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Identification of *Aspergillus* sp. from El-Baida marsh in Algeria: phenotypic and genotypic characterization and industrial enzyme production

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ABSTRACT. The aim of this study was to compare between morphological and molecular identification methods. Two strains (A1, A2) of *Aspergillus* sp. isolated from El-Baida marsh, were subjected to be identified using morphological characterization, and molecular analysis performed by amplification of the ITS1 and ITS4 regions of rDNA. Morphological analysis indicated that both strains were identified as *Aspergillus oryzae*. However, by molecular methods A1 strain was identified as *Aspergillus alliaceus* and A2 strain as *Aspergillus oryzae*. Our results showed that phenotypic methods were insufficient for correct identification, and the use of genotypic methods is the most reliable. Enzymatic activity of the A2 isolate was evaluated using a plate assay for Lipase, Amylase and Protease production; the results illustrated the capacity of the fungus of the production of the three enzymes, which are mostly used in the production of pharmaceuticals, foods, beverages, and textile. They are found in a vast variety of sources such as animals, plants and microorganisms but microbial enzymes are more stable than the others. In addition, the reality that this fungus is known as a safe microorganism in several domains (food, beverage, cosmetic, and pharmaceutical industries), this opens the research in the future.

Keywords: phenotypic identification; molecular identification; *Aspergillus*; phylogenetic tree; enzymatic activity.

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Introduction

Saline soils are characterized by physical properties that are typically inhospitable to most living organisms. However, research has demonstrated that certain microorganisms, particularly fungi, are capable of growing and adapting to such conditions due to the development of various physiological mechanisms (Kogej, Ramos, Plemenitaš, & Gunde-Cimerman, 2005). Fungi play a crucial role in maintaining and managing environmental systems, including sustaining natural cycles by converting dead organic matter into usable materials through their extracellular enzymes. For an extended period, humans have harnessed the analytical capabilities of fungi in diverse fields, including the food industry, production of acids and organic alcohols, antibiotics, and proteins. The genus *Aspergillus* is one of the most widespread fungal groups on Earth, encompassing approximately 300-350 species with highly diverse lifestyles (Varga et al., 2014). Some species within this genus are utilized in the food industry and for the production of raw materials used in food manufacturing, such as citric acid and other organic acids. On the other hand, while most *Aspergillus* species are saprophytes, some can cause diseases in animals, humans, and plants (Paulussen, et al., 2017). Additionally, many species have the potential to produce toxic compounds known as mycotoxins, including aflatoxins, ochratoxins, gliotoxins, fumagillin, helvolic acid (fumigacine), fumitremorgin A, asphemolysins, and others (Al-Fakih & Almaqtri, 2019). Researchers employ a wide range of *Aspergillus* strains across various fields, including medicine, pharmaceuticals, cosmetics, and industry, due to their production of numerous biotechnologically significant secondary metabolites (Li, Song, Liu, & Ma, 2005). Notable examples include (3-methylbutyl) petrolactone, which exhibits antifungal activity and anti-fungal properties, and astrogilepeptide (Tian et al., 2015; Ma et al., 2017). Other valuable compounds include antioxidants such as 2-hydroxyserchimate, iso-aspylvanone E, and anti-diabetic agents like aspirinone (Dewi, Tachibana, Fajriah, & Hanafi, 2015), as well as anticancer agents like 4-(2-methoxyphenyl)-1-piperazine and 1-methyl-1-4-

indole-3-yl (He et al., 2019). Additionally, *Aspergillus* species produce various enzymes including lipase, alpha-amylase, protease, as well as probiotics and statins (Al-Fakih & Almaqtri, 2019).

The morphology of filamentous fungal colonies, characterized by cylindrical, thread-like hyphae 2–10 µm in diameter and extending up to several centimeters, is traditionally used for identification based on colony features such as color, size, and shape visible to the naked eye. Microscopic examination further reveals the arrangement of spores (Alsohaili & Bani Hasan, 2018). Molecular identification methods, involving the extraction of fungal DNA, offer a precise means of species-level identification. This is achieved through PCR amplification and sequencing of the 18S rRNA gene using universal primers (Landeweert et al., 2013). Recently, the ITS region has been proposed as a primary fungal barcode marker by the Consortium for the Barcode of Life (Taverna et al., 2013).

Microbial enzymes represent a growing area in biotechnology, offering advantages over those derived from animals and plants, which are dependent on livestock and less in demand. Consequently, there is an increasing focus on microbial enzymes (Suza et al., 2015). This study aimed to compare classical phenotypic methods with rDNA sequencing for the identification and differentiation of two *Aspergillus* species isolated from saline soil, and to evaluate their enzymatic activity.

