
Characterization, molecular identification, and phylogenetic analysis of microfungi isolated from the Landfill Site of Bangar, La Union, Philippines

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Abstract Ten indigenous microfungal species were characterized and identified belonging to four genera such as *Actinomucor* sp., *Acrophialophora* sp., *Aspergillus candidus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. oryzae*, *A. terreus*, and *Penicillium oxalicum* and *Penicillium* sp. Seven microfungal species were successfully sequenced, and phylogenetic analysis revealed monophyly in *Aspergillus* spp. while *Acrophialophora* sp. is distantly related to the other microfungal species. After establishing their biological information, their biofunctional potential can be explored.

Keywords: BLASTn, ITS region, Phylogeny

Introduction

Fungi represent the second largest group of eukaryotic organisms on earth, with estimates ranging from 1.5 to 5.1 million species (Raja *et al.*, 2019). The continued search for more potential fungal species with a wide range of benefits or applications is necessary. Aside from this, the need to collect, identify, and conserve fungal species for fungal diversity is imperative because no one knows when and how these valuable life forms might be lost forever (Ram *et al.*, 2010). Fungi are among the most diverse microorganisms that inhabit different environments like soil, plant parts, water, or even food sources. Fungi are essential soil microorganisms, for they play an important part in nutrition and processes affecting the growth and development of plants and other organisms. Hence, it is then necessary to isolate and discover fungal species which might not yet be known to science.

Many researchers have proven the accuracy and reliability of molecular methods in ascertaining the identity of various fungal species (Elbashiti *et al.*,

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2010; Alwakeel, 2016; Raja *et al.*, 2019). The use of various molecular methods in the identification of the organism is proven to be fast, specific, and sensitive. To date, it is still powerful in resolving disputes among erroneous morphological features of closely related organismal species (Ezeonuegbu *et al.*, 2022 and Zeng *et al.*, 2022). Nuclear ribosomal DNA (nrDNA), especially the internal transcribed spacer (ITS) region, has been an excellent target in the study of genetic relationships of fungal species. It is because the ITS region is highly conserved among interspecies but variable between interspecies as well as it is easy to amplify even at a small amount of DNA (Appiah *et al.*, 2017).

Meanwhile, an open landfill site is one of the indispensable parts of solid waste management systems in many countries, including the Philippines. Despite the presence of toxicants, the solid wastes disposed of or dumped in landfill sites serve as substrates or sources of nutrients for the proliferation of different organisms, particularly microbes, including bacteria and fungi (Nair, 2021). It is also undeniable that open landfill sites pose potential threats and hazardous impacts to nearby communities or agricultural lands, as landfill leachates usually contain toxic substances, including heavy metals (Iraivanian and Ravari, 2020). Interestingly, tolerant microbes from the area can be isolated and tapped for potential biofunctional applications such as mycoremediation, degradation of complex pollutants, or plant growth promoters. Several studies have been made on the isolation of fungi in landfill sites. For example, Verma and Gupta (2019) isolated potential *Aspergillus* species and assessed their polythene degradation potential. *Nectriella pironii*, an ascomycete isolated from the dye industry waste, was used for the degradation of phenanthrene (PHE), benz[a]anthracene (B[a]A), and benz[a]pyrene (B[a]P) (Binkowska *et al.*, 2022). The *Penicillium* spp. isolated by Lira *et al.* (2022) showed tolerance in heavy metals and degradation potential to pyrene. Ozyurek *et al.* (2021) reported the mycoremediation potential of *Aspergillus ochraceus* NRRL 3174 in the removal of petroleum hydrocarbons. The potential of fungi for bioremediation is undeniable with their extensive enzymatic systems, rapid adaptation to toxic organic pollutants, and to adverse environmental conditions. The use of microorganisms, including fungi, in bioremediation is a cost-efficient, sustainable, and natural approach (Deshmukh *et al.*, 2016). Li *et al.* (2020) stress that much research has been made on bacterial bioremediation on contaminated soils, however, much less is known about the potential of fungi. In this regard, the need to search and isolate potential microfungi for mycoremediation can be explored to help address many problems in polluted soils. However, it is but essential to first isolate, describe, and establish the biology of the fungal species of interest. Thus, this study aimed to characterize,

identify, and assess the phylogenetic relationships of the microfungial isolates from the landfill site of Bangar, La Union, Philippines.

