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Molecular characterization of fungi associated with stored soybean (*Glycine max* L) seeds

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Abstract

Soybean is an important legume that has high quality protein and oil for food and feed. Despite the importance of this legume, the crop is affected by several post-harvest diseases caused by fungi. A study was carried out to identify the fungal species associated with the seeds of soybean using molecular techniques. The DNA of the isolate, was molecularly characterized using Internal Transcribed Spacer 1 (ITS-1) molecular marker. The isolate DNA sequence, was aligned using the Basic Local Alignment Search Tool for nucleotide (BLASTN) 2.8.0 version of the National Center for Biotechnology Information (NCBI) database. The results showed that the isolate sequence was 98% identical to *Diaporthe* spp. Voucher VP51, 98% identical to *Diaporthe schini* isolate L5N71 and 98% identical to *Diaporthe schini* strain B125. These findings showed that *Diaporthe* spp. is one of the causal fungal pathogens of post-harvest diseases of soybean seeds. It is anticipated that these results will provide information on culturing *Diaporthe* species also provide the basis for further study to show their antibiotic and anti-cancerous, enzymes and secondary metabolites producing ability.

Keywords: Soybean, *Diaporthe schini* and RBCL marker
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Introduction

Soybean (*Glycine max* L) is one of the important legumes and has a source of high quality protein, for human and animal consumption (Palacios *et. al.,* 2004). They can produce at least twice as much protein than any other key vegetable or grain crop besides hemp. According to the US Food and Drug Administration, soybean is a good source of protein for vegetarians (Dotzel, 1999). Despite being one of the most important legumes in the world, soybean plants are vulnerable to a wide range of diseases for example, Phomopsis seed decay- *Diaporthe longicolla* and parasites (Herbert, 2009). To study the pathogens that cause diseases in soybean and to gather up information on how to management the diseases, there is need to identify these pathogens.

There are conventional methods that have been used to identify these pathogens; however, these methods are bulky and inefficient (Hill, 1996). Efficient identification of disease causing fungal pathogens of soybean, is proper because according to St-Germain and Summerbell (2003), diseases caused by fungi have become a noteworthy medical problem and are increasing at a disturbing rate. The increase in the number of patients that are not immuno-competent, have emphasized the significance of precise identification of fungi. To accurately identify these pathogens, molecular characterization technique has been employed to verify the identification of the fungi pathogens (Gontia-Mishra *et. al*., 2013; Bechem and Afanga, 2017). Therefore, this study was aimed at identifying the fungal pathogens associated with the post-harvest diseases of soybean using macro- and micro-morphological identification, and validating the identification using molecular characterization technique.

Materials and Methods

Source of plant material

Seeds were obtained from National Centre for Genetic Resources and Biotechnology (NACRAB) Ibadan, Oyo State.

Study Area

The study was conducted in Mycology/ Pathology laboratory of Plant Science and Biotechnology and Regional Centre for Biotechnology and Bio-fuel Research Laboratory were DNA extraction was carried out, University of Port Harcourt. Amplification and sequencing of the PCR products were done at the International Institute for Tropical Agriculture (IITA) Ibadan.

Isolation of Fungi from soybeans using Blotter Method
Standard Blotter Method recommended by International Seed Health Testing Association (ISTA, 2016) was used to isolate fungi pathogens associated with stored maize. Petri dishes were lined with 3 layers of Sterile Whatman's 9cm filter papers and moist with sterile distilled water. The stored maize used were sorted to remove diseased ones, then soaked in 70% ethanol for 2-3 minutes and rinsed twice in sterilized distilled water; after which they were placed in the Petri dishes equidistantly and incubated at 25± 2°C for 3-7 days at the Pathology/Mycology laboratory of the Department of Plant Science, University of Port Harcourt, Rivers State, Nigeria. The most common soyabean isolate was coded SB-3A and SB-3B.

Morphological and microscopic characterization and identification
The fungal mycelium of only two (2) unknown fungi was found and cultured on Potato Dextrose Agar medium at room temperature for 7 days. The morphological identification of isolates was conducted by visually observing the mycelium and compared with the pictorial guide by Snowdon (1990). Colonies were compared for their diameters, overall colors, colors of conidia, reverse colors, texture, zonation and sporulation. The isolate was also subjected to microscopic analysis for visual identification using an electron binocular microscope at X40.

