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Original paper

PCR-ITS-RFLP identification of pineapple spoilage fungi

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Abstract

The fungi associated with the spoilage of pineapple fruits in Abidjan market were studied. Spoilt and healthy pineapple fruits were used in the study with potatoes dextrose agar as the culture medium while the pour plate technique was employed in the fungal isolation. Restriction digestion analysis of the ITS products was tested as a simple method to identify isolates of filamentous fungi on pineapple fruits. Endonucleases *Sdu*I, *Hinf*I, *Hha*I, *Nla*III, *Hae*III, *Rsa*I were used. The studied 10 species generated different composite profiles.

Different fungal strains responsible for the spoilage were isolated from various markets in Abidjan and identified by this method. *Aspergillus* was the genus the most frequently isolated, while the genus *Penicillium* was absent. *Aspergillus aculeatus* was the most isolated species of *Aspergillus*. The *Candida sp.*, *Rhizopus oryzae* and *Geotrichum candidum* were also isolated.

To the best of our knowledge, is the first report of the presence of *Neurospora tetrasperma* on pineapple. This technique is a rapid and reliable method appropriate for routine identification of fungi. This can be used to screen large numbers of isolates from various environments in a short time. This is the first exhaustive study of fungal diversity at species level in pineapple fruits.

Keywords Pineapple, spoilage, moulds, PCR-ITS-RFLP

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Introduction

Pineapple (*Ananas comosus*) is a vegetative propagated fruit crop. It is one of the few crops in which cultivars are derived from spontaneous mutations and natural evolution without controlled breeding (F. OSEI-KOFI & al. [1]). Pineapples are rich in sugars important health nutrients but their low pH values make them particularly desirable to fungal decay. Ripening pineapple fruits are susceptible to microbial infection by a variety of species including fungi. In high production countries, losses are often severe due to inadequate storage and transportation facilities (S. DROBY[2], L. G. B.KHALI & K. B. MAZHER [3]). It has been reported (S. DROBY [2] & S. J. ZHU [4]) that about 20-25% of the harvested fruits are lost via pathogens attack during post-harvest chain. Pineapple infection may occur during the growing season, harvesting, handling, transport, post-harvest storage and marketing conditions or after purchasing by the consumer. (L. G.B. KHALI & K.B. MAZHER [3]). Many fungal species are capable of producing mycotoxins, which are secondary metabolites that are highly toxic to humans and animals (R.R AL-HINDI & al. [5]). The consumption of pineapples has been on the increase in Côte d'Ivoire. This is so because they are easily accessible, nutritious and relatively cheap (E. NWACHUKWU & al. [6]). The increase in consumption has been linked with a parallel increase in foodborne illnesses (P. MENSAH & al. [7]). Pineapple fruits are processed and sold by unlicensed street vendors with poor education and lack of training in food hygiene (N. BARRO & al. [8]). These fruits are usually displayed on benches and in baskets for prospective customers in the open markets until sold, thereby exposing them to further fungal infections. The most frequent fungal genera reported to be on pineapple fruits are *Rhizopus*, *Aspergillus*, *Fusarium*, *Penicillium* and *Candida* (T.S. EWEKEYE & al. [9], S.C. ONUORAH & al. [10]). However, the diversity of fungi on pineapple fruithas not been extensively investigated at species level.

Cultivation-dependent methods with macroscopic and microscopic examination are traditionally used to identify filamentous fungi, but this may fail to identify the complete diversity of fungi present. Morphological and physiological characteristics are influenced by culture conditions and consequently this approach can provide incomplete or ambiguous results. Moreover, these methods are also time consuming and laborious. Therefore, numerous molecular methodologies have been developed for more specific identification. Among those techniques, there are: random amplified polymorphisms DNA (RAPD) method, Restriction Fragment Length Polymorphisms (RFLPs) technique, Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, Amplified Fragment Length Polymorphism technique (AFLP) or fluorescent AFLP (fAFLP) analysis was used to characterize fungal. PCR-temporal temperature gradient gel electrophoresis (PCR-TTGE) or PCR-denaturing gradient gel electrophoresis (PCR-DGGE) methods were applied to describe fungal communities on pineapple fruits (O-O. CHANPRASARTSUK & al. [11]). Most of the developed methods are based on the analysis of the internal transcribed spacer (ITS) region. ITS sequences including the 5.8S rRNA gene (the coding region which is conserved) and two flanking regions ITS1 and ITS2 (non-coding and variable) show low intraspecific polymorphism and high interspecific variability and have proved useful for identification of different fungi and yeasts (F. ACCENSI & al. [12], B. ESTEVE-ZARZOSO & al. [13], S. LA GUERCHE & al. [14], S. LEE & J.W. TAYLOR [15]). Most of the methods described in the literature have been used for the identification of specific fungi present on fruits or in other environments, but few techniques have been developed for describing and identifying in the same time the different classes of fungi. Here, we describe a PCR ITS-RFLP method that we have developed to be a fast and easy method for identifying species of fungal genera present on pineapple fruits. We also report the use of this method to describe diversity of fungi on pineapple fruits collected from different market in Abidjan (Ivory Coast) and to realize an exhaustive study on fungi on pineapple. The objective of this work is therefore to isolate and identify by ITS-RFLP the fungi associated with the spoilage of pineapple fruits in four Abidjan market, Côte d'Ivoire.