Materials and methods

Collection of soil samples

A systematic spatial grid sampling technique was adopted in collecting soil samples from the landfill site of Bangar, La Union, Philippines, as depicted in Figure 1. Using the soil auger, about 1000 g of dry soil was collected from a depth of 50 cm in every spatial grid sampling location point. There were nine spatial grid sampling points. The collected soil samples were mixed in order to come up with a 9000 g composite soil sample. The 9000 g composite soil sample was placed in a clean paper bag and was immediately brought to the laboratory for fungal isolation.



Figure 1. The satellite map of the landfill site of Bangar, La Union, Philippines, showing the spatial grid points (guided by a GPS) where soil collection was done

Isolation, purification, and characterization of microfungial isolates

In the isolation of potential culturable soil-borne microfungi, approximately 0.02 g of dry soil samples were seeded on the surface of previously sterilized Petri plates with solidified potato dextrose agar (PDA) medium. The lid of the Petri plates was sealed with parafilm. The plated samples were incubated at ambient room temperature to allow the growth of

microfungal colonies within 3-5 days of incubation. Microfungal colonies started to grow as early as 3 days of incubation; hence at this point, the researcher started isolating distinct microfungal colonies from the plates. This is to prevent isolating from overlapping growing microfungal colonies. Based on the culture appearance of distinct fungal colonies on the plates, they were isolated by individually picking each isolate using a sterile inoculating needle and transferred aseptically into new Petri plates with PDA. The microfungal isolates were incubated at room temperature to allow mycelial growth in preparation for purification.

In assessing the purity of the isolates, a three-point inoculation technique was employed, in this manner, by seeding the microfungal isolates at three points (triangular) in Petri plates with solidified PDA medium. After incubation at ambient room temperature for several days, the equal cultural characteristics of the three growing mycelia indicate the purity of the culture. Once pure culture was obtained, it was inoculated in test tubes with media. These tubes served as stock cultures of the fungal isolates.

Meanwhile, the description and traditional identification of isolates were patterned on the methodology of Hong *et al.* (2010) with modifications. The Illustrated Manual on Identification of some Seed-borne Aspergilli, Fusaria, Penicillium and their Mycotoxins by Singh *et al.* (1991) was also used. The macro-morphological and micro-morphological features of the isolates were characterized. For macro-morphological analyses of the cultural characteristics, the isolates were grown in potato dextrose agar (PDA) using a single-point inoculation technique and incubated for seven days. After seven days of incubation, the color of the obverse side of the colony as well as the texture of the colony, were observed and recorded. For the micro-morphological characterization of the isolates, the agar block method was employed. After several days of incubation, the micro-morphological characteristics such as the types of hyphae, the color of mycelium, the presence of sexual spores, and the possible presence of other structures like apophysis, chlamydospores or sclerotia were observed and recorded. The observed and recorded macro and micro morphological characteristics served as the basis for the traditional description and identification of the microfungal isolates and were confirmed using molecular technique.

Molecular identification, and phylogenetic analysis of microfungal isolates

In molecular identification, pure culture of microfungal samples in tubes was sent to MACROGEN Inc. in Korea for the DNA extraction, PCR amplification, and sequence analysis of the different isolates. The primers used

in this study were ITS1 5' (TCC GTA GGT GAA CCT GCG G) 3' (forward) and ITS4 5' (TCC TCC GCT TAT TGA TAT GC) 3' (reverse). The nrDNA ITS sequence was used for BLAST analysis to determine the identity of the microfungal species with the highest percent similarity match in the database. The DNA sequences were aligned using MEGA 11, and phylogenetic analysis of the different isolates was also performed using MEGA 11 with a statistical method as maximum likelihood, the test of phylogeny as the bootstrap method with 1,000 replications, and substitution model as Kimura two-parameter correction method.

Results

Microfungal isolates

In this study, ten microfungal species were isolated and purified belonging to four genera such as *Aspergillus* with five species, namely *A. oryzae*, *A. terreus*, *A. flavus*, *A. fumigatus*, *A. candidus*, and *A. niger*; *Penicillium* with two species namely *P. oxalicum*, and another *Penicillium* sp.; *Acrophialophora* with one species namely *A. levis*; and a species belonging to the genus *Actinomucor*. It showed the observed cultural characteristics (color/texture), sporangiophores/conidiophores, and sporangiospores/conidia of the different microfungal species in this study (Table 1). On the other hand, the finding showed the obverse 1-point and 3-point cultures as well as the microscopic conidiophores and sporangiophores of the different microfungal species after 7 days of incubation in potato dextrose agar (Figure 2).

