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Characterization and screening of marine-derived fungi along the coastline of Alexandria, Mediterranean Sea, Egypt

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ABSTRACT

Egypt is considered as a white spot for fungal biodiversity thus, this study used ITS sequencing, and routine identification to analyze the fungal communities within different marine sources (sediments, water, algae, and wood) from the Egyptian Mediterranean Sea at Alexandria coastline, which helped to uncover fungal diversity and distribution pattern. Two hundred fifty-two marine fungal isolates were isolated from twenty-five sea sediment, seven water, eight algae, and eight wood samples, which were collected seasonally through sex trips during the year 2017/2018 from the Egyptian Mediterranean Sea, Alexandria, Egypt. In the present study, sediment samples contained the highest diversity of fungi, and the most dominant phylum was the Ascomycota (90.8% OTUs) followed by the Basidiomycota (6.7% OTUs). A small proportion of the OTUs was assigned to the Mucoromycota. Some available indices had been successfully followed during this study for estimating the fungal biodiversity, and these indices proved their effectiveness.

INTRODUCTION

Fungi are ubiquitous nutrient cyclers in marine, terrestrial and freshwater ecosystems **(Hassett *et al.*, 2019)**. Fungal degradation processes release CO_2 to the atmosphere and into the marine food web. Heterotrophic microbes associated with the marine environment are highly dependent on dissolved organic matter produced by photosynthetic microorganisms **(Kuipers *et al.*, 2000)** or they can act as biodegraders involved in the detoxification of chemical pollutants **(Abdel-Razek *et al.*, 2020)**. Marine fungi are an ecologically rather than taxonomically or physiologically defined group of organisms **(Cavicchioli *et al.*, 2019)**.

Various new compounds with interesting biological activities have been isolated from marine fungi with various biological activities **(Blunt *et al.*, 2016)**. Fungi had been

classified into nine phyla, most fungi are mycelial, but yeast stages are also common **(Raghukumar, 2017)**. Marine fungi have been classified according to morphological features as the ascomycete order Halosphaeriales comprises largely marine species with 59 genera and some 141 species **(Jones, 1995).** This can be roughly half of the marine ascomycetes **(Kohlmeyer and Volkmann-Kohlmeyer, 1991)**.

Fungi are essential components of the marine ecosystem, but very little is known about their diversity and global distribution in the marine environment **(Tisthammer *et al.*, 2016)**. On the side, fungi play an important role as a component of the marine ecosystem, but very little is known about their diversity in the marine environments **(Grossart *et al.*, 2019)**. Marine fungi are active and essential players in marine biological processes across realms **(Richards *et al.*, 2012)**. Fungi have been recovered from nearly every marine habitat searched, including water (**Gao *et al.*, 2013**), hydrothermal vents and deep-sea **(Edgcomb *et al.*, 2011),** anoxic habitats, wood substrate **(Jebaraj *et al.*, 2010)**, as pathogens of microorganisms **(Nagahama *et al.*, 2003)**, associated with marine invertebrates **(Yarden, 2014)**, and on marine plants and algae **(Jones and Pang, 2012)**.

In general, many factors influence marine fungal community composition such as water, temperature, salinity, and location **(Rämä *et al.*, 2014)**, physicochemical characteristics (sulfide, total organic carbon, and dissolved inorganic carbon) **(Orsi *et al.*, 2013)**, both realms and sampling date significantly differentiated fungal community composition **(Guo *et al.*, 2015)**, and finally environmental factors, particularly sample depth, nitrate, and oxygen. Furthermore, fungal biodiversity is essential for exploring diversity from a biogeographical perspective and view of bioactive secondary metabolites, which help to establish conservation policies **(Duarte *et al.*, 2012)**.

Our knowledge of marine fungi from the Egyptian shore of the Mediterranean Sea is not adequate, and this study was initiated to fill this knowledge gap. Therefore, this study is suggested to be the first to use traditional Sanger sequencing and morphology identification to comprehensively analyze the fungal communities within different marine sources (sediments, water, algae, and wood) from the Egyptian Mediterranean Sea at Alexandria coastline.

MATERIALS AND METHODS

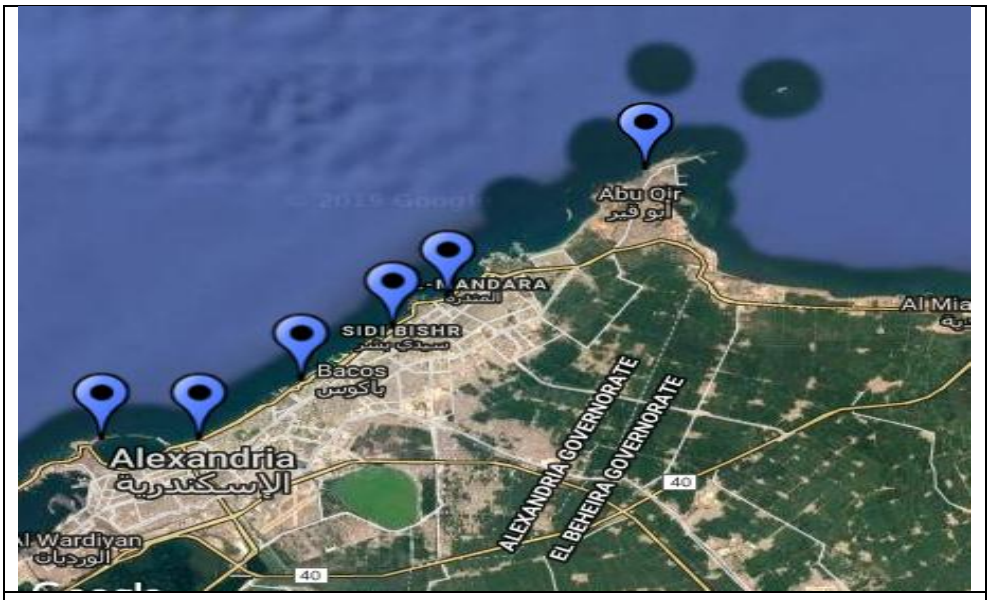
Media

Three media were set by aged seawater and adjusted to pH 5±0.1. They included: rose Bengal chloramphenicol agar (RCA) **(Jarvis, 1973)**, potato dextrose agar (PDA) **(Baka *et al.*, 2015)**, and glucose peptone agar **(Nagano *et al.*, 2008)**.

Study area and sample collection

Six locations were selected for the present study; Abo Qir, Elasafra, Mohamed Nagib, Estanly, Elebrahemia, and Eastern Harbor, and they are distributed along the

Alexandria coastline, Egyptian Mediterranean Sea, Alexandria, as shown on the map (**Fig. 1**). Basically, to obtain marine fungi, sampling was carried out seasonally from July 2017 to April 2018. Each season from each location, 25 sea sediment, 7 seawater, 8 algae, and 8 wood samples were randomly collected from different sampling sites. Seawater and sediment samples with depths ranging between 1.5 and 2.0 m which seawater were collected in 500 mL sterile screw-capped bottles, while sediment samples were placed in sealable sterile plastic bags underwater to prevent contamination (to reduce the likelihood of exposure to airborne spores). Wood and macroalgae samples buried deep in the sand or the sand grains were collected in sealable sterile plastic bags. All samples were directly kept on ice during transferring process to the laboratory for isolation, purification process, microscopic examination, and identification. It should avoid slow drying of samples that can cause discharge of fungal ascospores **(Bremer, 2000)**.



Site	Latitude	Longitude
Eastern harbor	31.212347	29.885246
Elebrahemia	31.2128	29.916842
Estanly	31.237239	29.949789
Mohamed Nagib	31.259197	29.978983
El Asfra	31.271855	29.997044
Abu Qir	31.324778	30.061835

Fig. 1. The map of sampling sites distributed along the Alexandria coastline.

