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Sequence Amplicon Analysis of 18S rDNA Variable Regions (V3, V4 and V9) in Natural Strains of *Sordaria fimicola* and Response to Different Carbohydrates

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ABSTRACT

Employing 18S hypervariable regions as molecular markers, we show a sequence analysis of (V3, V4 and V9) hyper-variable regions of the small sub unit (SSU) of different natural strains of the Pyrenomycete, *Sordaria fimicola*, to examine genetic diversity. Strains were taken from two contrasting environments (Natural and Harsh) from the south facing and north facing slopes of Evolution Canyon, Israel. Sequenced amplicons were introduced to the NCBI database as a query in BLASTN. The query revealed >100% resemblance with the Ascomycota genus *Sordaria* and the topmost species in this investigation was *fimicola*. Circular Phylogram showed close relatedness among the strains under study and potential association with the Ascomycetes. This study for the first time reports the variations at the molecular level between strains from two different environments. V3 and V4 regions showed genetic variations at two sites *i.e.,* 212 A (C); 213 G (A) in strains isolated from the stressful environment while V9 showed no polymorphism. Sequences were submitted to NCBI GenBank under the accession numbers; KY001568; KY000838; KY000839 for S3, N5 and N6 strains. 1% cellulose was found effective to enhance the mycelial and perithecial growth.

INTRODUCTION

In the past, common strategies for discovery and recognition of fungi have principally depended on different isolation techniques and consequent perceptions of morphological characters. These strategies are time-consuming and lengthy. Furthermore, it is difficult to grow a variety of fungi on a given medium. In previous studies, identifications of ascomycetes were based on morphological characters such as mycelia colour, growth pattern, hyphae septate or non-septate, perithecia shape or colour, ascospores arrangement in an ascus etc. The principal morphological character that differentiates the different species of the ascomycetes from members of another phylum is the presence of the sac like structure called the ascus (Alexopoulos *et al*., 1996). The ascus contains ascospores that formed after meiosis. It is difficult to classify the members of the ascomycetes based upon morphological characteristics only, since there are high variations in morphology, habitation and life cycles. However, they can be differentiated into two main groups, *i.e.* unicellular saccharomycetes and mycelia producing ascomycetes. Ascomycetes that have mycelia characteristically produce fruiting bodies called ascocarps (Barr, 2001). Ascocarps are multicellular and mount the asci in a specific way (Bistis *et al*., 2003). *S. fimicola* has a perithecia type fruiting body.

Identification of microorganisms from different substrates using PCR is becoming a more popular, reliable and advanced technique for fungal identifications employing universal primers (Zhihong *et al*., 2003). For the study of biodiversity, there are valuable genes about the length of one hundred nucleotides termed as "markers". In the modern age, molecular techniques are a basic tool for fungal study (Pavan *et al*., 2015; Jakub, 2017). The nuclear genome of eukaryotic cells is of a huge size consisting of different parts; rDNA with tandem repeats is one of them and remains highly conserved during evolution (Hillis and Dixon, 1991). Usually 18S, 26S, rDNA and ITS are thought to be conserved in eukaryotes, so are very common in molecular studies. Moreover, these parts are ubiquitous in nature and have vital functions (Swann and Taylor, 1993; Wilmottee *et al*., 1993). The ITS region is complex having the ITS1 and ITS2 regions with the 5.8S gene in between them. Moreover, the ITS region is enclosed by 18S (SSU) and 28S (LSU) genes (Vilgalys and Gonzalez,