Materials and Methods

1. Pineapples fruits sampling procedure

Samples were taken at the post harvesting stage from various market of Abidjan (Abobo, Yopougon, Plateau, Adjamé), Ivory Coast. Two hundred pineapple fruits (Extra Sweet variety) were collected from each fruits into plastic bags in aseptic conditions and conserved at 4°C before analysis. Pineapple fruits were swabbed in 70% ethanol for 2 min rinsed in two changes of sterile distilled water and the blotted dry with sterile filter papers. Necrotic lesions were aseptically cut, plated on sterile potato dextrose agar (Merck, Germany) and incubated at 28±1°C for 4 days (T.S. EWEKEYE & al. [9]) and each fungal colony considered to represent different genera and species was isolated and conserved on malt-extract agar (MEA). The isolates were screened by macroscopic and microscopic observations (J.I. PITT & A. D.HOCKING [24]) and the identifications were confirmed by PCR ITS-RFLP and sequencing.

2. DNA preparation

All fungal strains were grown on potato-dextrose broth (Difco) at 28ºC for 5 days. Cultures were centrifuged

to collect the mycelium which was frozen in liquid nitrogen and ground to a fine powder. About 150-200 mg of frozen mycelium was used for DNA extraction using the commercial EZNA Fungal DNA kit (Omega bio-teck, Doraville, USA) according to the manufacturer's instructions.

3. PCR reaction and DNA digestions

The 5.8S-ITS region was amplified by PCR using universal fungal primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3', ITS4 5'-TCCTCCGCTTATTGATATGC-3' and ITS5 5'-GGAAGTAAAAGTCGTAACAAGG-3' (T.J. WHITE & al. [16]).

PCR reactions were performed in 50 µl of 1.5 mM MgCl2, 0.2 mMdNTPs, 0.5 µM of each primer, 0.025 U of DreamTaq DNA Polymerase (ThermoFisher Scientific, USA) and 10-30 ng of fungal DNA per reaction. Annealing temperature was described by V. PHALIP & al. [17] and P.V. MARTÍNEZ-CULEBRAS & D. RAMÓN [18].

Amplicons were digested with the restriction enzymes *Sdu*I, *Hinf*I, *Hha*I, *Nla*III, *Rsa*I and *Hae*III (ThermoFisher Scientific, USA). These endonucleases were chosen after analysis of ITS sequences deposited in the EMBL (European Molecular Biology Laboratory) data libraryas allowing to differentiate between isolates.

DNA fragments were visualized under UV (254 nm) after electrophoresis in agarose gel 2%and evaluated using the DNA ladder (GeneRuler 100bp Plus DNA Ladder, ThermoFisher Scientific, USA).

4. DNA sequencing and sequence analysis

Sequencing was performed on 5.8-ITS DNA fragments generated by PCR. The primers ITS1/ITS4 were used. Microsynth, Switzerland *via* Biozyme Romania sequenced the PCR products in both orientations. The nucleotide sequences have been deposited in GenBank database. The sequences obtained were compared with sequences available in the EMBL database using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These sequences were aligned with ClustalW and restriction fragments with the http://biotools.umassmed.edu/tacg4/program. Also, in silico restrictions has been applied by the use of the Harry Mangalam's tacg 4.3 program (http://biotools.umassmed.edu/tacg4). MEGA6 was used to construct phylogenetic tree.

