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Morphological and molecular characterization of three fungi strains isolated from locals foods in Burkina Faso

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Abstract

In this study we aims to characterize three fungi isolates S_1, S_2 and S_3 respectively collected from maize, groundnuts and rice. These isolates have antibacterial activities against two (2) Gram negative and two (2) Gram positive bacteria. Firstly identification was performed using phenotypic methods including macroscopic colony characteristics and microscopic morphology by identification key. Secondly, molecular method was used by two (2) PCR based on 28S ribosomal sub unit (D1-D2 region) and the hyper variable ITS1-5.8S-ITS2 region. The morphological characterization of the isolates has been confirmed by molecular characterization. Phylogenetic trees obtained allowed us to clearly identify and classify the isolates. Indeed, according to phylogenetic trees fungi isolates S_1, S_2 and S_3 collected from local food in Burkina Faso were in the same clusters to those whose sequences are from to GenBank. As indicated by the results, isolates S_1, S_2 and S_3 are respectively *Aspergillus fumigatus*, *Aspergillus flavus* and *Penicillium citrinum*.

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Introduction

Fungi are multicellular eukaryotes, heterotrophs and ubiquitous organisms which several importance in medicine, agriculture and biotechnology. *Aspergillus* and *Penicillium* species represented the best source of biologically active metabolites. Theses molds are capable to produce many types of drugs like penicillin, fumagillin, compactin, kojic acid and citrinine with medical used. There are several methods available for taxonomic classification of fungi. Traditional methods for species identification are mainly based on morphological parameter, including colony diameter, growth time, colonies colors, diameter and texture of colonies, length and structure of conidiophores and the hyphae texture (Compaoré *et al.*, 2016a). The genus *Aspergillus* and *Penicillium* were identified using respectively Raper and Fennell (1965) and Pitt (1985) keys. In certain cases, traditional identification through morphologic characterization takes days to weeks of laborious, time-consuming work and requires significant technological expertise (Goltapeh *et al.*, 2007). Therefore fast and precise molecular methods has been developed and strongly used to discriminate several species in fungi kingdom. Nevertheless, the both method used together makes best identification of filamentous fungi (Lamrani *et al.*, 2008).

Molecular taxonomy of filamentous fungi was generally based on genes that encode ribosomal RNA (Frisvad *et al.*, 1998). Discrimination between species was rather based on the sequences of the region Internal Transcribed Spacers (ITS) because its non-coding and variable regions depending on the species (Chen *et al.*, 2001). According to Schoch *et al.* (2012), 28S region D1-D2 was best domain to makes comparison among *Aspergillus* and *Penicillium* species.

In this study three (3) isolates fungi collected from local foods were characterized on the basis of morphology and phylogenetic characters using two (2) PCR. We determined both the ITS1-5.8S-ITS2 and D1-D2 regions of the 28S nrDNA for all isolates.

Materials and methods

Sampling and fungi isolation

Five samples of maize, eight samples of groundnuts and four samples of rice were respectively collected from centers markets of Ouagadougou, Bobo Dioulasso and Ouahigouya and stored at 4 °C until analysis.

Samples were cultured on Potato Dextrose Agar (PDA) medium and incubated at 30 °C until 7 days. A total of one hundred to twenty five (125) fungi isolates were collected from local food. They were used in the antibiosis trial. Among them three isolates S_1, S_2 and S_3 were capable to produce antibacterial compounds against both Gram negative and Gram positive bacteria used as indicators strains (Compaoré *et al.*, 2016a; Compaoré *et al.*, 2016b). They were characterized using morphological and molecular methods.

Fungi characterization

Morphological method

The identification was based on the conventional key of Raper and Fennell (1965) for *Aspergillus* and of Pitt (1985) key for *Penicillium.* Thus the main criteria used are growth time, colonies colors during incubation time, diameter and texture of colonies. This identification was completed with microscopic characteristics for example length of conidiophores, the hyphae texture and the number of divergent metulae in a whorl. The number of phialides which bearing conidia was also described for tentative identification (Compaoré *et al.*, 2016a).

