Isolation and Characterization of Endophytic Fungi From Halophytes in Mangrove Community in AkwaIbomState, Nigeria.

Abstract

This study aimed at the isolation andcharacterizionof endophytic fungi inhabiting halophytes (*Nypafruticans*and*Rhizophoraracemosa*) growing in mangrove ecosystem ofAkwaIbom State, Nigeria. The physico-chemical parameters of the rhizospheric soils revealed EC (5.11, 5.41 and 5.01 dS/m), pH (5.1, 5.0 and 5.2), Na (2.26, 2.28 and 2.15 Cmol/kg) respectively. The identified strains were categorized into the phyla Ascomycota. At the genus level, *Aspergillus* (16 strains) accounted for the highest proportion followed by *Penicillium* (13 strains) and *Fusarium* (8 strains). The genera of the endophytic fungi isolated from the halophytic plants were*Talaromyces, Aspergillus, Neosartorya, Purpureocillium, Fusarium* and *Penicillium*. The six species isolated and characterized were; *Talaromycesalbobiverticillius*(1), *Aspergillus aculeatus*(2), *Aspergillus fumigatus*(3), *Fusariumequiseti*(4)and*Penicilliumcitrinum* (5 and 6). The result from this study demonstrates the existence of fungal endophytes which have great potential as plant productivity. Additionally, this work creates a baseline study in the said study area; this will enable further researches emphasizing the importance of endophytic fungi in agricultural sectors as an eco-friendly biofertilizer to improve plant growth performance and defense as an alternative to inorganic fertilizers which have detrimental effects on both plants and the environment.

Keywords:*Aspergillus,* Endophytes, Fungi, *Fusarium,* Mangrove, *Talaromyces.*

Introduction

Endophytesare an important group of widespread and diverse plant symbionts that live asymptomatically and sometimes systematically within plant tissues without causing symptoms or disease (Promputtha*et al.,* 2005; Porras-Alfaro and Bayman, 2011).Endophytes occupy micro niches within plant tissues and some have been found to be growth-promoting endophytes (Zhou *et al.*,2010; Senthilkumar*et al.*,2011).Endophytic fungi represent an important and quantifiable component of fungal biodiversity in plants that have an effecton plant community diversity and structure (Porras-Alfaro and Bayman, 2011).

Endophytic fungi are the functionally vital members of the plant microbiome, colonizing varied plant species (Porras-Alfaro and Bayman, 2011; U'Ren *et al.*, 2012) causing no visible damage or disease symptoms in their host (Rodriguez *et al.,* 2008). Endophytic fungi are considered as the main source of bioactive compounds and secondary metabolites that have potential applications in various fields such as agriculture, pharmaceuticals, environmental cleaning and the food industry (Strobel *et al.,* 2004; Verma *et al.,* 2009).

Chadha *et al.* (2015) stated that the characterization, diversity and the distribution of fungal endophytes across large geographical areas is still in the beginning, and only some general aspects can be affirmed; such as that the diversity of endophytic fungi is higher in the tropics than in higher latitudes. Additionally, a higher number of endophytic species are found in tropical environments and belong to a small number of classes.The characterisation of the endophytic fungi diversity in a host plant by cultivation-dependent methods is considered limited and can be influenced by several biotic and abiotic factors (Rashmi*et al.,* 2019; Chen *et al.,* 2020). Therefore, the introduction of the use of molecular tools to identify the endophytic community of different plant species has excelled in scientific surveys. The investigation of microbiomes has been performed through mass DNA sequencing from plant material, without needing cultivation and it allows the identification of a great number of uncharacterisedendophytic taxa (Brader*et al.,* 2017).