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1990). The 18S region is the most studied region for the molecular identification of different fungal species (Orsi *et al*., 2013). The small subunit, *i.e.* the 18S gene, is believed to be the distinctive marker with nine extremely variable regions from V1 to V9. To estimate the biodiversity in eukaryotes, V4 and V9 are the best candidates (Stoeck *et al*., 2010; Pawlowski *et al*., 2011; Behnke *et al*., 2011; Dunthorn *et al*., 2012). The aim of the present study was, the amplification of the smaller subunit of ribosomal DNA (18S rDNA) of *S. fimicola* strains and alignment of their sequences in Clustal O for identification of variations among strains from the south facing slope (SFS) and north facing slope (NFS) of Evolution Canyon (EC), Israel. The samples used in the present study have been collected from three stations of (SFS) *i.e.* station 1, station 2 and station 3 and are labelled as S1, S2 and S3. Similarly, the samples designated as N5, N6 and N7 have been collected from three stations of (NFS) *i.e.* station 5, station 6 and station 7, respectively (for more details about the strains and the site, see Saleem *et al*., 2001).

MATERIALS AND METHODS

Fungal strains and culturing

Eight strains, S1, S2, S3, N5, N6, N7, Sw17.2, and Sw92.1 (S stands for *Sordaria* and w for white mutant strains) of fungus *S. fimicola* were provided by the Molecular Genetics Research Laboratory, Department of Botany, University of the Punjab, Lahore, Pakistan. The F1 generation of each parental strain was raised by single spore isolation techniques. These original fungal strains were collected from the two slopes which are opposing each other and situated in the valley in Evolution Canyon (For further details about the strains, see Saleem *et al*., 2001).

Experimental design

The Petri-plates used during the whole experiments were 90 mm in diameter and contained 18 mL potato dextrose agar (PDA) culture medium. A 5 mm agar block was placed in the centre of the plates cut with the help of an aseptic cork borer from the side of the active culture plate of each parental strain of *S. fimicola*. All petri plates were incubated at 18°C in order to obtain perithecia after two weeks.

Effect of different carbohydrates as source of carbon on the growth and sporulation of S. fimicola

The effect of different carbohydrates (glucose, maltose, starch and cellulose) as a carbon source was observed on the growth and sporulation of *S. fimicola*. For final calculations, colony diameter was calculated by measuring the growth on Potato Dextrose Agar (PDA) media, three readings from three Petri plates were used to provide a mean. For a control, a carbohydrate-free PDA plate was used to compare the growth. All culture plates were placed in the dark at 18°C in a refrigerated incubator. Four carbohydrates were selected as the source of carbon i.e. maltose, glucose, starch and cellulose. 1% solutions of all these carbohydrates were used in a basal medium and all plates were incubated at 18°C for one week (7 days) for mycelial growth and 14 days for perithecial growth.

Effect of different nitrogen compound as source of nitrogen on the growth of S. fimicola

The effect of different nitrogen containing compounds (sodium nitrate, ammonium nitrate, casein hydrolysate and calcium nitrate) as a nitrogen source was observed on the mycelial growth of different strains of *S. fimicola*. 1 % solutions of all the nitrogenous compounds were used in the basal medium and all the plates were incubated at 18°C for 7 days to observe mycelial growth.

Genomic DNA extraction

Fresh fungal mycelia of all strains were collected separately for genomic DNA isolation by the CTAB method of Gardes and Bruns (1993), but without phenol were adopted. The extracted DNA was visualized on 1% agarose gel and the gel was observed under a gel documentation system (Ugenious 3 Syngene).

PCR amplification of 18S rDNA regions and sequencing

Two sets of universal primers were used for the amplification of the V3, V4 and V9 regions. One set was comprised of a forward primer having 5`-CAGCAGCCGCGGTAATTCC-3` (Macrogen, Korea) sequence and 5`-CCCGTGTTGAGTCAAATTAAGC-3' (Hadziavdic *et al*., 2014) sequence as a reverse primer for the amplification of the V3 and V4 region, while forward primer 5`-ATTGGAGGGCAAGTCTGGTG-3` and reverse primer 5`-CCGATCCCTAGTCGGCATAG-3` were used for region V9 (Loeffler *et al*., 2000).