Physicochemical analysis

Physical and chemical analyses were carried out for the collected seawater samples to measure the following variables: temperature which measured by an ordinary

thermometer accurate to 0.1ºC, the pH value, measured by a portable pH meter, total alkalinity, determined titrimetrically using dilute HCl **(Nessim *et al.,* 2005)**. Dissolved oxygen (DO) was analyzed through the modified Winkler method **(Grasshoff *et al.,* 2009)**, salinity (S%) was calculated from the electrical conductivity of samples measured by a Beckman salinometer **(Grasshoff *et al.,* 2009)**.

Isolation and purification of marine fungi

One milliliter of water samples was spread directly onto the three mentioned media. Sediments samples were diluted (1:10) using sterile seawater and then one mL of the diluted sample was spread onto the three mentioned media. All media were autoclaved at 121°C for 15 min, supplemented with antibiotics (Gentamicin 80 mg/L, Piperacillin, and Tazobactam 100 mg/L). All plates were incubated at 30 °C **(Amer *et al.,* 2019)**. Each thallus of algae samples was washed in sterilized seawater to remove unrefined sediments. They were homogenized in 20 mL of sterile filtered seawater. One mL of homogenate was plated onto the three mentioned media **(Bremer, 2000)**.

All wood samples were extensively washed by sterilized seawater to remove unrefined sediments and other contaminating debris, followed by dividing into two halves and each half was subsequently disintegrated to small cubes, the first group was immersed in the three mentioned media and incubated at 30 °C, while the cubes of the second group were placed in a sterile sealed plastic bag containing a paper towel wetted and examined weekly for the presence of fungal reproductive structures, with weekly spraying with sterile seawater to prevent sample desiccation. Occasionally, all samples were carried out in triplicate also, negative controls were applied during the isolation to detect true marine fungi.

Characterization of the isolated marine fungi

Morphological characterization and microscopic examination

Fungal cultures were identified based on morphological characteristics after individual isolates were transferred to 1.5% malt extract agar **(Hashem *et al.,* 2020)**. The macroscopic features of the cultures, such as colony color and mycelial habit, including whether the hyphae, were arial or growth within the medium (**Gow and Gadd, 2007)**. The slides were observed under a microscope (400X) by aiding **(Suleiman *et al.,* 2019)**. Conidiophores, conidia, chlamydospores, and branching patterns were identified under a microscope using the identification characteristics key by **(Domsch *et al.,* 1980)** and following the taxonomic arrangement described by **(Hibbett *et al.,* 2007)**.

Molecular identification

Extraction of DNA* and *ITS-PCR conditions

Fungal mycelium was inoculated in potato dextrose broth. Mycelium was harvested and converted to a fine powder in liquid nitrogen and DNA was extracted according to

(Abd-Elsalam *et al.*, 2007). The PCR amplifications were carried out in a total volume of 25 µL, containing 20 ng genomic DNA, 1X PCR buffer, 0.2 mM of each of dNTPs, 0.2 unit of Taq DNA polymerase (Roche Holding AG, Basel, Switzerland), and 10 pmol of the primers ITS1 (**5**`TCCGTAGGTGAACCTGCGG**3**`), ITS4 (**5**`TCCTCCGCTTA TTGATATGC**3**`) **(White *et al.*, 1990; Hashem *et al.*, 2021)**. The PCR amplification was carried out according to the following temperature profile: An initial step of 2 min at 94°C, 40 cycles of 60 sec at 94°C, 90 sec at 52°C, and 2 min at 72°C, and a final step of 7 min at 72°C.

PCR products purification

Amplified DNA was purified using AccuPrep PCR DNA Purification Kit (K-3034-1, Bioneer Corporation, South Korea). An aliquot of 48 mL of absolute ethanol was added to the washing buffer. Five volumes of buffer (PCR binding buffer) were added to one volume of PCR product (40 mL PCR product was removed and the volume was brought to 200 mL) and mixed thoroughly. Then a column of binding was inserted into the Eppendorf tube and the sample was transformed into the column. 500 mL, washing buffer was added and centrifuged for 15 minutes at 13.000 rpm at 25 ºC. The excess solution was removed and the second column was transferred in a new Eppendorf tube. The binding column was put within a new Eppendorf tube, and 40 µL samples were inserted in sterile distilled water two times in the middle column of binding placed on a metal rack were to remain constant. Tube and binding column were centrifuged for 15 min at 10.000 rpm. The column was removed and the DNA purified was collected in tubes at -20ºC for subsequent studies **(Hussain *et al.*, 2018).**

Sequencing and analysis

The PCR products were purified with the ExoSAP method and Sanger sequencing with PCR primers was performed at Macrogen Incorporation (Seoul, South Korea). The sequences were assembled using the DNA STAR SeqMan (DNA STAR Incorporation, Wisconsin, USA), and to establish the quality control of generated ITS sequences we have applied the guidelines listed by **(Nilsson *et al.*, 2012)**. Generated sequences firstly were submitted to the GenBank database. Sequence analyses obtained by BLASTN similarity search at the website (http://www.ncbi.nlm.nih.gov/BLAST) and secondly were multiply aligned using MEGA_X_10.1.6 software and the phylogenetic tree (Neighbor-joining) constructed by maximum- likelihood method with rapid 5,000 bootstrapping replicates. At last, species-level OTUs **(Blaxter *et al.*, 2005)** were determined based on a 97% sequence similarity cut-off threshold, which is inside the range of intraspecific ITS sequence similarity.

Statistical analyses

For the diversity indices (Species richness (S), Total number of individuals, Dominance_D, Simpson_1-D, Shannon_H, Evenness_e^H/S, Brillouin, Menhinick, Margalef, Equitability_J, Fisher_alpha, Berger-Parker, and Chao-1), PAST software version 3.15 (copyright Hammer & Harper, Natural History Museum, University of Oslo, Norway) was used. In our study, several estimates of total species number were used to confirmed the reliability of estimates richness (S) according to extrapolations from species richness values, namely Chao's method, first and second Jackknife and Bootstrap for each species using PAST software version 3.15 **(Hammer *et al*., 2001)** and matched them with observed total species number **(Oksanen *et al*., 2012).**

RESULTS AND DISCUSSION

1. Characterization of collected samples

The physicochemical properties included temperature, pH, salinity, alkalinity, and dissolved oxygen were significant predictors of the marine fungal community which played an essential role in the structuring of the marine fungal environments **(Tisthammer *et al.,* 2016).** However, the seasonal regional variations of different physicochemical water characteristics in the sex areas of sample collection during the four seasons are given in **Table 1**.

Variations in water temperature depend on sampling time, climatic conditions, position and mean the daily number of sunshine hours and also affected by turbidity, humidity, and wind force **(Ivanov *et al.,* 2007)**. As expected, high water temperatures were observed during the warm period from July to September. It is well established that the ranges of water temperature significantly affected the changes in physicochemical parameters. Temperature variations decreased from summer (28-29.2ºC) through autumn (23.9-24.4ºC) reaching the minimum level during winter (14.8-15.5ºC) and increase again during spring (26.1-26.7ºC).

The increase and decrease of pH values coincided with the drop in oxygen content due to the effect of discharge of brackish water **(Abdel-Halim and Aly-Eldeen, 2016)**. The surface water pH varied seasonally from a minimum of 7.40 in winter to 8.07 in summer. Salinity was measured seawater dilution mainly caused by land-based source subsequently and discharge, which reflects the degree of aquatic contamination in the marine environment **(Zyadah *et al.,* 2004)**. Salinity values of the study area increased from winter 33.71 % through spring 40.63% reaching a maximum level of 41.25% during summer. Increases in the spring-summer period may have been the result of evaporation **(Kükrer and Mutlu, 2019)**. Dissolved oxygen is very essential to the respiratory metabolism of most aquatic organisms. It affects the availability and solubility of nutrients, low levels of dissolved oxygen facilitate the release of nutrients from the sediments and therefore affects the productivity of aquatic ecosystems **(Abdelmongy and**

El-Moselhy, 2015). The dissolved oxygen values ranged from 3.73 to 7.68 mg/L in summer with an average value of 4.81 ml/L and from 3.42 to 7.85 mg/L in Autumn with an average value of 4.89 mg/L and from 4.54 to 8.98 mg/L in Winter with an average value of 5.99 mg/L and from 3.42 to 7.98 mg/L in Spring with an average value of 5.1 mg/L.

