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MOLECULAR DIVERSITY OF HALOPHILIC FUNGI ISOLATED FROM MANGROVES ECOSYSTEM OF MIANI HOR, BALOCHISTAN, PAKISTAN

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Abstract

To find the halophilic fungal biodiversity hundred soil samples were collected from mangrove ecosystem of Miani-Hor Balochistan. Miani-Hor lagoon is about 50 km long with a single inlet from sea. Out of hundred soil samples 14 different halophilic and halotolerant microorganisms were isolated. Growth characteristics along with microscopy delineated diversity of the halophilic fungal isolates. To determine the molecular level identification and classification, DNA extraction and consequently sequencing was done. Molecular biodiversity was determined by internal transcribed spacer region of fungus. Molecular identification along with phylogenetic tree determination was achieved by using ITS regions of the species belonging to the genus Aspergillus (4 isolates), Penicillium (4 isolates), Alternaria (3 isolates), Fusarium (1 isolate) and Pleosporaceae (2 isolates). Diversified halophilic and halotolerant fungi from mangroves ecosystem in Pakistan will open doors for the application of indigenous species as a biorefineries.

Key words: Mycology, Extremophiles, Molecular detection, Phylogenetics, Internal transcribed spacer.

Introduction

Environment where propagation and survival of organisms is difficult and sometime impossible is known as extreme environment. The abiotic factors like temperature, pressure, salinity, pH, radiations, nutrients availability and oxygen tension formulate the extreme environment. Exceeding and elevation of such abiotic factors make the environment extreme where organisms feel hard to live or survive (Oren, 2002). Scientist explored such unusual habitat with the advancement in research and technology. Earlier it was believed that only bacteria and archaea can resist extreme conditions. Organisms adopted to extreme conditions are known as extremophiles. Like other eukaryotic organisms, concept about fungus was that they cannot live in extreme salt conditions (Gunde-Cimerman *et al*., 2004; Ali *et al*., 2013). Isolation, detection and characterization of halophilic and halotolerant fungi is comparatively new and emerging field. Halophilic fungi are capable to grow and propagate in the natural environment prevailing the salt (NaCl) concentration of three molar (Gunde-Cimerman *et al*., 2009), while halotolerant microbes can tolerate such high concentration of salt (Ali *et al.,* 2014; Ali *et al.,* 2016). Though many halophilic fungal species are known but still more are yet to be identified.

Recognition of fungi as halophilic or halotolerant and isolation of such fungi from high salt environment happened in near past (Gunde-Cimerman *et al*., 2000). These fungi exhibit all their metabolic features in extreme condition of prevailing environment. The halophilic fungi survive in the presence of salt ranges from 20 to 30 % NaCl concentration (Ali *et al*., 2014). While moderate halophiles can withstand the NaCl salt concentration ranging from 5 to 20 % (w/v). Fungi which can tolerate salt concentration of 2 to 5% w/v are known as slight halophiles (Gunde-Cimerman *et al*., 2009; Ali *et al*., 2013).

Halophilic or halotolerant organisms living under extreme conditions of salt explored from the biological rich sites of mangroves. The hypersaline ecosystem occupies well diverse biotic and abiotic factors. Availability of organic matter in the form of plant organic cell components and variety of physical conditions make their habitat suitable to flourish. Fungus diversity is generally greater in such environment (Griffith, 1994; Ali *et al*., 2013). Fungal biodiversity of Miani-Hor is higher due to presence of mangrove forest. Various observations have been made on naturally existing high salt environments and man- made solar saltern. Number of species belonging to different genera have been reported. Study conducted on man-made solar saltern by (Ali *et al*., 2013) reported halophilic species of genera *Aspergillus* and *Penicillium*. Species of genus *Alternaria*, *Cladosporium* and *Wallemia* isolated from hypersaline environment (Gunde-Cimerman *et al*., 2000; Zalar *et al*., 2007).

Many fungal species show resemblance in their morphological and microscopic appearances. Their growth pattern and basic structure show coincidence. Molecular diversity is novel approach. This application is more specific and most sensitive comparatively to morphological and microscopic identification and classification. For the analysis of fungal diversity from an environmental sample, the internal transcribed spacer (ITS) has been chosen as a standard marker and barcode for fungal genetic material (Ali *et al*., 2018). Molecular identification of fungi from their DNA barcoding is precise and integral part of fungal ecology investigation. This tool is a way to find the fungal diversity at species level and even within the species, *(*Ali *et al*., 2016). Hence molecular approach has been applied to find out fungal diversity of the isolated extremophilic isolates from mangrove.