For amplification of DNA, a PCR master mix of 50 µl, devised and optimised during the present study was used (5 µl $MgCl_2$ + 5 µl PCR buffer + 1 µl *Taq* DNA polymerase + 29 µl dd H_2O and 5 µl dNTPs) (BIOLINE, UK). Then 25 µl of master mix was taken in an eppendorf tube with 2 µl primers and 3 µl concentrated gDNA. A Thermal Cycler (Veriti 96 Applied Biosystems) was used for amplification with the following reaction conditions: initial denaturation (for 5 min at 95°C) after 35 cycles of denaturation (for 1 min at 94°C), annealing (for 1 min at 55°C), elongation (for 1 min at 72°C), final elongation (for 5 min at 72°C) and a hold at 4°C. Amplified PCR product was observed on 1% agarose gel stained with ethidium bromide and sent

for direct sequencing to Macrogen, Korea.

18S rDNA variable regions amplicons were submitted to NCBI database for a homology search.

Construction of circular phylogram

Circular Phylogram was constructed by adopting the distance tree of BLAST pairwise alignments comparison to find out the relationship of the species under study with other members of the ascomycetes.

Statistical analysis

All experiments were designed in triplicates and repeated three times to measure the diameter of the colony and were also analyzed statistically applying one-way ANOVA. Duncan's multiple range test was used to measure the differences (p= 0.05). Standard error (SE) is also calculated as mean ± SE.

RESULTS AND DISCUSSION

Every organism present on the Earth needs food for its survival and to grow and different strains of *S. fimicola* are no exception. The results of the present investigation showed that all the strains produced compact mycelium on PDA media. These outcomes were comparable to the results stated by Mshandete and Mgonja (2009). For the culturing of many species of fungi such as *Pleurotus* species, PDA was the most suitable media to obtain a high level of mycelial growth (Munsur *et al*., 2012). Carbohydrates act as structural, storage components and a source of carbon for all living organisms and can be divided into three main saccharides (mono, di and poly-saccharides). Many fungi have high mycelia growth over a wide variety of carbohydrates in culture media.

In order to culture the different strains of *S. fimicola* under the aseptic conditions in the laboratory, all culture plates were supplemented with different carbohydrates to observe the response of mycelial growth, sporulation and to calculate the colony diameters. Table I showed that initially all the strains displayed a mycelial growth response to all the carbohydrates except the Sw92.1 strain that did not show any response towards glucose. A high response towards cellulose was observed for two strains isolated from the natural environment (N5, N6) as compared to the strains isolated from the harsh environment (S1, S2 and S3) and strain N6 showed a higher response to starch. Mycelial growth response towards glucose and maltose was comparable within strains and higher between the comparative groups. In case of perithecial development (Table II), traces of perithecia were observed in response to maltose in the case of S1, S2, S3, Sw92.1 and Sw17.2, while N5, N6 and N7 showed a better response to glucose. For further analysis (Table III), different concentrations of cellulose were used to check the effect on mycelial and perithecial growth. The rate of mycelial growth was increased by increasing the concentration of cellulose in the growth medium (0.1 to 1.0 %) while sporulation rate declined when concentration increased (1.5% to 2.5%), so 1.0% of cellulose concentration was used during subsequent analyses.

Table I.- Response of different strains of *Sordaria fimicola* towards different carbohydrates on mycelial growth.

Strains	Glucose	Maltose	Starch	Cellulose
S1	+	+	++	++
S2	+	+	++	++
S3	+	+	++	++
N5	++	++	++	+++
N6	++	++	+++	+++
N7	+	++	++	++
Sw92.1	-	++	++	+
Sw17.2	+	++	++	++

The + to +++ sign have been assigned to show the response of fungal mycelial growth to specific carbohydrate.

Table II.- Response of different strains of *Sordaria fimicola* towards different carbohydrates on sporulation.