Molecular method

Molecular identification of fungi isolates was carried out in two (2) PCR to amplify the ITS1-5.8S-ITS2 and D1-D2 regions of the 28S nrDNA.

DNA extraction from cultures

For fungi DNA extraction, isolates were sub cultured on Malt Extract Agar (MEA) medium and incubated at 30 °C until two or three days. The extraction was carried with a PrepMan Ultra Sample preparation reagent. Shake the reagent and let it settle until all the

bubbles disappeared. The edge of the filamentous fungi mycelia was removed with a loop and then introduced into 100 µl of reagent contained in a microcentrifuge screw-cap tube. The mycelium suspension was then vigorously vortex for 10 to 30 seconds and then boiled at 100 °C for 10 minutes in a heat block. The microtube was then allowed to cool to room temperature (±28 °C) for 2 minutes. The suspension was centrifuged at 10000 rpm for 2 minutes. Transfer 50 µl of the supernatant from the spin tubes into a second set of labeled-microcentrifuge screw-cap tubes and discard the remaining supernatant. The DNA solution was stored at - 20 °C until use. Before use, thaw, then vortex and centrifuge the stored supernatant.

D1-D2 and ITS1-5.8S-ITS2 regions amplification

Each PCR reaction contained2 µl of DNA extract, 2 µl of each primer, 0.6 µl of each dNTP, 0.2 µl of Fast Start *Taq* DNA polymerase, 2.5 µl of 10 X PCR buffer, 2.5 µl of 10 X GC Rich solution and 1.5 µl $MgCl_2$ adjusted to the final volume of 25 µl. The profile applied for amplification was as: 1 cycle of 5 min at 95 °C; 40 cycles of 30 sec at 95 °C, 30sec at 58 °C, 1 min at 72 °C; 1 cycle of a final extension for 10 min at 72°C. Amplification products were separated on a 0.8% agarose gel by electrophoresis, incubated with ethidium bromide, visualized and photographed under UV light.

Phylogenetic analysis and nucleotide sequence similarity

Sequencing of amplicons was performed by Genoscreen (Lille, France). The generated sequences were corrected with Chromas Lite 2.1.1 software and assembled with the Sequencer version 4.7 software. The similarity search of ITS1-5,8S-ITS2 sequences and those of the D1-D2 region with other sequences of fungi species was done by comparison with known sequences listed in the GenBank databases using Blast software version 2.2.31.

The phylogenetic trees was constructed with Seaview Version 4 software (Gouy *et al.*, 2010) from DNA sequences (ITS1- 5.8S-ITS2 and D1-D2) using PhyML method (Maximum Likelihood Phylogenetic) and robustness of the trees was evaluated by 100 bootstrap replicates.

Results

Morphological identification

Macroscopic characteristics

All fungi isolates grew well even up to 30 °C on PDA and MEA medium. Isolate S_1 can growth at 50 °C. They easily produce superficial and submerged hyphae with fruiting bodies. Colonies of isolates S_1 and S_2 attained 35 to 45 mm, as diameter in 7 days of incubation and its color changed with the incubation time.

Table 1. Primers of amplification of fungi ITS1-5.8S-ITS2 and D1-D2 regions.

Primers	Sequences (5'-----3')	Amplified regions	References
ITS1	TCCGTAGGTGAACCTGCGG	ITS1-5,8S-ITS2	(White *et al.*, 1990)
28SUniv	ACTTGTTCGCTATCGGTC		
NL1	GCATATCAAGCGGAGGAAAAG	D1-D2	(O'Donnell, 1992)
NL4	GGTCCGTGTTTGAAGACGG		

Those of isolate S_3 reached 15 to 20 mm. Isolate S_1 colonies were firstly white, blue- green and thereafter green dark to blackish-gray with a yellow reverse. Isolate S_2 colonies were firstly white, yellow-green thereafter dark green above all in the center where texture was velvety and denser, with yellow reverse. As for the isolate S_3, its colonies with a regular margin were white in the center and green dark frame, with yellow reverse, they produce exudate (Fig. 2.).