Present study aims to investigate halophilic fungal diversity from Miani-Hor mangrove site situated in province of Balochistan, Pakistan. This is the first study of its kind in targeted sampling area to explore its fungal biodiversity, this study will open doors for further scientific research and novel exploration.

Materials and Methods

Site description: Pakistan has total 990 Km of coast. This coastal area stretches between two provinces. Balochistan province contain 660 Km of coast. Balochistan coastal area consists of two coastal lagoons. The Miani Hor is located in district Lasbella. Miani Hor mangrove forest occupies about 8500 Acre land. The lagoon stretched to an area of about 363 km and 50 Km square long (Qureshi, 2005; Baig & Iftikhar, 2007). Soil samples were collected in pre-sterile sealable polyethene bags from Miani Hor and brought to the laboratory maintaining room temperature. The samples were processed in the laboratory within 24 h of its collection. The site is connected to Arabian Sea with an inlet of about 200 meters (Saifullah & Rasool, 2002).

Isolation of halophilic fungal isolates: Serial dilution assay was performed for each soil sample. All samples were analyzed for halophilic fungal isolation. Fungal growth media Potato Dextrose Agar (PDA) added with 15 % NaCl salt was used to support the growth of halophilic and halotolerant fungi from soil samples and to retard the growth of rest of fungi while following methodology adopted by Ali *et al*., (2013). Growth media was kept for incubation at 28 ºC temperature for 5-7 days. Halophilic fungal isolates were screened by allowing to grow without the addition of salt in PDA. The isolates were sub-cultured on salt (5-20%) added PDA for pure culture.

Morphological and microscopic analysis of halophilic fungal isolates: Morphological characteristics of fungal isolates were noted by examining growth feature on PDA supplemented with NaCl salt. Morphological characteristic of each fungal isolate observed from initial growth till to mature growth. Besides growth characters on solid media, microscopic examination performed by wet mount technique using cotton blue stain. Initial observations were made under compound microscope using objective lenses of high power field 10, then 20, and finally 40X. Isolates hyphal shape, color, and other features were observed. Similarly, spore shape, pattern of their organization and their originating bodies were observed following methodology adopted by Ali *et al*., (2013).

DNA extraction and molecular conformation of the isolates: To find the molecular diversity of halophilic fungal isolates obtained from mangrove sites of Miani-Hor were subjected to DNA extraction. The DNA from selected isolates were extracted by using Nucleo Spin Plant II kit (Macherey-Nagel, Duren, Germany) adopting the procedural protocol of (Ali *et al*., 2013)**.** Standard protocols of the kit were applied mentioned on the kit.

Molecular confirmation of fungal isolates: Extracted DNA were sent to Macrogen Korea to find out their sequences. Internal transcribed Spacer (ITS) was obtained for each DNA. ITS regions of rDNA (Benson *et al*., 2008; Ali *et al*., 2013) is popular for identification of fungi below generic level. This region is also attractive in taxonomy of fungi. The ITS1 and ITS2 have high intrinsic evolution rate that's why fit for species specific (Schoch *et al*., 2016). Isolates were identified with the help of National Center of Biotechnology Information (NCBI) web by using BLAST for the similarities between the sequences. Accession numbers were obtained after submission of sequences (Ali *et al*., 2018).

Phylogenetic analysis: The most similar species compared with isolates for their phylogeny. Neighbor joining method was used to find the phylogenetic tree with the help of DNASTAR Lasergene and MEGA 6 version 5.10 (Tamura *et al*., 2013; Ali *et al*., 2018).

Results

Total number of organisms isolated from the selected site were 14 with capability of growing on media supplemented with 15% salt NaCl (w/v). After screening for halophilic potential isolates were subcultured again on different salt (NaCl) concertation supplemented with PDA. Out of total (14) isolates 4 were from genus Aspergillus, 4 species were from Penicillium, 3 isolates were from Alternaria, one from Fusarium and two from family Pleosporaceae. All the isolates showed good growth at PDB added with different salt concentrations 5-20%. Growth pattern and morphological observations from primary to mature growth were noted.