Endophytes are largely unexplored components of biodiversity especially in the mangroves, and they are constantly exposed to environmental stresses especially in the mangrove. Although populations of endophytic fungi have been isolated and characterized in various parts of the world's Mangrove forests such as in India (Ananda and Sridhar, 2002), Brazil (Costa *et al.,* 2012) and China (Liu *et al.,* 2012), there is little or no available information describing the population of endophytic fungi isolated and characterized from dominant mangrove species in the Nigerian mangrove forest and particularly in Akwa Ibom State, which means there is a good opportunity of undocumented biodiversity loss as well as possibilities of finding new endophytes that colonize plants in different mangrove niches and ecosystems in Akwa Ibom State. Thus, this study aimed atthe isolation andcharacterizionof endophytic fungi inhabiting halophytes (*Nypafruticans*and*Rhizophoraracemosa*) growing in mangrove ecosystem ofAkwaIbom State, Nigeria.

Materials and Methods

Study Location

The plant samples (*Nypa fruticans* and *Rhizophora racemosa*) and rhizospheric soil samples were collected from Okorombokoh, Eastern Obolo Local Government Area, Akwa Ibom State and were brought to Microbiology laboratory and Soil Science laboratory, Akwa Ibom state University for preliminary culturing for fungal isolates and analysis of the physicochemical properties of the soil samples.

Physico-chemical Properties of Experimental Soils

Soil samples were analyzed following the standard procedures outlined by the Association of Official Analytical Chemist (AOAC, 2005) procedure for wet acid digestions.

Plant surface sterilization

Roots of N*ypa frutican* and *Rhizophora racemosa* were separated from the adhering soil, washed with 2% NaCl, dried using sterile filter paper, and weighed to establish biomass. The surface sterilization of roots was carried out using 70% ethanol (2 min), followed by washing with sterile 2% NaCl (three times), sterilized with 15% of H_2O_2 (5 min), and finally washed with sterile 2% NaCl (three times). The solutions obtained after the final washing (for each analyzed sample, 6 in total) were evaluated for surface sterilization efficiency by plating on agar and monitored for microbial growth. Only successfully sterilized root material was used for further analysis. From each sample (6 in total), 1 g of fresh biomass of sterile roots was stored in 2-ml Eppendorf tubes and lyophilized (Hrynkiewicz *et al.,* 2010).

Isolation of Fungi

The shoots and roots were dried using sterile filter paper and ground to a fine paste in a mortar. A series of serial dilutions were performed. Using the pour plate technique 1 ml suspension was mixed with potato dextrose agar (PDA) (Difco, BD Biosciences) supplemented with tetracyclin (30 µg/ml). To increase the probability of obtaining most of the culturable endophytic fungi, PDA was amended with different salt concentrations (0 mM, 100 mM and 200 mM NaCl). All the dilutions and plating were prepared in three replicates. The plates were incubated at 24 ± 2 °C and observed regularly for fungal growth and isolates were selected (selection was based on the colony morphological features such as colour, margin, mycelium form and microscopic slide preparations as described in Germain and Summerbell (2010) and transferred to fresh PDA plates during this period.

Molecular Characterization

The molecular analysis was carried out at the Genomics Training Center and Laboratory Limited, Uyo, Akwa Ibom. The fungi samples were labeled for easy identification and 60 mg that had been suspended in 200 ul of water were weighed.

DNA Extraction Protocol

The DNA extraction was carried out according to the manufacturer's protocol (Zymo Research Quick-DNA Fungal and Bacteria Kit). 60mg of fungal isolates was added to a ZR BashingBead lysis tube, also 750 ul of BashingBead buffer was added to the tube.A bead beater was used at maximum speed for not less than 5 minutes. The ZR BashingBead lysis tube was centrifuged in a microcentrifuge at 10,000g for 1 minute. Then up to 400 ul of the supernatant was transferred to a zymo-spin filter in a collection tube and centrifuge at 8,000g for 1 minute. 1,200 ul of genomic lysis buffer was added to the filtrate in the collection tube from the last step. 800 ul of the mixture from step 5 was transferred to a zymo-spin IICR column in a collection tube and centrifuge at 10,000g for 1 minute. The flow was discarded through and the last step repeated. Then 200 ul DNA pre-wash buffer was added to the zymo-spin ICCR column and centrifuge at 10,000g in a new collection tube for 1 minute. 500 ul g-DNA wash buffer was again added to the zymo-spin ICCR column and centrifuged at 10,000g for 1 minute. The zymo-spin ICCR column was then transferred to a clean 1.5 ml microcentrifuge tube and 100 ul DNA elution buffer was added directly to the column matrix and centrifuged at 10,000g for 30 seconds to elute the DNA.