Molecular characterization using the Internal Transcribed Spacer (ITS) marker and identification
The Genomic DNA of the 2 isolates SB-3A and SB-3B found on the seeds, was extracted following the protocol of Quick-DNA™Fungal/Bacterial MiniPrepKit (Zymo Research Group, California, USA) as described by the manufacturer, with modifications at the Regional Center for Biotechnology and Bioresources (RCBB), University of Port Harcourt, Choba, Rivers State, Nigeria. The SB-3B isolate DNA quantity and concentration were measured using Nano-Drop 2000c spectrophotometer (Thermo fisher Scientific Inc. Wilmington, Delaware, USA). The DNA purity was measured as a ratio of absorbance at 280nm to that of 260nm. The DNA sample of the SB-3B isolate was sent to the International Institute of Tropical Agriculture (IITA) Bioscience Center, Ibadan, Nigeria for amplification and sequencing. The primers used to amplify fragments of the nuclear ribosomal DNA (rDNA) of the SB-3B isolate were the Internal Transcribed Spacer 4 (ITS4) with the sequence TCCTCCGCTTATTGATATGS and ITS5 with the sequence GGAAGTAAAAGTCGTAACAAGG. The amplicons were sequenced using the ABI 3500 capillary electrophoresis sequencer. The DNA sequence file was saved in the Bioedit file with extension .ab1. The sequence was analyzed using the Molecular Evolutionary Genetics Analysis (MEGA) version 7.0.26 software, and aligned using the Basic Local Alignment Search Tool for nucleotide (BLASTN) 2.8.0 version of the National Center for Biotechnology Information (NCBI) database.

Results

Isolation, morphological and microscopic identification of fungi associated with Soyabean

Two unidentified fungal organisms SB-3A and SB-3B were isolated and found to be associated with Soyabean. On PDA, the isolates developed scanty mycelia and conidiomata had dark brown to black colour. The *c*onidia are rarely branched, tapering towards the apex. Then on the reverse, the isolate was observed to develop greyish to smoke-grey colour. From the microscopic, the isolate was identified as a *Diaporthe* species.

Molecular characterization using the Internal Transcribed Spacer (ITS) marker and identification
The genomic DNA of the isolates SB-3A and SB-3B of Soybean (*Glycine max* L) were successfully extracted and showed good quality. The Nanodrop result (Table 1 and Fig. 1) showed that the concentrations of the DNA of the isolates were 18.4ng/ul and 19.9ng/ul respectively. While the absorption peak of the 260nm/280nm readings were 1.96 and 2.12 respectively and the 260nm/230nm readings were 0.69 and 0.74 respectively. However, to reduce the cost of sequencing, the isolate SB-3B with the highest DNA concentration was selected.

Table 1: Concentration of DNA Extracted from Fungal isolates of Soybean SB-3A and SB-3B using Nanodrop (2000c)Spectrophotometer

Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor

3	RCBBR-UNIPORT	6/26/2018 3:08:19 PM	18.4	ng/µl	0.368	0.188	1.96	0.69	DNA	50
3	RCBBR-UNIPORT	6/26/2018 3:09:02 PM	19.9	ng/µl	0.398	0.188	2.12	0.74	DNA	50

#	Sample ID	User name	Date and Time	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
6	3b	RCBBR-UNIPORT	6/26/2018 3:09:02 PM	19.9	ng/µl	0.398	0.188	2.12	0.74	DNA	50.00

