Fungal Diversity Present at a Hypersaline Environment in Cabo Rojo, Puerto Rico Determined by Characterization of Isolates and Analysis of Environmental rRNA Genes Clones Libraries

by

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Abstract

Solar salterns are a thalassohaline environment where sea water is evaporated to obtain salt for commercial purposes. The prokaryotic diversity on these places has been extensively studied world wide. However, there are few reports that focus their attention on the fungal diversity present on this extreme environment. The main objective of this research project was to isolate and characterize halotolerant filamentous fungi from the salterns of Cabo Rojo, Puerto Rico. To determine the occurrence of fungi in these solar salterns, water samples were filtered, plated on three different media (Sehgal and Gibbons, malt extract, and potato dextrose) each at 15% NaCl and incubated at 29ºC. Selected isolates were characterized using light and scanning electron microscopy, and some physiological properties like secondary metabolite profiles, optimal NaCl concentration, pH and temperature were determined. Molecular analysis using RFLP patterns of a PCR amplicon containing the 5.8S rDNA and ITS1-ITS2 regions was also used to classify the isolates into groups. Taxonomic results indicated that several groups of fungi can be obtained from this hostile environment. A total of eight genera and seventeen species were reported. These include the filamentous fungi *Alternaria* sp. *Aspergillus elegans, A. sydowii, A. terreus, A. versicolor, A oryzae, A. japonicus, A. niger aggregate, Cladosporium cladosporioides, C. sphaerospermum, Eurotium amstelodami, Penicillium chrysogenum, P. expansum, Stemphylium* sp. and an unknown species. The dimorphic yeast, *Hortaea werneckii,* was also isolated at a high frequency. Many isolates tolerated NaCl concentrations up to 30% and showed a wide range of growth temperatures and pH. The ability of these isolates to grow at high salt concentrations

might indicate that they are part of the biological diversity of the saltern. The fungal diversity was also analyzed by non-culture dependent methods like 18S rDNA clone libraries. The amplification of fungal DNA was very difficult to achieve due to the complexity of the saltern community. However, after protocol optimization many clones were obtained and an *in silico* analysis was performed. Environmental sequences belonging to the genus *Aspergillus* were the most frequent Operational Taxonomic Unit (OTU), but other ascomycetes and basidiomycetes OTUs were also obtained. The combination of morphological, physiological and molecular approaches was very useful to describe the fungal community at the solar salterns of Cabo Rojo. This survey represents one of the first approaches to study fungal diversity on extreme environments in the Caribbean.

Resumen

Las salinas artificiales son un ambiente talasohalino en donde el agua de mar se evapora para obtener sal con propósitos comerciales. La diversidad procariótica de estos lugares ha sido extensamente estudiada. Sin embargo, muy pocos estudios enfocan su atención en conocer la diversidad de hongos presentes en aguas hipersalinas. El objetivo principal de este estudio fue aislar y caracterizar hongos filamentosos halotolerantes presentes en las salinas de Cabo Rojo, Puerto Rico. Para determinar la ocurrencia de hongos en estas salinas, se filtraron muestras de agua y se colocaron en platos conteniendo tres medios de cultivo diferentes: medio de Seghal y Gibbons, agar de papa y dextrosa y agar de extracto de malta cada uno con 15% de NaCl. Las muestras se incubaron a 29˚C. Se caracterizaron los aislados seleccionados utilizando microscopía de luz y microscopía electrónica de rastreo, propiedades fisiológicas como la producción de metabolitos secundarios, concentración óptima de NaCl, así como condiciones óptimas de pH y temperatura. Se realizó una caracterización molecular utilizando los patrones de restricción de la región que contiene la ITS1-5.8S-ITS2 del rADN para clasificar los aislados. Los resultados taxonómicos indican que se pueden recuperar hongos de este ambiente hostil. Un total de 8 géneros y 17 especies fueron encontradas. Entre los hongos filamentosos aislados se encuentran: *Alternaria* sp. *Aspergillus elegans, A. sydowii, A. terreus, A. versicolor, A oryzae,* agregado de *A. niger*, *Cladosporium cladosporioides, C. sphaerospermum, Eurotium amstelodami, Penicillium chrysogenum, Penicillium* sp., *Stemphylium* sp. y una especie desconocida. también se aisló en alta frecuencia la levadura dimórfica *Hortaea werneckii*. Muchos de los aislados toleraron hasta una concentración de NaCl de 30% y mostraron crecimiento en una gama de valores de

temperatura y pH . La habilidad de las cepas de tolerar altas concentraciones de sal podría indicar que éstas pueden ser parte de la diversidad biológica de las salinas. La diversidad presente en las salinas también se estudio utilizando métodos no dependientes de cultivo como las bibliotecas de 18S del rADN. La amplificación del ADN de hongos fue muy difícil de lograr debido a la complejidad de la comunidad de las salinas. Sin embargo, luego de la optimización de los protocolos se obtuvo muchos clones y se realizó un análisis *in silico*. Secuencias ambientales pertenencientes al género *Aspergillus* fueron las unidades taxonómicas operacionales (UTO) obtenidas en mayor frecuencia, pero también se obtuvo UTOs relacionados a otros ascomicetos y basidiomicetos. La combinación de las técnicas morfológicas, fisiológicas y moleculares fue útil para describir la comunidad de hongos en las salinas de Cabo Rojo. Este catastro representa uno de los primeros esfuerzos en estudiar la diversidad de hongos en un ambiente extremo en el Caribe.

Dedication

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Introduction

Microscopic fungi are distributed worldwide. They are important components of the ecosystems and play important biological roles such as nutrient cycling, soil formation, providing nutrition to plants through their roots, and transformation of waste material into useful products (Christensen, 1989; Tuomela et al., 2000). Depending on the type of environment in which fungi live in, they have shown different adaptations that allow them to complete their functions in the ecosystem. One type of environments to which fungi have adapted are those that have low water activity (a_w). Fungi that can grow under these conditions are considered osmophiles or osmotolerants.

Osmophiles or osmotolerant fungi can be classified into two different groups: xerophilic/xerotolerant or halophilic/halotolerant. The first group includes those who can grow at low a_w imposed by organic ions such as sucrose and glycerol. Meanwhile, halophilic or halotolotolerant organisms can grow in environments dominated by inorganic ions such as sodium (Na^+) and magnesium (Mg^{2+}). Although the stress caused by both types of environments is not necessarily the same, fungi thriving on both types of habitats share many of the adaptations.

Many studies are concentrated on xerophilic/xerotolerant yeasts and moulds because they are a frequent cause of food spoilage (Wheeler and Hocking, 1993; Halouat and Debevere, 1997; Abellana et al., 1999a-b; Vytrasova et al., 2002) The most common genera in this group are *Aspergillus* and *Penicillium* or their perfect forms (Abellana et al., 1999a-b). On the other hand, halophilic and halotolerant fungi have been studied recently because they are an excellent eukaryotic model for salt resistance (Prista et al., 1997; Petrovic et al., 2002). Halophilic and halotolerant fungi can be found growing at hypersaline waters where the total salt concentration is very high (saturation point).

These saline waters are named athalassohaline or thalassohaline depending of their origin.

Athalassohaline waters are influenced by the geology of the place where they develop and they form mainly from salt deposits originated from evaporative events. Thalassohaline environments are originated from the evaporation of sea water and, therefore, have an ion composition dominated mainly by sodium and chloride. Solar salterns are an example of this last type of environment. The NaCl concentration of these man-made pools can range from 3% to saturation (35%). Like other salterns, the Cabo Rojo system in Puerto Rico consists of a series of interconnected ponds in which the concentration of salt increases as sea water evaporates by solar radiation (Davis, 1978). Even though these conditions might appear hostile for life, many microorganisms are able to thrive in this extreme environment (Montalvo-Rodríguez et al., 1997; 1998; 2000; Gunde-Cimerman et al., 2000; Zalar et al., 1999a).

Reports on the diversity and role of fungi on hypersaline environments are scarce. One of the first studies on this area came from Cronin and Post (1977), where they reported the isolation of halophilic fungi from a natural athallasohaline hypersaline environment (the Great Salt Lake, Utah). Another habitat where the interest in extremophilic fungi has been focused on is the Dead Sea (Buchalo et al., 1998). A novel species of halophilic fungi (*Gymnascella marimortui*) belonging to the Ascomycota was identified by Buchalo et al., (1998) from these waters (where there is a high concentration of magnesium ions). Recently, the isolation and characterization of different species of fungi growing in the solar salterns of Spain (Zalar et al., 1999a) and Slovenia was reported (Gunde-Cimerman et al., 2000).

In recent decades, researches have paid attention to understand the role of fungi in the degradation of organic materials in marine and hypersaline ecosystems (Buchalo et al., 1998). The solar salterns of Cabo Rojo, Puerto Rico have been the subject of study of the prokaryotic diversity for the past years (Montalvo-Rodríguez et al., 1997; 1998; 2000). As a result from these studies, several novel extremely halophilic archaea were isolated and characterized (Montalvo-Rodríguez et al., 1998; 2000). To date, there are no reports about halophilic and halotolerant fungi from the Caribbean, especially in Puerto Rico. For this reason, the main goal of this research project consists of a taxonomical analysis of the fungi isolated from the solar salterns of Cabo Rojo, using traditional methods such as morphologic and physiological studies combined with molecular techniques. This taxonomical study constitutes the first report of extremophilic fungi for the West Indies. The isolation and characterization of these microorganisms could provide new sources of products of biotechnological importance. In addition, a preliminary survey of fungi present in these hypersaline waters using environmental genomics to access the cultured and uncultured community was also performed. This is the first attempt where this methodology has been implemented for fungi in a solar saltern.

Literature Review

Hypersaline Environments

Athalassohaline and thalassohaline are two kind of hypersaline environments. In the first, the ionic content is influenced by the geological composition that surrounds the waters. The Great Salt Lake in Utah and The Dead Sea in Israel are typical examples of this type of environment. The Dead Sea has an ionic composition dominated by magnesium. The brines of the Red Sea and lakes of the Atacama Desert, Northern Chile, are typical athalassohaline waters too. By contrast, thalassohaline environments are originated mainly from sea water evaporation, hence having a composition dominated by sodium and chloride ions. This condition can be achieved artificially by a gradual concentration of the sea water in consecutive evaporative ponds (solar salterns). As evaporation occurs, the precipitation process of calcite, gypsum, halite, sylvite and finally carnallite occurs sequentially. The system is designed so NaCl precipitates during the last stage of salt production (Brock, 1979). There are many solar salterns around the world such as the Marine salterns Sečovlje of Slovenia, the Mallorca and Santa Pola in Alicante, Spain, the Salinas Chicas in Argentina, San Francisco Bay in USA, Shark Bay in Australia, Los Roques in Venezuela, Eilat in Israel, Alexandria in Egypt, Margherita d′ Savoia in Italy, and Cabo Rojo, Puerto Rico. One characteristic shared by athalassohaline and thalassohaline environments is that their pH values are near neutrality. Another type of hypersaline environment is the alkaline soda lake where the high salt concentration is combined with high pH due to carbonate. The Baer Soda Lake in China and Manyara Lake, Africa, are examples of hypersaline alkaline waters. Other high salt environments can also have high temperatures such as the Tunisian Salt Lake.

Diversity at Hypersaline Environments: Archaea, Bacteria and Algae

A diversity of microorganisms can thrive in hypersaline environments. Life is represented by the three domains in both athalassohalinne and thalassohaline conditions. In the Dead Sea, an athalassohaline ecosystem, there are reports on the characterization of members of Archaea, Bacteria and Eucarya (Oren, 1995; 2001; Blum et al., 2001). A novel species of the genus *Halorubrum* was isolated in the Atacama Saltern, Chile (Lizama et al., 2002).

Many taxonomical analyses had been performed on thalassohaline systems. A new genus belonging to the Archaea domain, *Halogeometricum,* was isolated and characterized by Montalvo-Rodríguez et al., (1998). Several new species belonging to the genera *Haloterrigena*, *Haloferax* and *Haloarcula* have been also reported (Montalvo-Rodríguez et al., 2000; Asker and Ohta, 2002; Ihara et al., 1997). Representatives from the Bacteria domain can also be found on this environment. The genus *Thermohalobacter* was isolated from a saltern (Cayol et al., 2000). A new genus of halophilic bacteria, *Salinibacter*, was isolated and characterized (Antón et al., 2002) from a similar environment. Today, a huge list of new species of prokaryotes have been isolated from hypersaline waters.

Eukaryotic microorganisms can also be present in solar salterns. The green algae, *Dunaliella,* can be found in high salt natural environments like the Dead Sea (Oren and Shilo, 1985) and artificial systems like salterns (Richmond, 1986). *Dunaliella salina* was reported growing at a solar saltern (Salina Fortuna) from Lajas, Puerto Rico (Burkholder, 1967). A study of variations in their population in Salina Botoncillo, Puerto Rico, showed

a significantly density of the algae. This density was affected by salt production management (Rosado, 1990).

The water of the salterns is generally red colored due to the presence of high densities of haloarchaea, green algae *Dunaliella salina*, and bacteria from the genus *Salinibacter* (Oren, 2001). It was believed that eukaryotic microorganisms (with few exceptions) are not being capable to adapt extreme conditions like hypersaline waters (Oren, 1999; Pedrós-Alió et al., 2000).

Fungi in Saline and Hypersaline Environments

Salt tolerant fungi have been isolated from many sources. A lot of fungi can be isolated from seawater (Kohlmeyer and Kohlmeyer, 1979). The presence of fungal hyphae in the interior of living corals from the Caribbean and South Pacific was illustrated by Kendrick et al. (1982). Fungi associated with coral reef have been investigated for years. Some species are: *Halographis runica*, an endolithic lichenoid, *Corallicola nana* that are attached to dead coral slabs, *Xenus lithophylli*, a parasite of calcified algae, and *Lulworthia calcicola*, another coral-inhabiting species, (Kohlmeyer and Kohlmeyer 1988, 1989, 1992).

Some “terrestrial” fungi had been isolated from sea water or sea water flora and fauna. *Aspergillus* and *Cladosporium* were the most frequent isolates found in samples of sand, wood and mangrove in la Parguera, Puerto Rico (Acevedo-Ríos, 1987). Particularly in sand, other isolates were: *Cephalosporium, Diplodia, Fusarium, Helmithosporium, Penicillium, Trichoderma, Penicillium* and *Scopulariopsis.* Also, the genus *Pleospora* was found. Another study in La Parguera, Puerto Rico, performed by Calzada-Cordero

(1988) involves the fungus *Lindra thalassiae,* which is associated with the sea grass *Thalassia testudinum*. An important case is the fungus *Aspergillus sydowii.* Recent studies of a sea fan pathogen resulted in the isolation of this common terrestrial mould (Geiser et al. 1998). Only the marine strains of this fungus are pathogenic. That fact was demonstrated by the inoculation of sea fans with cultures of both, terrestrial and marine origin

A study of coastal mycology in Puerto Rico was performed by Nieves-Rivera (2005) including many aspects of fungi in marine environments. Also, a study of arenicolous filamentous fungi in the Mayagüez Bay shoreline was performed by Ruiz-Suárez, (2004) who reported the presence of *Aspergillus, Cladosporium, Dreschlera, Fusarium, Geotricum, Penicillium*, *Trichoderma*, *Mucor* and *Rhizopus*. The genus *Aspergillus* represented the 80% of total fungal abundance. Her data suggested that salinity concentration may regulate the abundance of fungi in the shoreline.

Fungi can tolerate higher salinities than the one of sea water. Pitt and Hocking (1977), examined the growth of xerophilic fungi (*Aspergillus ochraceous, A. flavus, Chrysosporium fastidium, Eurotium chevalieri, Xeromyces bisporus* and *Wallemia sebi*) in the presence of glycerol, sodium chloride and glucose/fructose mixture.

Salt marshes, a moderate salt environment, are another habitat for halotolerant fungi (Abdel-Hafez et al., 1978). Fungi have an important role in the decomposition of salt marsh grasses. Halophytes from salt marshes in Europe were examined for the presence of mycorrhizal fungi (Hildebrandt et al., 2001). Large quantities of spores were found in saline soil; 80% of them were identified as *Glomus geosporum*. Also, they have found that several plants from the salt marsh were mycorrhizal Another study suggests

that these fungi confer salt tolerance to plants based on the occurrence of *G. geosporum* and *G. caledonium* spores in salt marshes (Landwehr et al., 2002). This suggestion was supported later by Carvalho et al., (2004). They indicated that fungi in salt marsh plants may have the potential to confer salt tolerance to them and may influence the distribution of this vegetation. This study was made in a salt marsh from Portugal and showed that fungal adaptation is one of the possible reasons for the existence of mycorrhizal fungi in salt marshes (Carvalho et al., 2004).

Other types of soils can be saline environments (Abdel-Hafez et al., 1977;1989b). Some dessert soils have salts as one of their components. Osmotolerant and osmophilic fungi can be found here. A new species, *Exserohilum sodomii*, was isolated from soil near Dead Sea (Guiraud, 1997). In soils from Aswan (Osi and Sarah regions) and EL-Sharkia, Cairo were isolated *Penicillium* sp., *Mucor hiemalis, Aspergillus flavus, A. ochraceus, A. niger, A. terreus, Stachybotrys chartarum, Acremonium fusidioides, Acremonium* sp., *Cladosporium cladosporioides, Mucor* sp., *Absidia* sp., *Alternaria* sp., *Gliocladium* sp. *Pseudeurotium zonatum, Torulomyces lagena, Trichoderma* sp., *Fusarium* sp., *Cunninghamella echinulata, Botrytis* sp., *Paecilomyces* sp. and *Helminthosporium* sp. Several isolates, including *A. niger, A. terreus, A. flavus*, *Fusarium* sp., *Trichoderma* sp., *Penicillium* sp., *Mucor hiemalis*, and *Stachybotrys chartarum*, were tested for their halotolerance in medium with up to 10% of NaCl (Razak et al., 1999).

Fungi can be found in salted food. Water relations of xerophilic *Aspergillus candidus, A. sydowii, Paecilomyces variotii,* and *Eurotium amstelodami* isolated from salted fish were examined. *Eurotium amstelodami* germinated more rapidly than others (Wheeler and Hocking, 1988). Also, the interactions of xerophilc fungi were investigated

in order to define the species interactions and important environmental factors determining the dominant fungal species on dried salted fish (Wheeler and Hocking, 1993). More reports on xerophilic fungi were performed elsewhere. Pérez-Laguillo (1989) reported seventeen genera and thirty five species of moulds and yeasts as part of de mycobiota associated with the syrup used to prepare soft drinks in Puerto Rico. Filamentous fungi like *Alternaria alternata, Aspergillus flavus, A. niger, A. sydowii, A versicolor, Cladosporium herbarum, Eurotium chevalieri, Geotrichum penicillatum, Paecilomyces lilacinus, P. variotii, Penicillium citrinum, P. corylophilum, P. fellutanum, Phoma* sp., and *Syncephalastrum racemosum* were found. Several genera of yeast were also isolated: *Candida, Cryptococcus, Rhodotorula, Saccharomyces, Torulaspora, Schizosaccharomyces* and *Zygosaccharomyces.* From this research, he concluded that pH was the most important factor affecting the product quality.

Fungi can also grow in hypersaline waters. Xerophilic and halophilc fungi are able to grow in media with low water activities (a_w) and they can be expected to survive in this type of environment. In 1977, Cronin & Post reported the isolation of halophilic fungi belonging to the genus *Cladosporium* growing in a submerged piece of pine wood in the Great Salt Lake in Utah. In the same lake, non filamentous fungi from the genus *Thraustochytrium* were also found (Amon, 1978). Later, Butinar et al., (2005b) reported the occurrence of the yeasts *Debaryomyces hansenii* and *Metschnikowia bicuspidata* in this lake.

The first record for filamentous fungi in Dead Sea by Buchalo et al., (1998) reported a novel species, *Gymnascella marismortui* (Ascomycota). This fungus was shown to be an obligate halophile that grows optimally in a range of 10-30 % of Dead

Sea water. Moreover, they isolated *Ulocladium chlamydosporum* and *Penicilium westlingii* with salt tolerance in the range of 3 to 15 % NaCl at 26˚C. In another study, *Aspergillus versicolor, Chaetomium globosum, Eurotium herbariorum, E. amstelodami and E. rubrum* were isolated from Dead Sea waters and together with *Gymnascella marismortui* were tested for survival of spores and mycelia in Dead Sea water for prolonged time (Kis-Papo et al., 2001; 2003a). After the first record of fungi, Kis-Papo et al., (2003b) performed a study of genomic diversity of *A. versicolor* and their results suggested that genomic diversity was positively correlated with stress. The species *Trichosporon mucoides, Rhodotorula larynges,* a *Candida glabrata*-like strain and a *Candida atmosphaerica*-like strain were also isolated from these waters. *Candida glabrata* was not known for their halotolerance (Butinar et al., 2005b). *Candida atmosphaerica* was found associated with another extreme environment, the deep-sea hydrothermal systems of the Mid-Atlantic Rift (Gadanho and Sampaio, 2005).

The Mono Lake in California, an alkaline, hypersaline and closed basin, was the site of a study performed by Steiman et al., (2003). This lake is compared with the Dead Sea due to its high salinity. Those conditions permit the mineral formation named tufa. Steiman et al., (2003) isolated many species of the genera *Aspergillus, Achaetomium, Acremonium, Alternaria, Chaetomium, Cunninghamella, Ulocladium, Embellisia, Fusarium, Phoma, Sporothrix, Penicillium, Papulaspora, Geotricum, Curvularia, Mucor, Phialophora, Phaeoramularia, Sporormiella and Thelebolus*. Interestingly, in this study they were not able to isolate fungi from water using culture methods. They suggested that the salinity, the , and most important the pH (very alkaline), are not favorable for fungal growth (Steiman et al., 2003).

Most of the studies on halophilic or halotolerant fungi come from isolates of solar salterns ponds of Europe. In salterns of Seca, at the Adriatic coast in Slovenia, a new yeast, *Trimmatostroma salinum* was found (Zalar et al., 1999a). Other yeast species isolated from saltern waters were described by Zalar et al., (1999b). These strains belonged to the species *Aureobasidium pullulans, Phaeotheca triangularis,* and *Hortaea werneckii*.

Aureobasidium pullulans has been isolated before from Slovenian salterns (Gunde-Cimerman et al., 2000) and from *Spartina alterniflora* in a salt marsh. This fungus has a role on the decomposition of *Spartina* blades in the salt marshes. The tolerance and response to salinity and temperature of this fungus was studied by Torzilli, (1982;1997) and Torzilli and Managbanag (2002). The yeast *Phaeotheca. triangularis* was first reported by de Hoog et al., (1997) from a humidifier. Later, Zalar et al., (1999b) showed that this yeast might be an obligatory halophile because it grows better in saltern medium than *Hortaea werneckii. H. werneckii,* an agent of human tinea nigra, can tolerate up to 30% NaCl and that tolerance seems to be adaptive.