Molecular identification and phylogenetic analysis

This study showed the species list of indigenous microfungi that are identified using molecular methods, particularly the internal transcribed spacer (ITS) region (Table 2). For reference, GenBank accession numbers of the different microfungal species are presented. It can be observed from the table that there is a strong percent similarity (99%-100%) of the inferred microfungal sequences to the database present in the National Center for Biotechnology Information (NCBI) using nucleotide basic local alignment search tool (BLASTn). It can also be observed that the length of the sequences ranges from 571bp-634bp.

On the other hand, results on the phylogenetic analysis of the sequence dataset for ITS showed that *Acrophialophora levis* (KM995890) is distantly related to the other microfungal species but somehow related to *Penicillium*

oxalicum (KP780809) (Fig. 3). It is notable that the *Aspergilli* form monophyly, such that *A. candidus* (HQ607958) and *A. terreus* (MH047280) are closely related, having a bootstrap value of 99 and forming a single clade. *A. oryzae* (MK120548) and *A. flavus* (MH864264) are also closely related, forming a clade having a bootstrap value of 100. *A. fumigatus* (MG575498) was not within the clade of the previously mentioned *Aspergillus* spp.

Table 1. Observed morphological characters of the indigenous microfungi isolated from the landfill site of Bangar, La Union, Philippines

Species Name	Color/Texture (Obverse)	Sporangiophores/ Conidiophores	Sporangiospores/ Conidia
<i>Acrophialophora levis</i>	dirty white or light brown/flat and velvety	Single phialides attached to hypha, phialides are short, dense, and cylindrical; no conidiophores	ellipsoidal to semiglobose
<i>Actinomucor</i> sp.	white to dirty white/dense aerial mycelia	Sporangiophores are wide, erect, branched and verticilliate	Colorless, globose, large, and rough surface
<i>Aspergillus candidus</i>	white to slightly brown/cottony	thick and smooth stipe, biseriate	subglobose
<i>Aspergillus flavus</i>	white to light green/powdery	long, colorless, and rough stipe, uniseriate	globose
<i>Aspergillus fumigatus</i>	bluish green/cottony	short and smooth stipe, uniseriate and columnar head	small globose
<i>Aspergillus niger</i>	black/powdery	long, smooth, and colorless stipe, globose vesicle with an entirely fertile surface	globose
<i>Aspergillus oryzae</i>	yellow to dark green/velvety	rough and colorless stipe, uniseriate	spherical
<i>Aspergillus terreus</i>	light brown to brown/powdery	colorless and rough stipe, biseriate columnar head	globose
<i>Penicillium oxalicum</i>	grayish green/velutinous (dense)	long and smooth stipe, asymmetrically biverticilliate penicillus	large and smooth
<i>Penicillium</i> sp.	glaucous green/funiculose	long and smooth stipe, monoverticilliate	ellipsoidal
<i>Acrophialophora levis</i>	dirty white or light brown/flat and velvety	Single phialides attached to hypha, phialides are short, dense, and cylindrical; no conidiophores	ellipsoidal to semiglobose