DNA Quantification

DNA concentration was determined by using a Spectrophotometer (Gene Quant Pro). The concentration of DNA was 380 ng. The absorbance of total genomic DNA (gDNA) was quantified by measuring optical density (OD) at 260 nm and 280 nm. The concentration of DNA calculated from the absorbance at 260/280 nm as shown in the table below:

Table 1: DNA Concentration and Purity of Fungi Samples

Sample ID	DNA Concentration (ug/ul)	DNA Purity (260/280)
1	337	1.82
2	385	1.89
3	348	1.80
4	351	1.88
5	334	1.90
6	405	183

Gel Electrophoresis

The presence and quality of gDNA was also evaluated by agarose gel electrophoresis. DNA was quantified on 1.5 agarose gel. Electrophoresis was conducted in a 1X TAE (Tris-base glacial acetic acid, EDTA) gel buffer at 120 volts for 20 minutes. The gel was stained with 7 ul of Safe View dye. After the gel electrophoresis ran for 20 minutes, the TAE buffer was drained off the gel. The gel was then visualized under a UV trans illuminator.

PCR Amplification

PCR Reagents

The PCR master mix contains PCR amplification buffer, MgCl, DmSO, DNTPs and Taq polymerase. Other reagents include forward primer, reverse primer, ddH_2O and template DNA.

Cocktail Mix

The PCR final reaction volume was made up to 25 ul, using 12.5 ul of One Taq master mix, 0.5 forward primer, 0.5 reverse primer, 2 ul of sample and 9.5 nuclease free water.

PCR Conditions

PCR reaction conditions conducted at BIO-RAD thermocycler, were the following: initial denaturation at 94^{o}C for 30 seconds, 30 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 51^{o}C for 30 seconds, initial elongation at 68^{o}C for 1 minutes and final elongation at 68^{o}C for 5 minutes. Amplicons were separated on 1.5% agarose gel electrophoresis for 20

minutes at 120 V. DNA ladder of 100 bp was used as molecular weight pattern.Each amplicon was sequenced on a 3737xl genetic analyser at genewiz (USA).

Multiple Sequence Alignment

Each Sequence was blasted against the NCBI database to retrieve similar sequence. Search result that were >= 97% were selected and their sequences were retrieved from genebank. The sequences were assigned to a taxa based on the BLAST result. 10 sequences from the *Aspergillus* and *Penicillium* genus was added. ClustalW was used to conduct multiple sequenced alignments on Mega X software with default parameters.

Phylogenetic Analysis

Multiple sequence alignment was done on all the fragments using ClustalW algorithms. *Yarrowialipolytica* was used as the out-group. A maximum likelihood Phylogenetic tree was constructed on Mega X according to the Tajima-Nei model. Relative branch support was evaluated by 1000 bootstrap replicates, the branch lengths were calculated by pairwise comparisons of genetic distances, and missing data were treated by pairwise deletions of gaps. Phylogenetic studies were carried out using the Clustal W software (Larkin *et al.*, 2007) for sequence alignment and MEGA version 5.2 (Tamura *et al.*, 2011) for phylogenetic tree construction.

Results and Discussions

The physico-chemical parameters of the rhizospheric soils revealed EC (5.11, 5.41 and 5.01 dS/m), pH (5.1, 5.0 and 5.2), Na (2.26, 2.28 and 2.15 Cmol/kg) respectively (Table 2).