Table 1. Seasonal variations of different hydrographical parameters at different sites along the study area.

	Season						
Region/variable	**Summer**	**Autumn**	**Winter**	**Spring**	**Min.**	**Max.**	**Mean**
Temperature (ºC):							
Abu Qir	28.0±0.13	23.9±0.22	14.8±0.13	26.1±0.23	14.8±0.13	28.0±0.13	23.3±0.17
Elasafra	28.2±0.22	23.9±0.13	15.0±0.20	26.2±0.15	15±0.20	28.2±0.22	23.37±0.17
Mohamed Nagib	28.0±0.37	24.2±0.12	15.4±0.33	26.6±0.16	15.4±0.33	28.0±0.37	23.55±0.24
Estanly	28.3±0.34	24.3±0.25	15.3±0.24	26.6±0.37	15.3±0.24	28.3±0.34	23.62±0.3
Elebrahemia	29.0±0.29	24.4±0.13	15.5±0.12	26.6±0.23	15.5±0.12	29.0±0.29	23.95±0.19
Eastern Harbor	29.2±0.23	24.4±0.15	15.5±0.10	26.7±0.22	15.5±0.10	29.2±0.23	24.0±0.17
Min.	28.0±0.13	23.9±0.13	14.8±0.13	26.1±0.23			
Max.	29.2±0.23	24.4±0.13	15.5±0.12	26.7±0.22			
Mean	28.4±0.26	24.1±0.16	15.4±0.18	26.4±0.22			
pH value:							
Abu Qir	8.01±0.02	7.71±0.12	7.45±0.06	7.91±0.23	7.45±0.06	8.01±0.02	7.77±0.1
Elasafra	7.91±0.08	7.82±0.23	7.42±0.09	7.92±0.12	.42±0.09	7.92±0.12	7.76±0.13
Mohamed Nagib	8.03±0.03	7.76±0.52	7.40±0.07	7.95±0.14	7.40±0.07	8.03±0.03	7.78±0.19
Estanly	7.9±0.13	7.8±0.08	7.45±0.32	8.01±0.02	7.45±0.32	8.01±0.02	7.79±0.13
Elebrahemia	8.07±0.03	7.82±0.05	7.41±0.13	7.98±0.19	7.41±0.13	8.07±0.03	7.82±0.1
Eastern Harbor	8.03±0.02	7.85±0.04	7.4±0.12	7.94±0.27	7.4±0.12	8.03±0.02	7.80±0.11
Min.	7.91±0.08	7.71±0.12	7.40±0.07	7.91±0.23			
Max.	8.07±0.03	7.85±0.04	7.45±0.32	8.01±0.02			
Mean	7.99±0.08	7.79±0.17	7.42±0.13	7.95±0.16			
Salinity (%):							
Abu Qir	40.81±0.7	38.1±0.22	38.6±0.83	39.8±0.41	38.1±0.22	40.8±0.71	39.35±0.54
Elasafra	40.5±0.52	38.9±0.34	39.09±0.2	39.6±0.39	38.92±0.3	40.53±0.5	39.55±0.36
Mohamed Nagib	41.2±0.93	39.1±0.13	40.04±0.9	40.6±0.73	39.1±0.13	41.25±0.9	40.25±0.68
Estanly	40.9±0.32	38.8±0.36	39.19±0.6	40.42±0.9	38.8±0.36	40.9±0.32	39.82±0.56
Elebrahemia	39.98±0.4	38.3±0.52	38.74±0.6	39.8±0.67	38.3±0.52	39.98±0.4	39.2±0.57
Eastern Harbor	38.93±0.9	35.83±0.5	33.71±0.4	36.6±0.44	35.83±0.5	38.93±0.9	36.26±0.58
Min.	38.93±0.9	35.83±0.5	33.71±0.4	36.6±0.44			
Max.	41.25±0.9	39.1±0.13	40.04±0.9	40.63±0.7			
Mean	40.4±0.60	38.17±0.3	38.24±0.6	39.49±0.5			
Alkalinity (meq/L):							
Abu Qir	2.65±0.21	2.58±0.29	3.1±0.23	2.74±0.42	2.58±0.29	3.1±0.23	2.76±0.28
Elasafra	2.92±0.09	2.71±0.21	2.54±0.09	2.91±0.43	2.54±0.09	2.92±0.09	2.77±0.18
Mohamed Nagib	2.62±0.31	2.69±0.1	2.46±0.12	2.87±0.32	2.46±0.12	2.87±0.32	2.66±0.21
Estanly	2.6±0.23	2.51±0.1	2.32±0.31	2.64±0.24	2.32±0.31	2.64±0.24	2.51±0.22
Elebrahemia	2.59±0.12	2.56±0.02	2.36±0.07	2.73±0.12	2.36±0.07	2.73±0.12	2.56±0.08
Eastern Harbor	2.61±0.31	2.5±0.03	2.35±0.12	2.65±0.21	2.35±0.12	2.65±0.21	2.52±0.16
Min.	2.59±0.12	2.5±0.03	2.36±0.07	2.73±0.12			
Max.	2.92±0.09	2.71±0.21	2.54±0.09	2.91±0.43			
Mean	2.66±0.21	2.59±0.12	2.52±0.15	2.75±0.29			
Dissolved oxygen (mg/L):							
Abu Qir	7.68±0.93	7.85±0.70	8.98±0.97	7.98±0.32	7.68±0.9	8.98±0.9	8.12±0.73
Elasafra	3.73±0.43	3.42±0.83	4.54±0.95	3.42±0.56	3.42±0.56	4.54±0.9	3.77±0.71
Mohamed Nagib	3.74±0.86	3.6±0.93	5.32±0.56	4.06±0.67	3.6±0.93	5.32±0.56	4.18±0.75

Estanly	4.65±1.02	4.12±0.82	5.21±0.76	3.95±0.55	3.95±0.55	5.21±0.76	4.48±0.53
Elebrahemia	4.07±0.84	3.84±0.62	5.9±0.96	4.26±0.45	3.84±0.62	5.9±0.96	4.51±**0.71**
Eastern Harbor	5.01±0.53	6.54±0.61	6.02±1.03	6.97±0.35	5.01±0.53	6.97±0.35	6.13±0.38
Min.	3.73±0.43	3.42±0.88	4.54±0.98	3.42±0.56			
Max.	7.68±0.92	7.85±0.76	8.98±0.92	7.98±0.32			
Mean	4.81±0.60	4.89±0.75	5.99±0.87	5.10±0.49			

The highest values during spring and winter may be related to the decrease in the air temperature leading to an increase in the solubility of the photosynthetic activity, and the atmospheric oxygen due to the abundance of phytoplankton **(Ali and Khairy, 2016)**. Alkalinity is affected by several factors as respiration and photosynthesis, nitrification, denitrification, sulfide reduction, sulfide oxidation, and $CaCO_3$ dissolution **(Saad *et al.*, 2017)**. Total alkalinity in the surface water of the study area ranged from 2.59 to 2.92 meq/L in summer with an average value of 2.66 meq/L and from 2.5 to 2.71 meq/L in Autumn with an average value of 2.59 meq/L and from 2.32 to 3.1 meq/L in Winter with an average value of 2.52 meq/L and from 2.64 to 2.91 meq/L in Spring.