Hortaea werneckii, a black yeast, was described as *Cladosporium werneckii,* and later, the name was changed to *Exophiala werneckii*. Nishimura and Miyaji (1984) proposed the new genus *Hortaea*, while Mcginnis et al., (1985) proposed the genus *Phaeoannellomyces* to group all black yeasts that duplicate by anellospores like this yeast. Until the report of *Hortaea acidopila* (Holker et al., 2004), *Hortaea werneckii* was the only species known for this genus. In 1992, de Hoog and Gerrits van de Ende suggested that the salt can be the decision factor in the etiology of the tinnea nigra infection. Hyperhidrosis appears to be a risk factor for this disease. Abundant

transpiration and the raise of salt in the skin might facilitate the adhesion of the yeast (de Hoog and Gerrits van de Ende, 1992), that is lipolytic (Gottlich et al., 1995). The natural ecological niches for this melanized yeast are the hypersaline waters in salterns (Gunde-Cimerman et al., 2000). Previously, de Hoog and Gerrits van de Ende (1992) indicated that the natural niche of *Hortaea* should have higher electrolyte concentrations than normal ocean water.

Other yeasts were isolated in the Slovenian salterns. These isolates belong to *Pichia guillermondii, Rhodosporidium babjevae, R. sphaerocarpum, C. parapsilosis,* and a *C. atmosphaerica*-like strain. *Candida parapsilosis* was also isolated from Samouco salterns in Portugal. In addition to the Great Salt Lake in Utah, the yeast *Debaryomyces hansenii* was reported from salterns on the Atlantic coast of Namibia. Besides to the Dead Sea waters and Slovenian salterns, *Candida atmosphaerica*-like was isolated from waters in la Trinitat together with *Pichia philogaea*-like (Butinar et al., 2005b). The last yeasts species was isolated first from soil by van der Walt and Johannsen, (1975). In other salterns at Eilat, Israel, *Yarrowia lipolytica* and *Trichosporon mucoides* were isolated (Butinar et al., 2005b). The cellular processes involved in salt tolerance and osmotolerance in *Y. lipolytica* were previously studied by Andreishcheva et al., (1999) who demonstrated that this yeast has the potential to be found in hypersaline environments. A novel alkali-tolerant *Y. lipolytica* strain was isolated by Zvyagilskaya and Persson (2005) from leaves of a plant growing in Negev Desert, Israel.

Like the Dead Sea, the Salt Lake in Utah, and other saline environments, the filamentous fungi can be found in artificial salt ponds. *Alternaria alternata* and *Cladosporium cladosporioides* were identified in a study performed by Méjanelle et al.,

(2001). *Cladosporium* was detected with the highest frequency before halite concentration (Gunde-Cimerman et al., 2000). The genus *Eurotium*, an important mycotoxin producer (Samson et al., 2000), contains several species that have been isolated from moderate saline water and soils (Abdel-Hafez et al., 1978; 1989a-b; Grishkan et al., 2003). A recent study reported the spatiotemporal ocurrence of *Eurotium* spp. isolated from hypersaline waters around the world (Butinar et al., 2005a). Diversity of *Eurotium* in eutrophic salterns is higher in contrast with oligotrophic waters. *E. amstelodami* was the most frequent isolate in the salterns. A potential new species, *"E. halotolerans"*, was isolated during this study. They suggested that the fungal diversity present at hypersaline environments is composed by air-borne contaminants and the halotolerant indigenous community. The contaminants are not able to survive for long times. By contrast, the members of indigenous community are adapted to vegetative growth and long-term survival (Butinar et al., 2005a).

Fungal Adaptations to Hypersaline Conditions

Halotolerant and halophilic microorganisms are found in three Domains of life: Bacteria, Archaea and Eucarya. To date, two principal strategies to handle with osmotic stress have been described. There are the salt-in strategy and the "compatible-solute" strategy. In the last years, several issues concerning the preference, importance and cost of these strategies to organisms were reviewed (Yancey et al., 1982; Oren 1999; Welsh, 1999)

The first strategy is use by the archaea of the order *Halobacteriales* (Lanyi, 1974; Bayley, 1978) and bacteria of the order *Haloanaerobiales* (Oren, 1986; Rengpipat et al.,

1988). Their intracellular environments are distinguished by the presence of KCl in molar concentrations (Lanyi, 1974; Oren, 1986). They have salt adapted proteins that contain many acidic amino acids and a low content of hydrophobic amino acids. These proteins need the high salt concentration to maintain their structure and function.

The term “compatible solute” was introduced first by Borowitzka and Brown in 1974. Later, Brown (1990) defined them as solutes when present at high concentration permit the enzymes to work properly. These solutes can be synthesized by the cell or taken up from the environment. An advantage of this strategy is that is not necessary to have special adapted proteins. Many halophilic and halotolerant microorganisms use quaternary amines, sugars and their derivates, amino acids and their derivates, and polyols. “Compatible-solutes” are accumulated by the organism to be important carbon and nitrogen sources.

Filamentous fungi exhibit adaptive responses to various stresses which make them excellent survivors in nature (Markham, 1992). Certainly, the germination of fungi can be affected by the presence some solutes (Pitt and Hocking, 1977). For osmotic stress, fungi use “compatible-solutes” as the main strategy. Mycorrizal fungi from a sodic site in Canada were assessed for their growth under different NaCl quantities (Bois et al., 2005). The ascomycetes studied used mechanisms other than carbohydrate accumulation to handle with NaCl stress. A *Hymenoscyphus* strain, in response to NaCl treatments, accumulated proline. A *Phialocephala* isolate may have used compounds such as melanin, and the basidiomycete species accumulated mainly mannitol and/or proline in response to increasing concentrations of NaCl (Bois et al., 2005). Trehalose, a disaccharide, was reported as a “compatible-solute” for *Saccharomyces cerevisiae* under

NaCl stress (García et al., 1997). *Aspergillus nidulans* can produce trehalose, which is found in high concentrations under some types of stress. The results from the study of trehalose by Fillinger et al., (2001), are consistent with the role of this disaccharide in the acquisition of stress tolerance. The methods to handle with stress in *A. nidulans* have been extensively studied. Besides, *Aspergillus nidulans* produces glycerol and erythritol in response to salt stress (Beever and Laracy, 1986). Glycerol has a role in lipid biosynthesis and other processes (Dijkema et al., 1986). Redkar et al., (1995) demonstrated that sodium chloride can induce an alternate pathway that involves the $NADP^+$-dependent glycerol dehydrogenase I utilizing dihydroxyacetone (DHA) and $_{DL}$-glyceraldehyde ($_{DL}$-GAD). Those substrates are used in normal conditions by the fungi (Hondman et al., 1991). They studied the response in salt adapted and salt shocked strains of *A. nidulans*. Redkar and others found more induction in the salt adapted strains than in salt shocked strains. Their results suggest that the constitutive expression of the enzyme is up-regulated or modulated by NaCl but the mechanism in unknown. A study demonstrates the constitutive production of $NADP^+$-dependent glycerol dehydrogenase in *A. nidulans* and *A. niger* (Schuurink et al., 1990). Later, it was demonstrated that glycerol dehydrogenase is essential for osmotolerance in *A. nidulans* (de Vries et al., 2003).

The high osmolarity glycerol response (HOG) pathway responds to hyperosmotic stress. This pathway has been involved in the transcription of proteins for glycerol biosynthesis (Norbeck et al., 1996). This pathway was studied before in the yeast *S. cerevisiae* (Albertyn et al., 1994; Norbeck et al., 1996). A homolog of the gene involved in HOG was isolated from the black yeast *Hortaea werneckii*. Regulation of the gene activation occurs at posttranscriptional level in response to an increase in salinity (Turk

and Plemenitas, 2002). Compatible solutes probably are the principal method of this yeast to osmotic adaptation. In a recent study, Kogej et al., (2005) assumed that the accumulation of ions probably does not contribute significantly.

Other adaptations to osmotic stress include some changes to the cellular membrane (Russell, 1989). In the halotolerant *Aspergillus repens* several morphological and ultrastructural changes are induced by NaCl (Kelavkar et al., 1993). Those changes include a considerable difference in growth rate and an expansion in cell surface area caused by the decreases in oxygen solubility. In their observations, the fungal cells became larger, more septate, and had an increased branching. The mitochondria size and numbers increased as well the number of cristae per mitochondria. Also, the cristae face changed in their appearance. The study revealed the presence of pinocytic vesicles involved in the uptake of Na^+ ions.

Another study with the black yeast *Hortaea werneckii* showed a regulation for sterol biosynthesis in their membranes (Petrovic et al., 1999). The regulation is different to that performed by *Saccharomyces cerevisiae.* The regulatory enzyme, a coenzyme reductase (HMG R), is also present in Archaea. Melanized fungi isolated from salterns in Spain have several biosynthetic pathways that converge into ergosterol (Méjanelle et al., 2001). Ergosterol (sterol) is an important constituent of fungal membranes.

Many fungi like the yeast *Saccharomyces* and *Aureobasidium pullulans* can produce proteins in response to heat or salt stress (Gröpper and Reising, 1993; Torzilli, 1997). Fungal metabolites and proteins produced at high salt concentrations can provide a source of halophilic enzymes and other products to be use in medical, biotechnological, and industrial applications. For example, the development of crop plants with increased

salt tolerance requires the study of organisms with natural salt tolerance (Petrovič et al., 2002).

The Study of Fungal Communities in Hypersaline Environments

The study of fungal communities at this extreme environment depends on the techniques applied for that purpose. Many culture-dependent techniques like the use of culture medium have many limitations if we want to know real diversity in the studied environment. So, many types of media have been used in the studies focused on diversity of xerophilic/xerotolerant and halophilic/halotolerant fungi. These media select for some fungi and will eliminate the possibility of the isolation of many species.

In order to perform a more complete analysis of fungal communities in different environments, many culture-independent techniques were developed. In the last years the use of molecular characterization of fungi using chromosomal and mitochondrial genes has become one of the most used approaches. Many studies were performed to improve taxonomy and establish phylogenetic relationships between groups of fungi. The target for species level can include: (1) the ITS regions separated by the 5.8S gene and (2) the 18S rDNA region. (Cappa and Cocconcelli, 2001; Henry et al., 2000). Some studies included analysis of mithochondrial cytochrome *c* gene (Wang et al., 2001; Yokoyama et al., 2001). The molecular analysis is an important tool to fungal taxonomy (Henry et al., 2000; Turenne, 1999; Pařericová et al., 2001; Zhao et al., 2001; Cappa and Cocconcelli, 2001), because it is based in the study of important genes which are conserved during evolution.

As mentioned previously, the studies on fungal diversity using environmental genomics include the analysis of ITS_1-5.8S-ITS_2 region and 18S rDNA gene. That analysis can be applied in the study of fungal communities in different environments. A study to test a molecular strategy to be applied to environmental samples was performed by Kowalchuk et al., (1997). They developed a nested PCR system and coupled it with Denaturing Gradient Gel Electrophoresis (DGGE) for the analysis of fungi associated with Marram grass in coastal dunes. The technique could separate sequences based on their nucleotides composition. Fungal communities on a church window were analyzed using a combination of DGGE and the construction of 18S rDNA clones libraries (Schabereiter-Gurtner et al., 2001). The use of 18S rDNA sequences as phylogenetic markers for the study of fungal diversity is still in development.

Other investigations used automated rRNA intergenic spacer analysis (ARISA) for the characterization of bacterial and fungal communities in soil. This method is very effective for the detection of differences between fungal and bacterial communities (Ranjard et al., 2001). To determine the fungal diversity in soil a group (Valinsky et al., 2002) described a new method named oligonucleotide fingerprinting of rRNA genes (OFRG). Their results showed that the OFRG provides an inexpensive and efficient way to study of fungal diversity. Another research to study the composition of a fungal community was performed by Nikolcheva et al., (2003). They combined DGGE and terminal restriction fragment length polymorphism (T-RFLP).

For saline environments molecular studies are very scarce. Changes in archaeal, bacterial and eukaryal communities were analysed along a salinity gradient in a solar saltern of Spain (Casamayor et al., 2002). That was the first attempt to elucidate the

eukaryotic community composition in waters of this extreme environment. They used three fingerprinting techniques: DGGE, ARISA and T-RFLP. In 2002, an analysis of fungal diversity in a salt marsh in United States was performed, but not in the water (Buchan et al., 2002). Buchan and others construct an ITS clone library to the analysis of ascomycetes in *Spartina alterniflora* blades. They also studied the dynamics of fungal and bacterial communities (Buchan et al., 2003). An analysis of the ascomycete diversity in blades of *S. alterniflora* from a salt marsh was performed using lacasse genes (Lyons et al., 2003). To our knowledge, no studies to determine the fungal diversity directly from the saltern waters had been performed before.

Part I

Optimization of the Sampling Procedure and Isolation of Filamentous Fungi from the Solar Salterns

Material and Methods

I. Description of the Solar Salterns of Cabo Rojo, Puerto Rico

The Solar Salterns are located right across from Bahia Salinas at Cabo Rojo, Puerto Rico (Figure 1.1). The saltern system includes an estuary that is surrounded by natural mats. These mats feed a series of artificial salt ponds with seawater. Also, the system includes saline lagoons, salt flats and mangrove swamps. The constant high solar radiation and the lack of rain during most of the year promote the formation of a hypersaline environment. The salt flats have been functioning since the Spaniard conquest. Since 1974, the U.S. Fish and Wildlife Service established the Cabo Rojo National Wildlife Refuge that is located adjacent to the salt flats. The Cabo Rojo Solar Salterns are the most important stop site for migratory shorebirds in the Caribbean.

II. Physico-chemical Parameters of Saltern Water

Temperature was measured using a standard thermometer. NaCl percentage in saltern water was measured using a S-28F refractometer. Water samples were sent to Dr. Gunde-Cimerman's laboratory in the University of Slovenia to perform the several analysis. The pH values were measured using the ISO 10523 system. The Ca, Mg, Na, K values were measured using ICP – MS system. The anions Cl^-, SO_4^{2} were measured using the ISO 10304-2 system. Nitrogen and NH_4^+ content was measured using the ISO 5664 system, the macro-Kjeldahl method, and EN 25663.

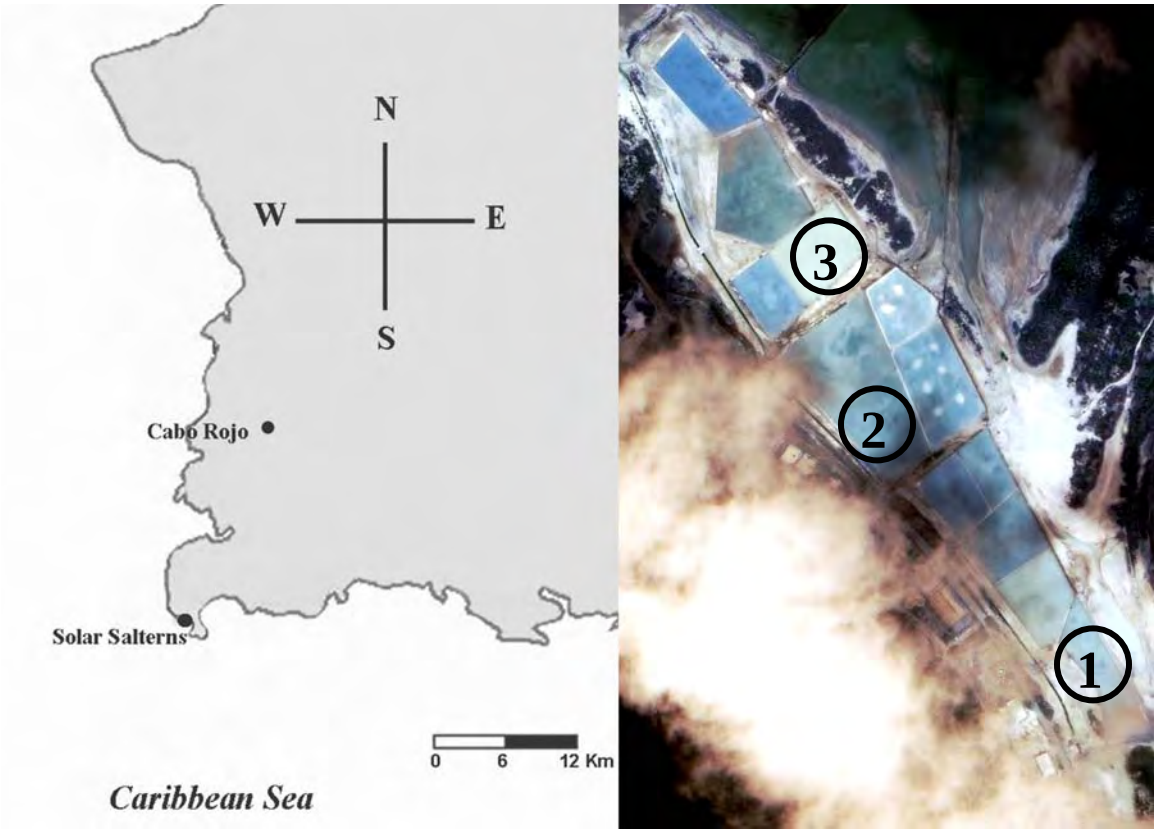

Figure 1.1 Location of the Solar Salterns of Cabo Rojo, Puerto Rico. The sampling areas are numbered 1 to 3.

III. Sample Processing and Isolation of Filamentous Fungi

Samples containing 250 ml of saltern water were collected (in triplicate) from each of the randomly selected ponds (Figure 1.1) at the Solar salterns of Cabo Rojo, Puerto Rico, using sterile plastic bags (Whirl Pak ®). Fifty ml of each sample were filtered through 0.45 µm nitrocellulose membranes (Millipore) and transferred on to three different media: Sehgal-Gibbons 15% NaCl (SG-15), Malt Extract 15 % NaCl (ME-15) and Potato Dextrose 15% NaCl (PD-15).

The SG-15 medium had the following composition (g/L) NaCl (150), $MgSO_4$ (20), KCl (2.0), sodium citrate (3.0), $FeSO_4$ (0.0023), casaminoacids (7.5), yeast extract (1.0) (Sehgal and Gibbons, 1960). ME-15 had the following composition (g/L): malt extract (15) and NaCl (150), while PD-15 was composed of (g/L): potato and dextrose (15) and NaCl (150). HCl was used to adjust the pH to 5. Solid media was achieved by adding 20 g/L agar. Plates were incubated at 30˚C. Isolated colonies were transferred periodically into plates at 29˚C until pure cultures were obtained. A preliminary characterization to genus level was performed to group the isolates. All the strains, except SC24 and SC28, were deposited in the Culture collection of the National Institute of Chemistry (MZKI) in Slovenia.

Results

Physico-chemical Conditions of Saltern Waters

The physico-chemical parameters of water from the Solar Salterns of Cabo Rojo are presented in Table 1.2. Temperature and salinity values were constant among all the

three ponds and also the pH values were near neutrality. Interestingly, NH_4^+ values in these waters were relatively lower compared to Slovenian salterns (N. Gunde-Cimerman, pers. comm.).

Table 1.1 Physico-chemical parameters of saltern waters, Cabo Rojo, Puerto Rico. Values correspond to one pond.

Parameter	**Value**
pH	7.7
Ca (g l^{-1})	0.33
Mg^{2+} (g l^{-1})	18.2
Na^+ (g l^{-1})	95.7
K^+ (g l^{-1})	5.3
Cl^- (g l^{-1})	188.0
SO_4^{2-} (g l^{-1})	25.1
NH_4^+ (mg l^{-1})	3.84
N (mg l^{-1})	17.7
Temperature (˚C)	35.5
Water activity (a_w)	0.773
Salinity (%)	28+

Table 1.2. CFU numbers obtained per 50 ml filtered water on the different media used.

Pond	**PDA-15**	**MEA-15**	**SG-15**
1	111	112	46
2	28	32	32
3	46	54	19
Total*	185	198	97

* represents the total number of CFU in all plates from two samplings

Isolation of Fungi

After 4 days of incubation several fungal colonies were observed growing on the inoculated membranes (Figure 1.2). The highest umber of colony forming units (CFU) were obtained in PD-15 (Table 1.2). However, the SG-15 medium showed a greater number of morphotypes (Table 1.3). The numbers of CFU obtained from the two samplings were very similar (175 and 180 CFU, respectively). The amount of morphotypes was similar too (87 and 75, respectively). The highest CFU numbers were recorded from pond # 1, while pond # 3 showed the highest diversity in both samplings.

Table 1.3 List of isolates as a function of the culture medium.

Isolates	**Culture medium**
Alternaria spp.	
SC39	PD-15
SC59	PD-15
SC61	PD-15
Aspergillus spp.	
SC7	SG-15
SC11	SG-15
SC17	SG-15
SC20	SG-15
SC22	SG-15
SC26	SG-15
SC27	SG-15
SC31	ME-15
SC34	ME-15
SC35	ME-15
SC37	ME-15
SC40	PD-15
SC41	PD-15
SC43	PD-15
SC44	PD-15
SC46	PD-15
SC49	PD-15
SC53	SG-15
SC54	PD-15
SC56	ME-15
SC57	SG-15
SC58	PD-15

Table 1.3 (continue)

Isolates	Culture medium
SC60	PD-15
SC62	PD-15
Cladosporium spp.	
SC21	SG-15
SC23	SG-15
SC33	ME-15
SC51	ME-15
SC52	PD-15
Eurotium spp.	
SC2	SG-15
SC3	SG-15
SC4	SG-15
SC5	SG-15
Hortaea spp.	
SC1	SG-15
SC8	SG-15
SC9	SG-15
SC10	SG-15
SC12	SG-15
SC13	SG-15
SC14	SG-15
SC15	SG-15
SC16	SG-15
SC18	SG-15
SC19	SG-15
SC29	SG-15
SC42	PD-15
SC47	PD-15
SC55	PD-15
Mycelia sterilia	
SC24	SG-15
SC45	PD-15
SC28	SG-15
Penicillium spp.	
SC30	ME-15
SC32	ME-15
SC36	ME-15
SC38	ME-15
SC48	PD-15

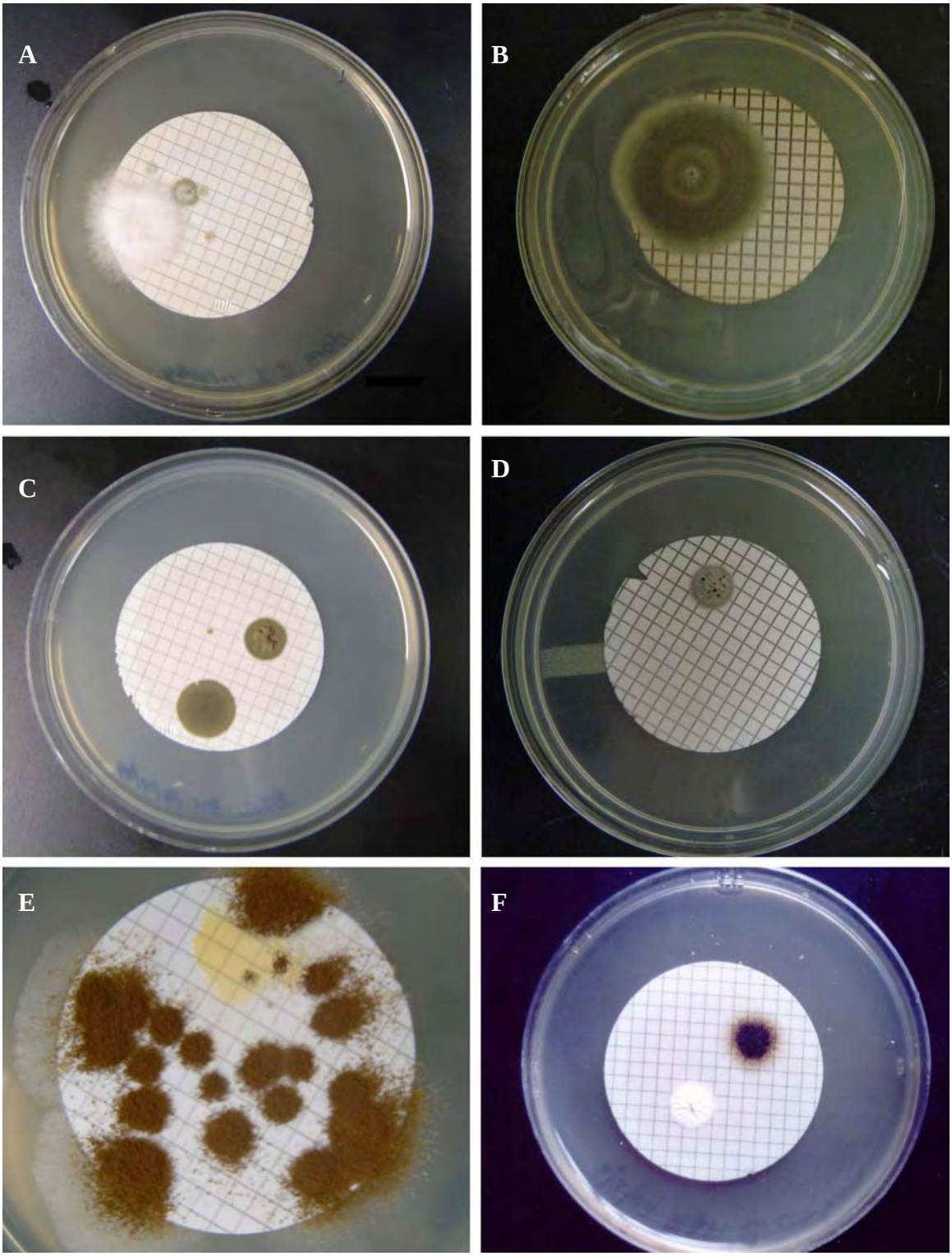

Figure 1.2 Isolated colonies from different media: MEA-15 (A-B), PDA-15 (B-C) and SG-15 (E-F).