Table 2: Physico-chemical Properties of the Rhizospheric Soil Samples

S/N	Sample no.	Dept (cm)	pH	Particle size distribution (%)			Texture	%OC	%OM	%TN	Ex. Cations (Cmol/kg)				ECEC (Cmol/kg)	%BS	P (Mg/kg)	TEA	Al3	H+	EC (dS/m)
				Sand	Silt	Clay					Ca	Mg	Na	K							
1	A	0-15	5.1	76.0	7.0	17.0	LS	6.02	-	0.41	0.55	0.85	2.26	0.72	5.35	44	9.65	2.97	1.98	0.99	5.11
2	B	0-15	5.0	76.3	6.7	17.0	LS	6.01	-	0.40	0.51	0.80	2.28	0.74	5.22	45	9.62	2.89	1.95	0.94	5.41
3	C	0-15	5.2	81.0	8.9	10.1	LS	6.06	-	0.43	0.40	0.91	2.15	0.46	3.22	58	9.77	1.40	1.13	0.27	5.01

Ex – Exchange, ECEC – Effective cation exchange capacity, EC – Electrical conductivity

Results gotten from this study showed that fungal samples subjected to genome sequencing for confirmationwere identified by obtaining the nucleotide sequences. The fungal-specific ITS4-ITS5 universal primers pair was used in amplifying the internal transcribed spacer (ITS) region from the DNA of all the isolates. The amplicon lengths and purity were estimated by gel electrophoresis and found about 600 bp in size (Figure 1 and 2). The sequences were analyzed and were compared with available sequences from the NCBI database. The sequences showing 99% -80% similarity were retrieved by Nucleotide Basic Local Alignment Search Tool (BLASTn) program available at the National Center for BiotechnologyInformation (NCBI) BLAST server (www.ncbi.nlm.nih.gov/BLAST). The numerical codes before the species names refer to their accession number at the GenBank (NCBI) (Table 3).

Table 3: Summary ofIdentifiedEndophyticFungalIsolatesfrom the roots of mangrove plants

Samples	Accession number	Name	% Similarity
1-FungiITS4F	MZ227499.1	*Talaromycesalbobiverticillius*	80
2-FungiITS4F	MT422091.1	*Aspergillusaculeatus*	94.788
3-FungiITS4F	MH865336.1	*Aspergillusfumigatus*	80.679
4-FungiITS4F	NR_121457.1	*Fusariumequiseti*	93.348
5-FungiITS4F	NR_121224.1	*Penicilliumcitrinum*	97.714
6-FungiITS4F_R	NR_121224.1	*Penicilliumcitrinum*	100

Constructing phylogenetic tree is crucial in molecular identification, since BLAST search alone cannot overcome possibilities of statistical errors. Bootstrap consensus is applied to the constructed tree so as to read maximum sequence replications. Neighbour joining tree with bootstrapping gave a clear picture for identifying the six (6) fungal isolates. And since the % similarity fell within 80-100% and more,BLAST hits shows the samples belonged to the genera, thus strongly recommending the isolates as a members of the groups as shown in Table 3 and Figure 3.

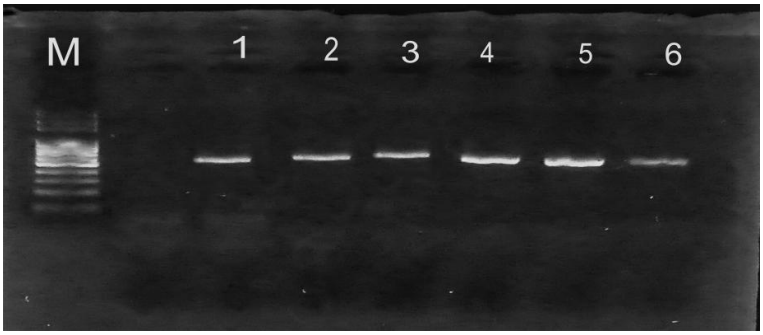

Figure 1:PCR product for fungal isolates. M = 100bp ladder

PCR provides a sensitive and specific method of identifying a known DNA sequence, and the PCR product is of a lighter band compared to the DNA, hence the flow in Figure 1.