Strains	Glucose	Maltose	Starch	Cellulose
S1	++	Trace	++	+++
S2	++	Trace	++	++
S3	++	Trace	++	+++
N5	+++	+	++	+++
N6	+++	+	++	+++
N7	+++	+	++	+++
Sw92.1	++	Trace	++	++
Sw17.2	++	Trace	++	+++

The + to +++ sign have been assigned to show the response of fungal sporulation to specific carbohydrate.

Data (Table IV) demonstrated that the observed mycelium colony diameter of S1 and N7 strains of *S. fimicola* on PDA media containing four different sources of carbon significantly differed at ($p < 0.05$). After seven days of incubation, (DI) the highest mycelium colony diameters of S1 strains of *S. fimicola* were attained from the culture media containing cellulose (3.71 ±0.21a) and the lowest diameter was calculated for media containing glucose (1.54 ± 0.12c). Starch containing media gave results comparable (3.45 ±0.18b) to that of cellulose.

Table III.- Response of different strains of *Sordaria fimicola* towards different concentrations of cellulose on mycelial growth and sporulation.

Strains	0.1 %		0.5 %		1.0 %		1.5 %		2.0 %		2.5 %	
	m	p	m	p	m	p	m	p	m	p	m	p
S1	+	++	++	++	+++	+++	+++	+	+++	-	+++	-
S2	+	++	++	++	+++	+++	+++	+	+++	-	+++	-
S3	+	++	++	++	+++	+++	+++	+	+++	-	+++	-
N5	+	++	++	++	+++	+++	+++	-	+++	-	++	-
N6	+	++	++	++	+++	+++	+++	-	+++	-	++	-
N7	+	++	++	++	+++	+++	+++	-	+++	-	+++	-
Sw92.1	+	++	++	++	+++	+++	+++	+	+++	-	+++	-
Sw17.2	+	++	++	++	+++	+++	+++	+	+++	-	+++	-

Table IV.- Effects of carbon sources on the mycelial growth of S1 and N7 strains of *Sordaria fimicola* of two contrasting slope.

Carbon source	Diameter of colony (cm) S1 strain of SFS	Diameter of colony (cm) N7 strain of NFS
Control*	0.81 ± 0.07d	0.78 ± 0.06c
Glucose	1.54 ± 0.12c	3.52 ± 0.11a
Maltose	1.76 ±0.09b	2.86 ±0.08b
Starch	3.45 ±0.18a	2.95 ±0.18b
Cellulose	3.71 ±0.21a	3.00 ±0.21b

*Growth medium without carbon source; Values are taken as the mean ± SE and different letters are represented significant differences at p= 0.05.

Table V.- Response of different strains of *Sordaria fimicola* towards different nitrogen sources on mycelial growth.

Strains	Sodium nitrate	Ammonium nitrate	Casein hydrolysate	Calcium nitrate
S1	+	+	++	+++
S2	+	+	++	+++
S3	+	+++	+++	+++
N5	++	++	+	++
N6	++	++	+	++
N7	+	-	++	++
Sw92.1	-	-	++	+++
Sw17.2	+	++	++	+++

The + to +++ sign have been assigned to show the response of fungal mycelial growth to specific nitrogen containing compound. + (minimum) ++ (optimum) and +++ (maximum) – (no response).

Reciprocal observations were made in case of N7 strain where the highest mycelium colony diameter was observed from the culture media containing glucose (3.52 ± 0.11a) and the lowest in case of maltose (2.86 ±0.08b). Present findings are in contrast to that of Hoa and Wang (2015) who found a high yield of mycelium of two oyster mushrooms (*Pleurotus ostreatus* and *Pleurotus cystidiosus*) on glucose.

The effect of 4 different nitrogen containing compounds was observed on mycelial growth in case of different strains of *S. fimicola* and the results are described in Table V. Calcium nitrate promoted maximum growth for all the strains isolated from S-slope and mutant strains while N-strains showed optimum response towards calcium nitrate. The N5 and N6 strains showed better growth in most of the media except casein hydrolysate where they showed minimum response. Mutant strains Sw92.1 did not show any response to sodium nitrate and ammonium nitrate. N7 strain failed to show any response to ammonium nitrate. The S3 strain was the only strain that showed better growth in ammonium nitrate and the lowest growth in sodium nitrate.