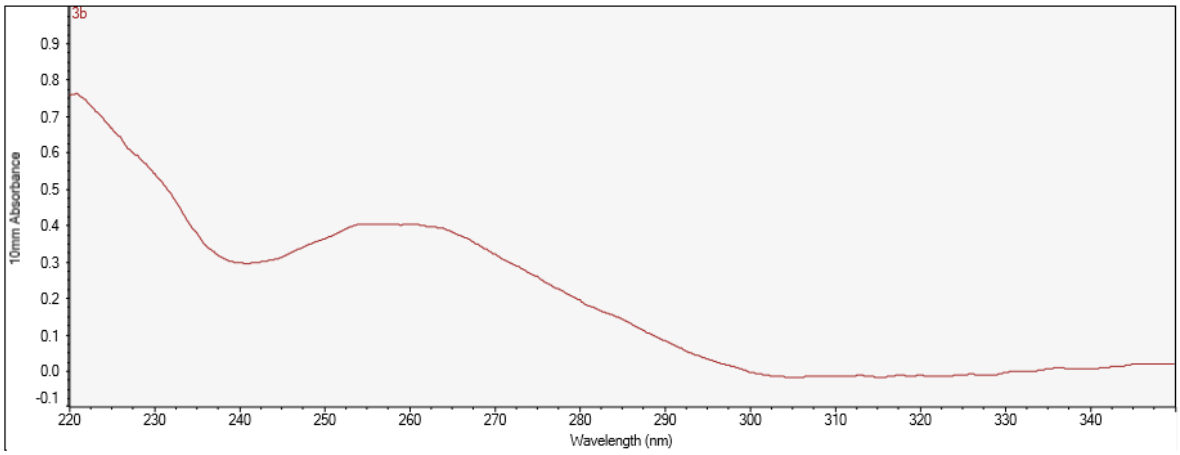

Figure 1: Graph showing Concentration of DNA Extracted from fungal Isolate SB-3B

Polymerase Chain Reaction (PCR) and Gel Electrophoresis

From the result of the amplified DNA or PCR band of the isolate SB-3B, the amplified DNA showed bands on gel when observed under UV light. From the result, the ladder used indicated that the SB-3B isolate sequence had over 572 base pairs.

DNA Sequencing

The sequencing result after alignment are shown in Figures 2 and 3. Figure 2 showed the beginning while Figure 3 showed the end of the DNA sequence of the isolate SB-3B. Figure 3 specified that the sequence length was 572 base pairs. This result authenticated the DNA amplification result. Also, from the results, it was noticed that the colours of the bases of the nucleotides were existing in four colours [green: adenine (A), red: thymine (T), blue: cytosine (C), black: guanine (G). These diverse colours allow for easy interpretation of the sequence.