Material and methods

Study area

The El-Baida Marsh is a natural saline wetland located between longitudes 5°53'20" E and 5°53'30" E, and latitudes 35°57'80" N and 35°54'20" N, near the Hammam El-Sukhna area in Setif region of Algeria. The marsh covers an area of approximately 12.223 hectares. The soil surrounding the site is characterized by varying degrees of salinity and is predominantly alkaline clay (Aliat & Kabeeche, 2013).

Sampling

Soil samples were collected from various locations within the marsh at a depth of 12 cm under sterile conditions and were subsequently preserved at 4 °C.

Isolation and purification of fungi

Soil fungi were isolated using Potato Dextrose Agar (PDA) medium through both direct plating and dilution methods.

Direct Method: One gram of the soil sample was placed in a sterile Petri dish, mixed

thoroughly with the Potato Dextrose Agar (PDA) medium, and then incubated at 28 °C until colonies appeared.

Dilution Method: One gram of the soil sample was suspended in 9 mL of sterile

distilled water to create a dilution series (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). The diluted samples were then plated onto PDA and incubated at 28 °C until colonies appeared (Suhail, Irum, Jatt, Korejo, & Abro, 2007; Admassu, Zhao, Yang, Gasmalla, & Zhang, 2015).

Fungi were purified by subculturing on PDA repeatedly until pure colonies were obtained.

Phenotypic Identification of *Aspergillus* sp. Isolates

Phenotypic identification of the two *Aspergillus* strains was performed following the method described by Pitt and Hocking (2009). Developing colonies were observed after incubation at 30 °C for 7 days, with attention to features such as color, shape, size, hyphae, and the microscopic characteristics of the conidia.

Identification was further supported by using specific media: Glycerol Nitrate Agar (G25N), Czapek Yeast Agar (CYA), and Malt Extract Agar (MEA). Colonies were also examined after incubation at 5 °C, 25 °C, and 37 °C for 7 days, focusing on colony diameter, and both morphological and microscopic characteristics (Pitt & Hocking, 2009).

Molecular Identification

Genomic DNA Extraction and PCR Amplification

Genomic DNA was extracted using the Quick-DNA™ Fungal/Bacterial Kit (Zymo Research). The extracted DNA was quantified using a Qubit® 2.0 Fluorometer (Life Technologies) with the Quant-iT dsDNA BR assay, following the manufacturer's instructions. The DNA concentration of the sample was 39.3 ng μL^{-1}. The sample was then diluted to a concentration of 2 ng μL^{-1} for PCR. PCR amplification was performed using the undiluted

and diluted DNA samples with the Taq DNA Polymerase (New England Biolabs) and NEB ThermoPol Buffer. Universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') were used. The PCR protocol consisted of an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of amplification: denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 68 °C for 1 minute, with a final extension at 68 °C for 5 minutes. Amplification products were analyzed by electrophoresis in a 0.8% agarose gel, stained with ethidium bromide, and visualized under UV light. After PCR, the sample, with a concentration of 38.1 ng μL^{-1}, was purified using microplate filtration and sent for sequencing at GATCBiotech (Nacef, Belhattab, & Larous, 2020).

Phylogenetic analysis

The alignment of ITS region sequences and the construction of the phylogenetic tree were conducted using MEGA-X software.

Screening of extracellular enzymes by plate assay

Extracellular enzyme production, including lipase, amylase, and protease, was screened using specific indicative agar media as described by Sunitha, Nirmala Devi, and Srinivas (2013). After incubation for 3-5 days at 30 °C, zones of enzymatic activity were observed.

Lipase

The fungus was cultured on Peptone Agar medium (peptone 1%, NaCl 0.5%, $CaCl_2 \cdot 2H_2O$ 0.01%, and agar 1.6%) supplemented with 1% sterile Tween 20. Following the incubation period, a visible precipitate around the colony indicated lipase activity.

Amylase

Amylase activity was tested by growing the fungus on Glucose Yeast Extract Peptone (GYP) Agar medium (0.1% glucose, 0.01% yeast extract, 0.05% peptone, and 1.6% agar) supplemented with 2% soluble starch. At the end of the incubation period, the plates were flooded with 1% iodine solution in 2% potassium iodide.

Protease

Protease activity was assessed by growing the fungus on GYP agar medium supplemented with 0.4% gelatin solution. After incubation, the plate was flooded with of 100% ammonium sulfate solution.