Among the 59 morphotypes obtained during the samplings, several strains were morphologically classified as *Hortaea*, *Aspergillus*, *Cladosporium*, *Alternaria* and *Penicillium.* Some strains were isolated as Mycelia Sterilia until transferred to a low salt medium. The species of *Hortaea werneckii* (15 isolates) had the highest frequency of isolation among all the fungi obtained from the solar salterns. This dimorphic yeast was selected as a morphotype of interest because it was in the filamentous form at the conditions tested. *Aspergillus* was represented by several morphotypes growing in all media used for isolation (Table 1.3). The most frequent were the strains preliminary identified as *Aspergillus sydowii.* Most of the species reported in this study appeared in both samplings except *Alternaria* sp. and *Eurotium* sp. The genus *Cladosporium* was found in all the ponds sampled and two different strains were observed. Also, two morphospecies of *Penicillium* were isolated (Table 1.3).

Discussion

Physico-chemical parameters from the saltpans at Cabo Rojo showed very similar values to salterns with comparable salinities in Slovenia (Gunde-Cimerman et al., 2000); Butinar et al., 2005a). The most remarkable difference occurs in NH_4^+ values that are considerably lower than the ones reported for the Slovenian salterns. Another difference between these two systems is temperature values. While temperature in Slovenian salterns is variable and increased up to 26˚C in July, the Cabo Rojo system had temperature values between 30-40˚C all year. For this reason the incubation temperature selected for this study was 29˚C.

Three different media were used in this study to see which one was most suitable for the isolation of fungi at this extreme condition. Two of these (MEA and PDA) are commonly used in mycology but they were modified by the addition of 15 % NaCl. This amount was selected because it represents a high salt concentration for common fungi and at the same time allows the growth of more morphospecies than 25% NaCl media based on previous samplings. The Seghal and Gibbons medium is commonly used for the isolation of halophilic prokaryotes (Montalvo-Rodríguez et al., 1997). However, this medium was used first in our preliminary samplings and showed good fungal growth. Therefore, SG medium was also selected for isolation of fungi in our samplings. The original medium uses NaCl at 25% (w/v) and this content was reduced as explained previously. Previous studies on hypersaline environments have employed media that is normally used for the isolation of food-borne fungi (Gunde-Cimerman et al., 2000). These media had showed differences on the number of CFU L^{-1} obtained. In our study, the numbers of CFUs obtained were similar using ME-15, PD-15 and SG-15. The differences were attributed more to the pond's characteristics than the medium used for isolation. The most important differences between the media used were found in the number of morphospecies obtained. PD-15 showed more diversity but the highest number of CFUs was obtained with SG-15. The ion composition of SG is proportional to sea water but at a higher concentration. Although, this medium is not commonly used for fungi, it might be appropriate for the isolation of these eukaryotes specially those who can grow at low water activity or of marine origin. Fifty mL of saltern water was enough to detect the presence of fungi.

Based on macroscopical characteristics, 59 morphotypes were further analysed. Preliminary observations (macroscopical and microscopical) indicated the presence of several genera in the saltern waters. The most frequent, *Hortaea,* was selected because it was in the filamentous form at the conditions tested. Their occurrence through all media and ponds is another reason for being interesting (Díaz-Muñoz and Montalvo-Rodríguez, 2005). *Aspergillus* strains were the most diverse being present in all ponds and grew in all the media tested. They are represented by several species with different characteristics. These strains showed good growth in medium with 15% NaCl. They appeared early in the membranes in comparison with other strains like *Alternaria*. Some isolates grew at 15% NaCl as Mycelia sterilia. When the NaCl content was lowered these strains showed the development of reproductive structures. This might indicate that the saltern conditions represent a stressful state for these strains compared to the other isolated fungi. The growth of yeast-like colonies was also observed in filtered membranes. Studies that have been focused in yeasts have reported a great diversity present at hypersaline environments around the world (Butinar et al., 2005b). Considering this, the yeast diversity that might exist in the Cabo Rojo salterns can be very interesting for future studies.

Part II

Characterization of Isolates

Materials and methods

I. Macroscopic Features

For each isolated strain, macroscopic colony characteristics such as color, border, texture, presence of pigmentation, exudates and color changes of the media from cultures growing on SG-15, ME-15, PD-15 were recorded. In addition, Czapek Yeast Agar (CYA) (Pitt, 1988) was used for the characterization of *Aspergillus* and *Penicillium* strains. *Stemphylium* and *Alternaria* strains were grown in V8 juice agar and Potato and Carrot agar (PCA) for further characterization.

II. Microscopic Features

a. Light Microscopy

Microscopic characteristics were determined by the moist chamber technique. Samples were prepared by inoculating each isolate into the appropriate media and incubating it at their corresponding optimal growth conditions. Analysis of somatic structures using light microscopy (bright field and Nomarski) was performed by preparation of semi-permanent slides.

b. Scanning Electron Microscopy (SEM)

Detailed analysis of spores and other fungal structures was performed using SEM. Colonies were fixed using glutaraldehyde 4%-paraformaldehyde 2% on a phosphate base at pH 7.2. Samples were dehydrated in an ethyl alcohol gradient using the following concentration (in %): 15, 25, 35, 45, 55, 65, 75, 85,

95 (30 min. each) and a final treatment with 100 (three times 30 min. each).The preparations were dried using the critical point dried technique and all samples were placed on an aluminum stub to be coated with argon-gold.

III. Physiological Features

a. Secondary Metabolites Profiles

Spore suspensions were sent to Dr. Nina Gunde-Cimerman's laboratory in the University of Slovenia to perform the secondary metabolite profiles.

b. Determination of Optimal Growth Conditions

For each isolate, optimal NaCl concentration and temperature were first determined. Determination of optimal pH was performed for each isolate at their optimal temperature and salinity.

a. Percentage of NaCl and Temperature

Optimal NaCl concentration and temperature for growth was determined by inoculating the colonies in the medium where the fungi were isolated, containing 0, 5, 10, 15, 20, 25 and 30% (w/v) of NaCl and at temperatures of 25, 29, 35 and 40ºC. Comparison and reproducibility of results was achieved by always inoculating 10^6 spores. The spore suspension was prepared as previously described (Stretch et al., 2001). Data was recorded after 7 days of incubation. The combination that showed the greatest colony diameter in the less amount of incubation time was considered the best growth condition for every isolate.

b. pH

Optimal pH was determined by growing the isolates in the proper medium plates at pH values of 3, 5, 7, and 8, and at their optimal temperature and % of NaCl. For some isolates, pH values of 2 and 10 were also used. Spore suspensions for inoculation were prepared as previously described (Stretch et al., 2001). Data were recorded after 7 days of incubation.

IV. Molecular Characterization of the Isolated Strains

a. Genomic DNA Isolation

The fungal isolates were incubated at 30ºC to obtain mycelial mass for genomic DNA isolation. Genomic DNA extraction was performed using Q-Biogene Fast DNA Kit according to the manufacture's protocol. A modified method from Saghai-Maroof et al., (1980) had to be used for several isolates. The extracted DNA was resuspended in 50 µl of pure deionized water and treated with RNAse (final concentration of 20µg/µl) for 30 minutes at 37ºC. The DNA quality was checked on 0.8% agarose gels after staining with ethidium bromide.

b. Polymerase Chain Reaction (PCR) and Gel Electrophoresis

Fungal molecular taxonomy have been based on the used of conserved regions like 18S and variable regions like ITS. Based on these previous findings, four fungal primers described by White et al. (1990) were used for the amplification of ITS_1, 5.8S and ITS_2 regions (Figure 2.1). The set of primers were ITS1 5′-TCC GTA GGT GAA CCT GCG G-3′ and ITS4 5′-TCC TCC GCT TAT TGA TAT G-3′. PCR reactions (small and large scale volume) were performed

using a protocol by Zhao et al., (2001) modified by Giovanni López (pers. comm.).

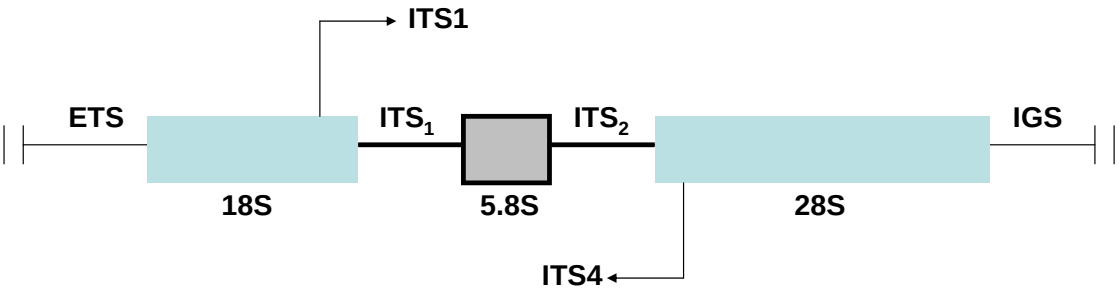

Figure 2.1 Schematic representation of ribosomal genes organization present in most fungi. Targets areas in the amplification of ITS and 5.8S rDNA regions are in bold.

The reactions had ≈ 10 ng of template, 1X PCR buffer, 300 µM dNTP's, 3 mM $MgCl_2$, 0.66 pmol/µl of each primer and 0.025 U of AccuTaq LA DNA Polymerase (Sigma ®). Thirty cycles of amplification were performed after initial denaturation of DNA at 95ºC for 5 minutes. Each cycle consisted of a denaturation step at 95ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension step at 72ºC for 1 minute, and a final extension at 72ºC for 2 minutes. 18S rDNA PCR reactions for SC24 were performed using primers described by Borneman et al., (2000). PCR reactions (small and large scale volume) were performed as described above but using 0.05U of Taq DNA Polymerase in Storage Buffer B (Promega ®). Thirty five cycles of amplification were performed after initial denaturation of DNA at 95ºC for 3 minutes. Each cycle consisted of a denaturation step at 95ºC for 30 seconds, annealing at 56ºC for 1 minute, extension step at 72ºC for 2:30 minutes, and a final extension at 72ºC for

10 minutes. PCR products were cleaned by using the MinElute PCR purification Kit (USA QIAGEN Inc.) according to the manufacture's protocol. PCR products were verified on 1% agarose gels after staining with ethidium bromide. PCR product concentration was determined by using a spectrophotometer at 260 nm.

c. Restriction Fragment Length Polymorphism (RFLP)

A double digestion was performed on the amplified PCR products using the restriction endonucleases *BsaHI* and *HaeIII.* In order to distinguish between similar patterns obtained with these enzymes, additional digestions using *Hinf I, HpaII* and *DdeI* were performed. All digestions were performed for an hour (twice) at 37ºC in a final volume of 10 µl. RFLP patterns were verified on 3% low melting agarose gels after staining with ethidium bromide.

d. DNA Sequencing

Selected PCR products were sent to the University of Iowa DNA Sequencing Facility and Macrogen, Korea, for DNA sequencing. Samples were prepared according to the facilities instructions.

e. Phylogenetic Analysis

Consensus sequences were used for *in silico* similarity analysis using the NCBI BLAST program (Altschul et al., 1997). Selected sequences were used to determine the isolates phylogenetic position with respect to similar strains. Sequences were aligned and edited using Clustal W and the Bioedit programs, respectively. Sequence similarity values were calculated by pairwise comparison of the sequences within the alignment. Seqboot was used to generate 100 bootstrapped data sets. Distance matrices were calculated with dnadist. One

hundred trees were inferred by using neighbor joining analysis. Any bias introduced by the order of sequence addition was minimized by randomizing the input order. Consense was used to determine the most frequent branching order. The final trees was drawn using treeview program (Page, 1996). The consensus DNA sequences obtained for each isolate were deposited in Gen Bank from the National Center for Biotechnology Information (NCBI).

Results

Morphological Characterization of Fungi

Macroscopical and microscopical observations were used for a preliminary classification of all isolates. Light microscopy and SEM revealed important details to discriminate between strains. SEM was very useful to examine the conidial ornamentation. After macroscopical and microscopical observations, a total of twenty three (23) strains were further analyzed. The selected strains were SC3, SC17, SC20, SC22, SC24, SC27, SC28, SC29, SC30, SC31, SC33, SC34, SC35, SC40, SC41, SC45, SC46, SC48, SC51, SC57, SC58, SC59 and SC60. These strains were transferred to different media in order to perform a better characterization for each genus. Strain SC45 was very difficult to cultivate and it could not be maintained.

Physiological Characterization of Isolates

Secondary metabolite profiles were performed to selected *Aspergillus* and *Penicillium* strains in order to identify them at species level (Table 2.1). These results were used to reduce the group to seventeen strains.

Table 2.1 Results of Secondary Metabolite Profiles for *Aspergillus* and *Penicillium* strains.

Strain	Secondary Metabolites	Proposed Classification
SC3	physcion, echinulin	*Eurotium amstelodami*
SC17	xanthomegnins, cycloechinuline	*Aspergillus elegans*
SC20	sydowin	*Aspergillus sydowii*
SC22	sydowin	*Aspergillus sydowii*
SC27	sterigmatocystin, nidulotoxin	*Aspergillus versicolor*
SC30	patulin, citrinin, chaetoglobosin A, B, communesin A, roquefortine C, expansolide A, fumaryl-d	*Penicillium expansum*
SC31	sterigmatocystin, nidulotoxin	*Aspergillus versicolor*
SC34	terrein, patulin, citrinin, citreoviridin, territrem	*Aspergillus terreus*
SC35	ochratoxin A, malformin	*Aspergillus niger* aggregate
SC40	sterigmatocystin, nidulotoxin	*Aspergillus versicolor*
SC41	ochratoxin A, naphtho-ã-pyrones, malformin	*Aspergillus niger* aggregate
SC46	kojic acid, cyclopiazonic acid, 3-nitropropionic acid	*Aspergillus oryzae*
SC48	penicillin, roquefortine C, meleagrin, chrysogine, secalonic acid, sorbicillin, PR-toxin	*Penicillium chrysogenum*
SC57	sydowin	*Aspergillus sydowii*
SC58	kojic acid, cyclopiazonic acid, 3-nitropropionic acid	*Aspergillus oryzae*
SC60	ochratoxin A, malformin	*Aspergillus niger*

Optimal growth parameters (NaCl percent, temperature, and pH) were determined for each strain after 7 days of incubation (Table 2.2). The majority of the strains had an optimal NaCl concentration of 5%. Strains SC3, SC29 and SC24 showed an optimal NaCl concentration of 10%. Only strain SC59, an *Alternaria* isolate, had an optimal growth at 0% of NaCl. Since the Sehgal and Gibbons medium has other salt components, in addition to NaCl, all of the strains tested in this medium for optimal NaCl percent were transferred to PDA or MEA without salt to ensure their ability to grow without any kind of salt.

Table 2.2 Optimal %NaCl, temperature and pH of the isolates.

Strain	Medium	NaCl (%)	Temperature (˚C)	pH
SC59	PDA	0	25	5
SC17	SG	5	29	5
SC60	PDA	5	29	3
SC41	PDA	5	29	3
SC35	MEA	5	35	3
SC46	PDA	5	29	3
SC20	SG	5	29	5
SC34	MEA	5	35	8
SC40	PDA	5	29	5
SC3	SG	10	29	3
SC51	MEA	5	29	5
SC33	MEA	5	25	10
SC29	SG	10	29	5
SC48	PDA	5	29	8
SC30	MEA	5	29	8
SC24	SG	10	29	5

It was found that the maximum NaCl percent for growth (in 7 days) was very variable among the isolates. Some strains can grow up to 15% of NaCl (SC24, SC35 and SC59). Other strains can grow up to 20% (SC41, SC48, SC51 and SC60) and 25 % of NaCl (SC30, SC33 and SC40). However, after approximately 10-15 days, many of the *Aspergillus* isolates (SC17, SC20, SC29, SC34 and SC46) and *Eurotium* (SC3) showed growth up to 30% NaCl. Due to difficulties in achieving sporulation for strain SC28, the physiological tests were not performed on this isolate. Eventhought the strain was transferred to several media, this fungus needed alternate cycles of light and dark for proper growth which was not possible to achieve. This strain was isolated as mycelia sterilia from SG-15 and probably the optimal NaCl percent is below this value (15%) or none at all.

The temperature range tested for growth of above-mentioned was 25˚C-40˚C. With the exception of several isolates, most had an optimal temperature for growth of 29˚C. Strains SC33 and SC59 (*Cladosporium* and *Alternaria* respectively) grew optimally at 25˚C. Some isolates had more tolerance to high temperatures. *Aspergillus* strains SC34 and SC35 grew optimally at 35˚C. The *Hortaea* strains grew up to 40˚C. Our results indicated that the tropical isolates may prefer higher temperatures than previously reported for this genus (Gunde-Cimerman et al., 2000).

The optimal pH for the isolates was tested in a range from 3 to 8. Table 2.1 shows the optimal values for each strain. Some isolates were tested also at pH 2 and pH 10. Strain SC33 (*Cladosporium sp.*) had an optimal pH of 10.

Other strains with a preference for an alkaline pH were the *Penicillium* isolates SC30 and SC48. All strains grew at pH 10 with the exception of SC3 (*Eurotium* sp.) which grew up to pH 8. Many strains grew at pH 2. Isolates of *Hortaea* (SC29), *Aspergillus* (SC17), and *Alternaria* (SC59) had a preference for acidic pH values (pH 5). SC35, SC41 SC46, and SC60, belonging to the *Aspergillus* strains, grew optimally in a even more acidic medium (pH 3).

Molecular characterization

The ITS_1-5.8S-ITS_2 region was used for molecular characterization. Two methods were used to performed DNA extraction. The PCR amplicons obtained varied in size from 500 to 550 bp (Figure 2.2-A) with the exception of strain SC24, which showed a product of more than 600 bp (Figure 2.2-B). It was necessary to perform optimization of the PCR parameters for some strains.

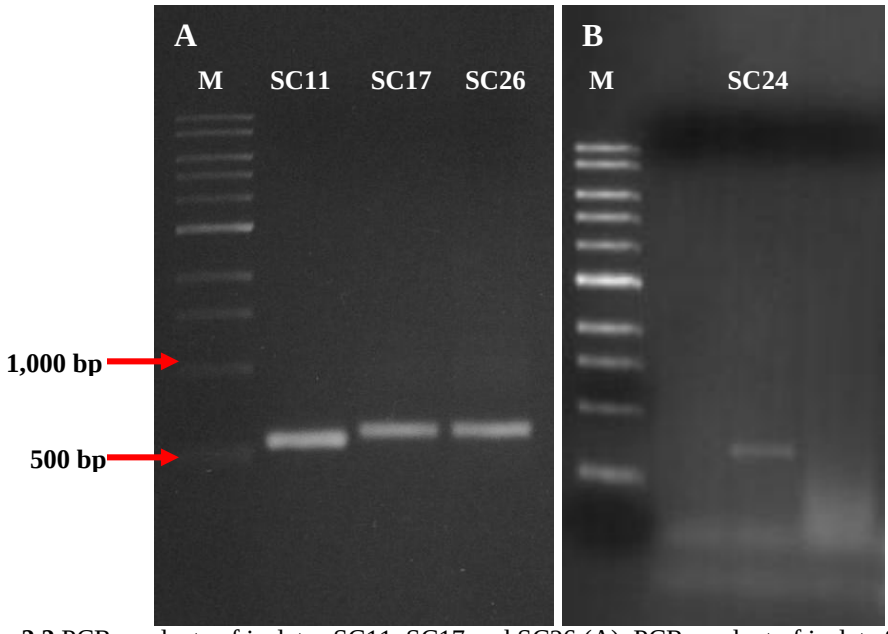

Figure 2.2 PCR products of isolates SC11, SC17 and SC26 (A). PCR product of isolate SC24 (B). M= 1Kb ladder (New England).

RFLP analysis on the ITS PCR product was performed to classify the isolates into groups. Restriction patterns were observed in 3% low melting agarose gel. The *Hortaea* isolates (from the first sampling) showed the same restriction pattern when digested with *BsaHI and HaeIII* (Figure 2.3). Other *Hortaea* strains, including SC29, and isolates from the second sampling were also digested. They also showed the same pattern as the other strains from the first sampling. Filamentous fungi belonging to *Aspergillus, Penicillium, Cladosporium*, and *Alternaria* were preliminary classified based on their RFLP patterns. Seven groups were observed for the *Aspergillus* strains after digestion (Figure 2.4). All these strains can be divided into groups that are consistent with the proposed characterization based on morphological and physiological properties, except strains SC27, SC31, SC40 and SC57.