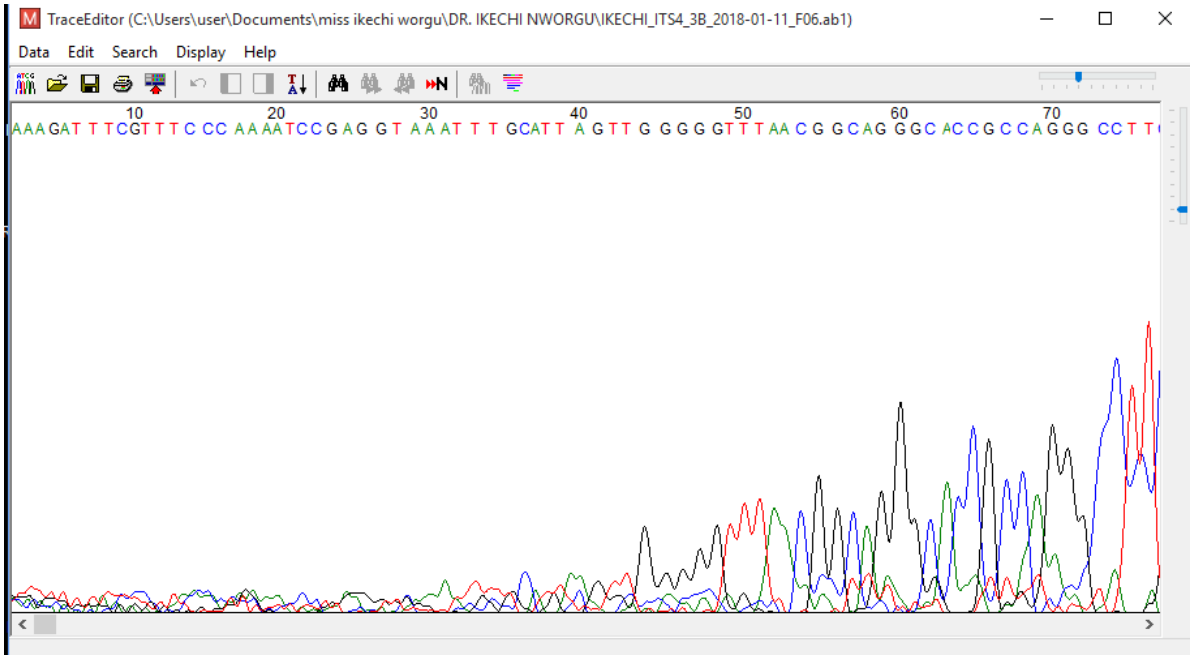

Fig. 2: Beginning of sequence alignment of the DNA of Soyabean after alignment

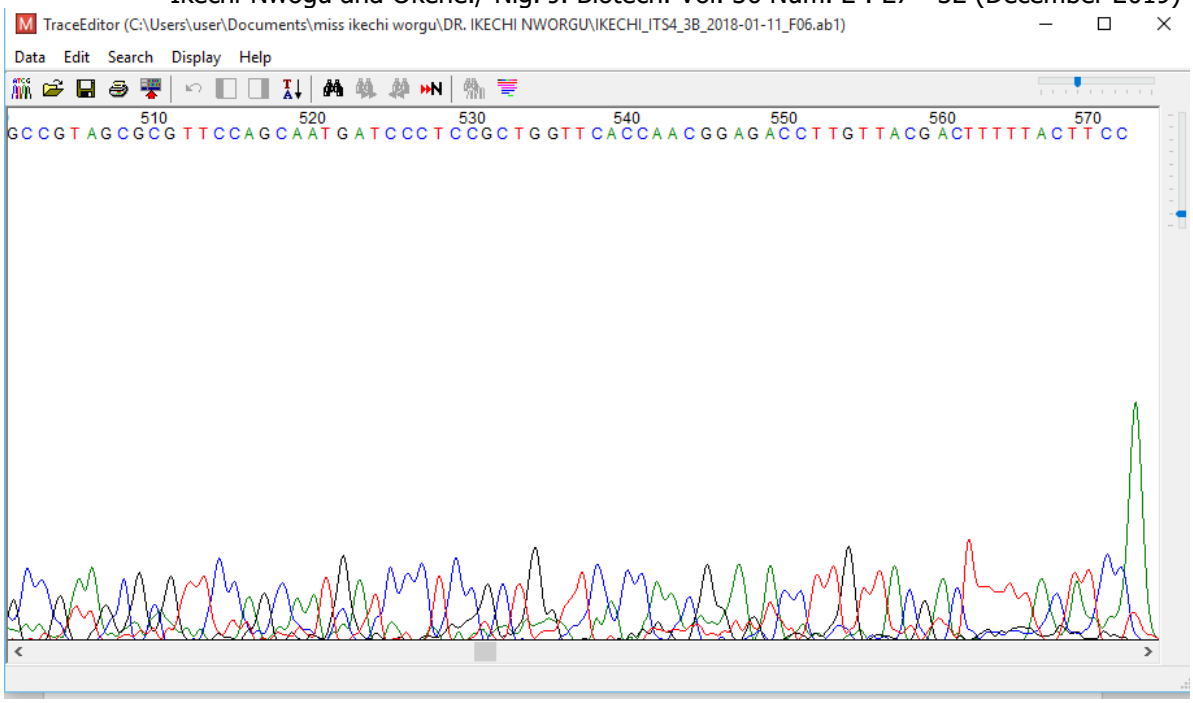

Fig 3: The End Part of Sequence Alignment of the DNA of Soyabean after Alignment

Sequence Alignment

The alignment results are presented in figure 4-5. Figure 4 displayed the alignment scores presented as red lines. The scores of the alignments of all aligned sequences were greater than 200.