Microscopic characteristics

In optical microscopy, isolate S_1 showed numerous round and refractive conidia. Its hyphae were septate and hyaline. Conidial heads were uniseriate with compact columnar, up to 100 µm long. Conidiophores were upright, simple, uncolored and smooth terminating in a globose swelling.

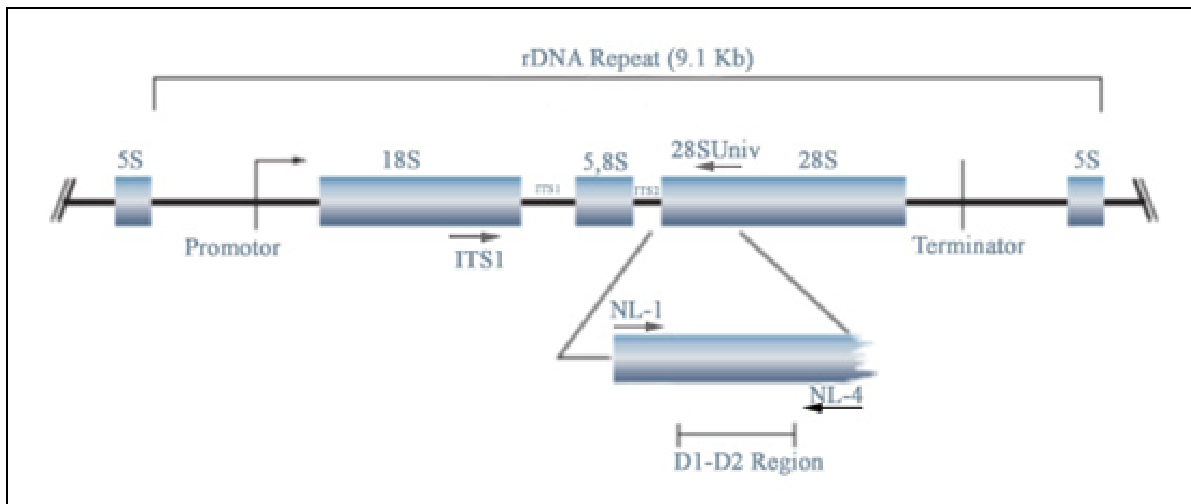

Fig. 1. Schematic representation of rDNA region with primers approximate localization (Bruce *et al.*, 2006) modified.

They bear phialides at their apex; up to 300 µm long, without metulae. Conidia were subglobose-globose to ellipsoidal, green with 2.5 to 3 µm diameter. Isolate S_2 showed long and dense felt of yellow conidiophores, which were non partitioned hyalines up to 0.85 mm in lenght.

Aspergillaires heads were biseriates and radiaires; they have globose to subglobose vesicles, 30-40 µm in diameter. Phialides borne directly on the metulae which bear numerous round and refractive conidia. Isolate S_3 showed uncommon conidia, conidiophores were biverticillate and partitioned with 3-5 divergent metulae in a whorl. Each metulae bear 4-6 phialides (Fig. 3.).

Molecular identification

Molecular characterization of three fungi isolates by the fragment consisting of ITS1, 5.8S and ITS2 allowed us to have two (2) sizes sequences between 800 bp and 840 bp for isolates S_1 and S_3. Unfortunately, sequence of the isolate S_2 contained many unknown nucleotides N, for this raison it is unusable.