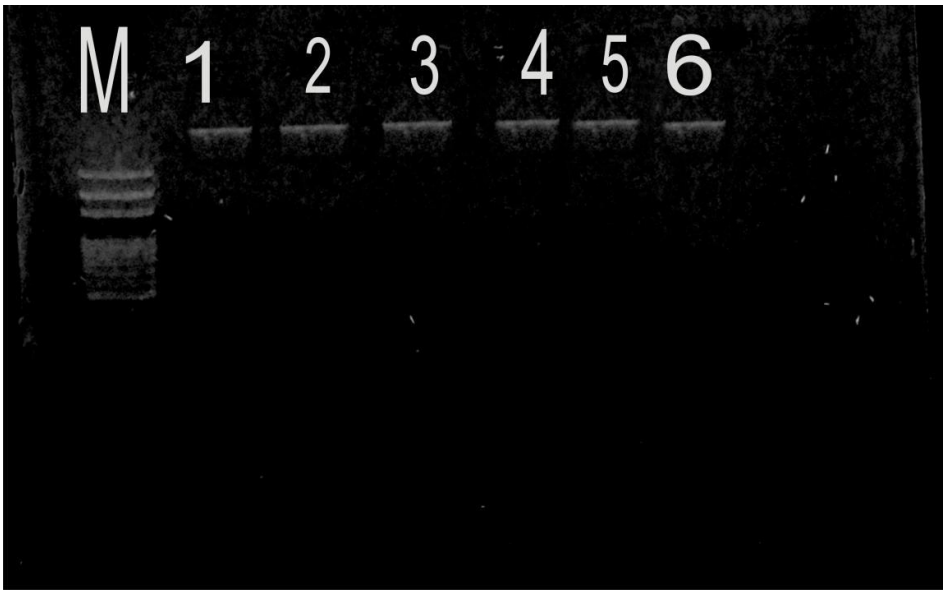

Figure 2: Isolation of Genomic DNA from fungal isolates and their ITS Amplification in Gel. (DNA ladder product for six fungal isolates. M = 100-bp ladder)

These DNA products showed a bit of diversity on the gel. And also there was variability on the sizes of the DNA (Figure 2). Some had bigger fragments compared to others. Genetic variability analysis based on sequences variation in ITS region of all the isolates showed the existence of either insertions or deletions of the nucleotides in the ITS region has led to the observed the variation in a clade-specific manner.

The sequences of the genomic regions of ITS, revealed slightly different phylogenetic structures and all the isolates is grouped into 11 clades in the derived dendrogram. Sample 2 and Sample 4 forms a distinct cluster but sample 1 was found in the same cluster as ascensions of *Asperagillus aculeatus.* Sample 3 belong to a clade with *Asperagillus flavus,* and *Neosartoya sp*. While sample 5 and 6 belongs to the same with *Penicillium* species (Figure 3).