2. Isolation and screening of marine fungi

This study is considered the first to use traditional Sanger sequencing and morphology identification to comprehensively analyze the fungal communities within different marine sources (sediments, water, algae, and wood) from the Egyptian Mediterranean Sea at Alexandria coastline, which helps to uncover fungal diversity and distribution patterns in marine sources of the Alexandria coastline. Fungi are essential components of marine ecosystems, when marine fungi strains are compared to strains of terrestrial fungi, they will be frequently overlooked and poorly understood **(Gladfelter *et al.*, 2019)**.

Based on locations, 252 marine fungal isolates were isolated from twenty-five sea sediments, seven water, eight algae, and eight wood samples which were collected seasonally through sex trips during the year 2017/2018 from the Mediterranean Sea, Alexandria, Egypt. The six locations selected for this study included Abu Qir, Elasafra, Mohamed Nagib, Estanly, Elebrahemia, and Eastern Harbor. Also, based on the percentage of locations, the most common fungal isolates were isolated from Abu Qir (33.73%), Elebrahemia (19.44%), Elasafra (19.05%), Estanly (15.48%), Mohamed Nagib (7.14%), and Eastern Harbor (5.16%) **(Table 2)**. Abu Qir harbored more numbers fungal taxa than the other stations, suggesting that it may be more polluted.

The sediment samples generated 145 fungal isolates (57.54%), 46 fungal isolates (18.25%) were obtained from the water samples, 34 fungal isolates (13.49%), 46 were obtained from the algae samples and 27 fungal isolates (10.71%) were generated from the wood samples (Table 2). Previous investigators also have found that fungal taxa in seawater were low **(Amer *et al.*, 2019)**. Fungi are very successful inhabitants in soil **(Frąc *et al.*, 2018)**. Correspondingly, in our study sediment samples have the highest diversity of fungi. A similar result **(Lee *et al.*, 2019)** had been observed when investigating the marine fungal communities associated with the mangrove tree *Avicennia*

alba throughout Peninsular, Malaysia, and Singapore where the sediment showed high numbers of fungal taxa. The most likely explanation is that the sediment-rich with organic and inorganic compounds that increases the fungal community.

For wood and algae, samples with a softened and roughened surface may suitable for the fungal growth while, the sandy beaches, macroalgae, and wood buried in the grains of the sand occurring near these organic substrata are idealistic **(Overy *et al.*, 2019)**. However, two different algal species were collected and identified as; *Ulva lacttuca* (green algae) and *Surgassum acinaciforme* (brown algae).

Table 2. The different percentages of fungal isolates from different locations and sources along the coastline of Alexandria, Mediterranean Sea, Egypt, (total number of isolates are 252).

Locations	**% of fungal isolates**	**Samples**	**% of fungal isolates**
Eastern Harbor	5.16	Sediment	57.54
Elebrahemia	19.44	Water	18.25
Estanly	15.48	Algae	13.49
Mohammed Nagib	7.14	Wood	10.71
Elasafra	19.05		
Abu Qir	33.73		

3. Preliminary identification of marine fungal isolates

The identifications of the 252 marine fungal isolates were initially based on gross macroscopic features as colony color and mycelial habit, including whether the hyphae were arial or growth was within the medium. Characterization of the isolated marine fungi indicated that 252 comprising 27 species of fungi. Microscopic examination is very essential in identifying or confirming the classification of the isolated fungus especially at the point of initial inoculation **(Overy *et al.*, 2019).**

The isolated fungi were examined based on cultural, microscopic, and morphological characteristics. Data in **Fig. 2**, show 27 fungal species isolated and identified. In this study, microscopic examination showed that members of the phylum Ascomycota were the most common group in this study. Twenty-two fungal species belonged to this group, while four fungal species belonged to Basidiomycota, and one only fungal species belonged to Mucoromycota. Ascomycota is the largest group of the Kingdom Mycetae with more than 32,000 species, of which more than 500 species are obligate marine origin **(Raghukumar, 2017)**. A similar result **(Abdel-Wahab *et al.*, 2019)** had been observed in the Red Sea mangroves in Saudi Arabia, twelve fungal isolates often belong to ascomycetes (ten ascomycetes, one basidiomycete, and one asexual fungus).

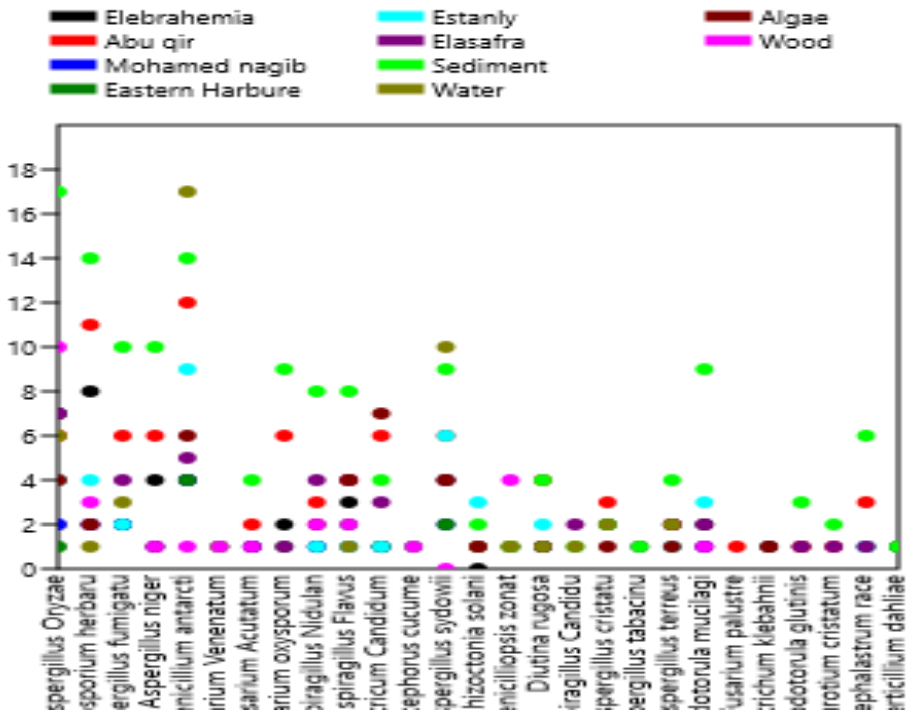


Fig. 2. Fungal species isolated and identified according to microscopic and morphological characteristics.

3.1. Numerical classification of marine fungal isolates

Representatives of all fungal morphotypes observed from each sample were picked from primary isolation. 252 colonies were selected for numerical classification according to location, sources of samples, macroscopic and microscopic examination. The similarity matrix was constructed based on the positive or negative location, sources of samples, macroscopic and microscopic examination test for each isolate which was scored as 1 and 0, respectively (**Fig. 3**). The cluster analysis of 35 isolates was carried out using PRIMER MeV 4.9.0 software according to their various characters. The dendrogram for 252 isolates was carried out using MEGA_X_10.1.6 software. Traditionally, numerical taxonomy according to morphological and physiological descriptions has been applied for fungal taxonomy leading to reliable improvements in isolates identification **(Amer *et al*., 2019)**.

