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Full Length Article

Genotyping of Ribosomal Proteins in *Sordaria fimicola* to Study Genetic Polymorphisms and Phosphorylation Modifications

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Abstract

The aim of the experiment was to look for natural genetic variation in different natural strains of *Sordaria fimicola* isolated from natural and stressed ecological conditions. It was challenging because there was no sequence information at the time, and we planned to design a set of PCR primers that would reliably amplify in *S. fimicola* and hoped that the amplified products were polymorphic. Hence we decided to target ribosomal protein genes, putting primers in the exonic parts to amplify the intron(s) they flank. We used *S. macrospora* sequence information to strategically place the "exon–primed, intron–crossing" primers in a couple of Rp genes (*RpS29*, *RpS23*, *RpS26*, *RpL17*, and *RpL30*) and to determine nucleotide variations in fifty strains of *S. fimicola* by sequencing PCR amplicons. As compared to the strains isolated from natural environment, strains isolated from stressed environment exhibited point mutation on three positions C (122) A; G (127) A and T (137) G for *RpS29* gene, at two positions G (257) T; T (327) G for *RpS23* gene and one base substitution A (152) T in case of *RpS26* gene and polymorphisms on four nucleotides were observed for RpL30 gene while 100% homology was found for *RpL17* ribosomal proteins. In this study, we have also explored the phosphorylation status of ribosomal protein *S29* and predicted the phosphorylation sites using NetPhos3.1 server. Serine–phosphorylation on two residues Ser9 and Ser18 out of 56 amino acids was found evolutionarily conserved in five model organisms while Ser18 modification is not present in *Sordaria* species. © 2019 Friends Science Publishers

Keywords: Genetic diversity; High resolution melt analysis; Real–time PCR; SNPs; *Sordaria*

Introduction

Genomics play an important role to determine the practical importance of genetic polymorphisms to determine the genetic diversity and evolution (Hedrick, 2006). Polymorphism at genetic level is common phenomenon that covers all types of differences present between homologous nucleotide sequences such as INDELs (insertion and deletion mutation), single nucleotide polymorphism (s), variable tandem repeats, duplications and rearrangement of genes etc. Though these mutations cover the small portion of the genome but ample of the diversity and variations mostly grounded on them (Burke, 2012). In GenBank and other genetic databases (NCBI), variety of amino acid and nucleotide sequences are available that make it possible to design certain sets of primers to amplify the specific genes across in many Ascomycetes. Introns might be a good source to find out genetic variations in the eukaryotes as they quickly gather variations without disturbing gene function. Exon–primed intron–crossing (EPIC) polymerase chain reaction (PCR) has been established and useful to fetch mutations (Lessa, 1992; Corte–Real *et al*., 1994; Palumbi and Baker, 1994; Daguin *et al*., 2001; Chow and Nakadate, 2004; Chow *et al*., 2007) in variety of organisms e.g. Copepoda, Echinodermata, and Teleoste (Chow *et al*., 2015).

In eukaryotes, ribosomes are the organelle that are principally involved in the synthesis of different essential proteins from mRNA; contains four species of rRNA and many other ribosomal proteins (Xiang *et al*., 2015). Most ribosomal proteins are somewhat conserved over diverse domains of life. For example, 35 ribosomal protein (s) own homologs in eukaryotes, bacteria and eubacteria and only 12 proteins are specific for eukaryotes (Jinek *et al*., 2013). Over the last few years, numbers of ribosomal proteins have been found to play an important role in the synthesis of ribosomes, translation of proteins, to control other biological processes and have also been used as biomarkers for strain identification. The 80S ribosomes encompassed two subunits i.e. 40S small subunit (SSU) and 60S large sub–unit (LSU) in eukaryotes. The 40S ribosomes consist of the 18S rRNA

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and about 33 small subunit ribosomal proteins (rps) while the 60S contain the 47 large ribosomal proteins (rpL) subunits (Gerbasi *et al*., 2004). Ribosomal proteins also facilitate the connection of ribosome with different factors involved in translation. Furthermore, numerous rp(s) (*L17* and *L30*) of the LSU form a ring like configuration at the peptide exodus channel that is necessary for the excretion of proteins (Halic *et al*., 2004).