Results and discussion

Isolation of fungi and phenotypic identification of *Aspergillus* strains

Twenty-one fungal genera, along with three yeasts, were isolated from El-Baida Marsh. Among these, the genus *Aspergillus* was the most dominant, comprising 43% of the isolates, followed by *Penicillium* at 24%, and *Alternaria* at 7%. Two strains of *Aspergillus* (A1 and A2) were selected for identification using both morphological and molecular analyses.

The morphological characteristics observed on Potato Dextrose Agar (PDA) medium (Figures 1 and 2) indicated that both strains (A1 and A2) are *Aspergillus oryzae*. These strains formed dense colonies with prominent white sclerotia on Czapek Yeast Extract Agar (CYA), while on Malt Extract Agar (MEA) the colonies appeared yellow with aerial mycelium. In contrast, on Glycerol Nitrate Agar (GN25) medium, mycelial growth was weak at all tested temperatures (Figure 3).

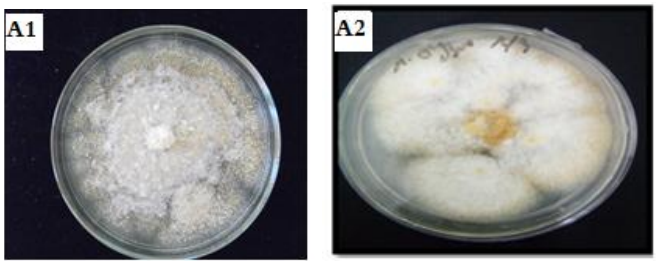

Figure 1. *Aspergillus* sp. A1 and A2 colonies grown on PDA medium at 30 °C.

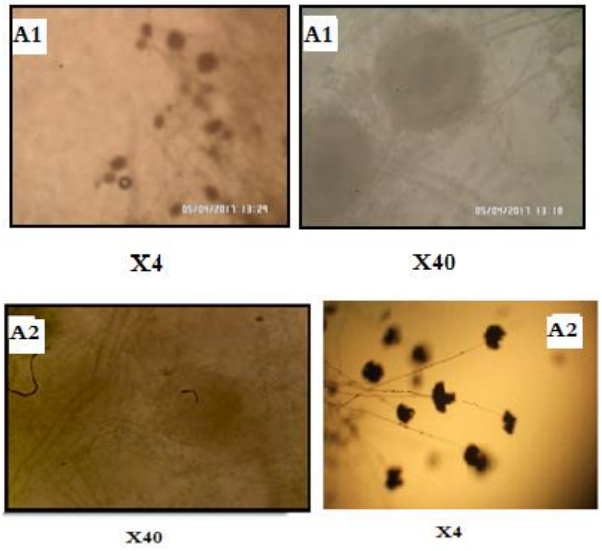

Figure 2. *Aspergillus* sp. A1 and A2 under microscope.

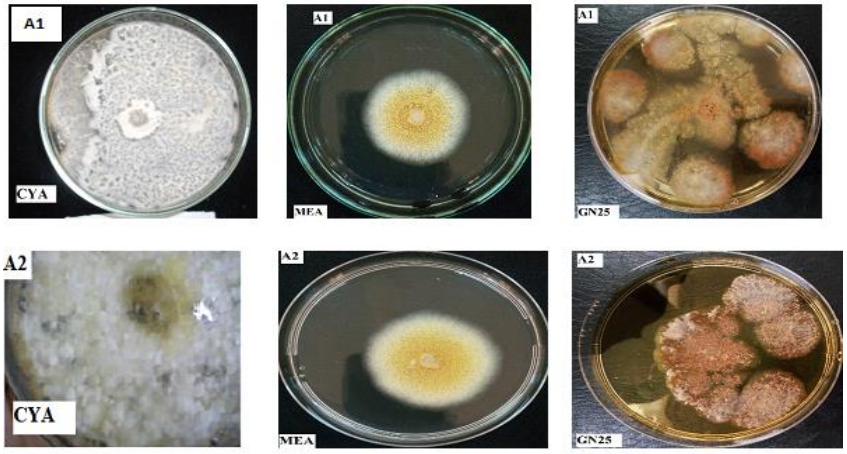

Figure 3. Cultural characteristics of *Aspergillus* A1 and A2 strains developing on diagnostic media.

Molecular identification

PCR amplification of the ITS regions using the primers ITS1 and ITS4 was successful for both A1 and A2 isolates (data not shown). BLASTN analysis confirmed that the isolate A2 is *Aspergillus oryzae* (GenBank accession MH793844.1) with a similarity of 99.61%. In contrast, isolate A1 exhibited 99.33% similarity to *Aspergillus alliaceus* (GenBank accession MH279440.1) according to the BLASTN results.