Morphological and halophilic characteristics: Morphology and growth characteristics of isolates on PDA supplemented with 5-20% NaCl salt and incubation period at optimum temperature were observed. Each isolated fungal isolates were subcultured on salt added media and growth pattern was observed from initiation of mycelium till mature growth. Surface details on PDA after optimum incubation period revealed that Aspergillus species were bluish, greenish, light grey to dark grey, velvety to granular appearances. Following Ali *et al*., (2013) the growth of isolates was observed and recorded, Penicillium species surface were light to dark grey, velvety uniformed growth on salt added PDA. Fusarium species were yellowish in color, fluffy raised in appearance over the culture plate. Alternaria species were light grey in color while Pleosporaceae appeared as compact golden yellow. Aspergillus and Penicillium species peripheries were whitish during development. Initially, during journey to mature growth there were no color appearances on growth media. Reverse color of all petri plates for all fungal isolates also observed. Reverse of plates were haline to light yellow in case of Aspergillus and Penicillium while Pleosporaceae was black and dark brown. Plate reverse color of Fusarium species were blackish and Alternaria was found light brown (Fig. 1; Table 1).

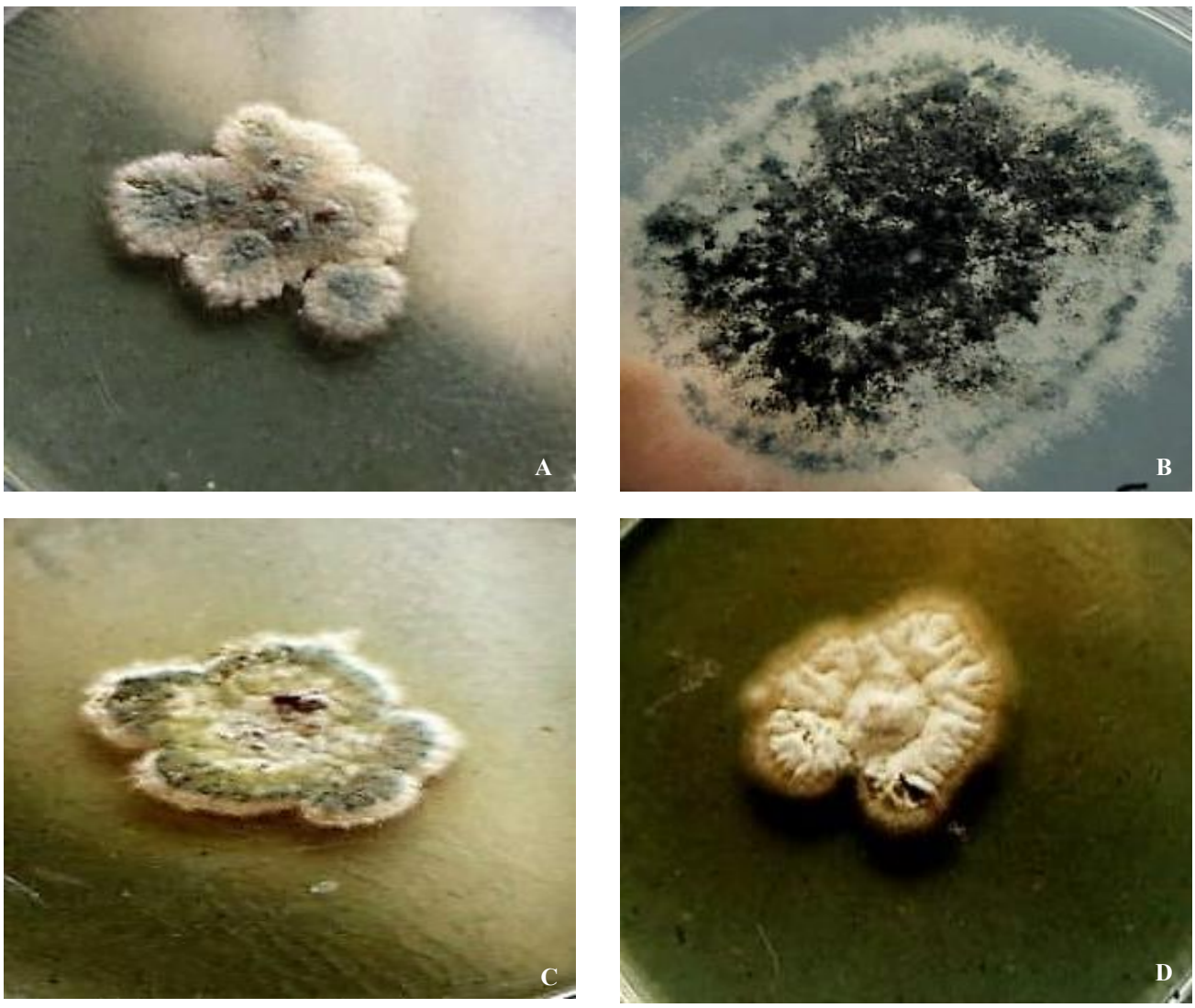

Fig. 1. Morphology of different halophilic isolates on 10 % NaCl supplemented potato dextrose agar *Penicillium citrinum* **(A),** *Aspergillus tonophilus* **(B),** *Aspergillus flavipes* **(C),** *Aspergillus terreus* **(D)**.

Microscopic characters of fungal isolates: The structural details of the isolates were observed under compound microscope (Fig. 2 and Table 1). Cotton blue stained Aspergillus species were found consisted of conidia densely present over the phialids. In case of biseriate metulae were supported by vesicle. Phialids were conical in shape. The whole structure formed over a conidiophore (Fig. 2 and Table 1). Penicillium species recognized by their unique branched like appearance. Broom like phialids occupied by conidia. Metulae were also branched. Fusarium species revealed sickle shaped thin walled Macroconidia. Conidia were septate internally forming 3-5 small units inside a single conidium. Alternaria species were identified by their specific oval, centrally swollen and thin at margin conidia. Hyphae were thin and newly originating conidium appeared on the tip of older one. The cells were centrally divided into two or three partitions. Two isolates were from family Pleosporaceae having thin abundant hyphae and somewhat long spores internally furcated.