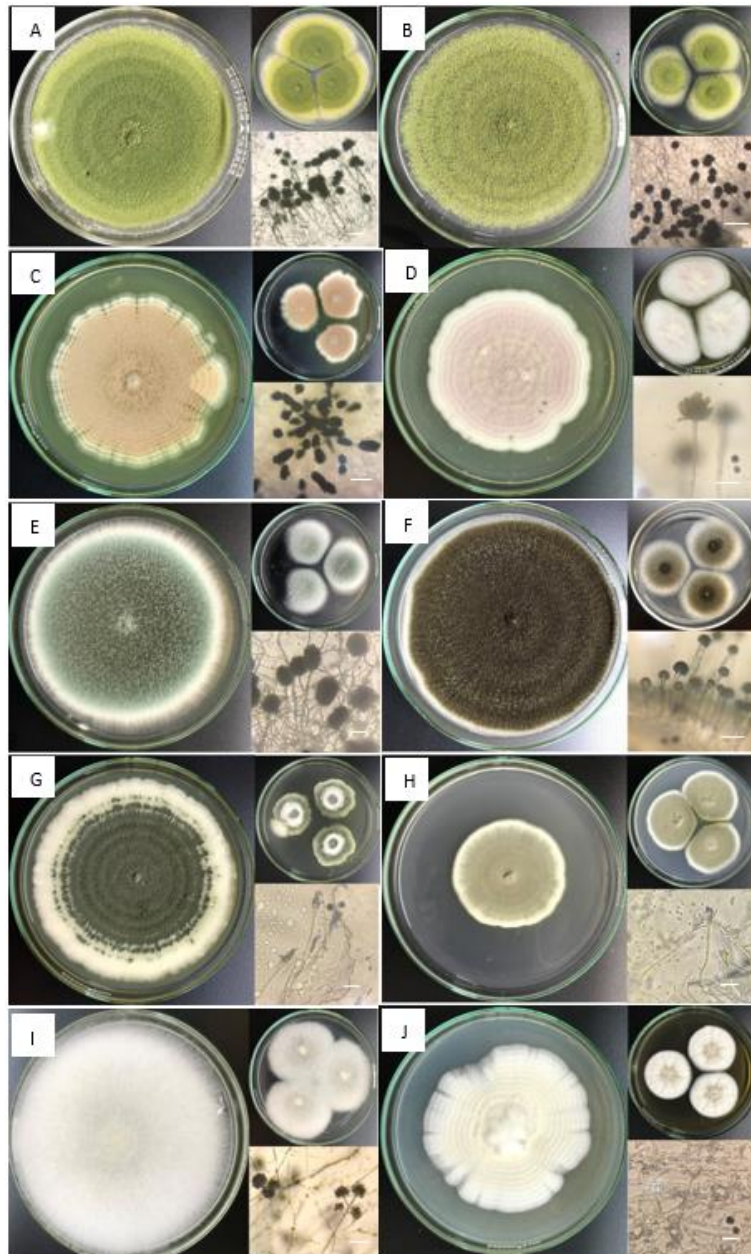


Figure 2. (A) *Aspergillus oryzae*, (B) *A. flavus*, (C) *A. terreus*, (D) *A. candidus*, (E) *A. fumigatus*, (F) *A. niger*, (G) *Penicillium oxalicum*, (H) *Penicillium* sp., (I) *Actinomucor* sp., (J) *Acrophialophora levis* showing 1-point and 3-point cultures on PDA after 7 days of incubation as well as its microscopic conidiophores. Scale bars: A,C,F,I = 20 μm , B = 30 μm , G,H,J = 15 μm

Table 2. Species list including isolate number, GenBank accession number, bp length, and percent similarity used in this study

Species Name	Isolate No.	Genbank Accession No.	bp length	% similarity
<i>Acrophialophora levis</i>	MF07	KM995890.1	571	100
<i>Aspergillus candidus</i>	MF04	HQ607958.1	627	99
<i>Aspergillus flavus</i>	MF11	MH864264.1	600	100
<i>Aspergillus fumigatus</i>	MF21	MG575498.1	602	99
<i>Aspergillus oryzae</i>	MF08	MK120548.1	587	100
<i>Aspergillus terreus</i>	MF10	MH047280.1	634	100
<i>Penicillium oxalicum</i>	MF12	KP780809.1	605	100