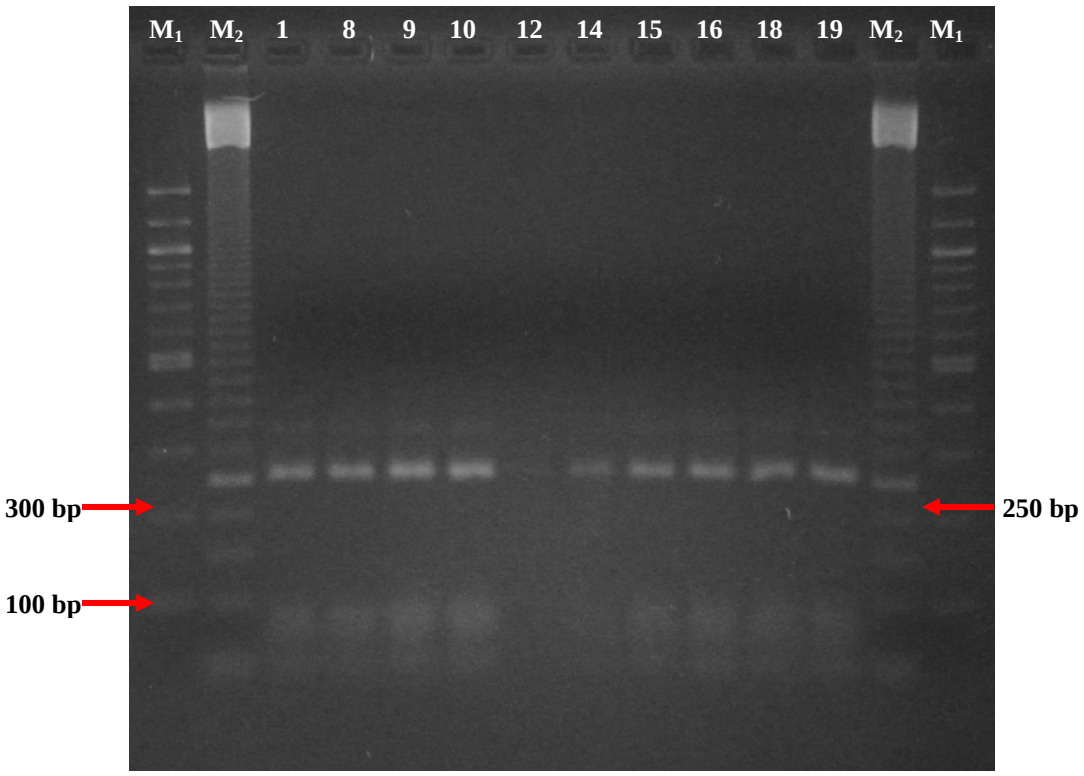

Figure 2.3 RFLP patterns of some *Hortaea* strains using restriction enzymes *BsaHI* and *HaeIII*. M1= 100 bp DNA ladder and M2= 50 bp DNA ladder (New England).

Strain SC57 was very similar morphologically to *Aspergillus sydowii* strains (SC11, SC20, SC22 and SC44) but had a different RFLP pattern as shown in Figure 2.4. Strains SC27, SC31 and SC40 had many morphological similarities and they were classified preliminary as *Aspergillus versicolor*. SC27 and SC40 were grouped into the *A. sydowii* RFLP group, but SC31 showed a different RFLP pattern. The group composed by SC34 and SC56 was classified as *A. terreus*. Strains SC41 and SC60 were classified into the *A. niger* complex based on secondary metabolite profiles, eventhough they formed a different RFLP group. Strains SC46 (*A. oryzae*) and SC7 showed each an unique RFLP profile. SC7 was similar to SC17. Other *Aspergillus* strains (SC17, SC26,

SC35, SC37, SC49, SC53, SC54, SC58 and SC62) were double digested and compared with strains from the seven RFLP groups.

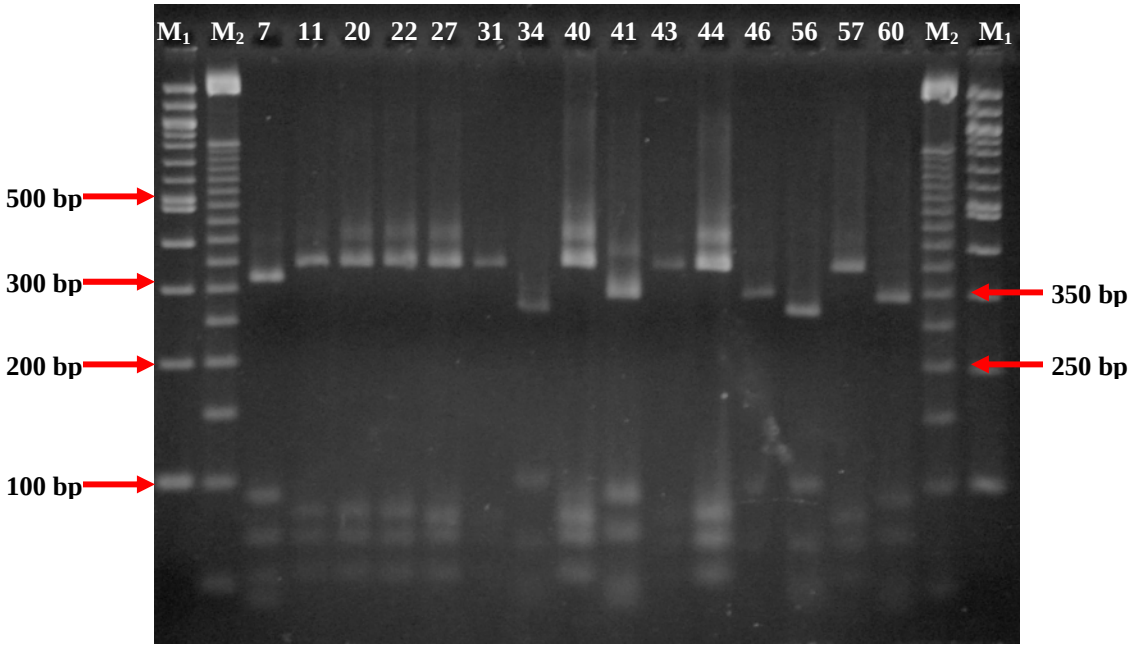

Figure 2.4 RFLP patterns of some *Aspergillus* strains using restriction enzymes *BsaHI* and *HaeIII*. M1= 100 bp DNA ladder and M2= 50 bp DNA ladder (New England).

Strains from the *Penicillium* and *Cladosporium* genera formed three different RFLP groups (Figure 2.5). The first was composed by the three strains preliminary classified as *Penicillium expansum* (SC30, SC32 and SC36) by secondary metabolite profiles. The second group was for SC48, a strain classified as *P. chrysogenum*. The third group was composed by strains SC21, SC33 and SC50. They were classified as *C. sphaerospermum* based on morphological data.

Strains classified as *Alternaria* sp. formed only one type of pattern when double digested with *BsaHI* and *HaeIII* (Figure 2.6). The group was composed by SC39, SC59 and SC61.

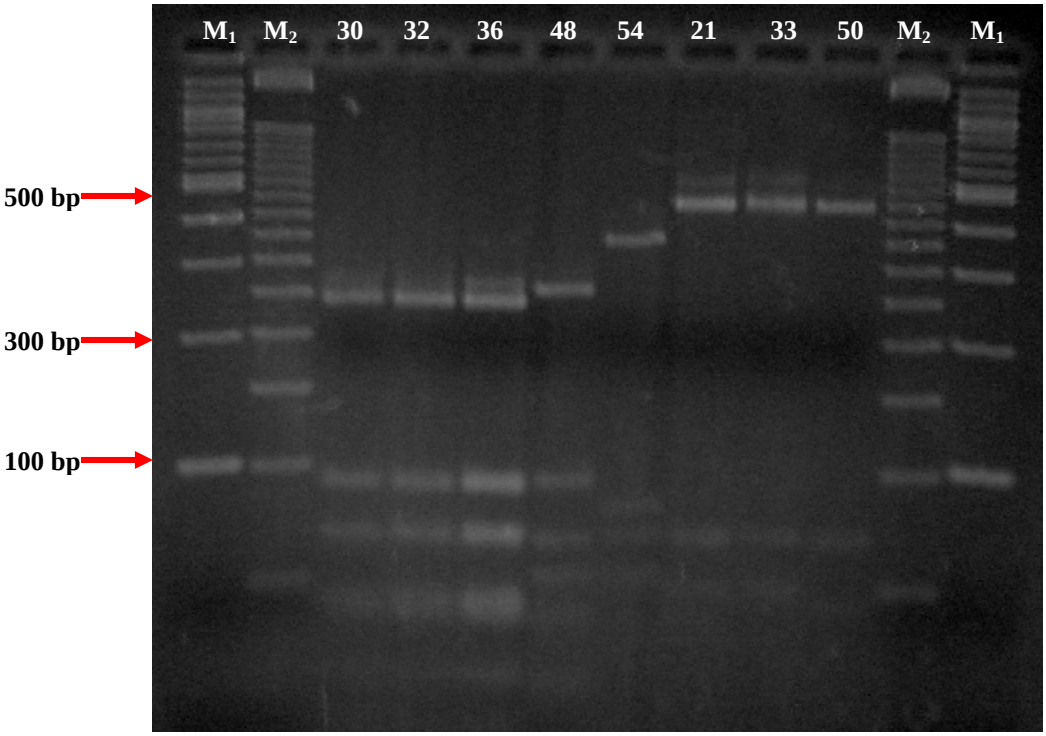

Figure 2.5 RFLP patterns of some *Penicillium* and *Cladosporium* strains using restriction enzymes *BsaHI* and *HaeIII*. M1= 100 bp DNA ladder and M2= 50 bp DNA ladder (New England).

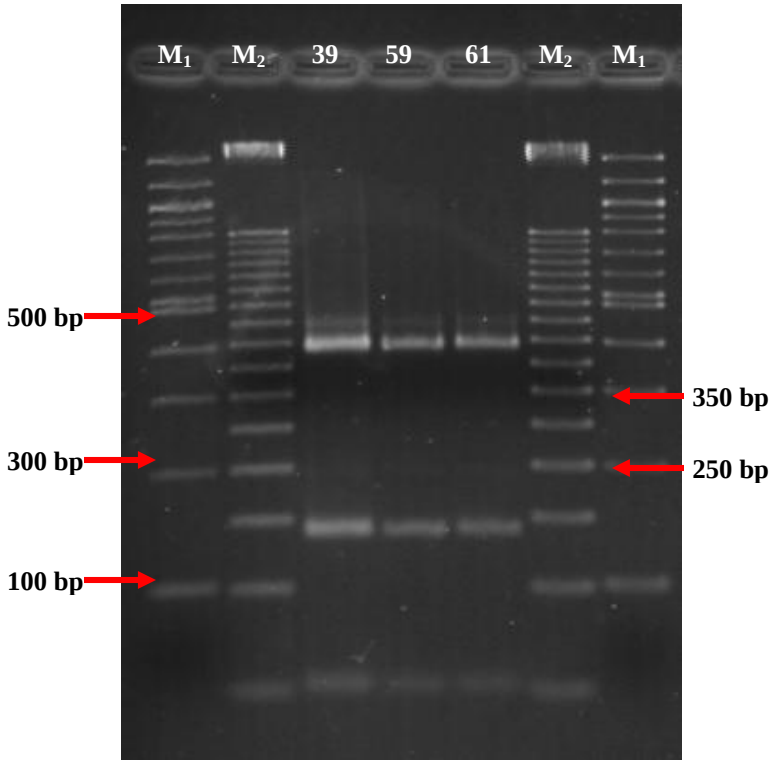

Figure 2.6 RFLP patterns of *Alternaria* strains using restriction enzymes *BsaHI* and *HaeIII*. M1= 100 bp DNA ladder and M2= 50 bp DNA ladder (New England).

Additional digestions were performed using enzymes *Hinf I*, *HpaII* and *DdeI* to improve resolution on the groups already obtained. Restriction analysis showed no significant differences for strains of *Aspergillus*, *Cladosporium*, *Penicillium* and *Alternaria* and the same groups were formed with few exceptions. Only digestion with *HinfI* showed some variation among the *Hortaea* strains. This observation was done before by Zalar et al., (1999b). Strains representing each of the RFLP groups were selected for sequencing. Additional strains with particular properties (morphological or physiological) were also sequenced.

A phylogenetic study of these sequences was performed using neighbor joining analysis. According to this analysis, the *Aspergillus* strains can be classified into several species. Strain SC40 was in the A. *versicolor* branch, while SC20 was in the *A. sydowii* group (Figure 2.7). The strain SC3 was grouped with the *Eurotium* branch and SC17 is more related to the *A. sclerotiorum* sequence in GenBank (Figure 2.7). SC46 was closer to a branch that included *A. flavus* and *A. oryzae* species (Figure 2.8). Several strains belonged to *Aspergillus* Section *Nigri* (Figure 2.9). Two of them (SC41 and SC60) were grouped with biseriate species belonging to *A. niger* aggregate. The other one, SC35, was more related to uniseriate species *A. japonicus*. Strain SC34 was clustered with *A. terreus* species (Figure 2.9). *Cladosporium* isolates (SC33 and SC51) formed a separate cluster apart to the species deposited in GenBank (Figure 2.10). Strain SC29 is related to the *Hortaea werneckii* cluster (Figure 2.11) as described before (Díaz-Muñoz and Montalvo-Rodríguez, 2005). Also, *Penicillium* isolate SC30 was closer to the *P. citrinum* and *P. westlingii* branch (Figure 2.12). Another *Penicillium* isolate, SC48 was related to the *P.*

chrysogenum group (Figure 2.13). The strain SC28 was grouped into a branch that includes several species of *Stemphylium* and it is difficult to be more precise about the possible species (Figure 2.14). Isolate SC24 formed a branch distant to the other species included in the analysis of the ITS regions (Figure 2.15). Additionally, a partial portion of 18S rDNA gene was used to construct a phylogram. Several of the related species belonged to the *Sordarial* group (Figure 2.16). Again, SC24 formed a separated branch completely apart from the others.

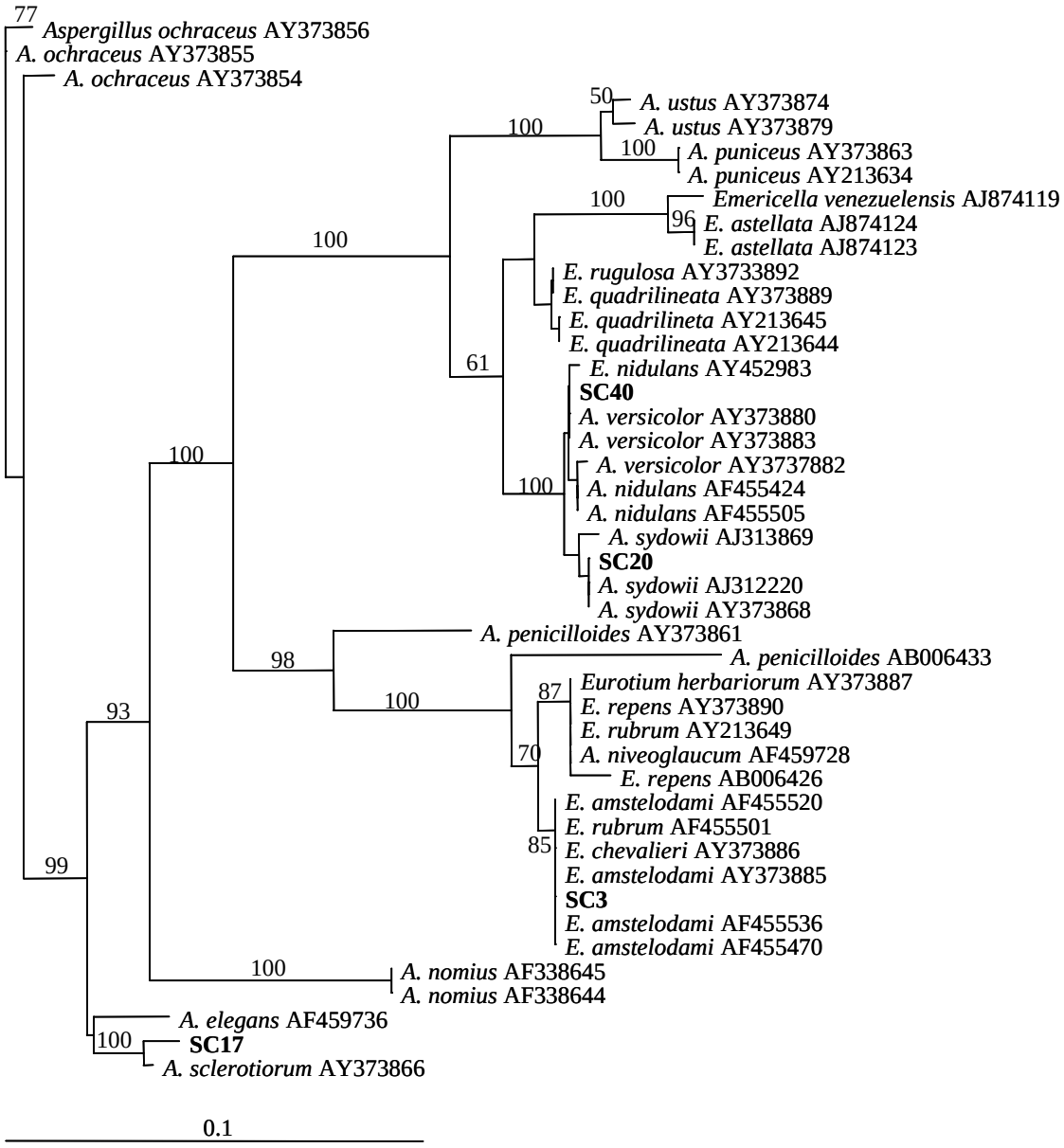

Fig. 2.7 Neighbor-joining distance tree of ITS1-5.8S rDNA-ITS2 sequences for *Aspergillus* strains SC3, SC17, SC20 and SC40. Bar represents 10 substitutions per 100 nucleotides. Significant bootstrap values are indicated.

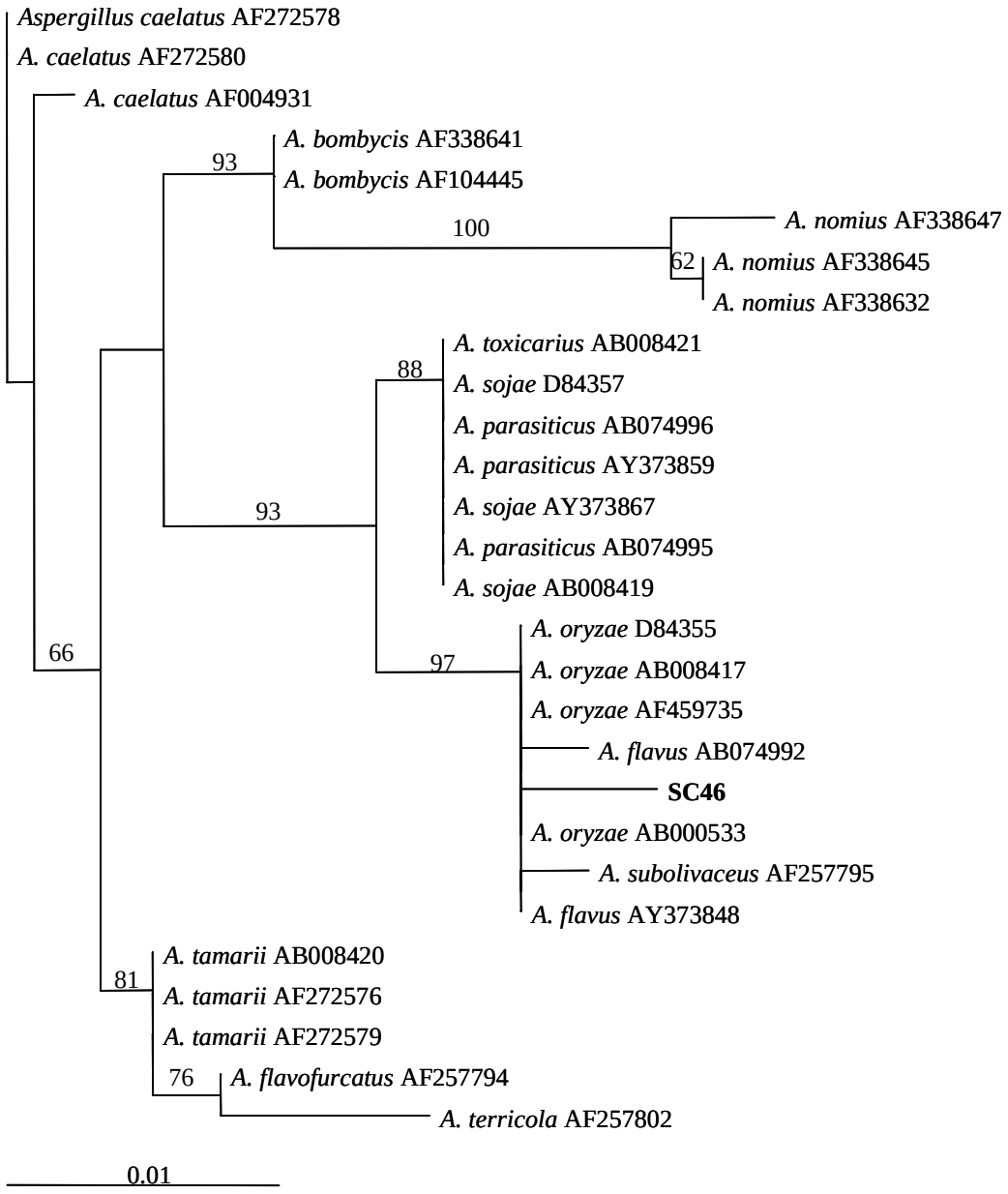

Fig. 2.8 Neighbor-joining distance tree of ITS1-5.8S rDNA-ITS2 sequences for *Aspergillus* strain SC46. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated.

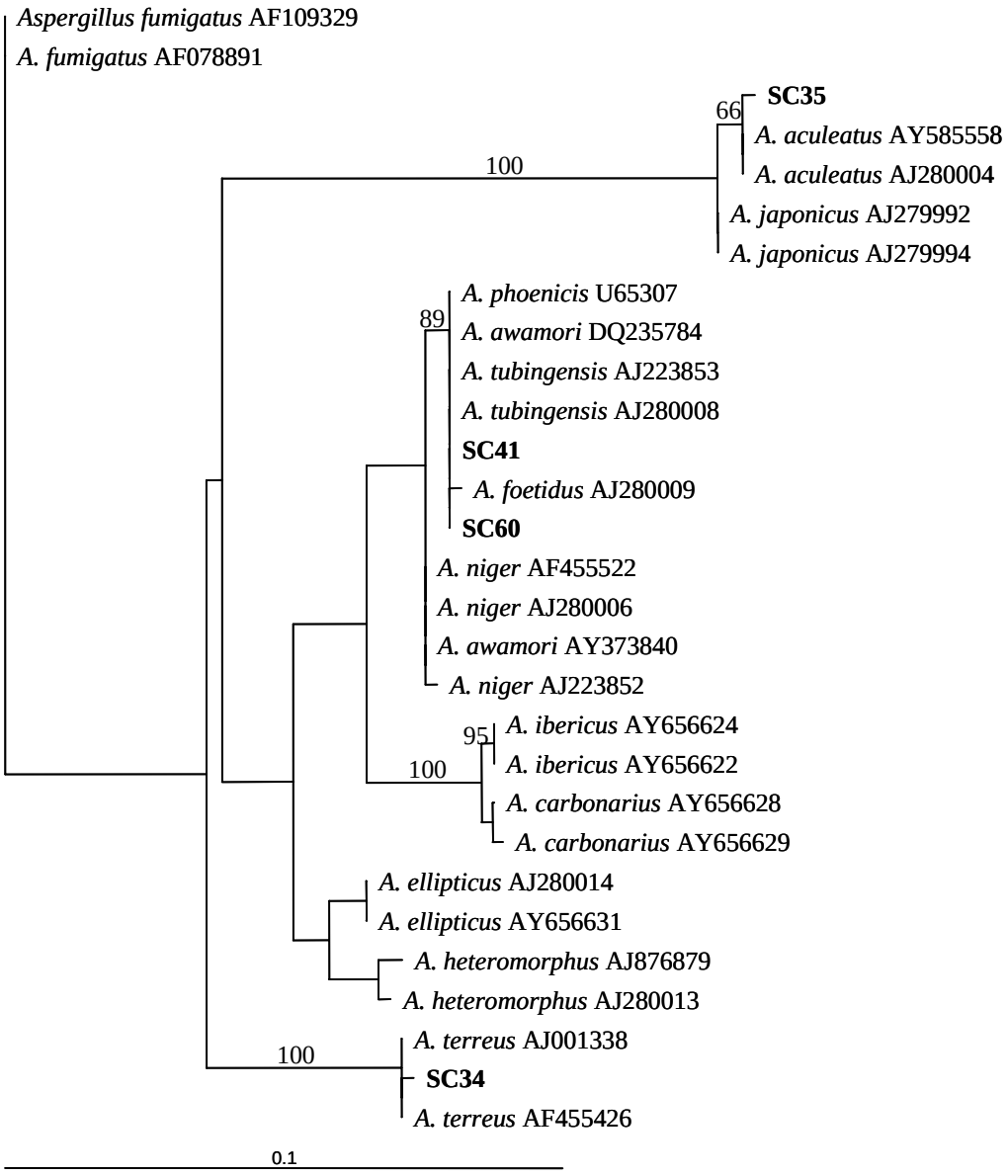

Fig. 2.9 Neighbor-joining distance tree of ITS1-5.8S rDNA-ITS2 sequences for *Aspergillus* strains SC34, SC35, SC41 and SC60. Bar represents 10 substitutions per 100 nucleotides. Significant bootstrap values are indicated.