Phylogenetic analysis

The phylogenetic tree of the ITS sequences (Figure 4) displays three distinct clusters representing different species.

The first cluster at the top includes sequences with 100% similarity to isolate A2, including *Aspergillus oryzae*.

The second cluster contains strain A1, which is notably distinct from A2, indicating that it is a different species.

The third cluster consists of sequences from *Armillaria aotearoa*, which is positioned as an outlier and belongs to the Basidiomycota phylum.

The most abundant genera isolated from the marsh were *Aspergillus* sp. and *Penicillium* sp., comprising 43 and 24% of the total isolates, respectively. These results align with several studies on soil-isolated fungi, although the frequency and timing of genera occurrence may differ (Rahmoun et al., 2012). The dominance of *Aspergillus* sp. in the soil can be attributed to its production of a variety of rehydration enzymes, which enhance its ability to survive on decomposing organic matter (Suhail et al., 2007). Furthermore, Sohail et al. (2009) noted that *Aspergillus* is widespread in nature, particularly in soil environments.

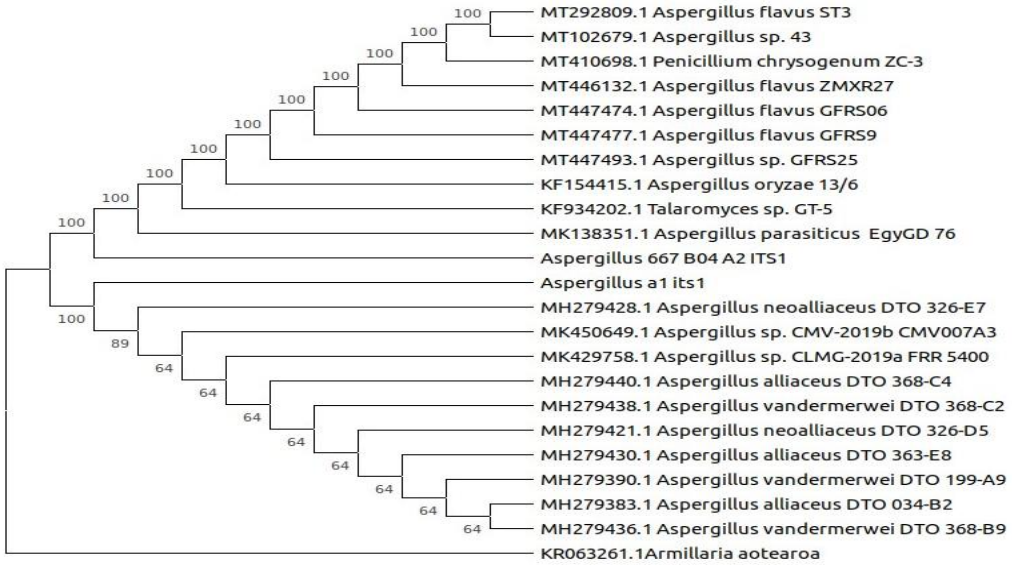

Figure 4. Phylogenetic tree for the ITS sequences of the tow isolates A1 and A2.

Some studies have indicated that morphological characteristics are not always reliable for fungal identification. While they may sometimes provide accurate results, many researchers find them insufficient due to the need for extensive experience, particularly when dealing with closely related fungal groups. Additionally, morphological identification requires considerable time and effort, as spore and colony sizes, shapes, and colors can be influenced by various environmental factors.

Molecular identification has addressed these limitations by enabling the detection and analysis of genetic differences among fungi, thereby overcoming the shortcomings of traditional methods (Sohail et al., 2009). The discovery of significant variations in the non-coding ITS regions of fungal DNA has been crucial for developing accurate and rapid methods for species and strain identification, as well as for understanding phylogenetic relationships and the origins of new species and strains. ITS regions are now widely recognized as a universal fungal barcode (Barbar, Abu-Duka, AL-Ghazali, & Ali, 2018).

Based on our comparative phenotypic and genotypic identification methods, isolate A1 was found to have been misidentified using phenotypic methods. This isolate corresponds to a species with a similar phenotypic profile to other species or cryptic species (Taverna et al., 2013). Previous studies have shown discrepancies between genetic and phenotypic analyses in various organisms, such as lactic acid bacteria and *Candida* species (Taverna et al., 2013).

In contrast, phenotypic identification was consistent with genotypic identification for other fungi isolated from the northeastern Jordanian Desert, including the genus *Aspergillus* sp. (Alsohaili & Bani Hasan, 2018).