bp - base pair

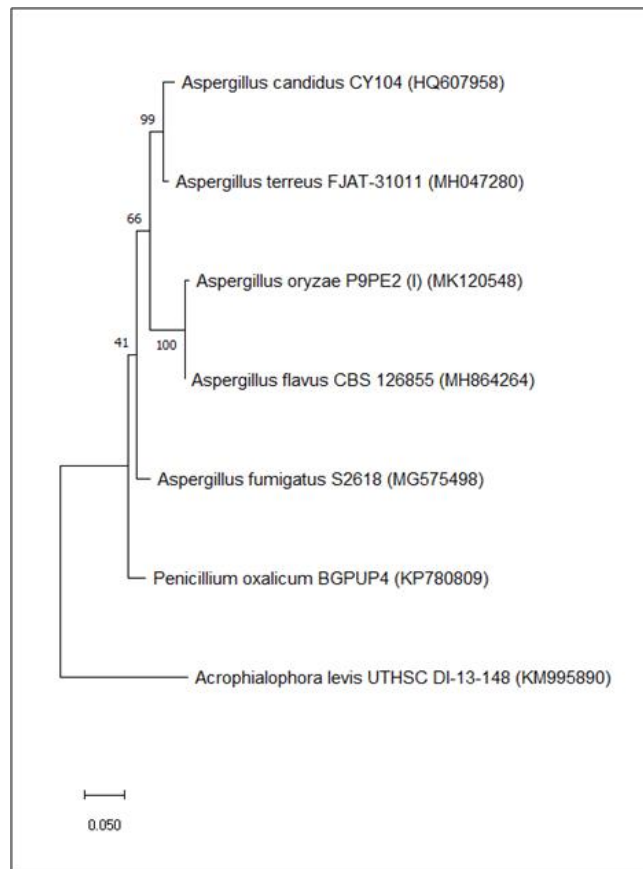


Figure 3. Phylogenetic tree based on maximum likelihood analysis of ITS sequences of the 7 microfungal species. Bootstrap support values of $\geq 50\%$ are indicated at the nodes, and the bar indicates the number of substitutions per position

Discussion

Identification of a species is traditionally done using morphological characters such as shape, size, and color of body parts, among others, as prescribed in a step-by-step instruction of an identification key and is later confirmed by an experienced professional taxonomist. When the specimen is damaged or is in a young stage of development, even an experienced professional taxonomist can have a hard time identifying it. Usually, morphological keys are effective only for a particular life stage or gender of an organism; thus, many are difficult to identify. Also, the number of professional taxonomists is declining (Hebert *et al.*, 2002).

Due to the advent of technology nowadays, a more powerful tool in fungal identification is in practice, i.e., molecular identification making use of the DNA sequence of the organism. In the case of fungi, the nuclear ribosomal internal transcribed spacer region serves as an appropriate barcode in ascertaining their identity (Schoch *et al.*, 2012; Dentinger *et al.*, 2011). Molecular tools provide more accurate methods for identification using molecular markers and DNA techniques, which are quick and reliable in establishing the identities of various fungal species (Rajaratnam and Thiagarajan, 2012).

The result of the current study agrees with the findings of Alwakeel (2016) in that *Aspergillus fumigatus* does not form a single clade with other *Aspergillus* spp. like *A. terreus*. Chang *et al.* (2006) stressed the cladal relatedness of *A. oryzae* and *A. flavus* isolates in S and L morphotypes. *A. oryzae* is morphologically similar to *A. flavus* and generally produces abundant conidiophores but few or no sclerotia. The observations of the present study on the cultural similarities of *A. oryzae* and *A. flavus* coincide with the observations of other researchers (Elbashiti *et al.*, 2010; Miyazima & Matsuura, 2011; Thathana *et al.*, 2017). The degree of similarity of cultural characteristics of *A. candidus* and *A. terreus* also coincides with the observations of several studies (Gautam and Bhadauria, 2012; McClenny, 2005). Phylogenetic analysis is done to elucidate the genetic relationship and to further classify the evolutionary relationships at the genus level (Cui *et al.*, 2015; Guan *et al.*, 2020). Phylogenetic analysis is considered to be a highly reliable and important bioinformatic tool in assessing evolutionary or genetic relationships among taxa (Roy *et al.*, 2014).

This study could significantly contribute to the discovery of soil-borne microfungi with mycoremediation potential in heavily contaminated soils which may help the area. Detection and isolation of microfungi from landfill sites could suggest resistance of these microfungi from hostile environmental conditions like the presence of excessive amounts of soil toxicants; hence, they

could also be tapped as soil amendments in poor soil quality or heavily contaminated agricultural areas, which could possibly contribute to plant or crop productivity. Aside from that, the mycoremediation potential of microfungi from the landfill site could be tapped in conjunction with other bioremediation agents like microbes and plants in addressing seriously contaminated soils. Moreover, this study could possibly identify microfungi isolates as potential soil amendments in heavily contaminated or poor soils, enhancing plant growth for greater agricultural productivity.

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