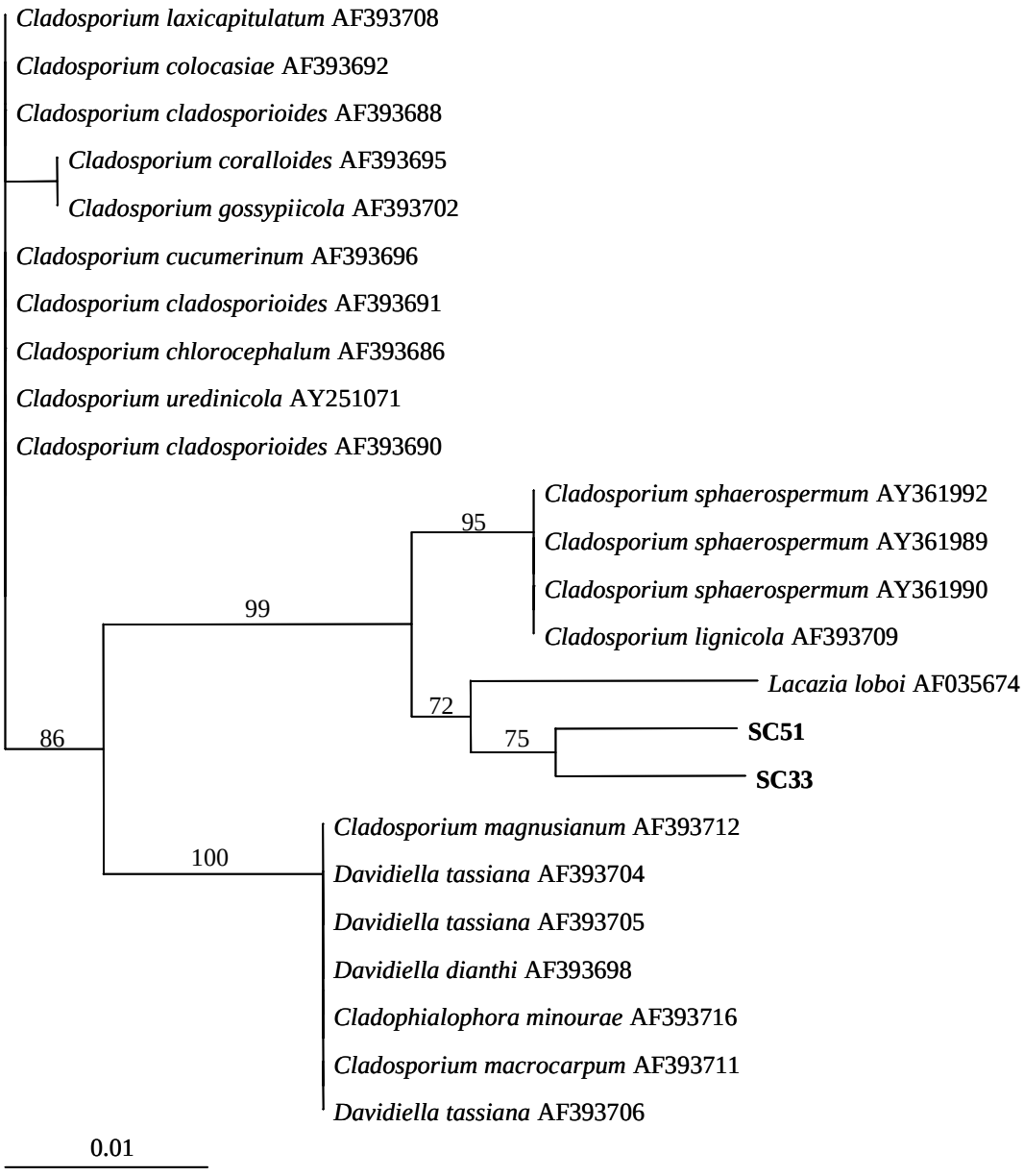

Fig. **2.10** Neighbor-joining distance tree of ITS1-5.8S rDNA-ITS2 sequences for *Cladosporium* strains SC33 ans SC51. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated.

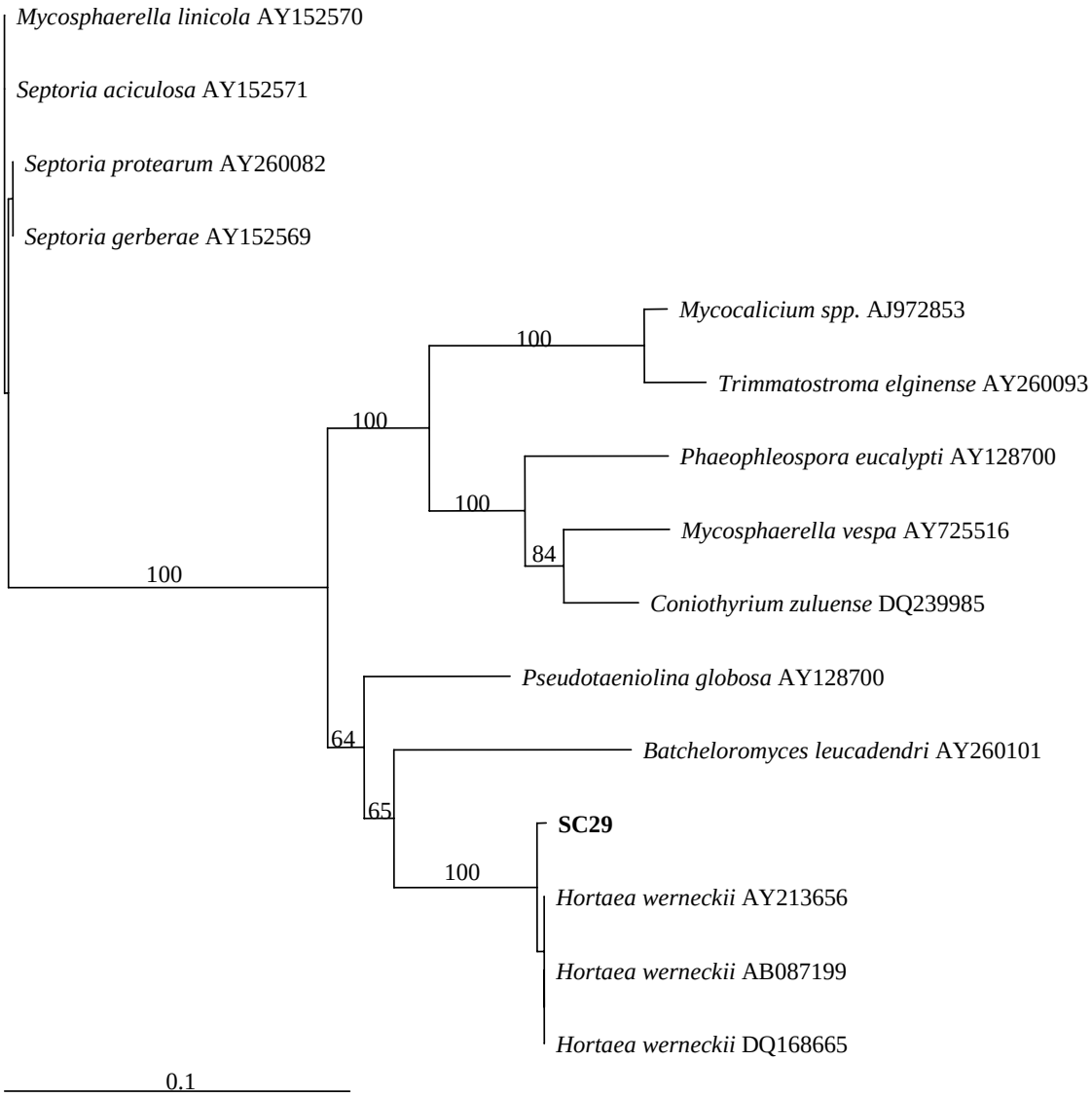

Fig. 2.11 Neighbor-joining distance tree of ITS1-5.8S rDNA-ITS2 sequences for *Hortaea* strain SC29. Bar represents 10 substitutions per 100 nucleotides. Significant bootstrap values are indicated.

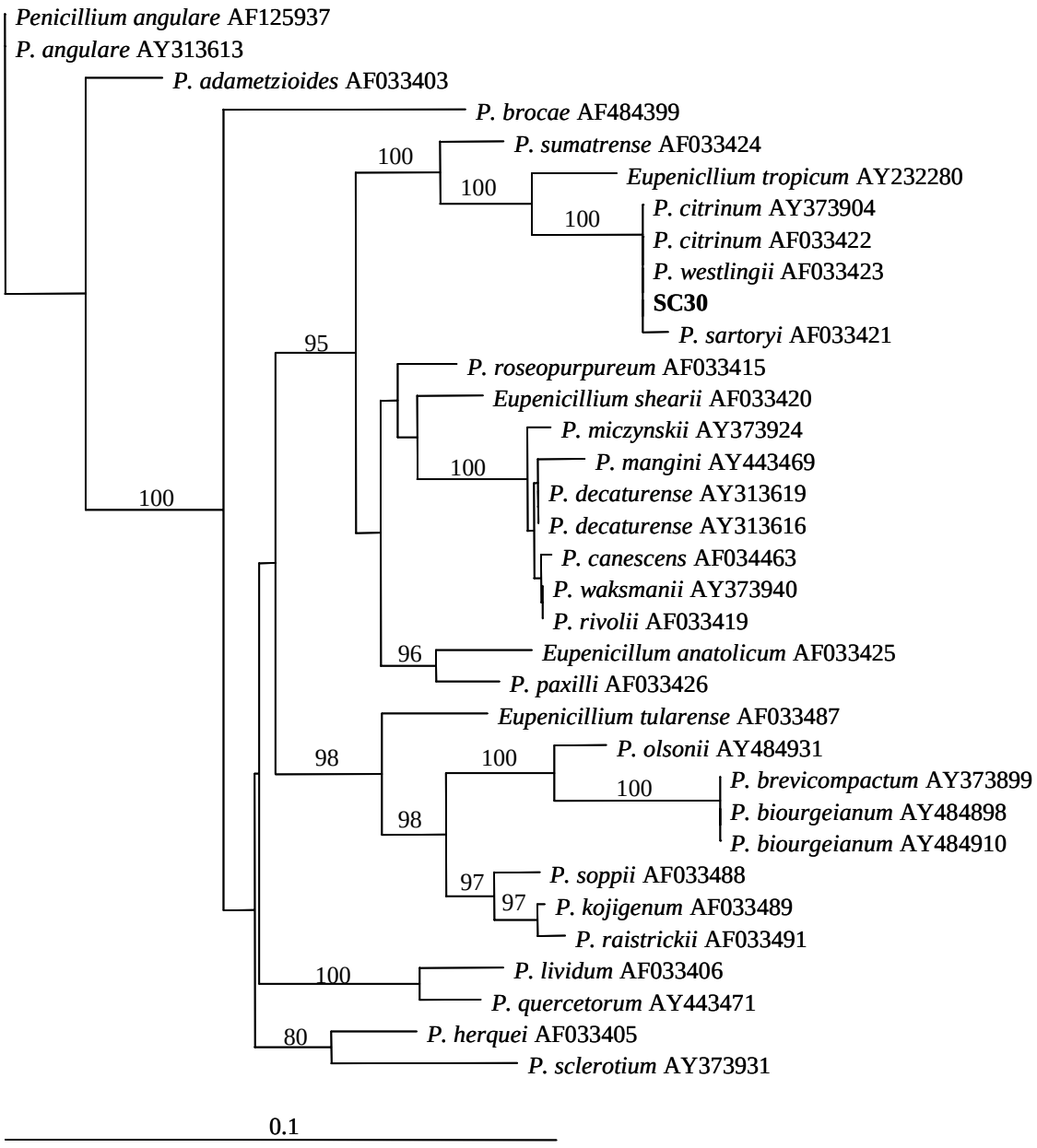

Fig. 2.12 Neighbor-joining distance tree of ITS1-5.8S rDNA-ITS2 sequences for *Penicillium* strain SC30. Bar represents 10 substitutions per 100 nucleotides. Significant bootstrap values are indicated.

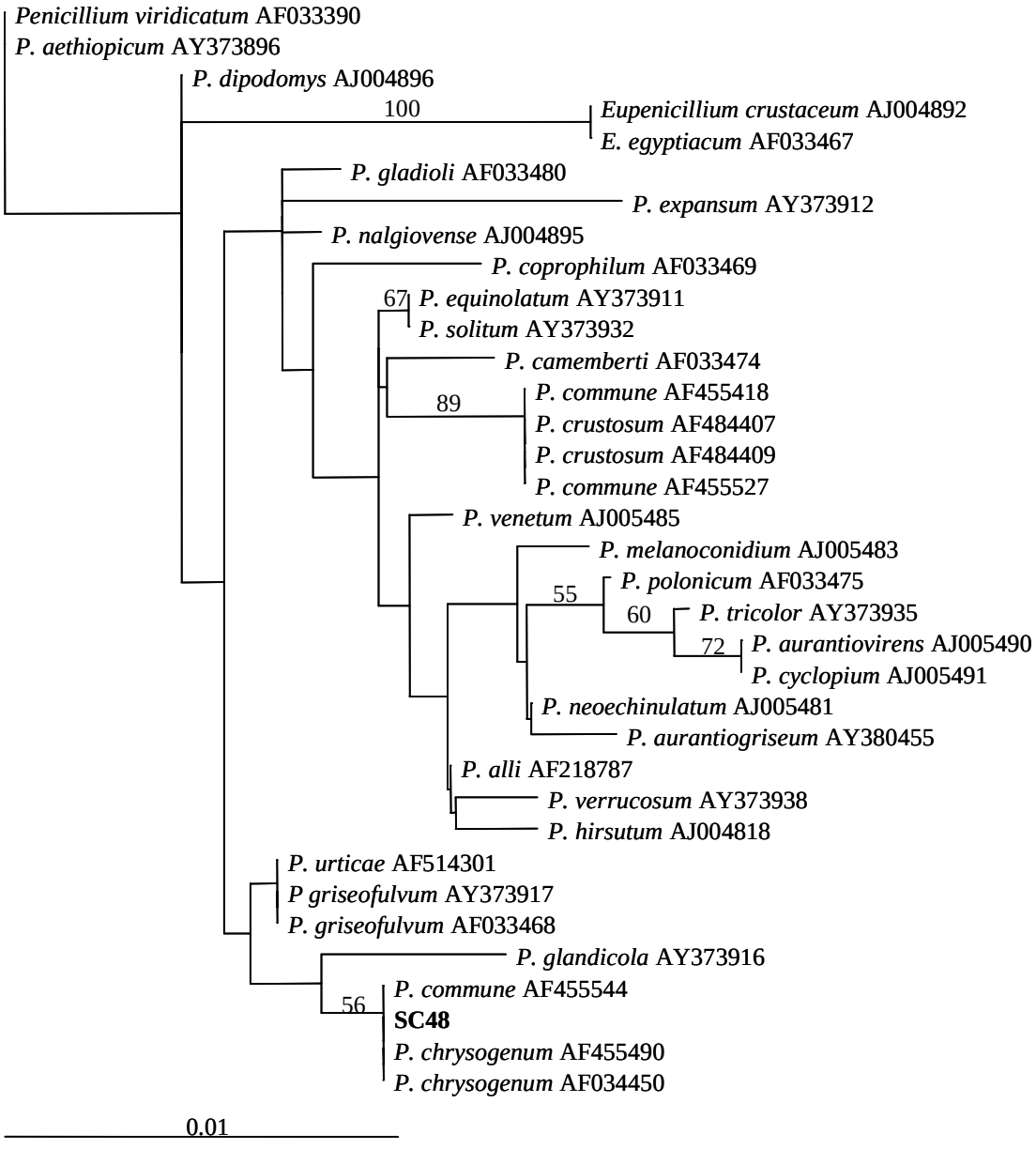

Fig. 2.13 Neighbor-joining distance tree of ITS1-5.8S rDNA-ITS2 sequences for *Penicillium* strain SC48. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated.

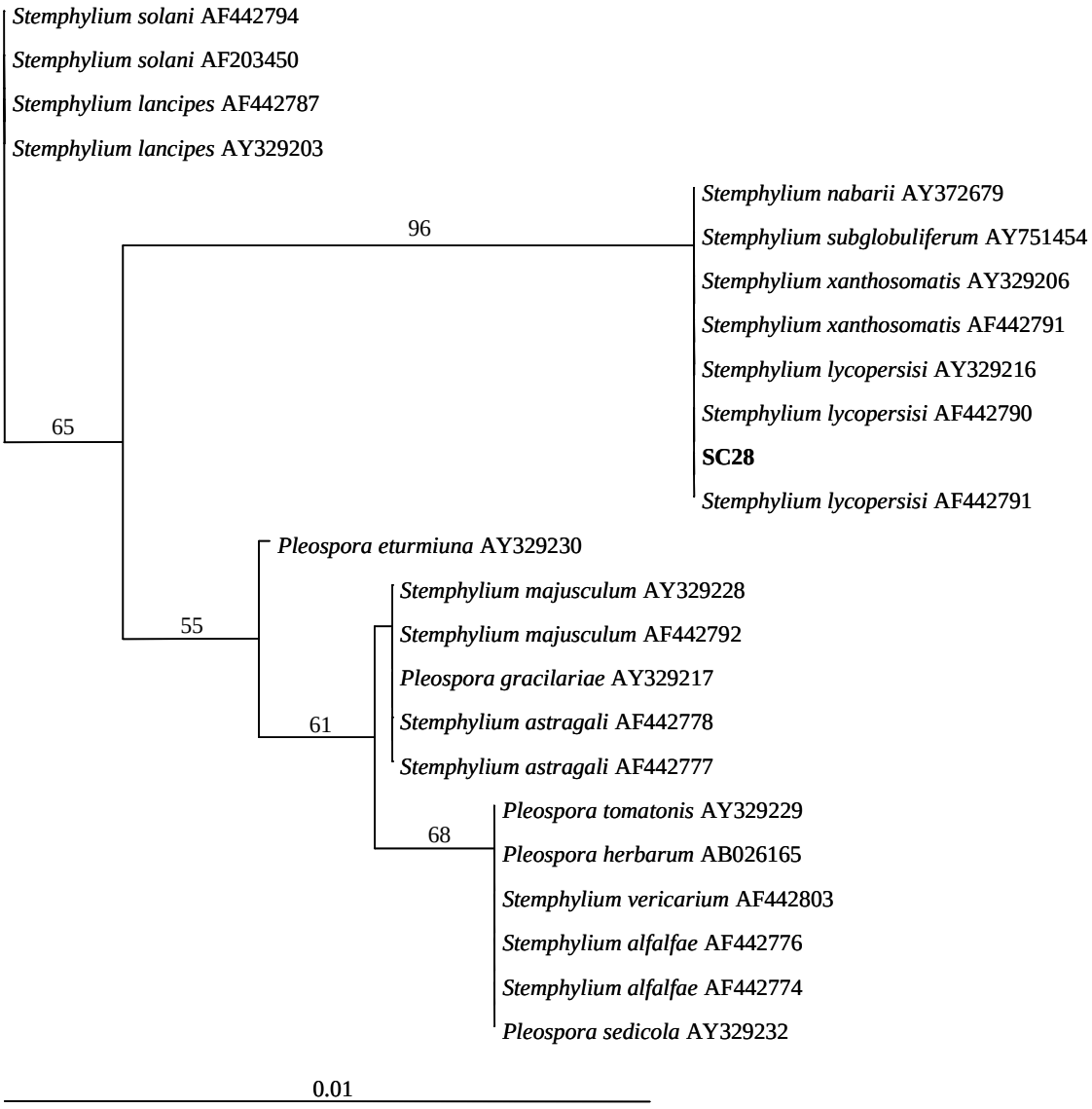

Fig. 2.14 Neighbor-joining distance tree of ITS1-5.8S rDNA-ITS2 sequences for *Stemphylium* strain SC28. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated.

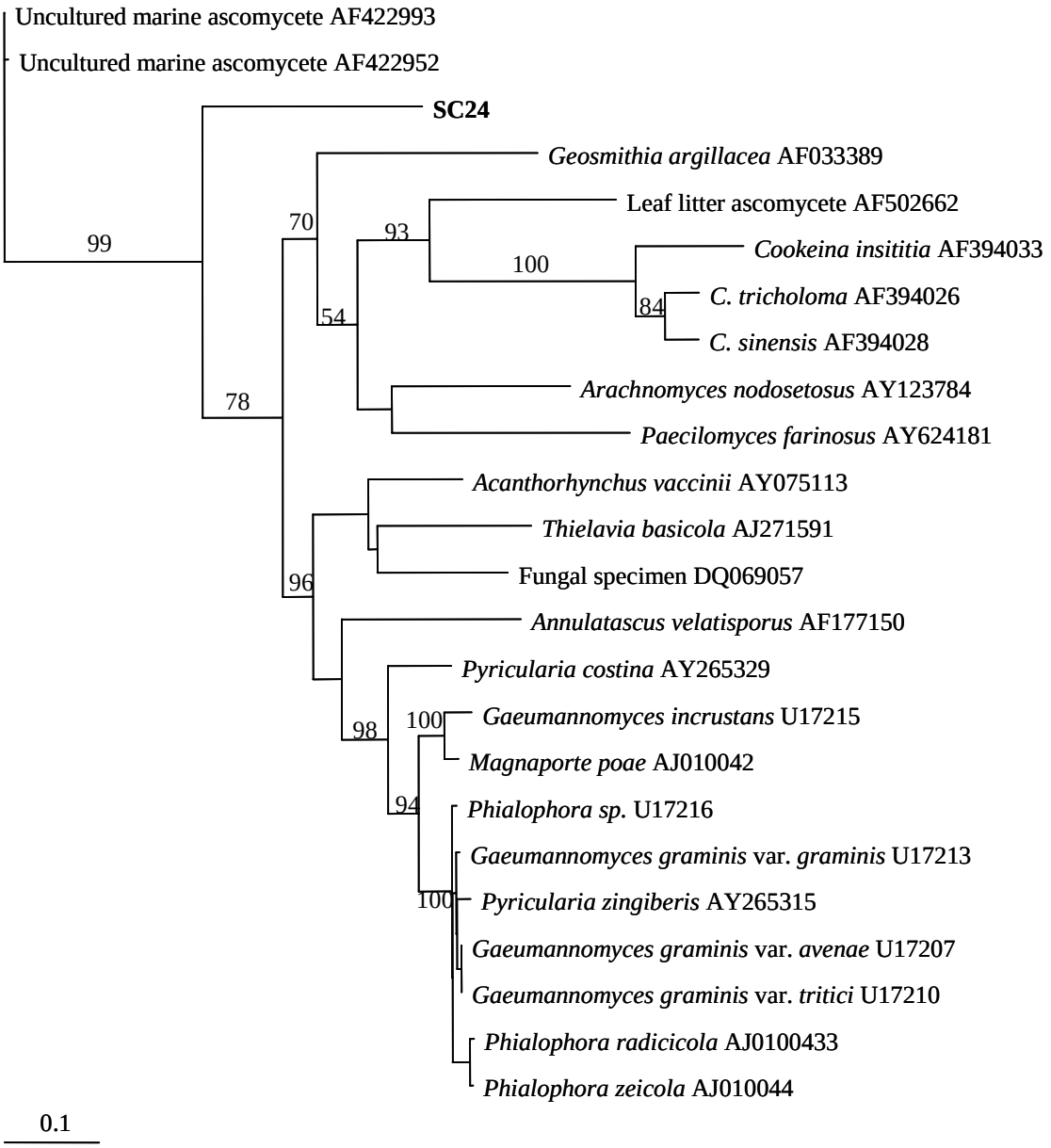

Fig. 2.15 Neighbor-joining distance tree of ITS1-5.8S rDNA-ITS2 sequences for strain SC24. Bar represents 10 substitutions per 100 nucleotides. Significant bootstrap values are indicated.