Ribosomal proteins *S29* exist in two forms in yeast i.e. *RpS29A* and *RpS29B* both are identical to each other. Of 56 amino acids, 51 amino acids are similar in both but can easily be differentiated during mass spectrophotometry and both proteins are equally expressed in yeast (Ghaemmaghami *et al*., 2003). *RpS26* is another important protein of small subunit of rp(s) that is encoded by *RpS26A* (present on chromosome 7) and *RpS26B* (on chromosome 5) proteins (Belyy *et al*., 2016). Both these protein by ~92% resemble to each other. It was first time isolated in 1977 from the liver of the rat and later on from human c–DNA (Vincent *et al*., 1993). Former investigation verified that *RpS26* interrelates with the 5' noncoding region of mRNA through the eukaryote–specific Y62–K70 motif. In eubacteria the ribosomal protein S18 is considered as the homolog of *RpS26* protein containing comparable rRNA fundamental motif (Malygin and Karpova, 2010).

In Drosophila, deficiency of rp genes leads to the developmental and morphological changes e.g., reduced body mass, late development of larvae, thin hair, female infertility and deformity of wings and eyes (Lee *et al*., 2013). Ribosomes are highly vigorous, and post translational modifications of Ser, Thr or Tyr amino acids is perhaps one of the most significant controlling process working inside ribosome and mitochondria (Hopper *et al*., 2006). Phosphorylation of ribosomal proteins might help as a molecular shift, affecting protein synthesis and cell physiology (Kim *et al*., 2007).

The EPIC primers are mostly aimed to target conserved areas may have a widespread effectiveness through a wide variety of organisms (Chow *et al*., 2007; Pinho *et al*., 2010; Jennings and Etter, 2011). The universality of these primers depends on the conservation of exonic regions, and the productivity of PCR amplification may be principally affected by gene copy number, including pseudo genes. Genes encoding ribosomal proteins (rps) may be highly suitable for designing universal primers, as ribosomal proteins are the very much conserved genes between eukaryotes and are normally determined by a single gene (Wool *et al*., 1995).

In this study, we have simply adopted an HRM technique along with Real time PCR to amplify five ribosomal protein genes in *S. fimicola* strains collected from two opposite slopes of Evolution canyon to group the genotypes if variation existed, and we sequenced representatives from each genotype to confirm the identity of the PCR products and the presence of nucleotide polymorphisms. So far little work has been done at molecular level for *S. fimicola*; hence little information is available regarding its genome. Hereafter, the emphasis of the paper is on the discovery of genetic variation in the EC populations.

Materials and Methods

Experimental Organism

S. fimicola is used as an experimental organism throughout this study. Fifty strains of this fungus were used for the identification of five ribosomal proteins (*RpS29*, *RpS26*, *RpS23*, *RpL30*) and *RpS17* and genetic variations (For strains details please see Arif *et al*., 2017). All the strains were subcultured under sterile conditions on plates containing solidified Potato Dextrose Agar (PDA) as growth medium and refrigerated at about 18°C for 8–10 days. When a thick mat of mycelia appeared, these plates were used for DNA extraction and subsequent genomic analysis.

DNA Extraction

Cultures of all the strains were homogenized in DEPC treated water (Bioline) and incubated with Lysis buffer PA1 along 10 µL of RNAase at 65°C for 10 mins to break the cell wall. DNA from all the samples was extracted using ISOLATE II Plant DNA kit (BIOLINE, Cat No. BIO–52069). Purity and quantification of DNA was done using UV–3000 spectrophotometer (Hamburg, Germany). Extracted DNA was also run on 1% agarose gel along with 1 kb plus size marker (Invitrogen) to measure the band size and photographed under the gel imaging system Syngene Ugenius3.

High–Resolution Melting Analysis in *S. fimicola*

Many techniques have previously been employed to genotype molecular markers. The subsequent example is a latest HRM methodology that has been used for rp(s) genotyping on light cycler (Roche Light Cycler® 480) in *S. fimicola*.