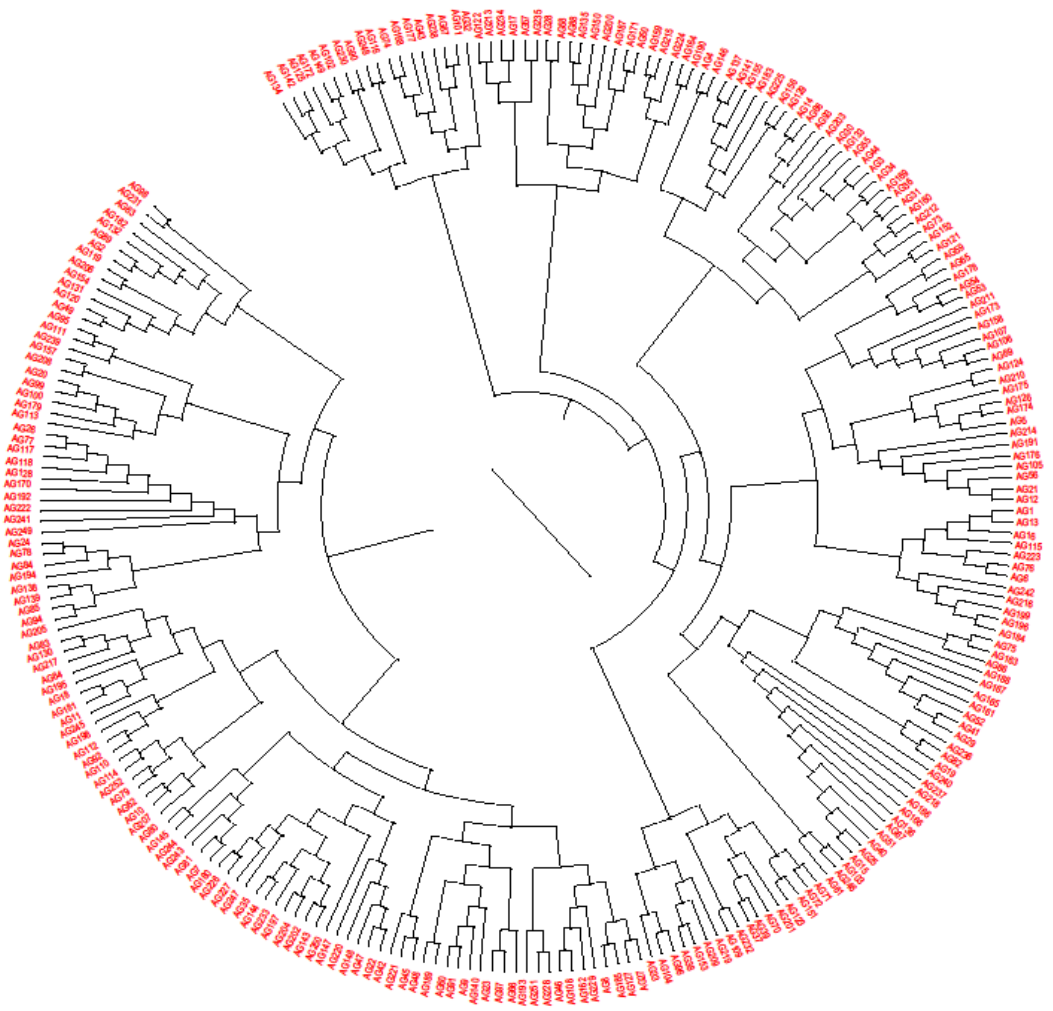


Fig. 3. Simplified dendrogram showing the relationship between fungal isolates using MEGA_X_10.1.6 software.

3.2. Isolates identification

- **ITS sequences**

The sequences of ITS-PCR products of 27 fungal isolates were obtained which helped in identification to the species level **(Arif *et al.*, 2013) (Table 3)**. Based on their similarity with ITS sequences obtained from GenBank, good quality ITS sequences could be confidently separated into five subphyla: Pezizomycotina, Saccharomycotina, Mucoromycotina, Agaricomycotina, and Pucciniomycotina.

ITS sequences from nineteen isolates in the subphylum Pezizomycotina were obtained, all of which were of good quality and formed contigs ranging from 437 to 811 base in length. ITS sequences from three isolates in the subphylum Saccharomycotina were obtained and formed contigs ranging from 389 to 696 bases. ITS sequences from

two isolates in the subphylum Agaricomycotina and Pucciniomycotina were obtained, which formed contigs 586, 591, and 679, 503 bases in length respectively. Only one isolate in the subphylum Mucoromycotina was obtained, which was formed contigs 617 bases in length. The current results indicated that Pezizomycotina was the most abundant subphyla. Similar results were obtained with previous studies that find that Pezizomycotina tends to dominate marine habitats **(Crous *et al.*, 2009)**.

The identification of fungus isolated during this investigation was based on ITS sequence data and typically supported by morphological observations; morphological features of fungus isolates were generally in agreement with descriptions of corresponding fungus type strains. Describing fungal cell morphology at a microscopic level limited the extent to which morphological observations were able to support the identification of fungal isolates.

Table 3. Isolates identification based on GenBank search results and phylogenetic analysis using ITS sequence data.

Subphylum	Highest identity sequence from GenBank					The closest neighboring organism from the phylogenetic tree
	Partial ITS sequence (bases)	Accession number	E-Value	Query cover (%)	Identity (%)	
Saccharomycotina	696	MN638741.1	0.0	100	100	*Geotrichum candidum*
Pezizomycotina	550	MW668463.1	0.0	100	100	*Penicillium antarcticum*
Mucoromycotina	617	MN638743.1	0.0	100	98.70	*Syncephalastrum racemosum*
Agaricomycotina	586	MW668464.1	0.0	100	97.95	*Thanatephorus cucumeris*
Pezizomycotina	507	MN638745.1	0.0	100	99.21	*Fusarium venenatum*
Pezizomycotina	558	MN638746.1	0.0	100	99.1	*Fusarium acutatum*
Pezizomycotina	811	MN638747.1	0.0	100	98.52	*Aspergillus oryzae*
Pezizomycotina	516	MN638748.1	0.0	99	99.61	*Fusarium oxysporum*
Agaricomycotina	591	MN638749.1	0.0	99	98.52	*Rhizoctonia solani*
Pucciniomycotina	679	MN638750.1	0.0	100	100	*Rhodotorula mucilaginosa*
Pezizomycotina	518	MN638751.1	0.0	100	100	*Cladosporium herbarum*
Pezizomycotina	554	MN638752.1	0.0	100	97.11	*Aspergillus terreus*
Pezizomycotina	590	MN638753.1	0.0	98	99.49	*Aspergillus niger*
Pezizomycotina	622	MN638754.1	0.0	100	100	*Aspergillus fumigatus*
Pezizomycotina	513	MN871428.1	0.0	100	99.42	*Aspergillus cristatus*
Pezizomycotina	609	MW668465.1	0.0	98	96.66	*Aspergillus sydowii*
Pezizomycotina	783	MW668466.1	0.0	99	98.59	*Aspergillus nidulans*
Pezizomycotina	549	MW668467.1	0.0	99	92.54	*Aspergillus flavus*
Pezizomycotina	437	MW668468.1	0.0	96	96.99	*Penicilliopsis zonata*
Saccharomycotina	389	MW668469.1	0.0	97	99.47	*Diutina rugosa*
Pezizomycotina	464	MW668470.1	0.0	100	99.57	*Aspergillus candidus*
Pezizomycotina	561	MW668476.1	0.0	99	99.28	*Aspergillus tabacinus*
Pezizomycotina	502	MW668472.1	0.0	96	96.40	*Fusarium palustre*
Pucciniomycotina	503	MW668474.1	0.0	99	95.47	*Rhodotorula glutinis*
Saccharomycotina	423	MW668473.1	0.0	99	99.18	*Geotrichum klebahnii*
Pezizomycotina	552	MW668477.1	0.0	96	97.6	*Verticillium dahliae*
Pezizomycotina	529	MW668475.1	0.0	97	96.41	*Eurotium cristatum*

- **Phylogenetic analysis**

Phylogenetic studies have been facilitated by DNA marker technologies, which offer a number of insensitive genetic markers such as random amplified polymorphic DNA, amplified fragment length polymorphism, and intersample sequence repeat **(Das *et al.*, 2017**). Phylogenetic studies of genera and species within fungi based on the sequence variability amongst 5.8S ribosomal RNA genes and internal transcribed spacer are widely used **(El-Sheikh *et al.*, 2014)**. Phylogenetic analysis was carried out using ITS sequences from a fungus isolated during this investigation and selected ITS sequences from GenBank search results. For each isolate with good quality ITS sequence data, a comparison was made between GenBank search results and the closest neighboring organism from phylogenetic trees (**Fig. 4**, **Table 3**).