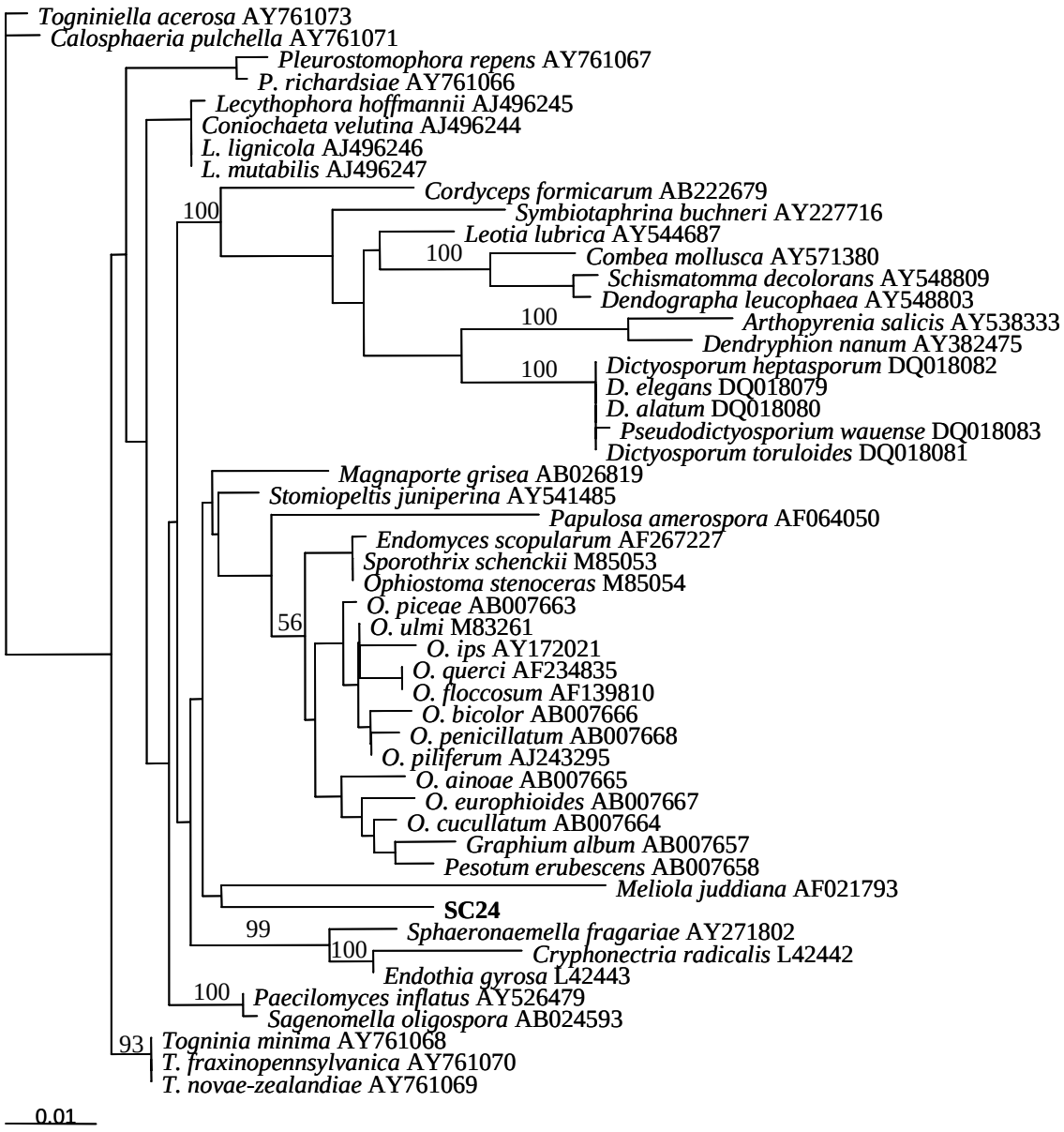

Fig. 2.16 Neighbor-joining distance tree of 18S partial sequences for strain SC24. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated.

Morphological, physiological and molecular data led to the classification of the strains into seven different genera, a total of seventeen species and an unknown morphospecies (SC24) (Table 2.3). All the species are described subsequently:

Table 2.3 Proposed classification of isolates, MZKI collection number and their GenBank (NCBI) accession numbers for the ITS1-5.8S-ITS2 region. N/A= not available.

Strain	Proposed Classification	Strain Designation in MZKI	Accession Number
SC59	*Alternaria* sp.	N/A	DQ372670
SC17	*Aspergillus* sp.	EXF 2405	DQ372667
SC60	*A. niger* aggregate morphotype 2	EXF 2417	DQ335978
SC41	*A. niger* aggregate morphotype 1	EXF 2413	DQ335979
SC35	*Aspergillus sp* (Section Nigri)	EXF 2411	DQ394077
SC46	*A. oryzae*	EXF 2414	DQ335983
SC20	*A. sydowii*	EXF 2406	DQ335981
SC34	*A. terreus*	EXF 2410	DQ335977
SC40	*A. versicolor*	EXF 2412	DQ335982
SC51	*Cladosporium cladosporioides*	EXF 2401	DQ335984
SC33	*Cladosporium sphaerospermum*	EXF 2400	DQ335985
SC3	*Eurotium amstelodami*	EXF 2404	DQ335980
SC29	*Hortaea werneckii*	N/A	AY820140
SC48	*Penicillium chrysogenum*	EXF 2403	DQ335974
SC30	*Penicillium* sp.	EXF 2402	DQ335975
SC24	unknown	N/A	DQ372668
SC28	*Stemphylium* sp.	N/A	DQ335976

***Alternaria* sp.- Nees**

Figure 2.17 - *Alternaria* had catenate (formed in chains) conidia. The conidia of SC59 was large, brown, multicelled and with transverse septa. The catenulation is characteristic of the group of *Brevicatenatae.* The strain has conidia in short chains with long beaks. The colony was dark brown in PDA. The reverse was brown. Diameter is up to 80 mm at optimal NaCl percent. The colony was tan with a cream reverse in Potato Carrot Agar (PCA).

Aspergillus sp.

Figure 2.18- Colonies on CYA, 50-60 mm of diameter. Colony reverse brown-greenish. Colony sulcate. Conidial heads had a radiating column arrangement and their color in mass was yellow. The stipes were ~250-500 µm and 7 to 10 µm. Stipe's walls rough. Vesicles elongate (10-25 µm width). Metulae present (10-15 µm of length). The phialide measures 5-10 µm. Conidia globose with length and width around 2 to 3 µm. Their walls smooth to slightly rough. SC17 was isolated from SG-15. Diameter up to 57 mm in optimal NaCl percent. Mycelium and conidial heads were tan to rose. Reverse of colony was cream.

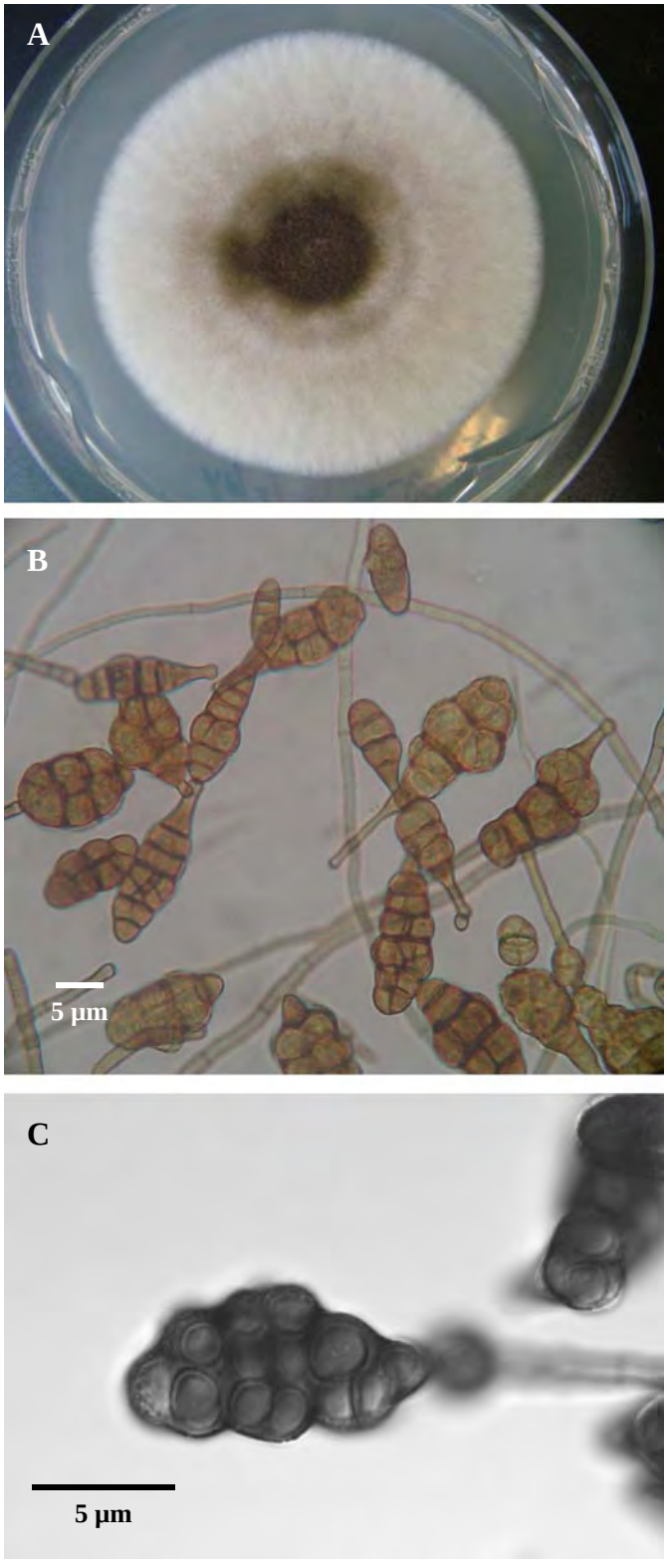

Figure 2.17 *Alternaria* sp. colony on PDA (A). Bright field microscopy (B) and Nomarski microscopy (C) of spores.

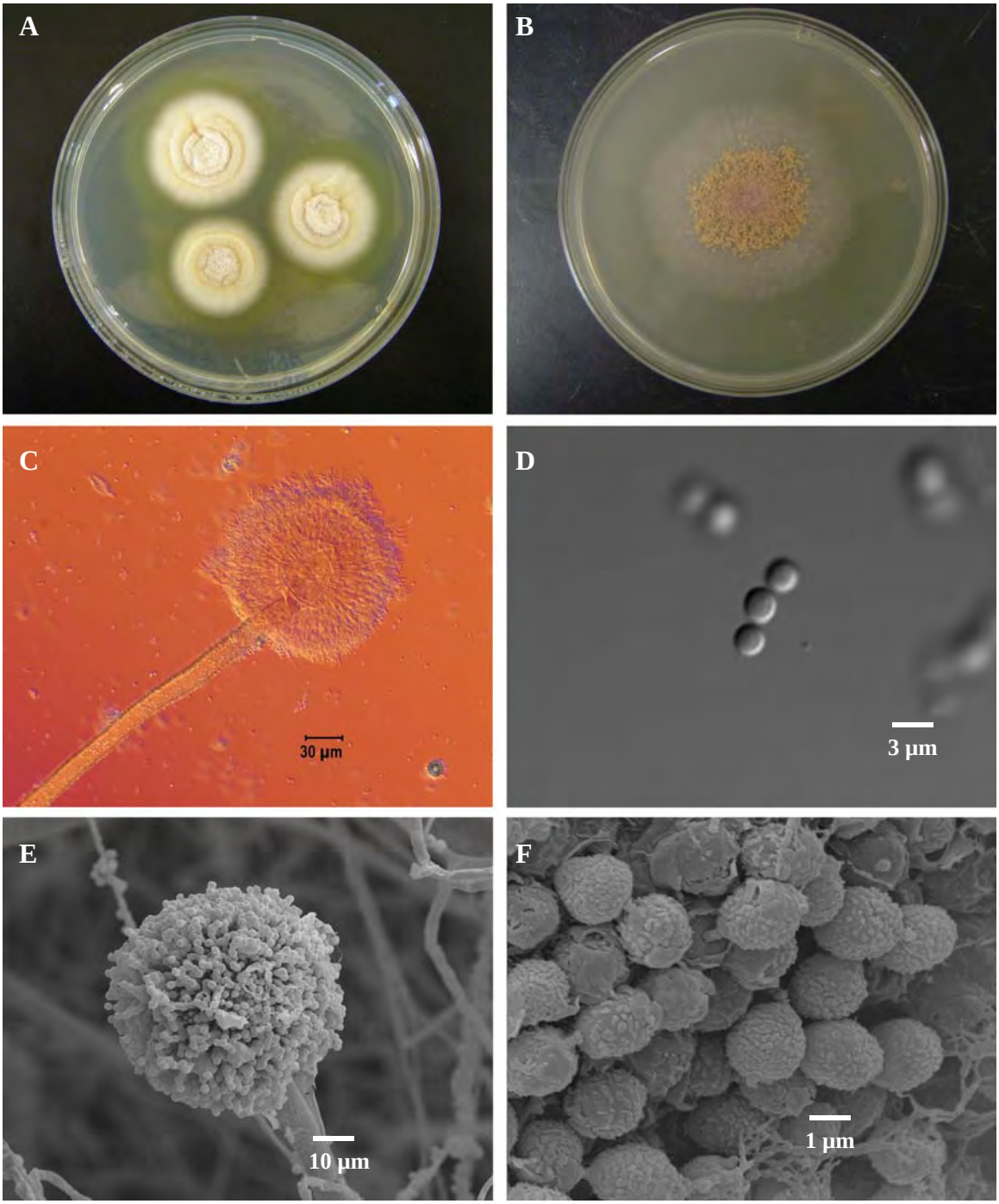

Figure 2.18 *Aspergillus* sp. colonies on CYA (A) and SG-5 (B). Conidiophore and spores, respectively, in Nomarski microscopy (C-D) and SEM (E-F).

***Aspergillus niger* aggregate (morphotype 1 SC41)**

Figure 2.19 - Colonies on CYA, 60-70 mm in diameter. The color of mycelium dark yellow. Colony reverse pale. Conidial heads had a radiating column arrangement and their color in mass was brown. The stipes ~600-900 µm and 10 to 12 µm. Stipe's walls smooth. Vesicles globose (10-50 µm width). Metulae present (10-15 µm length). The phialide 7-10 µm. Conidia globose with length and width around 3 to 5 µm; their walls spiny. It was isolated from PDA-15. Diameter up to 65 mm in optimal NaCl percent. Mycelium and conidial heads dark yellow. Reverse of colony cream.

***Aspergillus niger* aggregate (morphotype 2 SC60)**

Figure 2.20 - Colonies on CYA, 60-70 mm in diameter. Mycelium dark yellow. Colony reverse pale and uncolored. Conidial heads had a radiating column arrangement and their colour in mass was brown. Stipes ~700-900 µm and 7 to 15 µm. Stipe's walls smooth and thick. Vesicles globose (10-60 µm width). Metulae present (10-20 µm length). Phialide 7-10 µm. Conidia globose with length and width around 3 to 5 µm; their walls rough. It was isolated from PDA-15. Diameter up to 75 mm in optimal NaCl percent. Mycelium and conidial heads dark yellow. Reverse of colony cream.

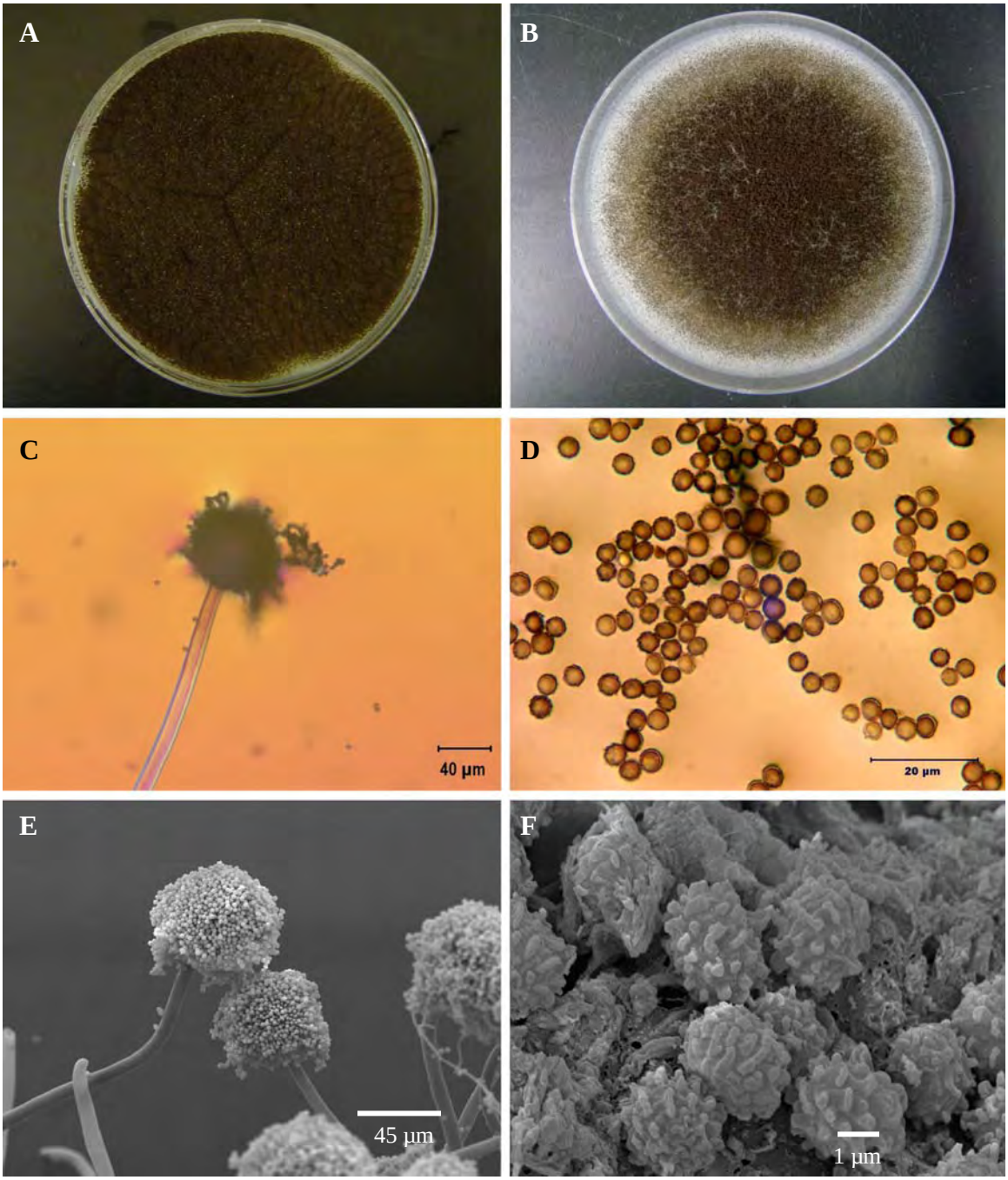

Figure 2.19 *Aspergillus niger* aggregate morphotype 1 colonies on CYA (A) and PD-5 (B). Coniophore and spores in Nomarski microscopy (C-D) and SEM (E-F).

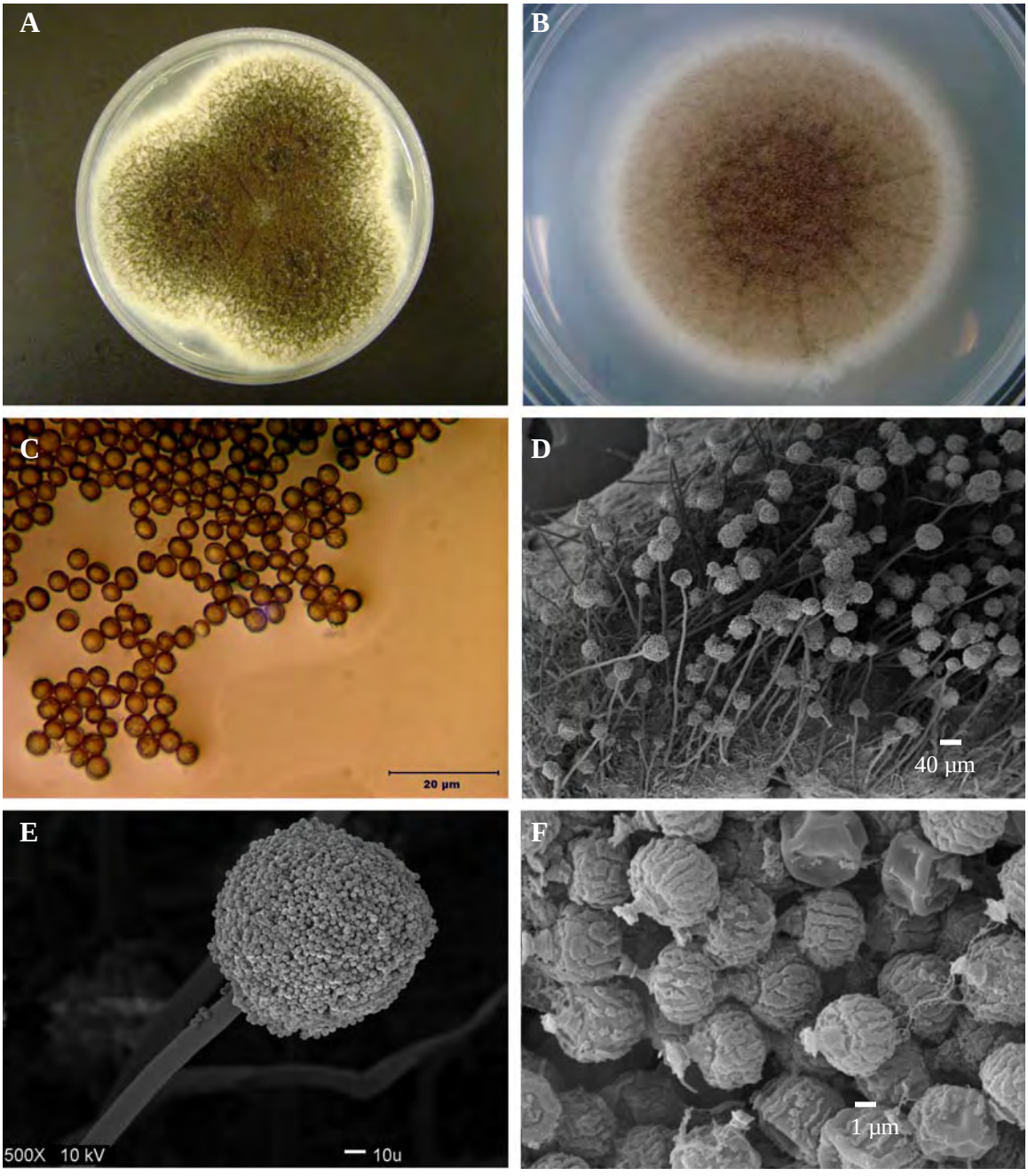

Figure 2.20 *Aspergillus niger* aggregate morphotype 2 colonies on CYA (A) and PD-5 (B). Nomarski microscopy of spores (C) and SEM of colony (D), conidiophore (E) and spores (F).