According to the phylogenetic tree, the two isolates belong to different groups. Isolate A2 shares 100% similarity with species in the *Flavi* section, such as *Aspergillus flavus* and *Aspergillus oryzae*. The genome sizes of *Aspergillus* species in the *Flavi* section are generally larger compared to other *Aspergillus* species, with an average of 37.96 Mbp versus 31.7 Mbp, as reported for *A. oryzae* (Fguiri, Ziadi, Atigui, Arroum, & Khorchani, 2015).

Previous studies using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to differentiate 200 isolates of *Aspergillus flavus* found that only 10.2% were accurately identified as *A. flavus*. In contrast, 19.9% were identified as *A. oryzae*, and 69.9% were classified as *A. flavus*/*A. oryzae*. Additionally, *Aspergillus pseudonomius* and *A. alliaceus* were misidentified as *A. flavus* and *A. parasiticus* (Kjærbølling et al., 2020). Similar results were observed by Huang et al. (2017), where *A. flavus* was misidentified as *A. oryzae*. Masih et al. (2016) also reported that *A. oryzae* can be easily misidentified as *A. flavus*, with a low discrimination rate of 38%.

In the present study, phylogenetic analysis was performed based on a single marker. However, single-gene analysis often provides insufficient information for resolving phylogenies (Taverna et al., 2013). For cases where the ITS region lacks sufficient resolution for species identification, as with *Aspergillus*, *Fusarium*, *Penicillium*, and *Trichoderma*, or when working with morphologically similar species exhibiting cryptic speciation, it is recommended to analyze other protein-coding gene regions (Masih et al., 2016).

Despite this, the ITS region has been widely adopted as a DNA barcode for identifying various fungi, including *Trichoderma*, *Zygomycetes*, *Fusarium*, and *Penicillium*. This region is useful because it displays

significant heterogeneity that varies among genera and species (Barbar et al., 2018). However, Raja, Miller, Pearce, and Oberlies (2017) noted that the ITS region may not be effective for some highly diverse genera such as *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichoderma*, which may have narrow or absent barcode gaps in their ITS regions. Conversely, other studies have shown that ITS regions, when combined with additional loci, can effectively distinguish species within *Aspergillus* by examining multiple DNA sequences. This approach has revealed evolutionary relationships among different species within the genus (Barbar et al., 2018). *Aspergillus oryzae* was capable of producing all three tested extracellular enzymes. For lipolytic activity, the fungus exhibited a visible precipitate around the colony, indicating the formation of calcium salts of lauric acid released by the enzyme, which demonstrates positive enzyme activity (Figure 5a). The fungus also displayed significant amylolytic activity on GYP agar medium (Figure 5b), and produced protease, evidenced by a clear zone of casein hydrolysis on the medium (Figure 5c). This enzymatic capacity suggests that *A. oryzae* could be a valuable asset for industrial applications due to the stability of its enzymes compared to others (Prathyusha, Sri, & Prasad, 2015).

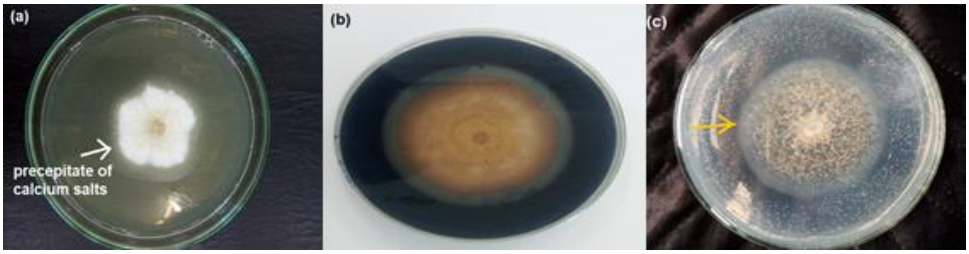

Figure 5. Enzymatic activity of *A. oryzae* (A2); (a) Lipolytic activity, (b) Amylolytic activity, (c) Proteolytic activity (the arrow indicates the clear zone of casein hydrolysis).

Conclusion

This study is pioneering in its application of molecular techniques to differentiate between similar fungal species, demonstrating significant advantages over classical methods. The results, supported by previous research, show that the ITS region is effective for species discrimination, though caution is advised when relying solely on GenBank BLAST for identification. This technique alone may not optimally distinguish between species like *A. flavus*, *A. oryzae*, and *A. alliaceus*. Comprehensive fungal identification should integrate both phenotypic and genotypic analyses using diverse molecular tools. *A. oryzae*, isolated from saline soil, exhibits high production efficiency of protease, lipase, and amylase, which are valuable in pharmaceuticals, food, beverage, and textile industries.

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