Composition of PCR Mixture and Cycling Conditions

A 10 (µL) PCR mixture comprised of following reagents (genomic DNA 2 µL; 10X PCR buffer (Bioline) 2 µL; 50 mM $MgCl_2$ 0.064 µL; 25 mM dNTPs 0.064 µL; the LightCycler® 480 High Resolution Melting Master solution (Roche) 0.25 µL; the IMMOLASE™ DNA polymerase (Bioline) 0.01 µL; the forward and reverse primers 0.04 µL and double distilled–water 6.26 µL.

The amplification was programmed as follows: initial DNA denaturing step of 95ºC for 10 min, followed by 50 cycles of denaturation (95ºC for 5 s), (65ºC for 15 sec) and (72ºC for 1 min), ending with a final elongation step at 72ºC for 5 min. Fluorescence acquisition was obtained after each 72ºC step. Products were heated to 95ºC for 1 min, cooled to

40ºC for 1 min. and raised to 78ºC for 1 sec. As temperature increased gradually from 78ºC to 95ºC, fluorescence data were acquired continuously.

The Roche Light Cycler® 480 software package was used to determine the crossing point (Cp) value and melting temperature (Tm) using Absolute Quantification and Tm calling modules. All amplicons were observed using melt curve genotyping and HRM genotyping present in the Light Cycler 480 1.5.1.62 software.

Results

Gene Sequencing and Mutations

Ten primers listed in (Table 1) were designed for the amplification of targeted genes using Primer3Plus software available at http://primer3plus.com. All genes were successfully amplified using designed primers and analysed by HRM analysis. To check the sequence homology alignments; all sequenced amplicons were subjected to BLAST tool of the "National Centre for Biotechnology Information" (http://www.ncbi.nlm.nih.gov/blast). The experiments were conducted in triplicates to obtain the reproducible melting peaks. Conditions for HRMA were optimized and tested on eight strains collected from two contrasting environments. Point mutations between different strains are shown in Fig. 1 and 2. Structure of *RpS29* is shown in Fig. 3.

Prediction of Post–translational Modifications

Phosphorylation on Serine/Threonine/Tyrosine residues were calculated using NetPhos 3.1 (www.cbs.dtu.dk/services/NetPhos/) servers for the prediction of post–translational modifications in six eukaryotic model organisms listed in (Table 2).

Discussion

Genes that code for ribosomal proteins are multigene families and have pseudo genes and one intron with functional gene (Doherty *et al*., 2010). Many RP genes and other conserved genes have been employed by many workers to recognize the different species of bacteria and fungi e.g. HRM analysis has been found to be effective for differentiating between different species of *Candida* by targeting *ITS* region 1 from rDNA (Gago *et al*., 2014). Here, we first time employed same advance technique; real–time PCR followed by HRMA together with sequencing amplicons for the amplification and detection of mutations for targeted genes of ribosomal proteins in different natural strains of *S. fimicola* isolated from two conflicting surroundings.

Sun *et al*. (2006) used 42 ribosomal sub proteins as biomarkers for the identification of new species of *Lactobacillus* by matrix–assisted laser desorption or ionization mass spectrometry (MALDI–MS).

Table 1: List of primers for the amplification of targeted ribosomal protein genes in different strains of *S. fimicola*

Primers	Sequences (5′-3′)	Region Targeted
RpS26_F	GCCGCGGCCACGTCAAGCCCATCCG	RpS26
RpS26_R	GTACTGCAGCTTGAGGTACATCTTG	
RpS29_F	TGGAACTCCCGCCCCCGCACCTACG	RpS29
RpS29_R	GCCTTCTCACGGAAGCACTGACGGC	
RpS17_F	TGAGAAGAAGGAGGCTGTCCCCATG	RpS17
RpS17_R	GCCTGGTTGACCTGGATGTGCTTG	
RpL30_F	CAAGTCTGAGCTTGAGTACTACTCC	RpL30
RpL30_R	AGGATGTCGGAGTCACCGGCATC	
RpS23_F	CAGCTCATCAAGAACGGCAAGAAGG	RpS23
RpS23_R	ACACCGGGAATATCACCCTTGGCC	