Results and Discussions

1. Fungal isolates

The isolation work has been finalised with the isolation of six different moulds colonies (codified as Asp2015; Asp'2015; Asp2016; Geo2016; Neu2015; Rhz2015) and three colonies of unicellular fungi (yeast like, codified as YP2015; YP'2016; YP2016).

Table 1. Morphological identification of the species

Fungal isolates	Morphological characteristics
Asp2015; Asp'2015; Asp2016	Colonies are dark brown to black; unbranched conidiophores; conidia are ellipsoidal
Geo2016	Colonies are white to cream, flat; hyphae are hyaline, septate and branched
Neu2015	Colonies are fast growing, flat, pale orange; hyphae were broad, hyaline, septate and branched.
Rhz2015	Colonies expanded quickly; grey colour; non fragmented mycelia; columella are globose to sub globose; sporangiospores are globose to ovoid
YP2015	Colonies are cream to yellow; cells are short-ellipsoidal
YP'2016; YP2016	Colonies are, circular white; cells are spherical to ovoid

Applying classical tools of morphological criteria (like color, size, mycelium and spore/cell structures), it was concluded that the six moulds may belong to different genera, like *Aspergillus* section *Nigri, Neurospora, Rhizopus* and *Geotrichum*, while the yeast like colonies showed aspects close to *Candida* sp (Table 1). To confirm these results, molecular tools have been applied in the identification, respectively PCR ITS-RFLP.

2. Results of ITS-RFLP analysis of isolated fungi

The main objective of this work was to identify the fungal strains isolated on pineapple fruits and responsible of spoilage. Identification was based on PCR-RFLP of the 5.8S-ITS region.

The restriction fragments of ribosomal DNA for the strains belonging to *Aspergillus* section *Nigri* after digestion with the endonucleases *Rsa*I and, respectively with *Hha*I is shown in Figure 1.When digesting with *Rsa*I two fragments were obtained (100+500), while applying digestion with *Hha*I led to four different fragments (70+140+180). To have more accurate results, another digestion with *Nla*III was applied which led to two different fragments (230+350) for all the isolates.

The obtained composite profile obtained for the three *Aspergillus* isolates are close to *Aspergillus aculeatus* on specie level. This result agrees with those of P.V. MARTÍNEZ-CULEBRAS AND D. RAMÓN [18] who have reported that these three endonucleases were necessary to differentiate *A. niger, A. tubingensis, A. carbonarius* and *A. aculeatus*. The result has been

confirmedby sequencing. The nucleotide sequence has been deposited in GenBank database: *Aspergillus aculeatus* Asp2016 (MH656795).

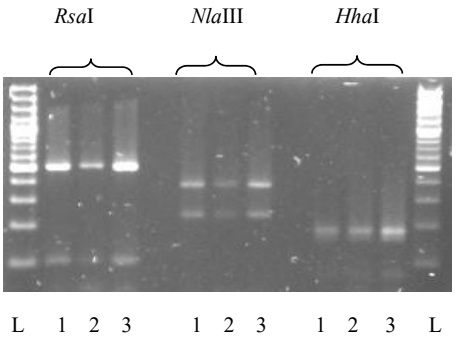

Figure 1. Restriction fragments of ribosomal DNA for *Aspergillus* strains after digestion with *Rsa*I, *Nla*III and *Hha*I endonucleases :**1 -** Asp '2015; **2 -** Asp 2015; **3 -** Asp 2016; **L**-GeneRuler 100bp Plus DNA Ladder

Among *Aspergillus sp., A. Niger* and *A. flavus* were found associated with deteriorating pineapple (*Ananas comosus*) (T.S. EWEKEYE & al. [9], O.O. AKINMUSIRE [23]). To our knowledge, *A. aculeatus* has never been isolated on pineapple.