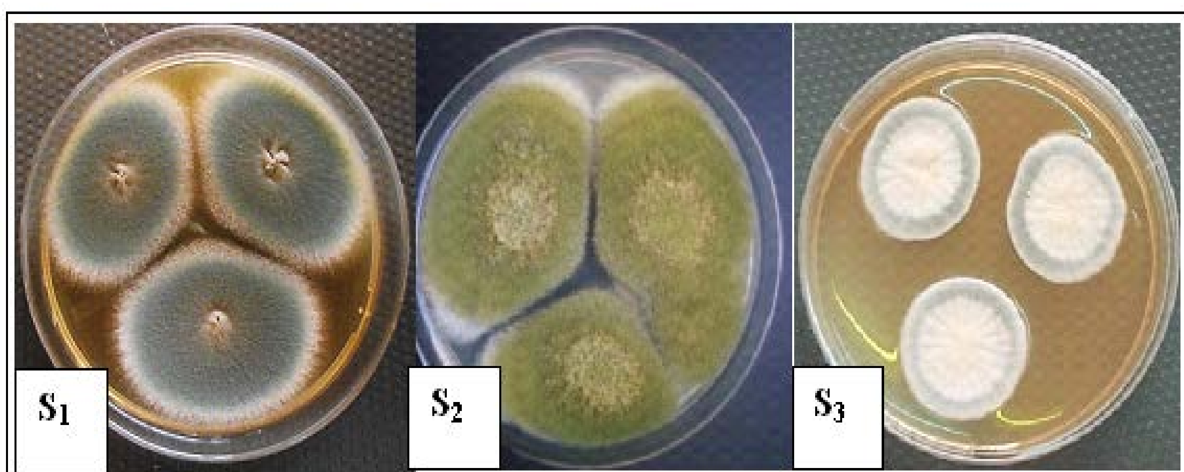

Fig. 2. Macroscopic aspects of isolates in PDA medium at 7 days of incubation at 30 °C (Compaoré *et al.*, 2016a).

The amplification of the D1-D2 region of 28S ribosomal large sub unit (LSU) of fungi isolates allowed us to have three (3) sequences for the isolates S_1, S_2 and S_3. The sizes of obtained sequences between 550 bp and 560 bp, were compared with the sequences registered in GenBank. For both PCR, the

taxonomic identification results were similar. Fig. 4 and Fig. 5 show the phylogenetic trees constructed by maximum likelihood PhyML with a bootstrap 100 from ITS1-5,8S-ITS2 and D1-D2 sequences for all strains. *Saccharomyces cerevisea* and *Candida temnochilae* sequences were used as outgroup respectively for tree 1 and 2. The phylogenetic trees show that our isolates belong to two genuses *Aspergillus* and *Penicillium*.

Discussion

From the above results of morphological characteristics, isolates S_1, S_2 and S_3 were close to *Aspergillus fumigatus*, *Aspergillus flavus* and *Penicillium citrinum* (Raper and Fennell, 1965; Pitt, 1985).

The determination of the isolate S_1 was confirmed by the Cooney and Emerson (1964) key for the identification of thermophilic filamentous fungi. Indeed *Aspergillus fumigatus* is the only species of the genus *Aspergillus* capable to grow at 50°C (Lamrani, 2009).

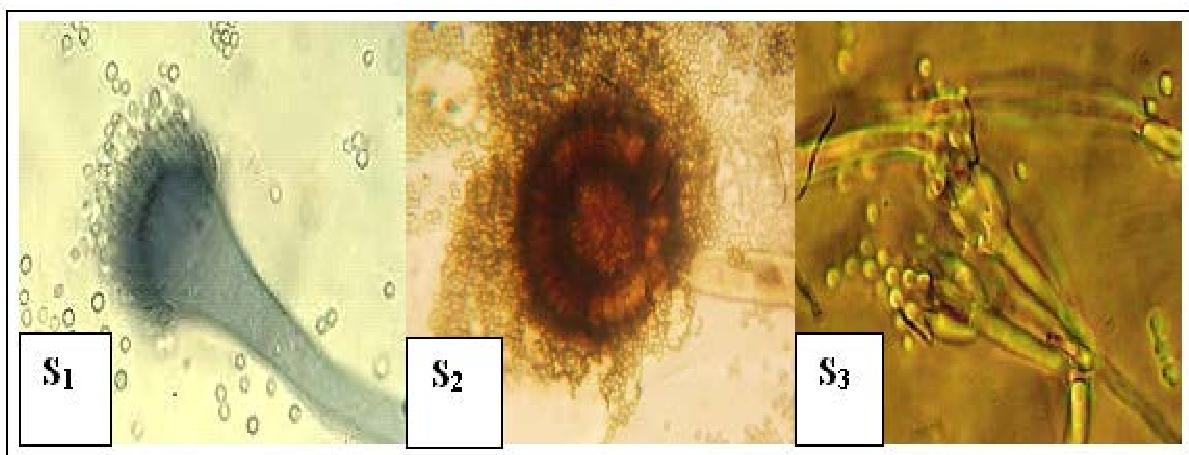

Fig. 3. Microscopic aspects of isolates (x100) (Compaoré *et al.*, 2016a).