All the isolates which were identified based on traditional, phenotypic methods were confirmed by molecular methods. The sizes of obtained PCR products were in the range of 389-811 bases. Results of molecular identification were completely consistent with those obtained by traditional methods. All the nucleotide sequences obtained with primers ITS1 and ITS4 together with the corresponding sequences to which these showed the closest match are deposited in GenBank under accession numbers. A BLAST search queried good quality ITS sequences from 27 isolates against the sequence database GenBank, and most sequences yielded BLAST results with high sequence identity (92.54-100%) and the Evalues were zero (0.0). For some ITS sequences, the BLAST result with the highest sequence identity was from a named species, allowing confident identification of the corresponding isolate based on GenBank search results alone.

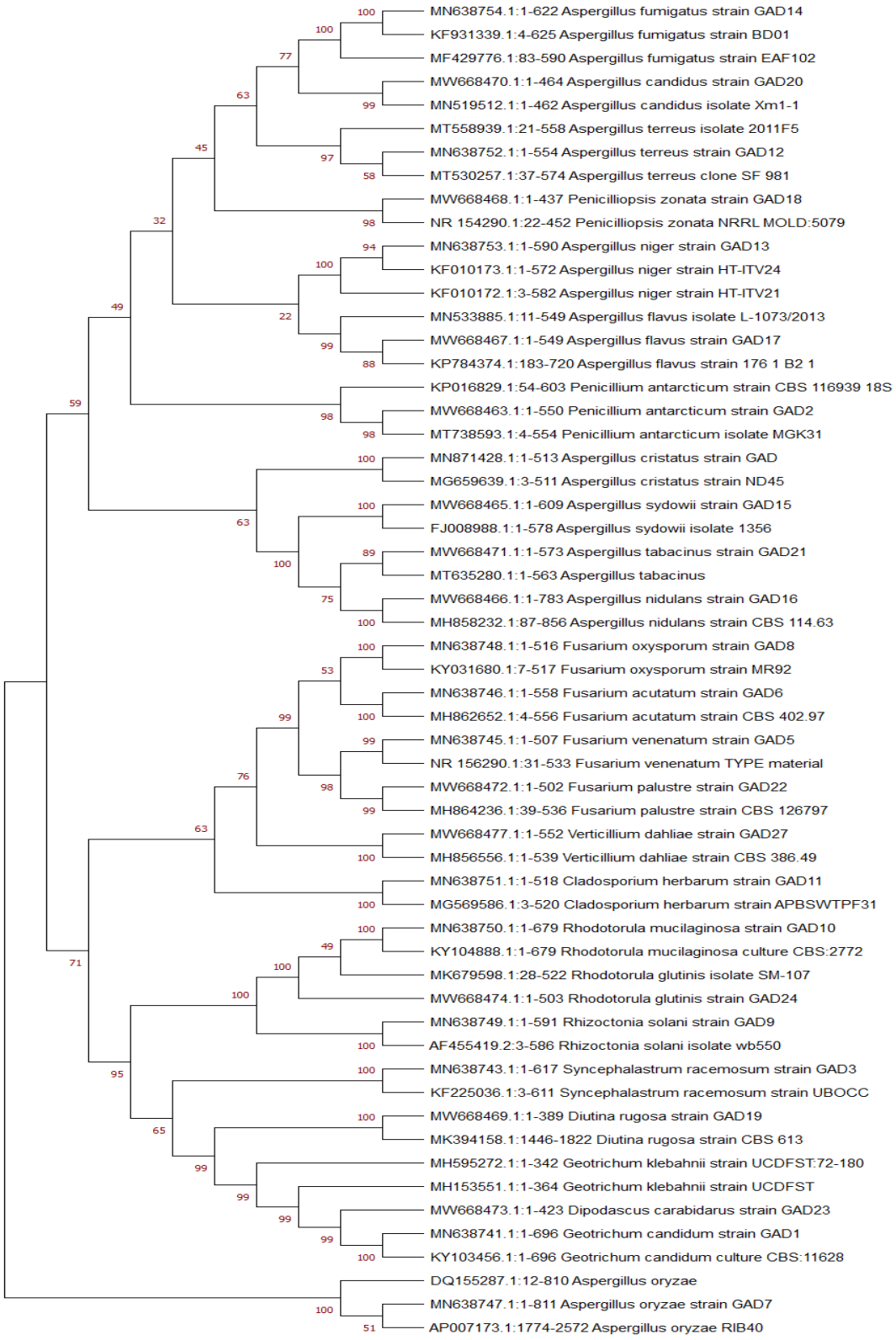


Fig. 4. Phylogenic tree of marine isolates using ITS sequence data.

4. Distribution of fungal isolates

Marine fungi are worldwide in distribution but certain taxa may be restricted geographically to the tropics, temperate, subtropics, or polar waters **(Schmit, 2003)**. The Mediterranean Sea is connected to the Atlantic Ocean and is considered a part of it. Tropical marine fungi are known from the Atlantic, Pacific Oceans, and Indian, from a wide range of substrates, supporting the greatest diversity **(Hassett *et al.*, 2020)**. However, there is little overlap between temperate and tropical regions in fungal species **(Bennett and Classen, 2020)**.

The distribution of the isolated species from variance locations is shown in **(Fig. 5)**. The results showed that *Aspergillus oryzae*, *Penicillium antarcticum*, *Cladosporium herbarum*, *aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus sydowii* were the most distributed species, and they were isolated from all locations, while *Thanatephorus cucumeris*, *Aspergillus Candidus*, and *Aspergillus tabacinus* were isolated only from Elasafra, *Fusarium palustre* was isolated from the Abu Qir only, while *Geotrichum klebahnii* and *Verticillium dahliae* were isolated only from Elebrahemia.

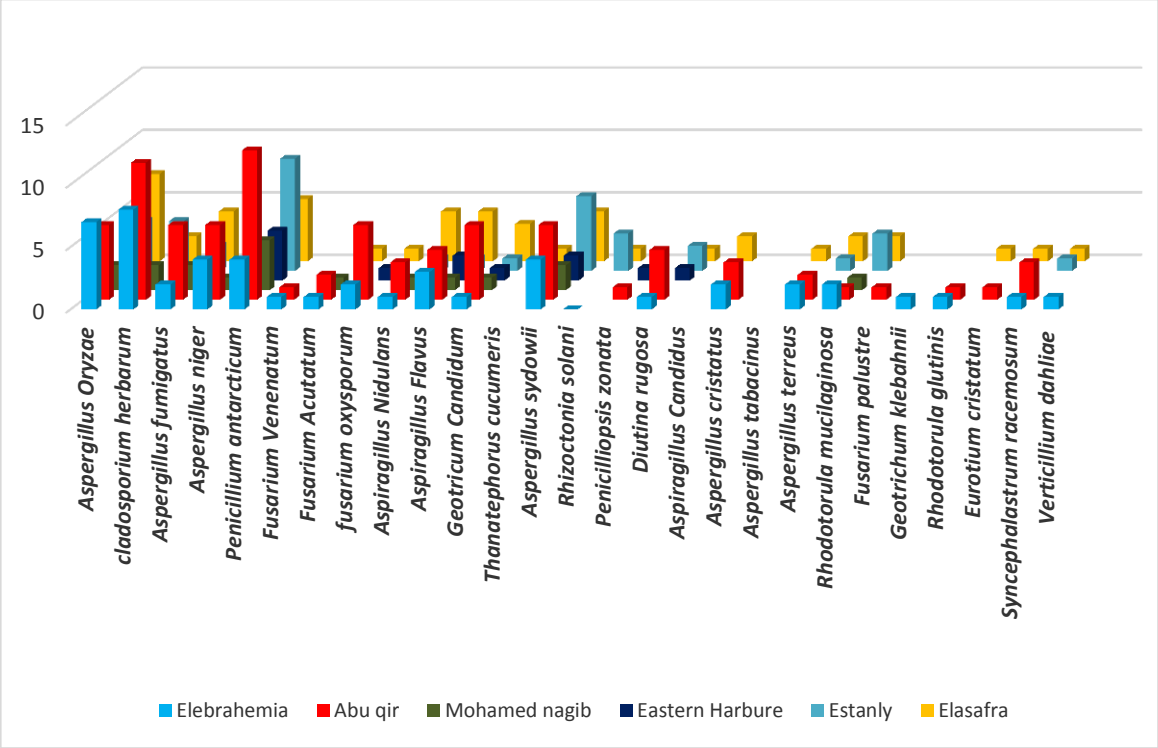


Fig. 5. Distribution of isolated species at different locations of the eastern coast of Alexandria, Egypt.