***Aspergillus sp.* Section Nigri**

Figure 2.21- Colonies on CYA, 50-60 mm in diameter. Colony reverse brown-greenish. Colony sulcate. Conidial heads had a radiating column arrangement and their color in mass was brown. Stipes ~300-1,000 µm and 5 to 10 µm. Stipe's walls smooth. Vesicles globose (10-45 µm width). Metulae sometimes present and absent. Phialide 5-10 µm. Conidia globose to subglobose, with length and width around 4 to 7 µm. Their walls slightly spiny. SC35 was isolated from MEA-15. Diameter up to 80 mm in optimal NaCl percent. Mycelium and conidial heads brown. Reverse of colony cream.

***Aspergillus oryzae*- (Ahlb, 1878) Cohn, 1884**

Figure 2.22- Colonies on CYA, 50-60 mm of diameter. Mycelium and colour in mass was yellow-greenish. Conidial heads columns had a radiating arrangement. The reverse was cream-yellowish. The stipes measures ~800-900 µm length and 10 to 15 µm width. Walls were rough. Vesicles were globose (20-75 µm width). No metulae present. The phialide measures 10-20 µm. Conidia were globose to subglobose with length and width of of 3-10 µm. Their walls were rough. Sclerotia were dark brown to black. SC46 was isolated from PDA-15. In optimal NaCl percent, diameter is up to 85 mm. Mycelium and conidial heads observed were green. Reverse of colony was cream-yellowish.

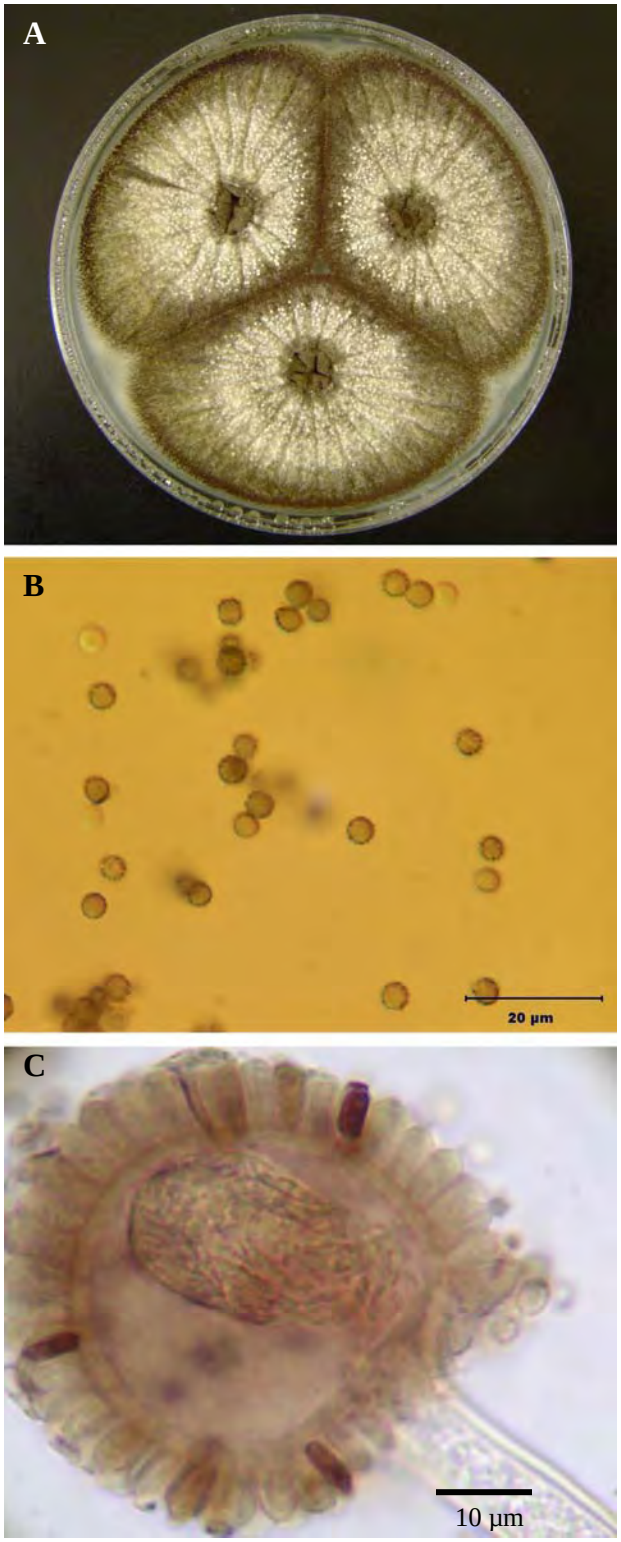

Figure 2.21 *Aspergillus* sp. colonies on CYA (A). Nomarski microscopy (B) and bright field microscopy (C) of spores and conidiophore, respectively.

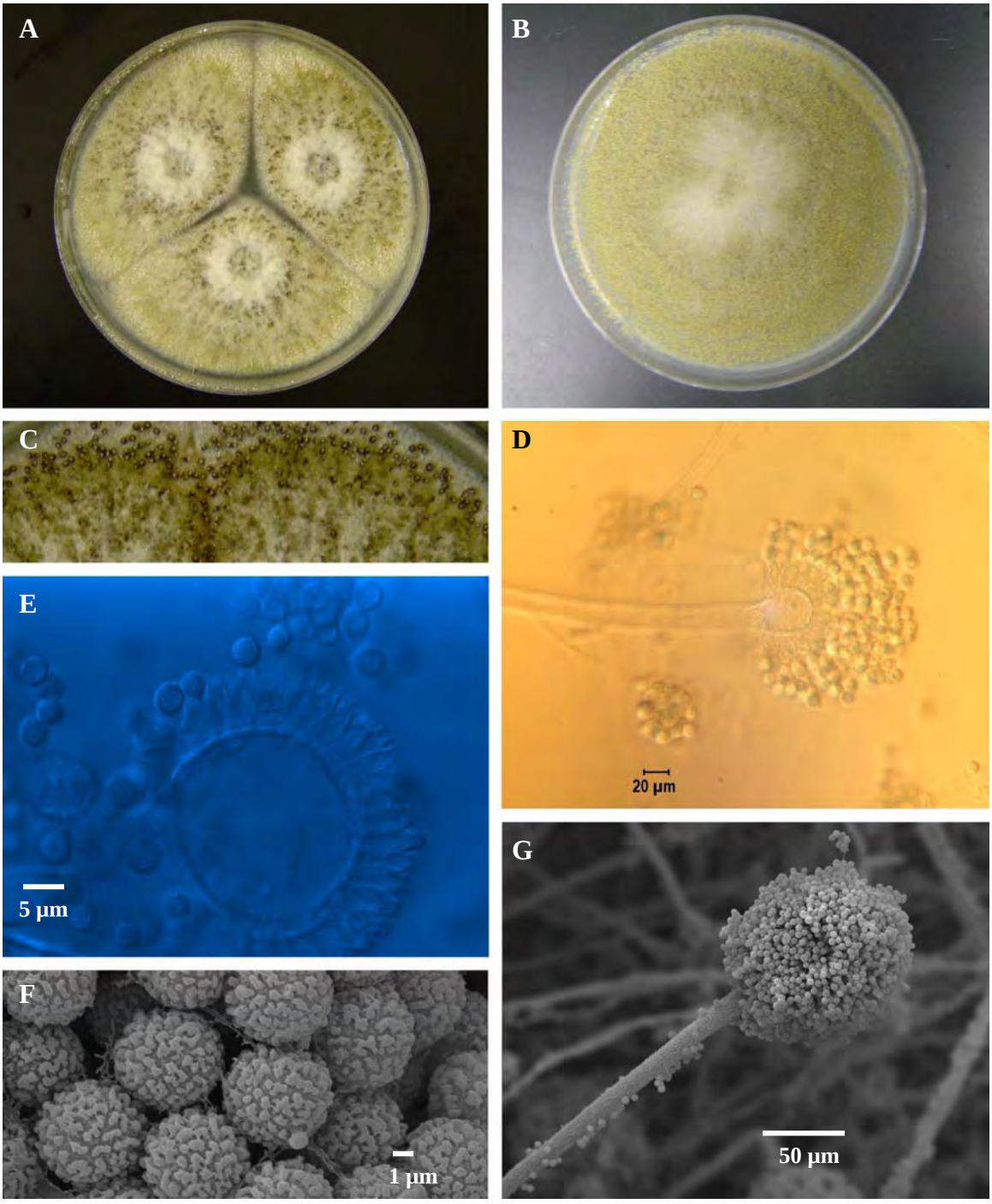

Figure 2.22 *Aspergillus oryzae* colonies on CYA (A) and SG-5 (B). Colony producing sclerotia (C). Nomarski microscopy (D-E) and SEM of spores (F) and conidiophore (G).

***Aspergillus sydowii*- (Barnier & Sartory, 1913) Thom & Church, 1926**

Figure 2.23- Colonies on CYA 20-22 µm of diameter. Colony sulcate and the reverse had a grey-greenish color. Mycelium brown with red exudates and brown-reddish soluble pigment. Color in mass green. The columns had radiating arrangement. The stipes ~100-500 µm of and 8-10 µm. Their walls smooth. Vesicles globose to spathulate and 10-15 µm width. Metulae present (5-7 µm) and phialide (5-10 µm length). Conidia globose of 2 to 4 µm of length and width; their walls rough or spiny. Strain SC20 was isolated from SG-15. Colonies on SG up to 45 mm of diameter in optimal salinity. Conidial heads brown. The reverse of colony cream.

***Aspergillus terreus*- (Thom, 1918)**

Figure 2.24- Colonies on CYA 30-50 mm in diameter. Mycelium and soluble pigment color was yellow. Reverse of colony yellow or brown. Colony sulcate. Conidial heads color brown with compact column arrangement. Stipes ~50-500 µm and 5-8 µm; their walls were smooth. Vesicles globose to hemispherical, 10-15 µm wide. Metulae present, 5-7 µm of long. Phialide length 5-10 µm. Conidia shape globose, 2-3 µm and 2-3 µm; their walls smooth. Strain SC34 was isolated from ME-15. In MEA-5 (optimal %NaCl) diameter is up to 80 mm. Conidial heads dark yellow. Colony reverse color was cream-yellowish.

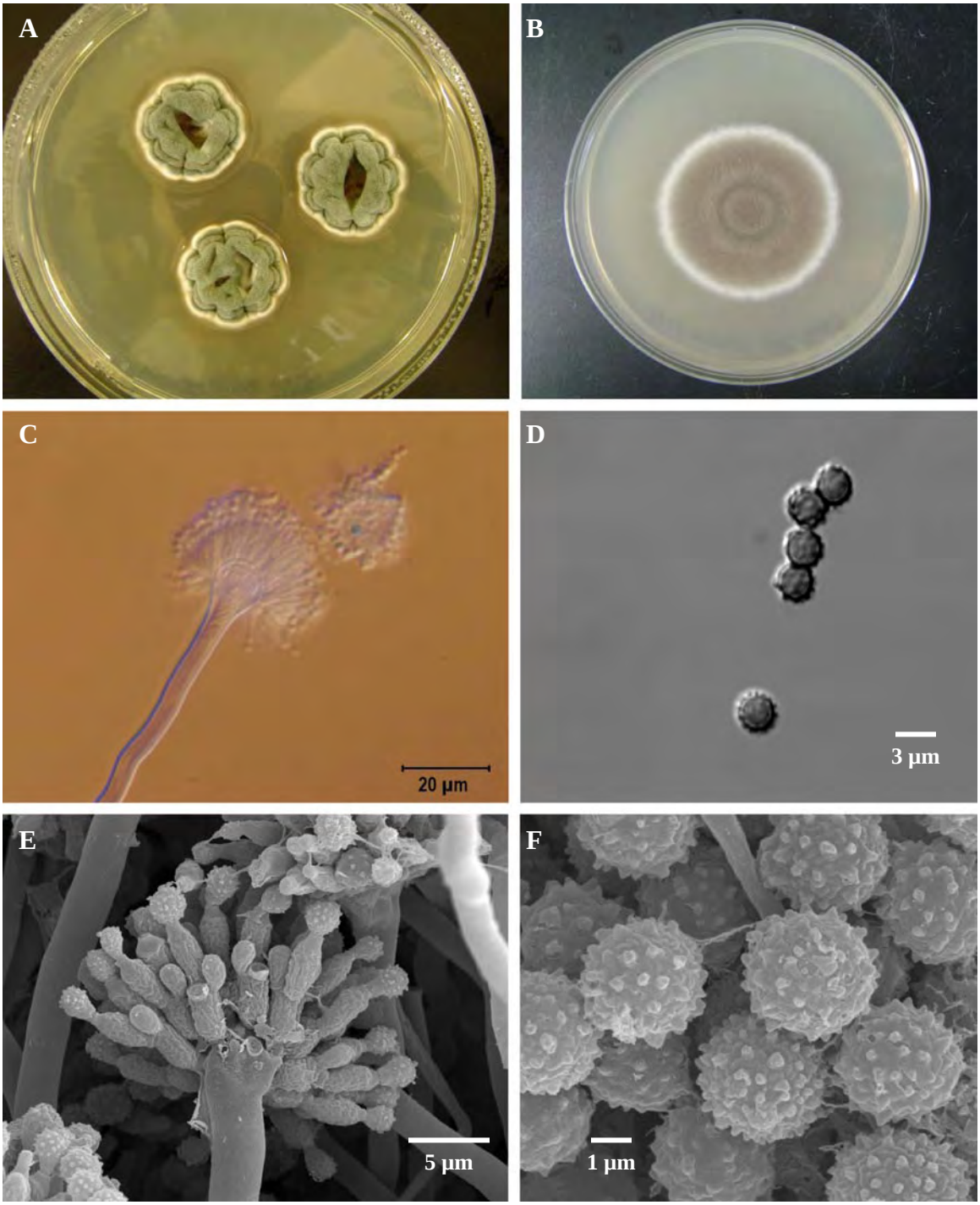

Figure 2.23 *Aspergillus sydowii* colonies on CYA (A) and SG-5 (B). Nomarski microscopy (C-D) and SEM (E-F) of conidiophore and spores.

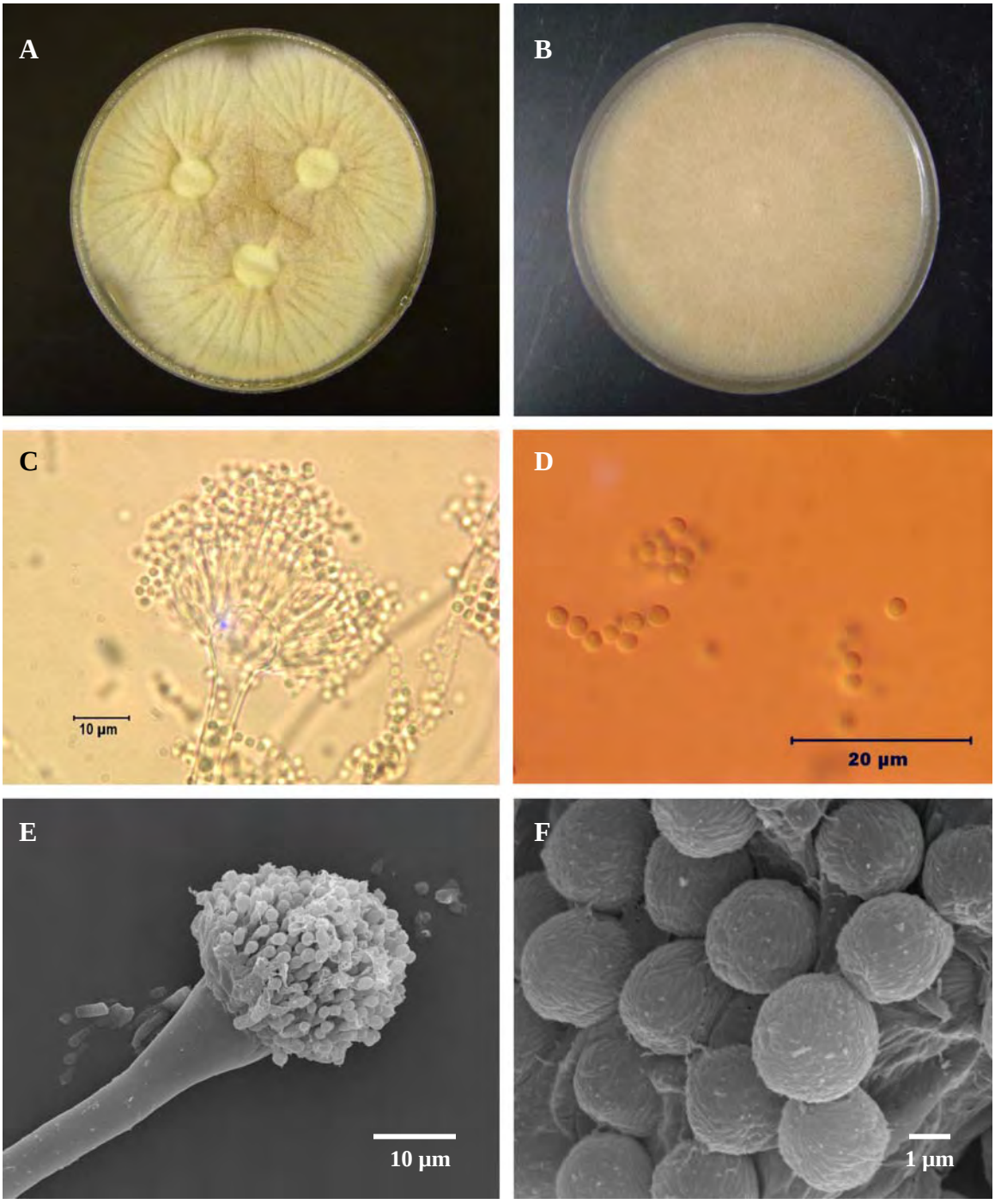

Figure 2.24 *Aspergillus terreus* colonies on CYA (A) and ME-5 (B). Bright field microscopy of conidiophore (C), Nomarski microscopy of spores (D) and SEM (E-F) of these microscopical structures.

***Aspergillus versicolor*- (Vuill, 1903) Tirab, 1908**

Figure 2.25- Colonies on CYA 15-20 mm of diameter. Mycelium white or pink. Reverse color brown. Production of uncolored exudate and yellowish soluble pigment. Colony sulcate. Conidial heads white with a radiating arrangement. Stipes ~100-700 µm and 3-7 µm width; their walls smooth. Vesicles shape elongate to clavate up to 15 µm wide. Metulae present with length up to10 µm. Conidial shape globose, of 2-4 µm and 2-4 µm; their walls rough. Strain SC40 was isolated from PD-15. Colony diameter 40-50 mm in optimal NaCl percent. Conidial heads were green and the color for colony reverse cream.

***Cladosporium cladosporioides*- (Fresen) de Vries**

Figure 2.26- Colonies on PDA olivaceous grey-green. Reverse black. Conidiophore branching widely. Conidia in chains, ellipsoidal of 3-4 µm and 2-3 µm. Prominent scars present. The strain SC51 was isolated from ME-15. In optimal salinity the colony had a diameter up to 22 mm. Colony was green, with a black reverse.

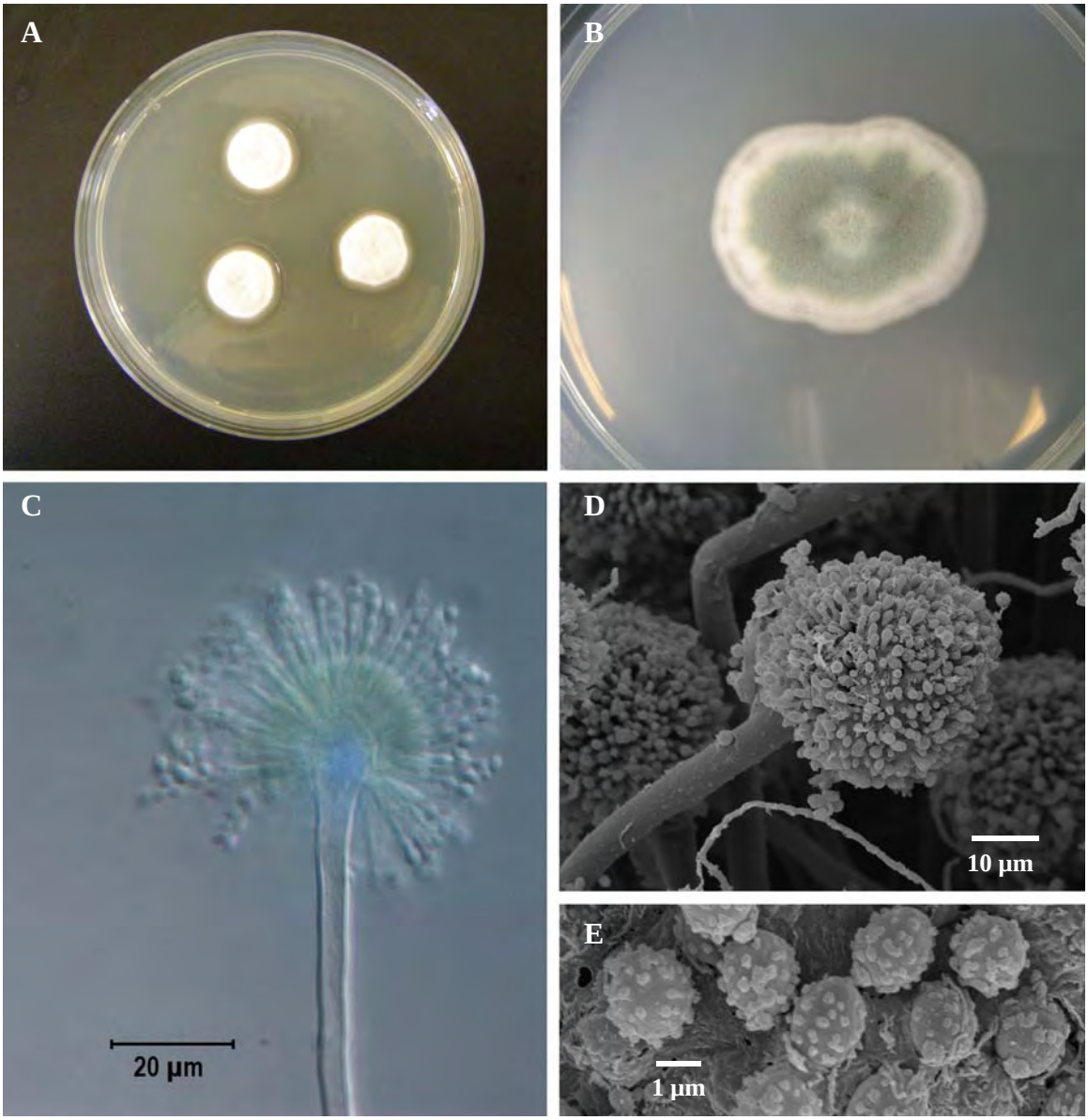

Figure 2.25 *Aspergillus versicolor* colonies on CYA (A) and PD-5 (B). Nomarski microscopy (C) and SEM (D-E) of conidiophore and spores.