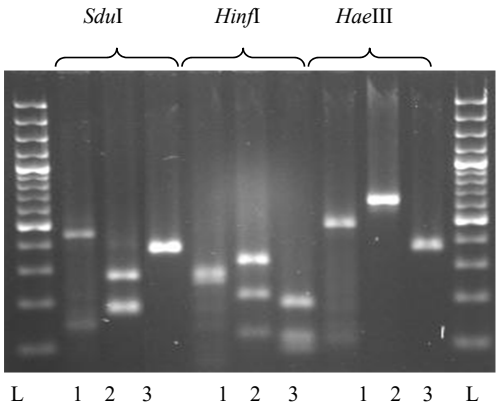

Figure 2. Restriction fragments of ribosomal DNA for fungal strains after digestion with *Sdu*I, *Hinf*I and *Hae*III endonucleases: **1 -** *Neu2015*; **2 -** *Rhz2015*; **3 -** *Geoz2016*; **L**-GeneRuler 100bp Plus DNA Ladder

The restriction fragments of ribosomal DNA for others fungalisolates after digestion with the *Sdu*I, *Hinf*I and *Hae*III endonucleases are shown in Figure 3 and synthesised in Table 2. The PCR-RFLP results have been confirmed by sequencing. The nucleotide sequence has been deposited in GenBank database: *Geotrichum candidum* Geo2016 (MH656796), *Neurospora tetrasperma* Neu2015 (MH656797), *Rhizopus oryzae* Rhz2015 (MH656798).

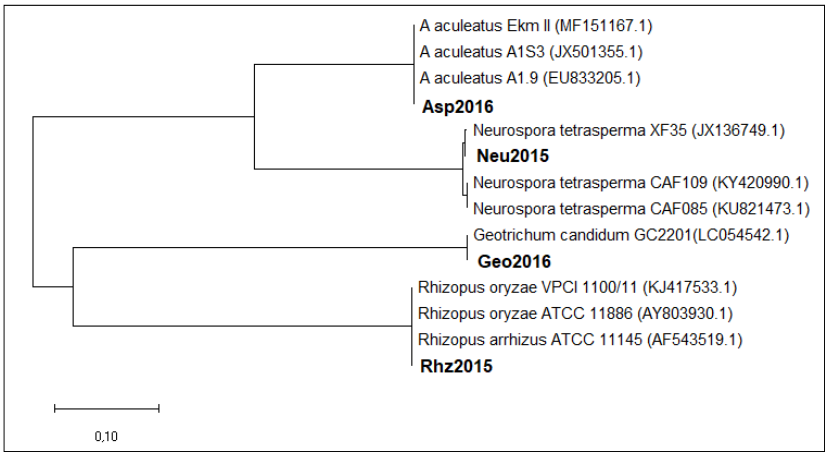

Figure 3. Phylogenetic tree of fungal isolates based on the sequencing 5.8-ITS DNA sequences using the Neighbor-Joining method. Sequences obtained during this study are presented in bold, as well as additional sequences from publicly available model strains with their Accession Numbers. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X.

Initially, the strain Neu2015 has been identified by classical morphological analysis as belonging to *Fusarium* genus [Y. F. KOFFI & al. 25]. In this study, this strain has been re-identified as *Neurospora tetrasperma* by PCR-RFLP and confirmed by sequencing.

Phylogenetic relationships between the fungal strains isolated in this study were constructed using MEGA6 software (Figure 3), including the reference sequences deposited in the NCBI using the BLASTN algorithm for species identification.

Table 2. Restriction fragments of different moulds isolates from pineapple after digestion with different restriction enzymes

No	Fungalisolates	Restriction fragments (bp)		
		***Sdu*I**	***Hinf*I**	***Hae* III**
1.	*Neu2015*	140+450	70+250+270	110+490
2.	*Rhz2015*	180+270	110+200+310	630
3.	*Geo2016*	370	90+110 +180	370

Also, we have used the PCR-RFLP analysis of the 5.8S-ITS region for yeast identification, as developed by B. ESTEVE-ZARZOSO & al. [13]. In our study, the restriction fragments of ribosomal DNA for the yeast strains after digestion with three endonucleases (*Hha*I, *Hinf*I and *Hae*III) led to two different composite profiles (Figure 4 and Table 3), which indicate the presence of two different species. Based on RFLP type, YP2015 isolate yielded the restriction pattern described for *Pichia guilliermondii* (anamorph as *Candida guilliermondii*) (B. ESTEVE-ZARZOSO & al. [13]). The restriction patterns of YP'2016; YP2016yielded described for *Pichia kudriavzevii.* Recently, A.P. DOUGLASS & al. [26] showed conclusively that pathogenic *Candida krusei* and environmental *Pichia kudriavzevii* are the same species, with collinear genomes 99.6% identical in DNA sequence. Pathogenic *Candida* species (such as *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis, C. guilliermondii* and *C. krusei*) cause invasive candidiasis at the immuno-suppressive patients (N. YAPAR, [27]). Future work will be focused on validation of these results by sequencing analysis, as well as to the pathogenic potential of the *Candida* isolates.

