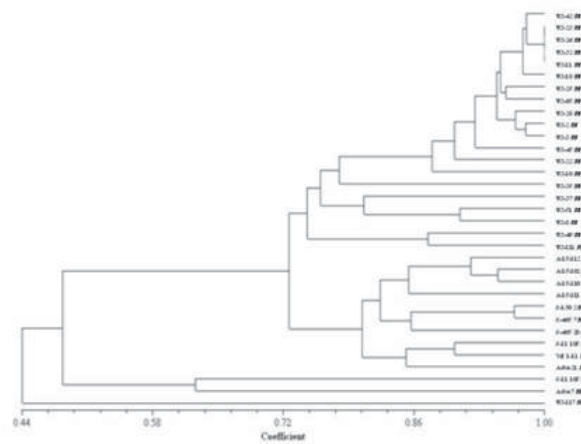


Fig. 7. Phylogenetic tree of ISSR data made by NTSys ver 2.2 showing high diversity in the non-fertile SSIs

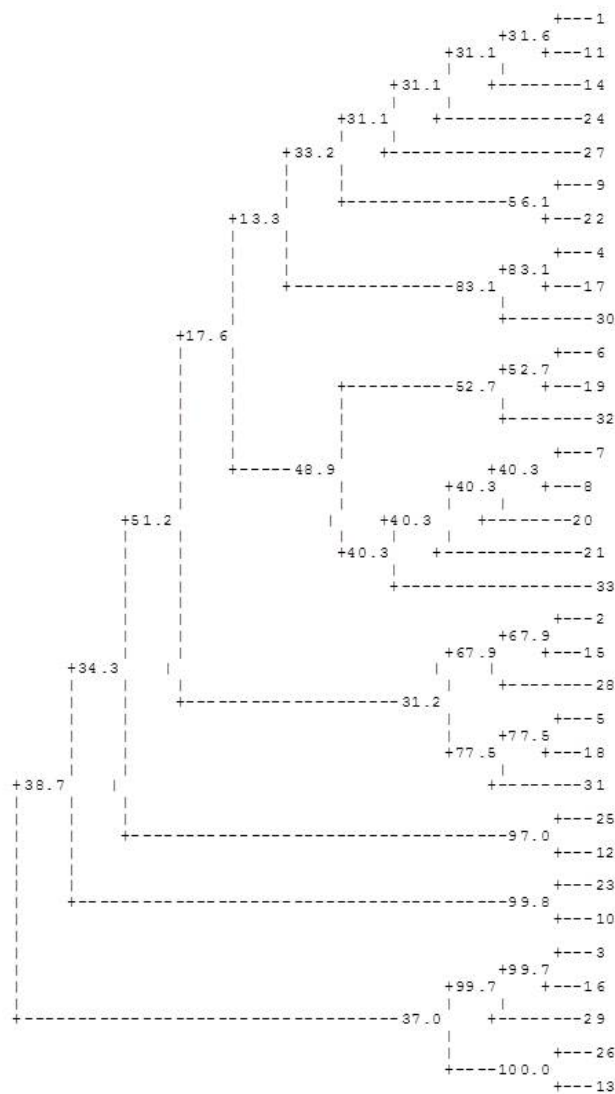


Fig. 6. Bootstrap analysis of RAPD tree showing strong bootstrap values

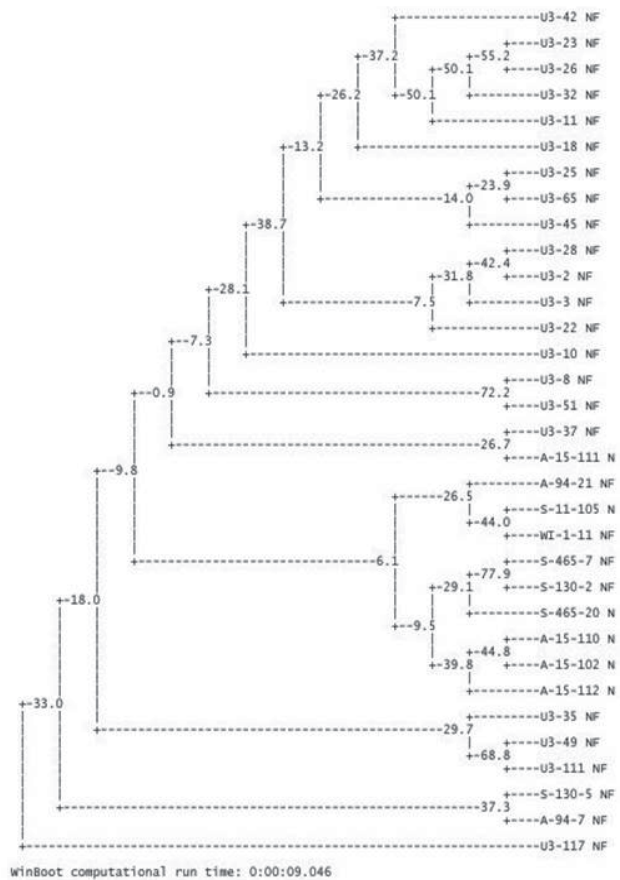


Fig. 8. Bootstrap analysis of ISSR tree showing strong bootstrap values

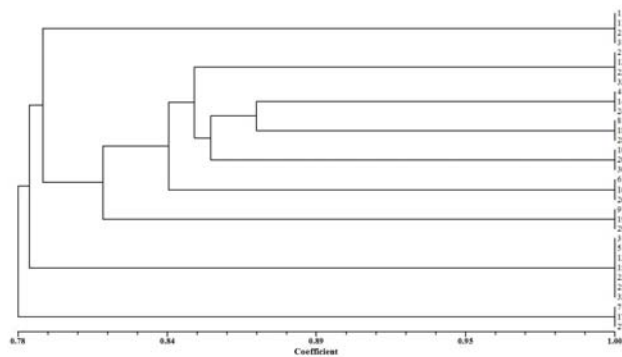