Extraction of DNA from fungal isolates and their molecular confirmation: Molecular confirmation made by DNA extraction from all selected 14 fungal isolates from soil samples taken from Miani-Hor. In molecular analysis of ITS 1-4 regions of DNA were sequenced. The sequenced data of the isolates subjected to NCBI, BLAST analysis for their similarities with most nearest species. Molecular sequenced data of the fungal isolates submitted to GenBank and accession numbers for nucleotide sequences were obtained. Samples coded with number C1, C2, C3, C8b were species of Aspergillus having accession number MH282504, 05, 06, and 08 respectively. Sample with code number C7, C9b, C13b, and E2 were Penicillium species having accession numbers MH282507, 09, 11, and 14. C12 and C14 isolates were from family Pleosporaceae with accession number MH282510 and MH282512. Samples code E3, E5a, and E10 have accession number MH282515, MH282516, and MH282517 were Alternaria species while MH282513 is the accession number of Fusarium species (Table 1).

Table 1. Fungal isolates characteristics on potato dextrose agar supplemented with 5-20% W/V NaCl

Isolates code	Accession number	Growth appearance	Background color	Mycelium	Spore type	Isolates name
C1	MH282504	Bluish grey, velvety, granular, uneven	Creamy, hyaline	Vesicle, metulae and Septate hyphae	Conidiospore over phialids	*Aspergillus chevalieri*
C2	MH282505	Dark grey, granular velvety, uniform	Creamy, hyaline	Septate hyphae	Conidiospore	*Aspergillus tonophilus*
C3	MH282506	Grey, greenish velvety, granular, rises and ridges	Light yellowish	Septate hyaline hyphae	Conidiospore	*Aspergillus terreus*
C7	MH282507	Grey velvety, rises and ridges	Off white	Septate and hyaline	Conidiospore	*Penicillium citrinum*
C8b	MH282508	Grey, velvety, granular	Off white	Hyaline and septate	Conidiospore	*Aspergillus flavipes*
C9b	MH282509	Similar to C7	Off white, light yellow	Septate haline hyphae	Conidiospore in chains	*Penicillium chrysogenum*
C12	MH282510	Velvety uniform compact	Dark brown	Septate	Macroconidia	Pleosporaceae spp.
C13b	MH282511	Grey velvety centrally curved	Off white	Septate	Conidiospore on branching conidiophores	*Penicillium commune*
C14	MH282512	Golden yellow, smooth velvety.	Dark brown	Septate	Long and short macroconidium	*Pleosporaceae* spp.
E1	MH282513	Yellowish fluffy	Blackish	Septate hyaline	Sickle shaped long macro conidia	*Fusarium culmorum*
E2	MH282514	Light grey velvety	Dark black	Septate	Conidiospore	*Penicillium oxalicum*
E3	MH282515	Light grey fluffy granular	Yellowish	Septate	Pencil shaped macro conidia	*Alternaria tenuissima*
E5a	MH282516	Light brown. Peripherally white	Yellowish	Septate Simple or branched	Pencil shaped macro conidia Internally septation	*Alternaria alternata*
E10	MH282517	Grey white cottony	Off white	Septate	Pencil shaped macro conidia	*Alternaria alternata*