Moreover, the distribution of the isolated species from variance marine sources is shown in **(Fig. 6)**. The results showed that *Aspergillus oryzae*, *Cladosporium herbarum*, *Penicillium antarcticum*, *aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus sydowii* were the most distributed species, and they were isolated from all marine sources, while *Rhodotorula mucilaginosa*, *Rhodotorula glutinis*, *Eurotium cristatum*, *Syncephalastrum racemosum*, and *Verticillium dahliae* were isolated only from sediment, *Fusarium palustre* was isolated from the wood, while *Geotrichum klebahnii* were isolated only from algae. The total 252 OTUs corresponded to three fungal phyla (Ascomycota, Basidiomycota, and Mucoromycota), the most dominant phylum was the Ascomycota (90.8% OTUs), followed by the Basidiomycota (6.7% OTUs). A small proportion was assigned to the Mucoromycota (2.3% OTUs). In our study, we were investigated marine fungi through the cultivation of fungal isolates and subsequent molecular analyses as in another study **(Rämä *et al.*, 2014)** with other parts of the North Norwegian coast has recovered a less or more similar fungal OTUs results, where Dikarya dominates.

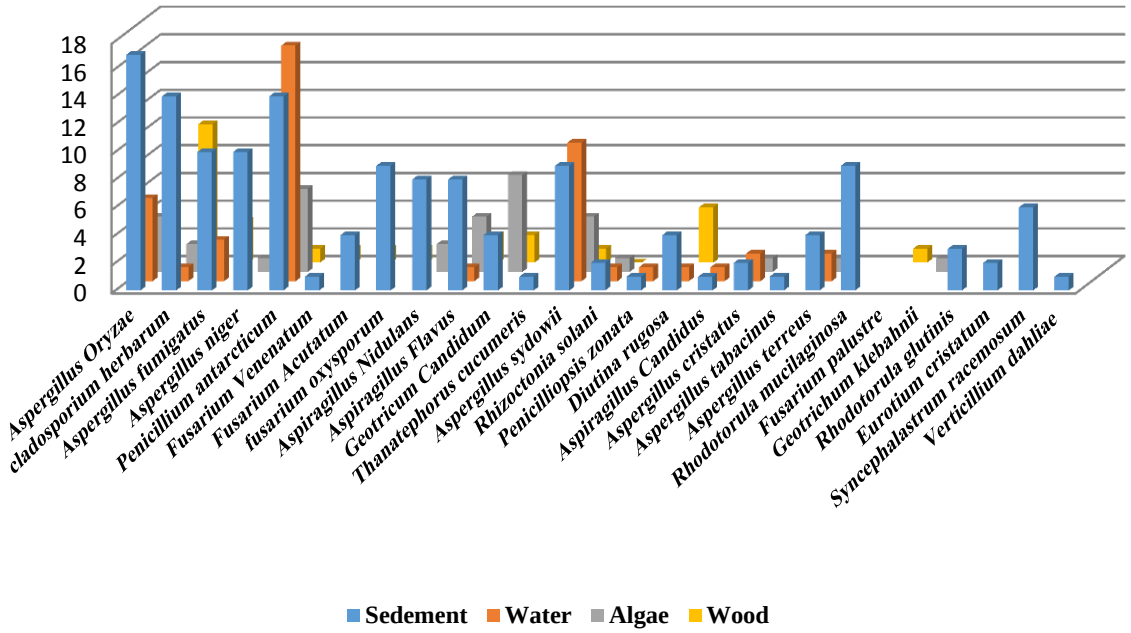


Fig. 6. Distribution of isolated species from variance marine sources of the eastern coast of Alexandria, Egypt.

4.1. Robustness of species richness

Estimators of species richness were applied to obtain reliable species numbers to be expected with the conventional cultivation techniques **(Pozo *et al.*, 2011)**. Estimates of the total species number clearly showed that our observed richness values likely underestimated total richness **(Table 4)**. Estimates of OTUs of fungal richness overlapped our observed values **(Morris *et al.*, 2014)** suggesting that these observations are robust.

Table 4. Observed and estimated the total number of OTUs by using four different estimators Chao **(Chao, 1987)**, first-order Jackknife, second-order Jackknife, and Bootstrap **(Smith and van Belle, 1984)**.

Original data set:		**SD**
Observed OTUs	27	
Chao 2:	27	0.305
Jackknife 1:	27	0
Jackknife 2:	23.44	NA
Bootstrap:	27.61	NA
Bootstrap replicates, means:		**SD**
Chao 2:	27.09	1.73
Jackknife 1:	28.03	1.70
Jackknife 2:	27.66	3.15
Bootstrap:	27.60	1.13

(SD) stands for standard deviation.

4.2. Biodiversity measures

Quantitative expressions of community structure are known as biodiversity measures. The number of species of a given taxon (species richness) and how similar species are in their abundances (evenness) are important measures of diversity **(Magurran, 2004)**. For estimating the biodiversity measures present many indices available that emphasize different components of diversity so that no unified biodiversity index is available **(Clarke and Warwick, 2001)**. So, in our study, we were measured fungal biodiversity by most of the indices available for estimating these measures. The fungal samples sediment, Abu Qir, Elebrahemia, Elasafra, with 25, 21, 20, and 20 OTUs, respectively, showed the highest richness of fungal communities. Eastern Harbor, Mohamed Nagib, wood, water, algae, and Estanly samples reveal 8, 11, 11, 12, 12, and 13 OTUs, respectively. Indices of Shannon (H) for diversity and Simpson (1–D) for evenness, and data were broadly in agreement with those reported with the richness index **(Table 5).**

The Shannon index has represented all species in a sample and that they are randomly sampled which the highest value is found in sediment (Shannon index=2.9) and similar in both localities Abu Qir and Elasafra (Shannon index= 2.77). Diversity regarded as evenness was found to be high in Sediment (Simpson's index: 0.93) and similar in

both localities Abu Qir and Elasafra (Simpson's index: = 0.92). According to Buzas and Gibson's evenness (Evenness_e^H/S), the water (Evenness_e^H/S=0.56) and wood (Evenness_e^H/S=0.68) samples proved to be less diverse which, as mentioned before, decreases in the value of the Shannon diversity index. Margalef index revealed the highest value for species richness in Elasafra (Margalef = 4.9) followed by Elebrahemia (Margalef = 4.88). The Brillouin index agrees that the highest diversity is found in Sediment (Brillouin = 2.64) followed by Abu Qir (Brillouin = 2.44) but the Fisher_alpha index shows a higher abundance of rare species in Elasafra (Fisher_alpha = 12.87) than in Elebrahemia (Fisher_alpha = 12.61). Calculated diversity indices revealed Menhinick index was found to be high in Elasafra (Menhinick = 2.88) followed by Elebrahemia (Menhinick = 2.85). the equitability index revealed the highest value for species evenness in Mohamed Nagib (Equitability=0.94) followed by Elasafra (Equitability=0.92). Berger-Parker and Chao-1 were also calculated **(Table 5)**.