Fig. 9. Phylogenetic tree of IRAPs and REMAP data showing high diversity in the non-fertile SSIs

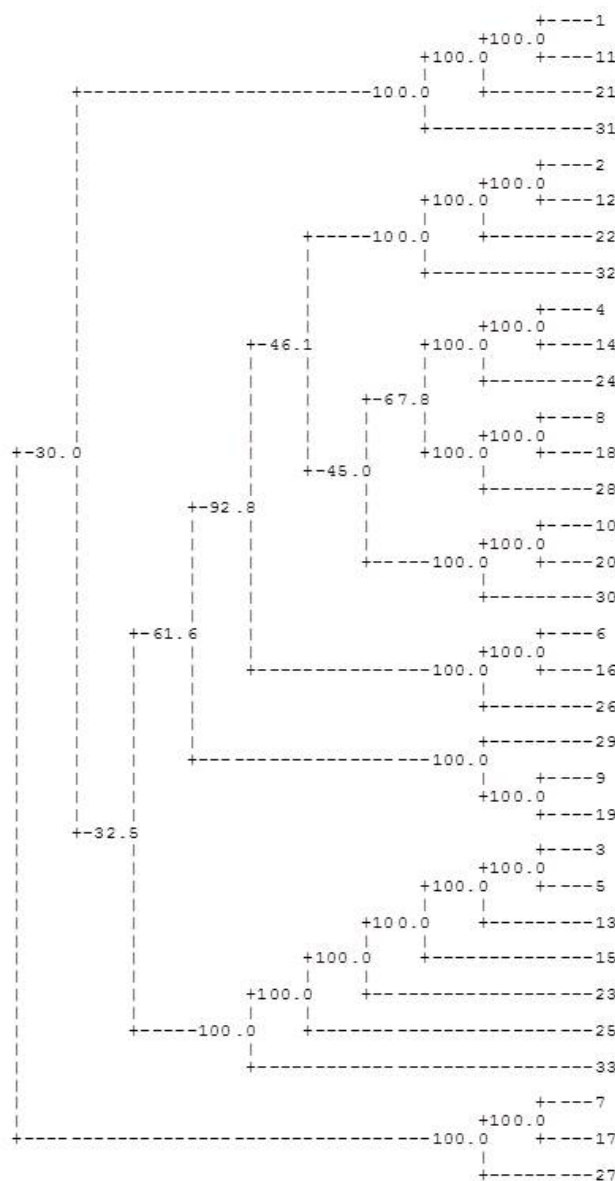


Fig. 10. Bootstrap analysis of IRAPs and ReMAP tree showing strong bootstrap values

to characterize non-fertile spores. ISSR markers are more promising than RAPD marker system. By using IRAP and REMAP marker system we can clearly differentiate non-fertile spores from fertile spores.

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