Phylogenetic tree analysis: The phylogenetic tree was constructed using neighbor joining method to find inter and intra relationship between and among the halophilic fungal species (Fig. 3). It was found that phylogenetic tree showed two nodes of which one node represented most of Aspergillus and Penicillium species, while the other having species from Pleosporaceae, Alternaria species predominantly.

Discussion

From the last two decades the general concept about the presence of organisms in high salt environment was changed. The prevailing concept was that the growth at high salt environment is sole ability of bacteria. Although fungi known to be ubiquitous in nature but before 2000, growth of fungi at high salt environment was not known (Gunde-Cimerman *et al*., 2000; Ali *et al*., 2013). Fungi evolved under extreme condition of salt and found the ways to cope with adverse conditions and to sustain their existence under high salt environment (Ali *et al.,* 2013; Wang *et al*., 2014). Majority of the isolated species from mangrove ecosystem belonged to genera Penicillium and Aspergillus which showed coincidence with study conducted on Red sea coast (Alwakeel, 2013). The genus Aspergillus has higher rate of adaptability to high salt environment (Kis-Papo *et al*., 2003).

A fungus belongs to well diverse eukaryotic organism. Fungi are highly resilient and capable to adjust itself not only in versatile condition but also under adverse environment. That's why the diversity of fungi is higher. Fungi composed of second largest group after insects (O'Brien *et al*., 2005). Number of various molecular approaches make easier the identification and differentiation up to species level (Anderson, *et al*., 2004; Ali *et al*., 2018). Various approaches like Restriction fragment length polymorphism of ribosomal, mitochondrial and nuclear DNA has been used to determine species identification (Anderson *et al*., 1987; White *et al*., 1998; Pérez *et al*., 1999). Isolates are identified by molecular approaches after sequencing their 18S rDNA (Ali *et al*., 2018). The technique used by Harrington & Wingfield (1995) consisted of PCR reaction, and RFLPs of Intergenic Spacer Region (IGS).

ITS region sequence comparison is favorable for molecular phylogeny and taxonomy. It shows high degree of variation even in closely related species, it could be detected from a small amount of DNA (Baldwin, *et al.,* 1995, Song, *et al.,* 2012). In molecular analysis the ITS region is extensively sequenced part of DNA. This region of DNA is a recommended universal barcode sequence of fungi (Yang *et al*., 2016*).* The ITS sequence not only signifies species level molecular classification, but also within the species (Schoch *et al*., 2016). The sequences of ITS remained conserved irrespective of evolution in plant (Chase & Fay., 2009; Bast & Felix. 2009). Sequences of all the isolates submitted to NCBI GenBank. Morphological observations and molecular confirmations revealed that majority of the isolates belonged to genera *Fusarium*, *Aspergillus*, *Alternaria* and *Penicillium*. These organisms were also isolated from other regions of high saline environment (Kis-Papo *et al*., 2003; Ali *et al.,* 2013). Researchers has explored the halophilic fungal species for the production of multiple biochemicals (Ali *et al*., 2014b; Ali *et al*., 2016). Batista-García *et al*., (2014) reported the production of cellulases, xylanases, manganese peroxidase (MnP) and esterases from halophilic *Aspergillus caesiellus*.