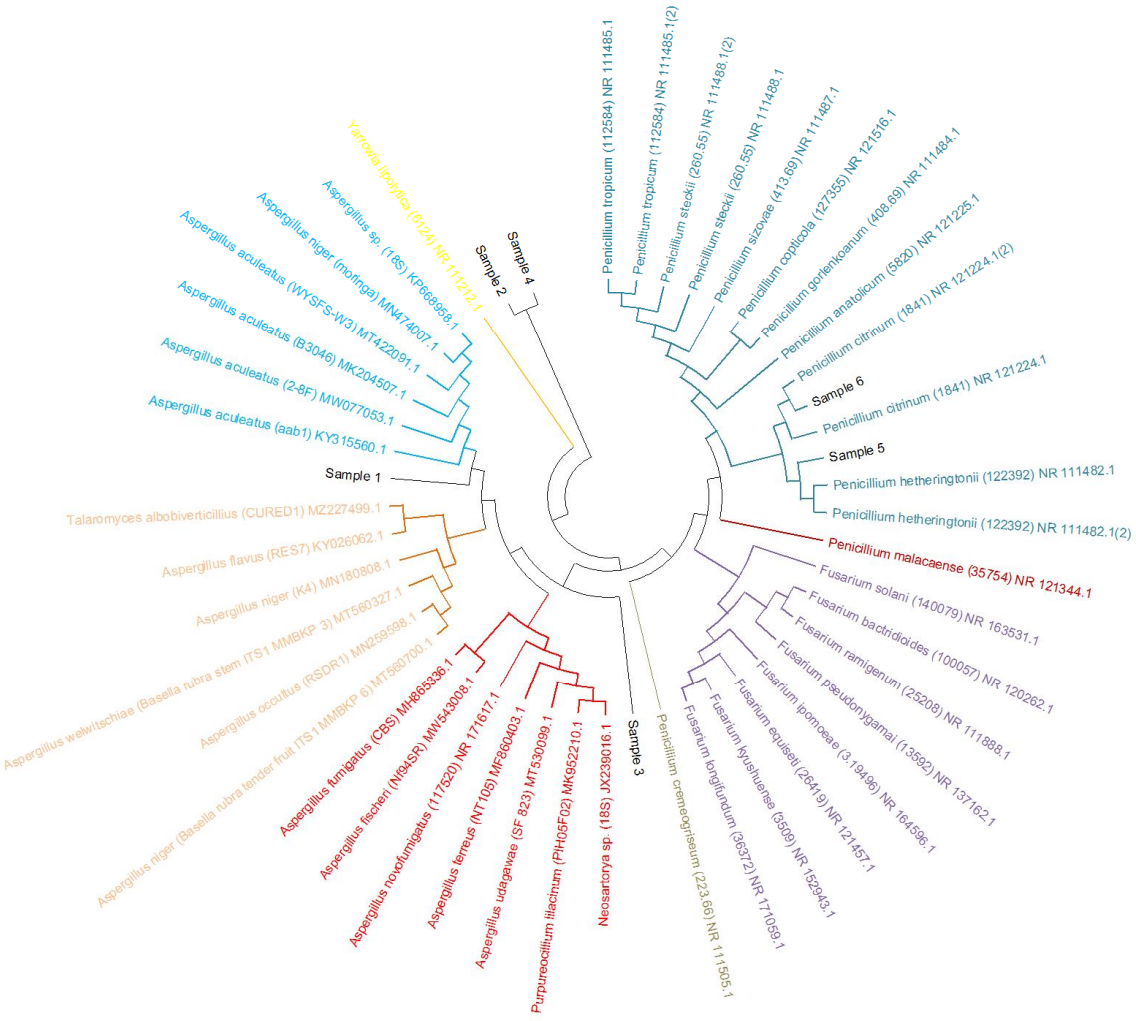

Figure3: The evolutionary history of the fungal isolates. This was inferred by using the Maximum Likelihood method and Tamura-Nei model.

The tree with the highest log likelihood (-1386.98) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. This analysis involved 48 nucleotide sequences. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option).

There were a total of 178 positions in the final dataset. Evolutionary analyses were conducted in MEGA X . The tree was rooted to *Yarrowialipolytica*. Bootstrap replication (1000 replications) was used for a statistical support for the nodes in the phylogenetic tree.Although many phylogenetic trees exist and are used for analysis, neighbour-joining method has been designated most reliable tree construction method especially when dealing with closely related strains under varying rates of evolution (Guo*et al.,* 2001; Yang and Khuri, 2003).

The identified strains were categorized into the phyla Ascomycota. At the genus level, *Aspergillus* (16 strains) accounted for the highest proportion followed by *Penicillium* (13 strains) and *Fusarium* (8 strains). In the present study, the majority of the isolated fungal endophytes belonged to the phylum Ascomycota. The genera of the endophytic fungi isolated from the tested halophytic plants were*Talaromyces, Aspergillus, Neosartorya, Purpureocillium, Fusarium* and *Penicillium*. These endophytes could play a role in plant development according to previous studies. Members of the genus *Penicillium* were represented in most of the studied plant samples.

Conclusion

Research done to date shows that fungal endophytes have various beneficial effects on their host plants and these fungal endophytes can be a significant component of sustainable agriculture, being safe, cost-effective, have ability to produce various compounds like phytohormones, defensive compounds, solubilize phosphates, extracellular enzymes, siderophore production, inhibiting plant pathogens, and promoting plant growth.The results from this study demonstrated the existence of fungal endophytes which have great potential as plant productivity. Additionally, this work creates a baseline study in the said study area, this will enable further researches emphasizing the importance of endophytic fungi in agricultural sectors as an eco-friendly biofertilizer to improve plant growth performance and defense as an alternative to inorganic fertilizers which have detrimental effects on both plants and the environment.

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