Data in **Fig. 7**, clearly shows that fungal marine samples (sediment and Elebrahemia) were detached from (Elasafra) and (Abu Qir) samples, thus suggesting that their marine fungal communities were different. Wood samples were replicated to verify that the sampling effort is reliable. Results in **Fig. 8**, showed that both replicates were grouped and present in the same branch cluster. As we expected wood is the most different which form various clusters of similarity. These different similarity values may be due to nature, properties, and the long period of fungus isolation from the wood. Further clusters were formed like Eastern Harbor and Mohamed Nagib; water and Estanly; sediment and Abu Qir; Elasafra; and Elebrahemia.

Table 5. Means of total fungal OTUs, diversity indices of samples from different locations and sources.

Locations and sources Diversity	Elebr-hemia	Abu Qir	Mohmd Nagib	Eastern Harbor	Estanly	Elasfra	Sedim-ent	Water	Algae	Wood
OTUs richness (S)	20	21	11	8	13	20	25	12	12	11
Total number of individuals	49	86	18	13	38	48	145	46	34	27
Dominance_D	0.082	0.074	0.1173	0.1716	0.124	0.075	0.065	0.211	0.126	0.190
Simpson_1-D	0.917	0.925	0.8827	0.828	0.875	0.925	0.934	0.788	0.873	0.809
Shannon_H	2.726	2.775	2.274	1.925	2.296	2.773	2.9	1.915	2.239	2.013
Evenness_e^H/S	0.7637	0.7635	0.8837	0.857	0.7642	0.8007	0.7271	0.5658	0.7818	0.680
Brillouin	2.258	2.443	1.691	1.384	1.902	2.293	2.642	1.621	1.84	1.596
Menhinick	2.857	2.264	2.593	2.219	2.109	2.887	2.076	1.769	2.058	2.117
Margalef	4.882	4.49	3.46	2.729	3.299	4.908	4.822	2.873	3.119	3.034
Equitability_J	0.91	0.9114	0.9484	0.9258	0.895	0.925	0.901	0.770	0.900	0.839
Fisher_alpha	12.61	8.856	12.01	8.855	6.975	12.87	8.708	5.277	6.61	6.92
Berger-Parker	0.163	0.139	0.2222	0.3077	0.236	0.145	0.117	0.369	0.205	0.370
Chao-1	26.00	26.00	14.00	11.33	16.33	27.20	28.75	17.00	15.33	16.00

As a further perspective, the fungal world is full of many interesting applications which encourage the researchers to attempt to recover new strains/isolates in particular that belong to extreme environments, fungi could contribute to finding new substances that act as antimicrobials in a similar finding with plants **(Suleiman, 2020)** to tackle the emerging problem of bacterial resistance to antibiotics **(Suleiman, 2017)**, production of valuable substances such as biodiesel **(Hashem *et al*., 2019)**, PUFAs from the oleaginous fungi **(Suleiman *et al*., 2018a; Suleiman *et al.*, 2018b)**. Our future studies will deal with the extraction of some fungal enzymes with inspiring biotechnological applications.

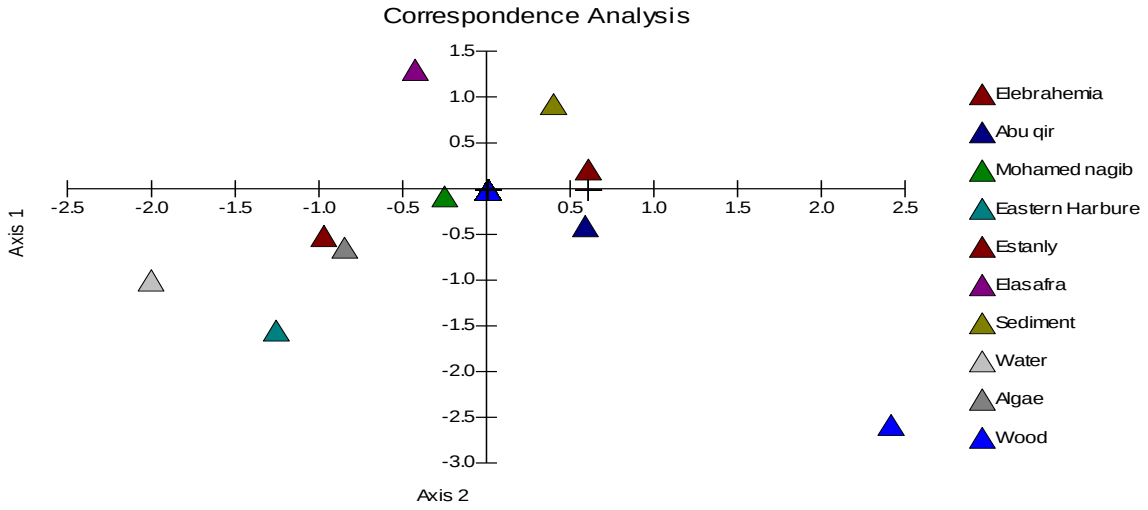


Fig. 7. Correspondence analysis of the different fungal communities in different locations and sources of the eastern coast of Alexandria, Egypt. Marine samples are positioned along the two axes.

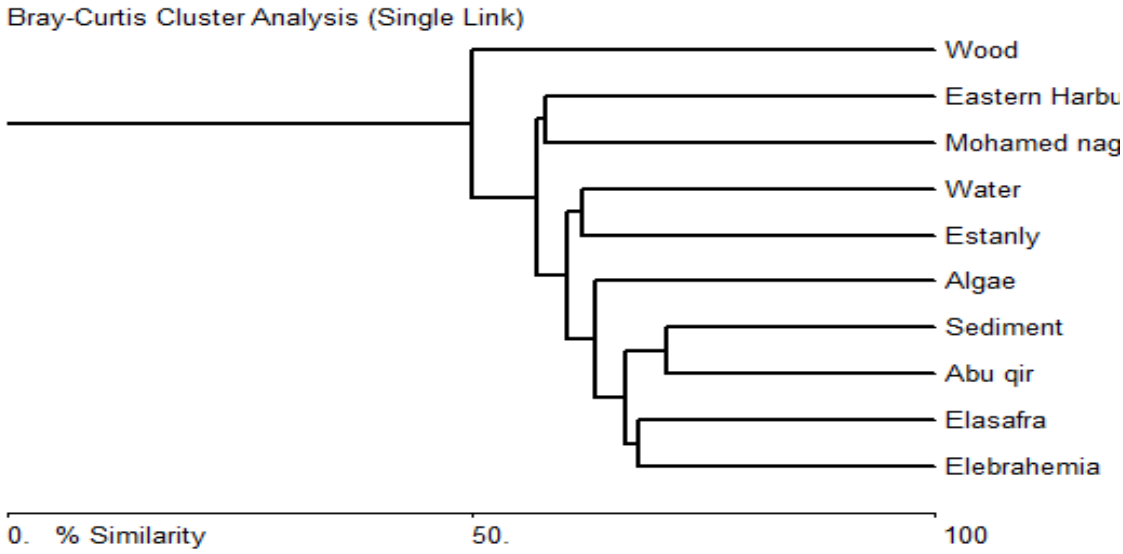


Fig. 8. Bray-Curtis cluster analysis of different fungal communities in different locations and sources of the marine eastern coast of Alexandria, Egypt.

CONCLUSION

According to our findings, the biodiversity measures of marine fungal isolates were isolated from sea sediment, water, algae, and wood samples present many indices available that emphasize different components of diversity so that, in our study, we had measured fungal biodiversity by most of the indices available for estimating these measures. The fungal samples from sediment, Abu Qir, Elebrahemia, Elasafra, with 25, 21, 20, and 20 OTUs, respectively, showed the highest richness of fungal communities. Estimates of total species number showed that our observed richness values likely underestimated total richness. All marine fungal isolates from this work will be an integral part of future work to produce efficient enzymes which can be used for industry.

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