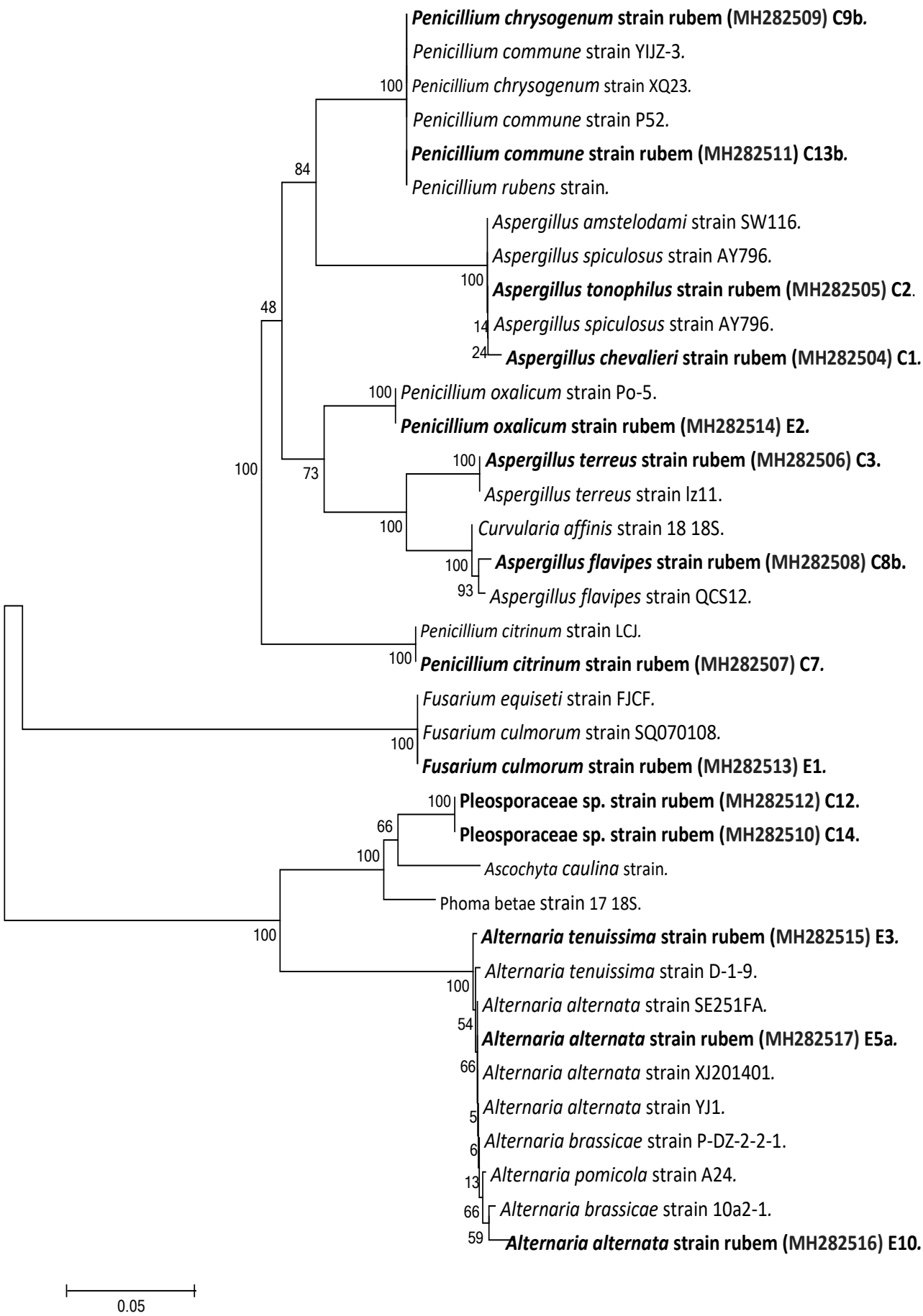

Fig. 3. Detailed phylogenetic tree of halophilic fungal species isolated form mangrove ecosystem.