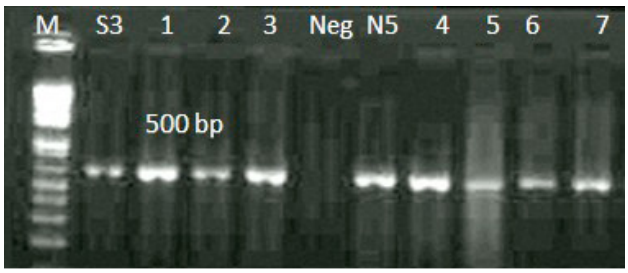

Fig. 1. V3 and V4 region analysis of 18S rRNA genes in natural strains of *S. fimicola* and their F1 progeny. M, DNA marker; S3, parental strain; 1, 2 and 3, F1 progeny of S3; N5, parental strain; 4, 5, 6 and 7, F1 progeny of N5; Neg., negative control without genomic DNA.

The primers enclosing the V3 and V4 regions yielded 481 bp long amplicons (Fig. 1) while the V9

region produced 181 bp long sequences in all strains. The nucleotide sequences were aligned using ClustalO. V3 and V4 regions in all strains were also completely aligned except on two points. To check polymorphism, whole 18S rDNA was also targeted by Machouart-Dubach *et al*. (2001), but the amplicon length was too long *i.e.* 1743/2136 with a single base alteration in the V2 region of moulds *Scytalidium dimidiatum* as compared to the current work. Both results were quite different from each other due to changed organisms and changed target sites. In the present work, V3 and V4 showed polymorphism on two nucleotides (Fig. 2), *i.e.* 212 A (C); 213 G (A). V9 region was deprived of any polymorphism. The query sequence showed 100 % homology with *S. fimicola* (Fig. 2).

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N5        GGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGG        60
S.F       GGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGG        60
S3        GGCTACTACATCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGG        60
          ************************************************************

N5        TAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACAAT        120
S.F       TAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACAAT        120
S3        TAGTGACAATAAATACTGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACAAT        120
          ************************************************************

N5        TTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATT        180
S.F       TTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATT        180
S3        TTAAATCCCTTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATT        180
          ************************************************************

N5        CCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTGG        240
S.F       CCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAACCTTGG        240
S3        CCAGCTCCAATAGCGTATATTAAAGTTGTTGAGGTTAAAAAGCTCGTAGTTGAACCTTGG        240
          ********************************  ***************************

N5        GCCCAGCCGGCCGGTCCGCCTCACCGCGTGCACTGGATAGGTTGGGCCTTTCCTTCTGGA        300
S.F       GCCCAGCCGGCCGGTCCGCCTCACCGCGTGCACTGGATAGGTTGGGCCTTTCCTTCTGGA        300
S3        GCCCAGCCGGCCGGTCCGCCTCACCGCGTGCACTGGATAGGTTGGGCCTTTCCTTCTGGA        300
          ************************************************************

N5        GAACCGCATGCCCTTCACTGGGTGTGTCGGGGAACCAGGACTTTTACTCTGAACAAATTA        360
S.F       GAACCGCATGCCCTTCACTGGGTGTGTCGGGGAACCAGGACTTTTACTCTGAACAAATTA        360
S3        GAACCGCATGCCCTTCACTGGGTGTGTCGGGGAACCAGGACTTTTACTCTGAACAAATTA        360
          ************************************************************

N5        GATCGCTTAAAGAAGGCCTATGCTCGAATACATTAGCATGGAATAATAGAATAGGACGTG        420
S.F       GATCGCTTAAAGAAGGCCTATGCTCGAATACATTAGCATGGAATAATAGAATAGGACGTG        420
S3        GATCGCTTAAAGAAGGCCTATGCTCGAATACATTAGCATGGAATAATAGAATAGGACGTG        420
          ************************************************************