The sequence analysis of the hyper variable region ITS1-5,8S-ITS2 showed that the isolated fungi of local foods are divided in two groups: *Aspergillus* and *Penicillium* (Fig. 2).

These two genuses are the most food contaminants. Indeed several authors have already isolated *Aspergillus flavus* from groundnuts seeds in Burkina Faso (Ouattara-Sourabie*et al.*, 2011), *Aspergillus fumigatus* from maize seeds and *Penicillium citrinum* from rice in Korea (Nguyen, 2007).

The identification of the fungi species was performed, initially, by sequencing the ITS1, ITS2 and 5.8S. Indeed, by single amplification of a one region, several species of fungi have been discriminated. The combined ITS1-ITS2 regions have been proposed as "barcode of life" for animals, plants and fungi (Schoch*et al.*, 2012).

Thus, with the use of specific primers ITS1-5,8S-ITS2 sequence, several *Aspergillus* species belonging to the section *Nigri* (*A. japonicus*, *A. heteromorphus*, *A. ellipticus*) and two species morphologically difficult to differentiate (*A. Niger* and *A. tubingiensis*) could be discriminated (Gonzalez *et al.*, 2005).

In addition, the work of Patino *et al.* (2005) allowed the differentiation of two ochratoxinogenic species belonging to the genus *Aspergillus*: *A. ochraceus* and *A. carbonarius*. From the same target region (ITS1), La Guerche *et al.* (2004) characterized by PCR different species of *Penicillium* isolated from grape berries.

However, single amplification of only region is not always possible to distinguish specifically different fungal species. According to Schoch *et al.* (2012), the important genus for pharmaceutical industries, such

as *Aspergillus*, *Penicillium*, *Cladosporium*, *Alternaria*, etc … the precise discrimination of the common species is not always possible with the sequences ITS1/ITS2 alone or combined.