Fig. 1: Multiple sequence alignment of parental strains of *Sordaria fimicola* for *RpS29* gene by Clustal Omega
Highlighted area indicating SNPs; * indicating conserved region; S stand for strains from south facing slope of Evolution Canyon; N stand for North facing slope of Evolution Canyon; 1, 2, 3, 5, 6, 7 are the assigned station from where samples were collected

Fig. 2: A Sequence Alignment produced by Clustal *O* of model organisms of *RpS29* proteins from 1-56 amino acids
Key: (*) conserved region between all organisms; (:) for conservative mutation; (.) semiconservative mutation

Similarly, Teramoto *et al*. (2007) identified different strains of *Pseudomonas* by using ribosomal proteins to determine the phylogenetic relationship among the sixteen strains of *Pseudomonas putida* by MALDI–MS technique. Ribosomal proteins are extremely conserved multifaceted housekeeping proteins and small differences in sequences can occur at the level of strains and are useful to measure rate of evolution. Ribosomal protein *S29* is highly conserved protein from *Saccharomyces* to *Homo sapiens* and is composed of 56 amino acids (Liu *et al*., 2005). *RpS29* of *S. macrospora*, *Neurospora crassa* and *S. fimicola* consists of 3 exons and 2 introns (Fig. 3) while that of *Aspergillus niger* and *A. fumigatus* consist of 5 exons and 4 introns. First three exons are found to be conserved while length of exon 4 is varied in different species of *Aspergillus* i.e. 57bp in *A. niger* and 61bp long for *A. fumigatus* (Nakamura *et al*., 2016).

In the present study, we also found highly conserved nature of *RpS29* gene between different strains of *S. fimicola* except on three positions. Polymorphism on position C (122) A; G (127) A and T (137) G was found in strains isolated from south facing slope (Fig. 1). *RpS29* protein was also found highly conserved between different model organisms when aligned. Of 56 amino acids 25 residues are evolutionary conserved between *H. sapiens*, *Drosophila melanogaster*, *C. elagans*, *Saccharomyces pombe*, *N. crassa* and *S. macrospora* (Fig. 2).

Table 2: Prediction of phosphorylation in six model organisms to find the conserved modifications of *RpS29* protein

Server	Organisms	Predicted Phosphorylation sites				
NetPhos 3.1		On serine	On threonine	On tyrosine	Residue modified	Kinases
	Drosophila	9, 18, 25	nil	46	YGQGSRCCR CRACSNRHG CFREYANDI	PKA, cdc2 cdc2 INSR
	Homo sapiens	9, 18, 20	nil	0	QLYWSHPRK FGQGSRSCR QGSRSCRVC	PKC, cdc2 PKAPKC, cdc2
	C. elagans	9, 18	nil	0	NLWFSHPRK FGPGSRSCR	PKC PKA
	S. pombe	9,18, 42	24	46	NVWFSHPRK YGKGSRQCA QCAHTGRRL ISRQSFREY SFREYANDI	PKC PKA, PKC PKC unsp INSR
	N. crassa	2, 9, 18,20	13	53	MSHESV SVWNSRPRT SRPRTYGKG YGKGSRSCR KGSRSCRVC DIGFTKHR	CKI, cdc2 DNAPK PKC Unsp
	S. macrospora	2, 9, 20	0	53	MSHESV SVWNSRPRT KGARSCRVC DIGFTKHR-	PKA,CKI, cdc2 PKC, cdc2 PKA

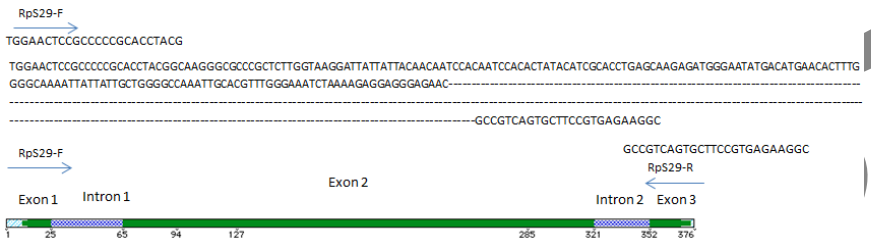

Fig. 3: Gene structure of *RpS29* exhibiting three exons and two introns
Key: Arrow indicating location of forward and reverse primers