Table 3. Restriction fragments of different yeast isolates from pineapple after restriction

	***Hha*I**	***Hinf*I**	***Hae*III**
YP2015	300+270	320+300	400+110+90
YP'2016; YP2016	230+200	210+150	380+90

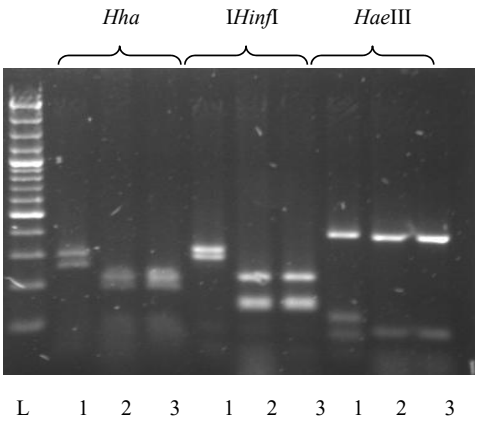

Figure 4. Restriction fragments of ribosomal DNA for fungal strains after digestion with *Hha*I, *Hae*III and *Hinf*I endonucleases: **1 -** YP20155; **2 -** YP'2016; **3 -** YP2016; **L**-GeneRuler 100bp Plus DNA Ladder

We used PCR ITS-RFLP method to identify the fungal strains isolated from pineapple fruits from different markets in Abidjan, Ivory Coast. Microbial population of pineapple fruits is important from a technological point of view because it determines the quality of products. If the ecology of yeasts has been studied extensively, few studies have focused on filamentous fungi of pineapple fruits. A better knowledge of the fungal diversity on pineapple fruits, particularly as concerns species responsible for spoilage, may help efforts to control their development (G.A. Stefanoiu et al., [28]). Y.F. KOFFI & al. [25] reported that the all fungal strains isolated in this study are responsible for the pineapple fruit rotting. *Aspergillus aculeatus* was dominant among our isolates. *Penicillium* species are more common in temperate and cold climates such as in northern Europe, whereas *Aspergillus* species are commonly associated with warmer and tropical regions (J. I. PITT & A. D. HOCKING [21], R. SERRA & al. [22]). Indeed, no strain of *Penicillium* was isolated in this study. T.J. EWEKEYE & al., [9] observed a similar distribution in other market in Nigeria. The strains *Candida* spp. and *Rhizopus* spp. were the most frequently isolated micro-organisms other than members of the genus *Aspergillus*. This is consistent with previous works which indicate that five genera are frequently identified on pineapple fruits: *Aspergillus* spp., *Candida* spp., *Penicillium* spp. and *Rhizopus* spp. (T.J. EWEKEYE & al. [9], S.C. ONUORAH [10], O.O. AKINMUSIRE [23]).

Nevertheless, culture independent methods, such as PCR-TTGE or PCR-DGGE (O-O. CHANPRASARTSUK, [11]), do not reveal genera and species other than those we

have identified. This is the first study of fungal diversity on pineapple fruits using a reliable and rapid molecular method. Moreover, this work has contributed to the enriching of the database of fungal ITS sequences.

Conclusions

Strains of fungi responsible for the spoilage were isolated from various markets in Abidjan and identified by PCR ITS-RFLP method. *Aspergillus* was the genus the most frequently isolated and no strains of the genus *Penicillium* was isolated. *Aspergillus aculeatus* was the most isolated species of *Aspergillus* group. The species *Candida sp, Rhizopusoryzae* and *Geotrichum candidum* were also isolated. On our knowledge, is the first report of the presence of *Neurospora tetrasperma* on pineapple. In conclusion, the PCR ITS-RFLP method we describe facilitates rapid and easy identification of fungal species isolated from pineapple fruits without sequencing. This assay is a routine, sensitive and reliable compared to morphological identification and can be used to screen vast numbers of isolates in a short time. Consequently, it could be very useful for studies comparing large samples of isolates where sequencing cannot reasonably be undertaken. This method could be applied to study the impact of pesticide treatments or cultural practices on the fungal flora of pineapple fruits. Moreover, this method allows thorough investigations of diversity of filamentous fungi on pineapple fruits, and particularly the distribution and the identification of *Aspergillus* species responsible for mycotoxins.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by allauthors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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