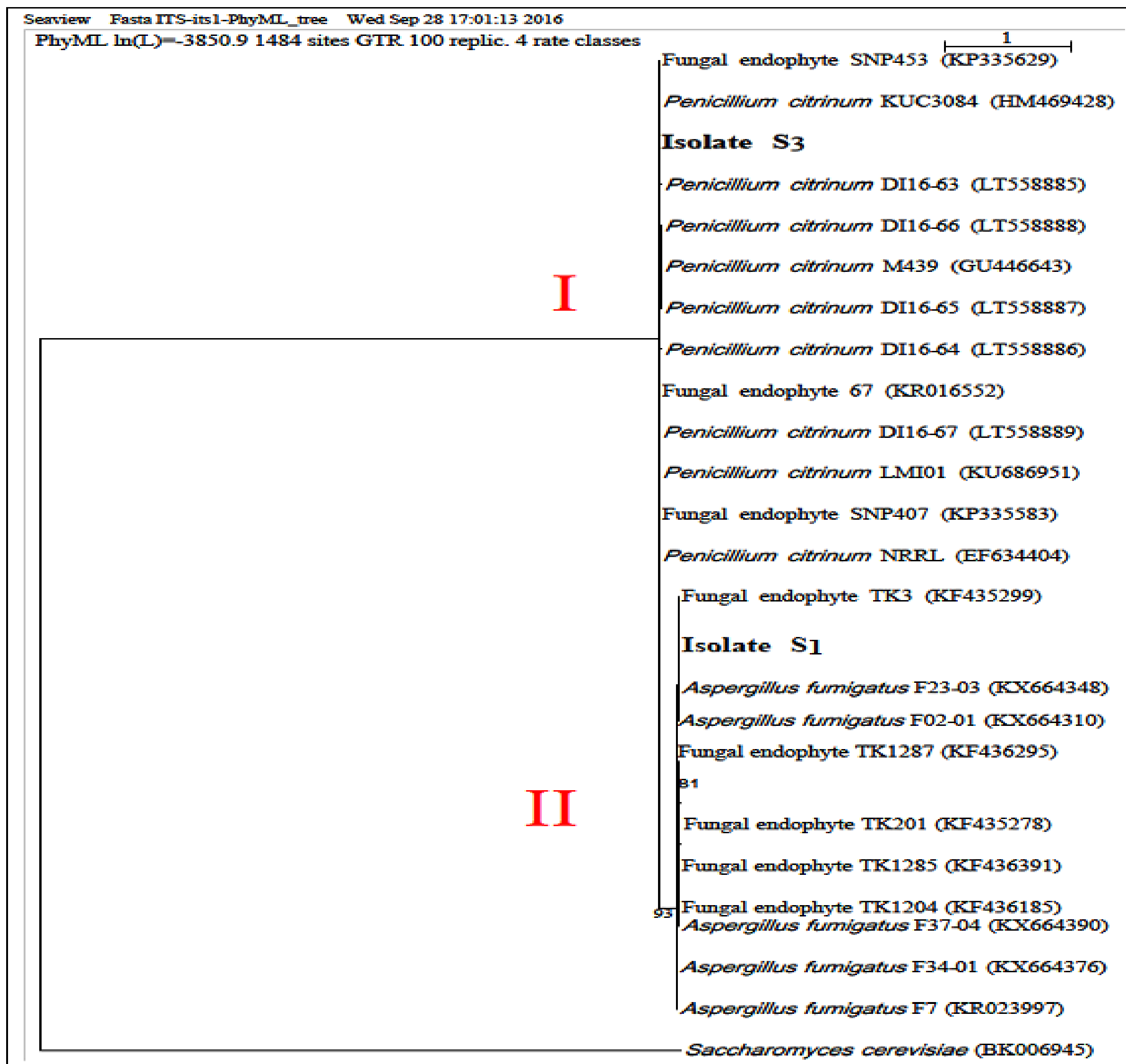

Fig. 4. Phylogenetic Tree PhyML based on ITS1-5,8S-ITS2 sequences of two fungi strains of local food and related reference strains from GenBank.

Secondly, to have best classification of our isolates fungi, we sequenced the D1-D2 region of ribosomal large sub unit (LSU) that allowed having a very good discrimination of the studied isolates. Thus, three strains are subdivided into three (3) clusters.

The isolates S_1 and S_2 are in the same group as the genus *Aspergillus*.

The isolate S_1 is closer to the species *Aspergillus fumigatus* and the isolate S_2 is close to the species *Aspergillus flavus*. As for isolate S_3, it is in the same group as the genus *Penicillium*, and closer to the species *Penicillium citrinum*. (Fig. 5). In fact this region is more informative in distinguishing species within the same genus (Kurtzman, 1993).

The 28S region D1-D2 refers to distinguish many species of Ascomycetes or Basidiomycetes fungi (Kurtzman, 1993). In most cases, the D1-D2 sequence is considered discriminant for the identification of fungi. The sequencing results allowed good identification.