N5        TGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGGGGCA        480
S.F       TGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGGGGCA        480
S3        TGGTTCTATTTTGTTGGTTTCTAGGACCGCCGTAATGATTAATAGGGACAGTCGGGGGCA        480
          ************************************************************

N5        TCAGT     485
S.F       TCAGT     485
S3        TCAGT     485
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Fig. 2. CLUSTAL O (1.2.3) multiple sequence alignment of different strains of *Sordaria fimicola* with reference sequence of *S. fimicola.*

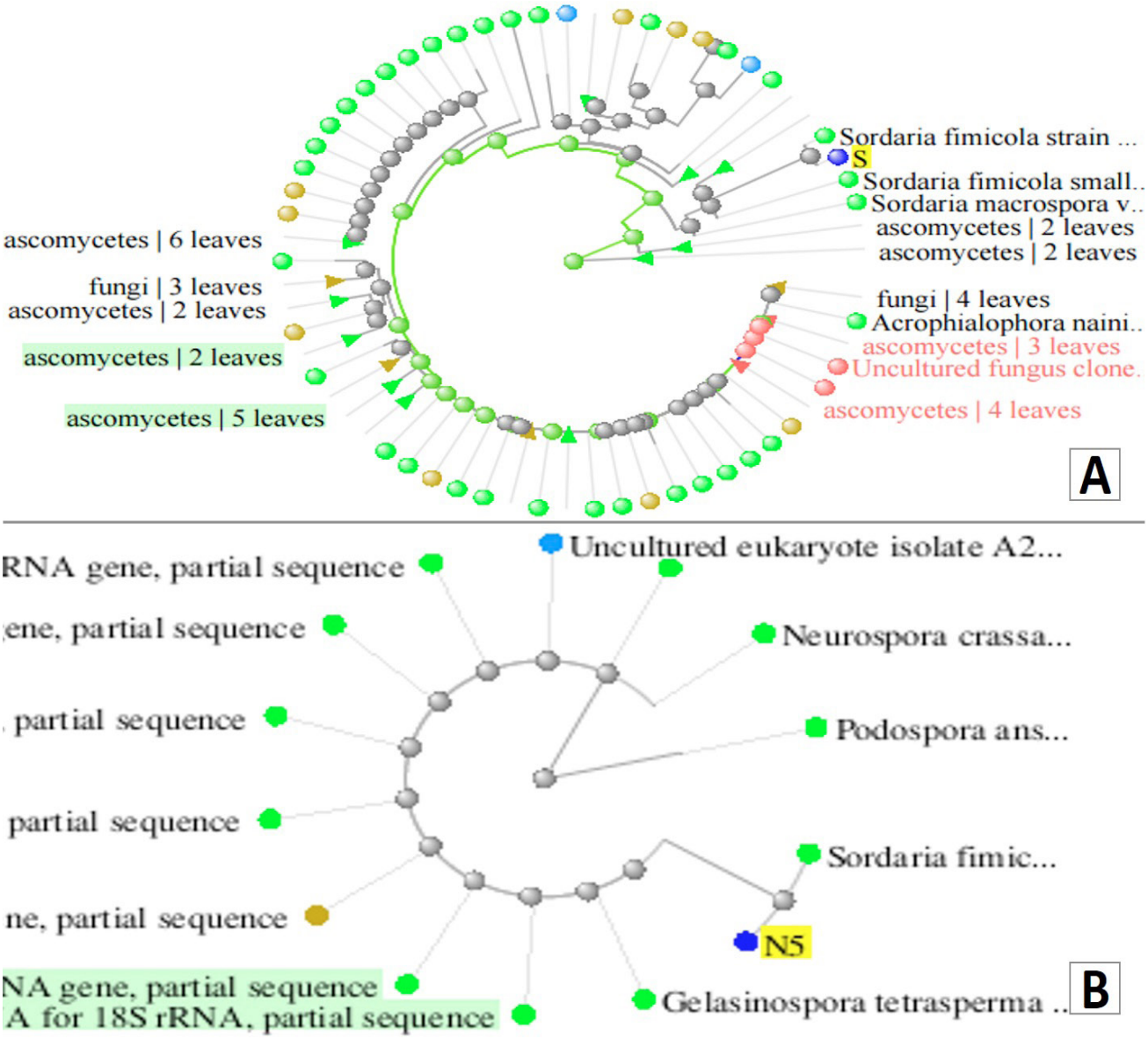

Fig. 3. Circular Phylogram to find phylogenetic relationship among ascomycetes and experimental strains reconstructed from the conserved region spanning V3 and V4 rDNA genes (A) and V9 rDNA genes (B) yellow highlighted colour indicating strain under study.

Similarly, Basem *et al*. (2012) also targeted the 18S region to recognize different isolates of fungi isolated from Red Sea water and found 98% similarity in the sequences while studying the 18S rRNA regions of different strains of *Scytalidium*. Machouart-Dubach *et al*. (2001) found an insertion sequence of 393 bp in two strains. Morakotkam *et al*. (2007) isolated 257 strains of fungi from bamboo plants of which 71 were categorized by sequencing the 18S and ITS regions. Most of these isolates were from the family *Sordariaceae*. Modern studies discovered that fungi can be used in biofilm development. A study conducted by Weber *et al*. (2009) identified oxygen consuming granules in different fungi that play an important role in biofilm production by sequencing 5.8S, ITS and SSU rRNA regions using universal primers of these targeted regions. Zhihong *et al*. (2003) identified 49 species of airborne pathogenic fungi from sequence analysis of small sub unit rRNA genes (rDNA) demonstrating 15 genera. Cluster analysis divided these fungi into two main classes (Ascomycetes and Zygomycetes). Hadziavdic *et*