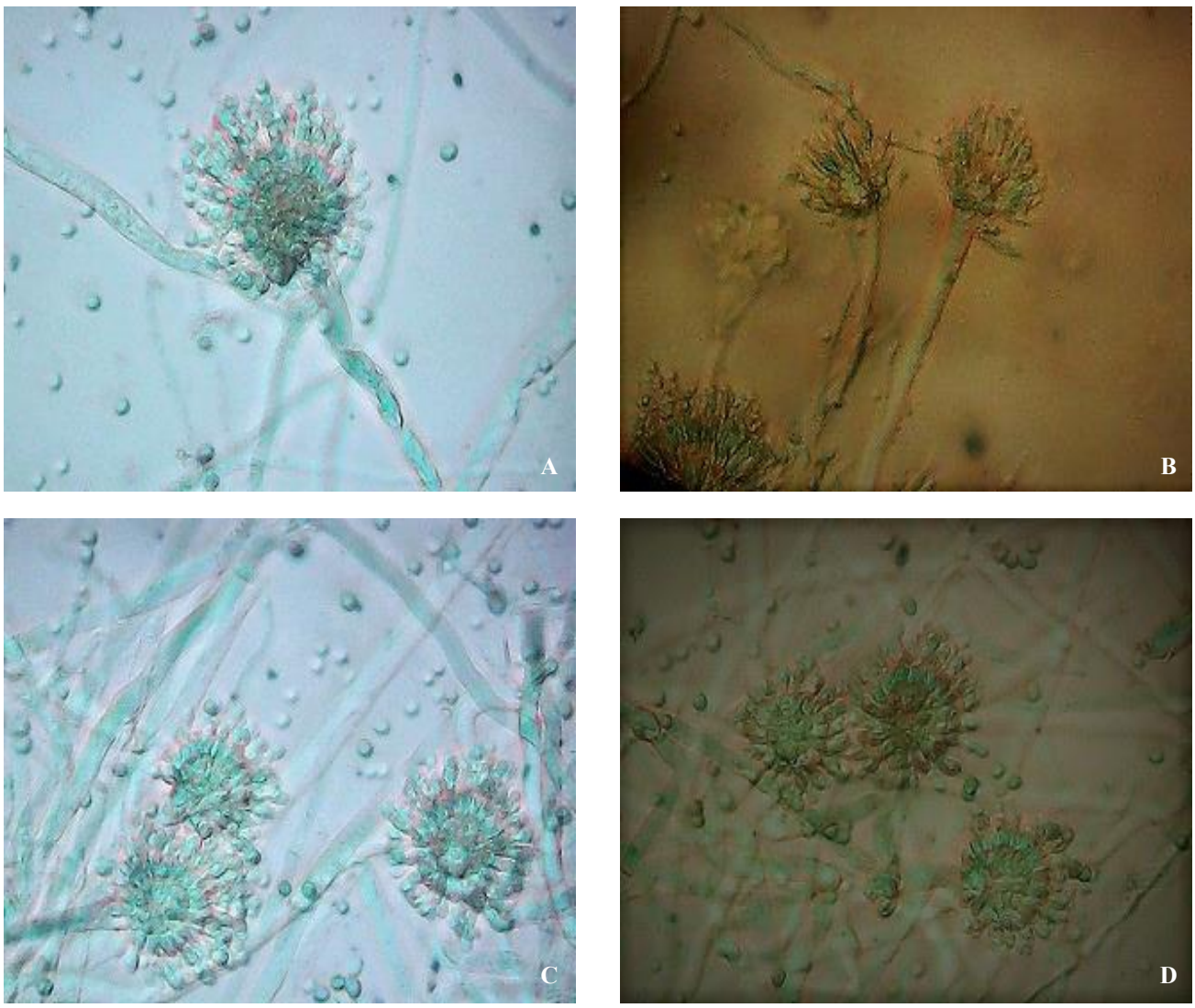

Fig. 2. Structure of fungal isolates under copound microscope used objective lense 20 X, *Aspergillus tonophilus* **(A)**, *Penicillium citrinum* **(B)**, *Aspergillus flavipes* **(C)**, *Aspergillus terreus* **(D).**

Conclusion

In this study a number of halophilic fungal species were isolated, which were confirmed molecularly. The isolates belonged to diversified genera of fungi. It was found that mangrove ecosystem of the Balochistan coastal areas were rich in microbial biodiversity. These microbes can be a useful biorefineries for the industrial uses in future. The molecular techniques along with basic microscopy was helpful in the identification of species in this study. The molecular techniques are rapid, sensitive and more specific. Mangrove ecosystem is rich in fungal diversity and needs more exploration for biologically potent biodiversity and biotechnological significance of the new and existing species. The indigenous species of halophilic fungi from local ecosystem can be better explored for the production of antibiotics and other useful biochemicals.

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