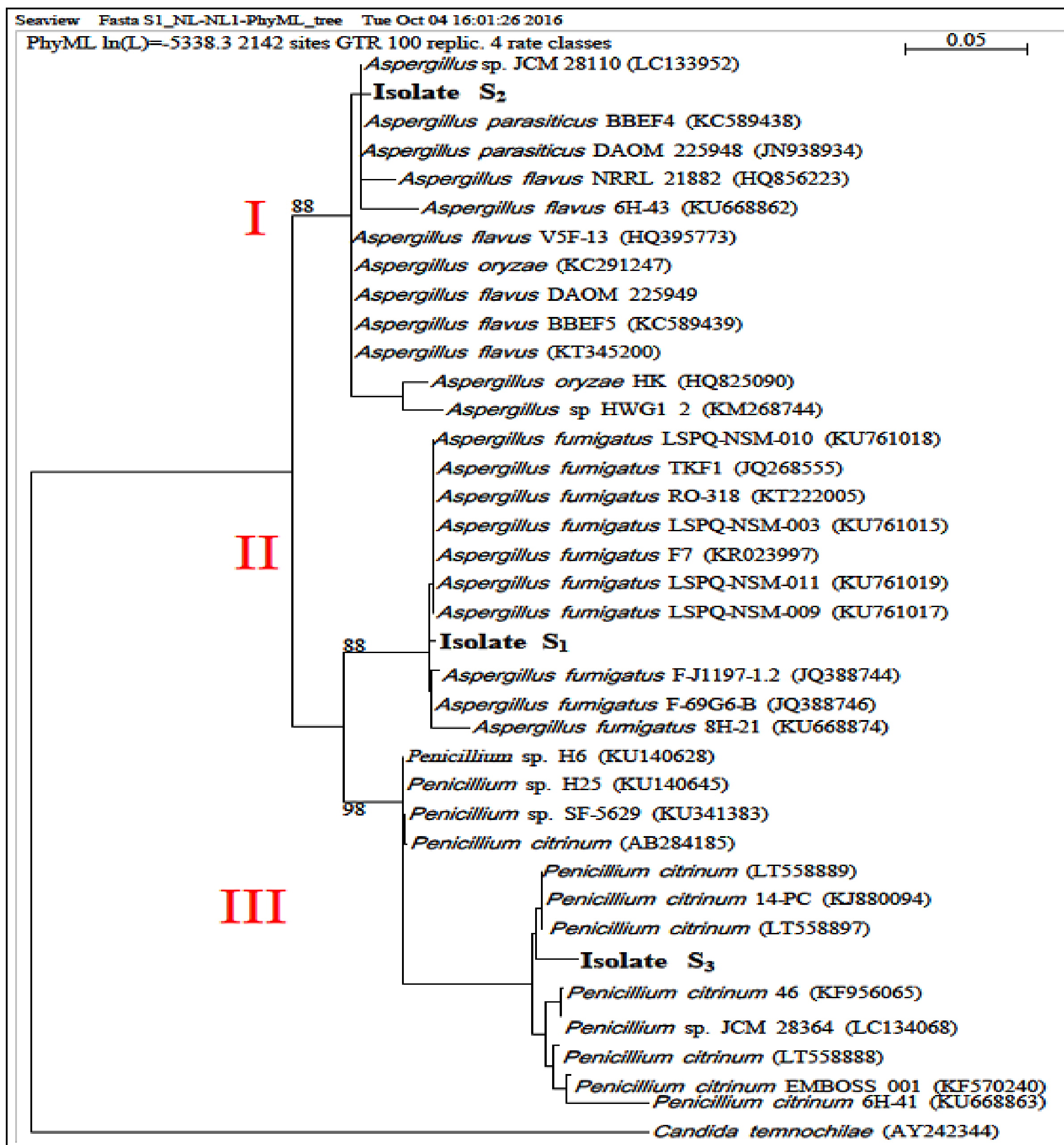

Fig. 5. Phylogenetic Tree PhyML based on D1-D2 sequences of three fungi strains of local food and related reference strains from GenBank.

Conclusion

The results show that the isolate S_1 collected from maize which could growth at 50 °C corresponds to *Aspergillus fumigatus*. Indeed, it is the only species of the genus *Aspergillus* capable to growth at this temperature. The isolate S_2 collected from groundnut is close to *Aspergillus flavus* and isolate S_3 from rice is related to *Penicillium citrinum*.

The morphological characterization was confirmed by molecular identification, according to phylogenetic trees, isolates S_1, S_2 and S_3 were in the same clusters to those whose sequences are from GenBank. Amplification of two regions ITS1-5,8S-ITS2 and D1-D2 has led to have good classification of strains. Nevertheless, identification must be confirmed by household genes sequencing such as betatubulin.

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