By using PCR, Filipenko *et al*. (1998) isolated the genomic sequence of *RPS26* gene. There are four exons of about 2kb which are transcribed into 438 bp long mRNA and poly (A) tail. It also has a transcription start that is present inside a poly–pyrimidine region, and an untranslated region of small 5–prime. According to Doherty *et al.* (2010) deletion of *RPS26* causes low production of 18S rRNA. *Rps26* is also interacted with initiation factors during protein synthesis (Sun *et al*., 2011). From literature it was noted that *RpS26* was acquiescently intricate in docking of the messenger–RNA to the ribosomal 40S unit in protein synthesis through the Y62–K70 amino acids (Sharifulin *et al*., 2012). Based on the data, it was speculated that *Rps26*, via the Y62–K70 segment, was directly involved in docking of the mRNA to the 40S subunit during translation initiation.

In prokaryotes and eukaryotes covalent post–translational protein modifications such as phosphorylation, acetylation and methylation have appeared as significant factors contributing to complex structure of the ribosomes. Protein translation in higher organisms is mostly measured at the level of initiation, which includes numerous events of protein phosphorylation (Jackson *et al*., 2010). Most of these covalent modifications are often conserved between different organisms. In eukaryotes phosphorylation and methylation modifications have a potential to change the function and synthesis of ribosomes. In *Arabidopsis* removal of methionine initiate the N–terminal covalent modifications (Adam *et al*., 2008).

In silico predictions of protein modifications of *RpS29* protein was predicted in 6 model organisms and during this predictions Ser9 and Ser18 modifications were found conserved between five model organisms except *S. macrospora* where Ser18 phosphorylation was not predicted (Table 3). Phospho–tyrosine modification Y46 is common between *Drosophila* and *S. pombe* while Y53 modification is similar between *N. crassa* and *S. macrospora*. No tyrosine modifications were observed in *H. sapiens* and *C. elagans*. Phosphorylation on threonine was predicted only for *S. pombe* and *N. crassa* but on different positions on thr24 and on thr13 respectively and absent in other organisms. In *Arabidopsis*, maize and many other plants ribosomal small subunit protein S6 is successively phosphorylated on five serine residues of the C–terminus and these modifications are highly conserved across eukaryotes (Enganti *et al*., 2018). Mythelation modifications occurred on five large subunit r–proteins (L11, L7, L12, L16 and L33) and S11 while acetylation occurred on S5, S18 and L7 proteins. Significantly, Turkina *et al.* (2011) recognized an earlier unknown phosphorylation site Ser–231 in the 40S ribosomal protein S6.

Web–based tools, such as NetPhos, was also used by (Blom *et al*., 1999) to recognize potential kinases, which may help as successful contenders to phosphorylate DAP3 *in vivo* and to categorize possible serine, threonine, and tyrosine phosphorylation sites in the human DAP3 sequence. They found cAMP–dependent protein kinase (PKA) and protein kinase C delta (PKCd) as the best kinases that play important role to phosphorylate DAP3. Similar, web–based tool (NetPhos 3.1) was used during the current study to investigate the kinases involved in the phosphorylation of ser/thr/Y modifications for *RPS29* protein in six model organisms (Table 3). For example, PKA and PKC were predicted to phosphorylate Ser9 and Ser18 in *H. sapiens*, *C. elagans* and *S. pombe*, while in *N. crassa* and in *Sordaria*

macrospora these kinases were predicted on Ser2 and Ser20. These kinases can be confined to the mitochondria from side to side via A–kinase anchoring proteins (AKAPs), and their enzymatic action take place on the matrix side of the inner membrane of mitochondria (Chen *et al*., 2004). In higher plants the variations in phosphorylation position of ribosomal protein were consider to be responsible for speedy regulations in their development configurations under ecological conditions (Reinbothe *et al*., 2010).

Conclusion

In conclusion, overall, the NetPhos prediction results indicated that four serine, one threonine, and one tyrosine residues were predicted to be phosphorylated with high probability in the six model organisms *RPS29* sequence. Although the physiological significance of *RPS29* remains unknown, our data provide a mechanistic basis for the future understanding of these modification events.

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