al. (2014) characterized the eukaryotic SSU 18S rRNA region to design the universal primers to study genetic variation among eukaryotes by using SILVA database for characterization. In short, the SSU-18S rDNA region can be used as molecular markers to recognize or differentiate different species of fungi isolated from different sources. The comparatively fewer variations and highly conserved nature of 18S rDNA make it a reliable molecular tool for determining homologous regions appropriate for identification of fungi at genus level. Circular Phylogram to find phylogenetic relationship among ascomycetes and experimental strains reconstructed from the conserved region spanning V3, V4 and V9 rDNA genes are shown in Figure 3. In Germany and the USA, it was found that in rainbow trout salmonid whirling disease is caused by *Myxobolus cerebralis* parasite. To check this, Andree *et al*. (1999) made a comparison between internal transcribed spacer region-1 and 18S region and found 14 polymorphic sites out of 1700bp long sequence and no difference was observed in ITS-1 region.

Ribosomal RNA exists in all organisms present on earth with an extremely conserved nature and only a few different modifications or variations are present between prokaryotes and eukaryotes. To study genetic diversity in prokaryotes, the hyper-variable 16S SSU-V6 region is mostly studied. Many workers have targeted the V4 region in eukaryotes for genetic analysis due to its varied nature while this region is very short in prokaryotes therefore not suitable for genetic diversity. However, in this study we have identified variations in two nucleotides on the V4 region while no base change was observed in V9 region. The V3 and V4 region successfully characterize the south-facing slope strains from north-facing strains of *S. fimicola*.

CONCLUSION

In the light of the above mentioned results, the V4 region analysis proved to be a powerful tool to differentiate the different strains of *S. fimicola* and cellulose was found to be an effective source of carbon to enhance the mycelium growth and rate of sporulation while calcium nitrate was found to be an active source of nitrogen.

Statement of conflict of interest

The authors declare that there is no conflict of interest regarding current studies.

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