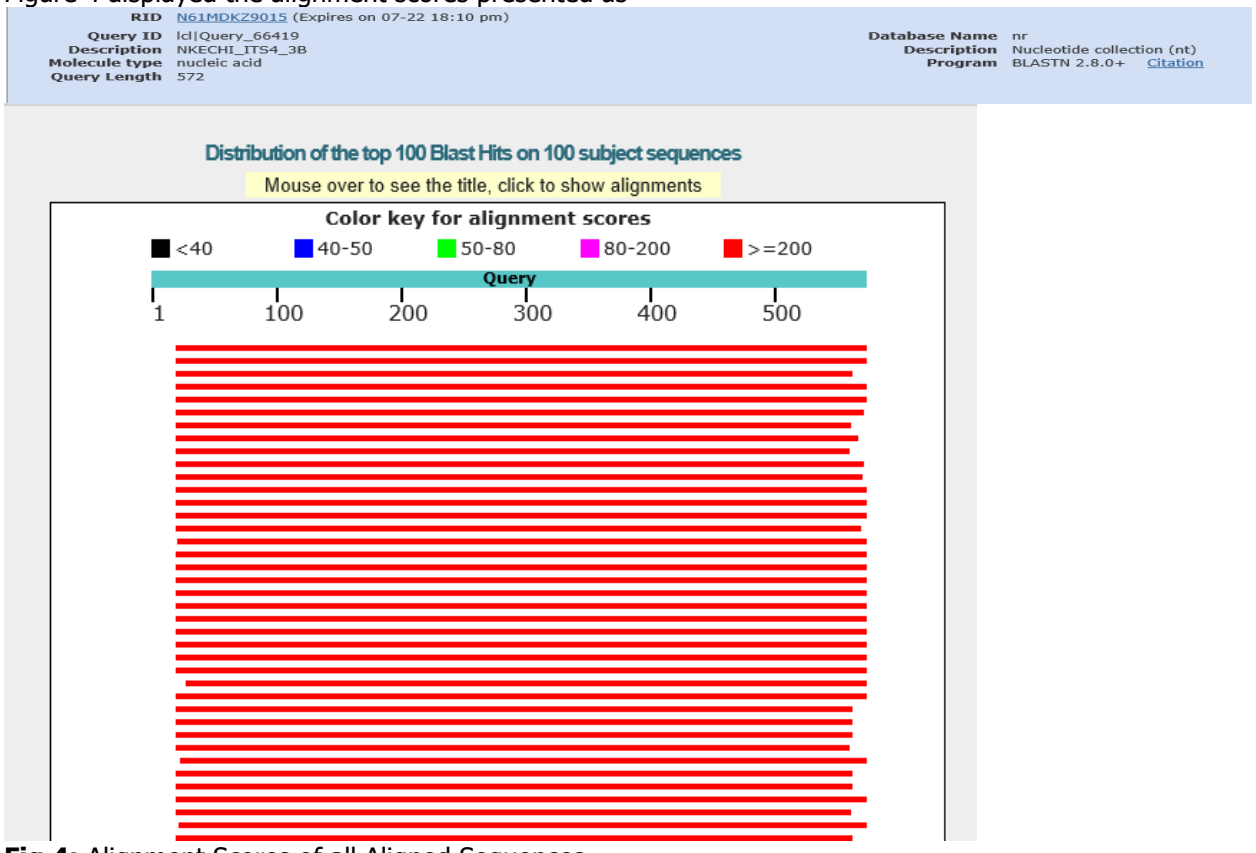

Fig 4: Alignment Scores of all Aligned Sequences

The result of the SB-3B isolate sequence alignments is presented below in Fig. 5. The result indicated that SB-3B isolates sequence aligned with 100 sequences deposited in National Center Biotechnology Information (NCBI) composite biological database. The percentage identity ranged from 97% to 98%. The results showed that the SB-3B isolate sequence was 98% identical to *Diaporthe* spp. Voucher VP51 (red arrow), 98% identical to *Diaporthe schini* isolate L5N71 (black arrow) and 98% identical to *Diaporthe schini* strain B125 (blue arrow). These findings showed that isolate SB-3B is a *Diaporthe* spp. *Diaporthe* spp. is one of the causal pathogens of post-harvest diseases of soybean seeds.

Discussion

Diaporthe belong to the Ascomycota, Pezizomycotina, Sordariomycetes, Diaporthomycetidae, Diaporthales, Diaporthaceae. They have also been recognized as a producer of enzymes and secondary metabolites (Dai *et. al*., 2005) with antibiotic (Lin *et. al.,* 2005) and have anticancer activity (Kumaran & Hur, 2009). Furthermore, *Diaporthe* species in the past, prevent herbivory (Vesterlund *et. al.,* 2011).

The Diaporthe species have often been reported to be plant pathogens, endophytes (they colonize internal plant tissues without causing immediate negative effects) or saprobes fungi, known to cause disease on a wide range of plants hosts that are economically important, causing root and fruit rots, dieback, cankers, leaf spots, blights, decay and wilt (Santos *et. al.,* 2011).

The species of *Diaporthe* occurring on soybean has been the subject of a considerable amount of research and discussion.

According to Sun *et. al.* (2003), in a Short Communication, studied two species of *Diaporthe* (*Phomopsis*) fungi from soybean plants that were identified by morphological and molecular characterizations they concluded that soybeans are known to harbour a complex of *Diaporthe* and *Phomopsis* species. The research by Petrović *et al.* (2015), identified *four Diaporthe* taxa found on soybean: *Diaporthe phaseolorum* var. *sojae*, the causal agent of pod and stem blight; *D. phaseolorum* var. *caulivora* and *D. aspalathi* (formerly referred to as *D. phaseolorum* var. *meridionalis* and they are responsible for serious diseases and significant yield losses.

In this study, *Diaporthe* spp. was found to be one of the causal fungal pathogens of post-harvest diseases of soybean seeds. From the DNA sequence alignment result (Fig. 3), it was observed that the SB-3B isolate sequence was 98% identical to *Diaporthe* spp. Voucher VP51, 98% identical to *Diaporthe schini* isolate L5N71 and 98% identical to *Diaporthe schini* strain B125.

Conclusion

This study will provide information on culturing *Diaporthe* species also provide the basis for further study to show their antibiotic and anti-cancerous, enzymes and secondary metabolites producing ability. It will also increase the knowledge of the fungal species associated with Soybean and enhance disease control, which will increase the production yield.

Diaporthe species not only reduces the quality of seeds for planting by affecting germination potential but also can reduce the quality of seeds which may also reduce flour and oil quality and change the composition of free fatty acids, and affect other quality factors.

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