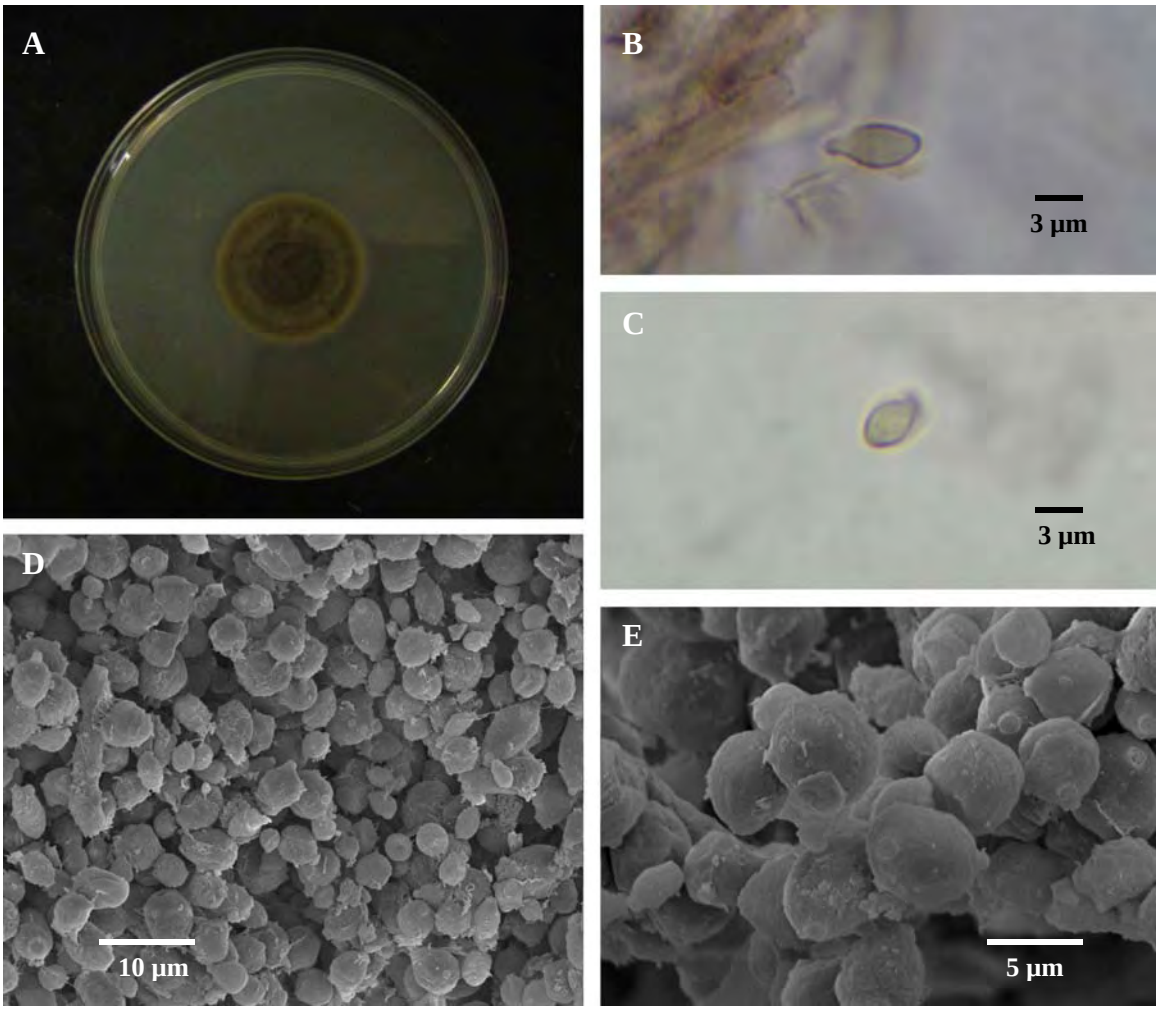

Figure 2.26 *Cladosporium cladosporioides* colonies on ME-5 (A). Bright field microscopy (B-C) and SEM (D-E) the spores.

***Cladosporium sphaerospermum*- (Penz)**

Figure 2.27- Colony had a green color on PDA. Conidia smooth to slightly rough, more rounded than *C. Cladosporioides*. Conidia in chains, ellipsoidal, 4 µm and 2-3 µm. Prominent scars present. The strain SC33 was isolated from PD-15. Colony diameter up to 23 mm in optimal salinity. Colony green, with a black reverse.

***Eurotium amstelodami*- (Mangin, 1909)**

Figure 2.28- Colony on CYA 15-20 mm of diameter. SC3 had dark yellow mycelium and colony reverse was green. Color in mass green and radiating arrangement of conidial heads. Stipes walls smooth, ~100-500 µm and 7-15 µm. Vesicles globose to spathulate (10-30 µm width). No metulae present. Phialide up to 10 µm long. Conidia globose to subglobose (3-7 µm of length and 3-7 µm width) with rough walls. Cleistothecial ascomata yellow (100-250 µm of diameter). Ascospores lenticular and with single furrow ornamentation with two or three ridges or crests. Ascospores uncolored, with rough walls, of 3-5 µm long and wide.

***Hortaea werneckii*- (Nishimura and Miyaji, 1984)**

Figure 2.29- On PDA colonies dark green, with black reverse. The strain was melanized, and showed different unicellular propagation types: meristematic enlargement, annellidic budding (bipolar or unipolar) and sympodial budding. Filamentous stage present at the same culture. Strain SC29 was isolated from SG-15. In optimal salinity (10%) the diameter was up to 10 mm.

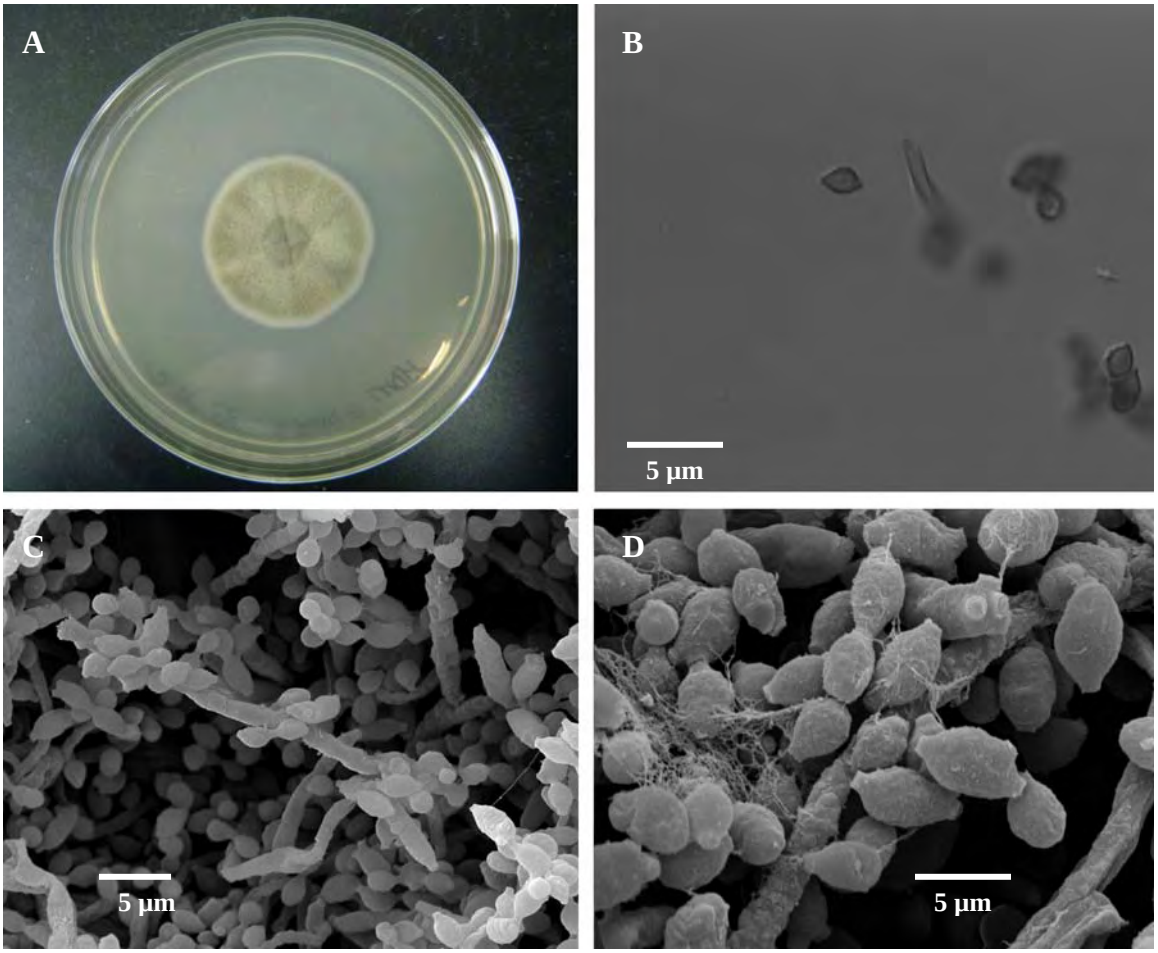

Figure 2.27 *Cladosporium sphaerospermum* colonies on ME-5 (A). Nomarski microscopy (B) and SEM (C-D) of the spores.

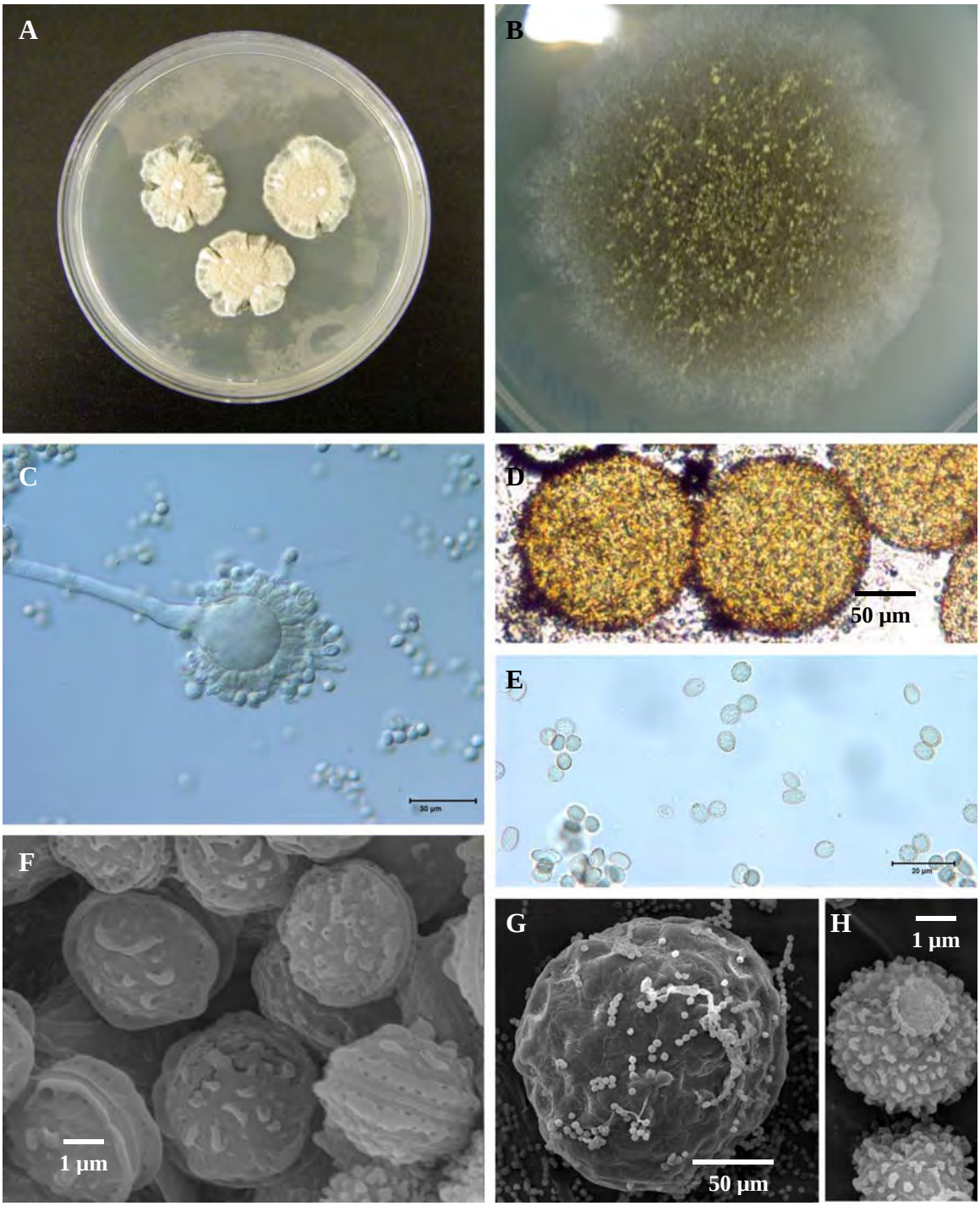

Figure 2.28 *Eurotium amstelodami* colonies on CYA (A) and SG-10 (B). Nomarski microscopy (C) of conidiophore, bright field microscopy of cleisthotecia (D) and spores (E) and SEM of ascospores, cleisthotecia and spores (F-G-H).

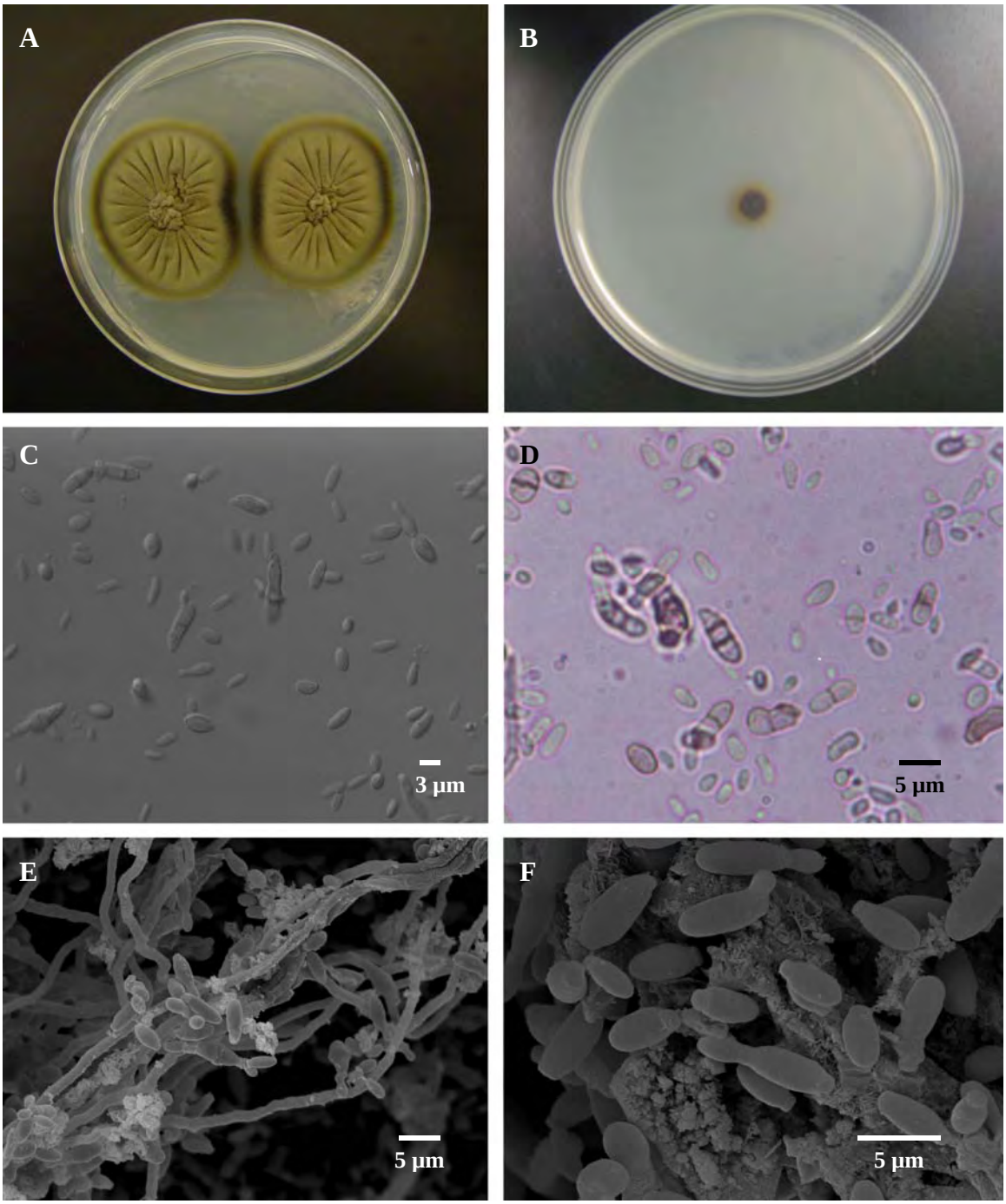

Figure 2.29 *Hortaea werneckii* colonies on PDA (A) and SG-10 (B). Nomarski (C), bright field (D) microscopy and SEM (E-F) showing different types of propagation: multilateral budding and annellidic budding (unipolar or bipolar).

***Penicillium chrysogenum*-(Thom, 1910)**

Figure 2.30 - On CYA colonies velvety, 25-30 mm in diameter. Colony green-yellowish, on reverse, soluble pigments and exudates yellow. The penicilli were predominantly Terverticillate. Mononematous conidiophore and single branches. Stipe not longer than 100 µm. Phialide short (8-10 µm). Conidia subglobose to ellipsoidal in some cases; their walls were smooth. Strain SC48 was isolated from PD-15. In optimal NaCl percent (5 %), colony green-bluish, up to 55 mm of diameter. Produced clear exudates at 0% of NaCL and 25˚C.

***Penicillium* sp.**

Figure 2.31- On CYA, colonies velvety, 25-30 mm in diameter. Colony green; reverse and soluble pigments yellow. Penicilli predominantly Terverticillate. Mononematous conidiophore. Stipe longer than 100 µm, smooth. Phialide short (5-10 µm). Conidia ellipsoidal to subglobose in some cases; their walls smooth to slightly rough. The strain SC30 was isolated from ME-15. In optimal NaCl percent (5 %) the colony green up to 42 mm in diameter.

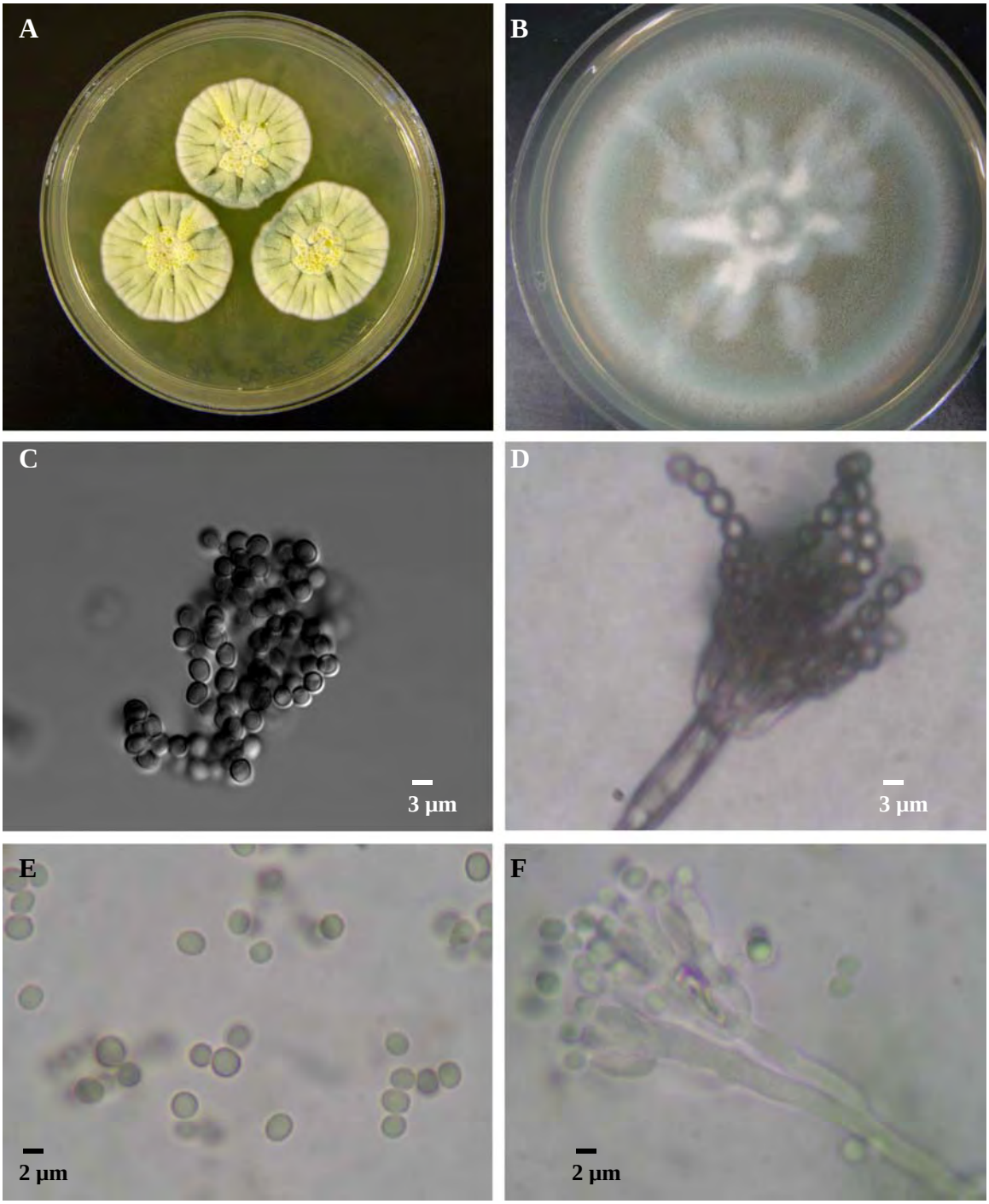

Figure 2.30 *Penicillium chrysogenum* colonies on CYA (A) and PD-5 (B). Bright field microscopy (C-F) of conidia and penicilli.

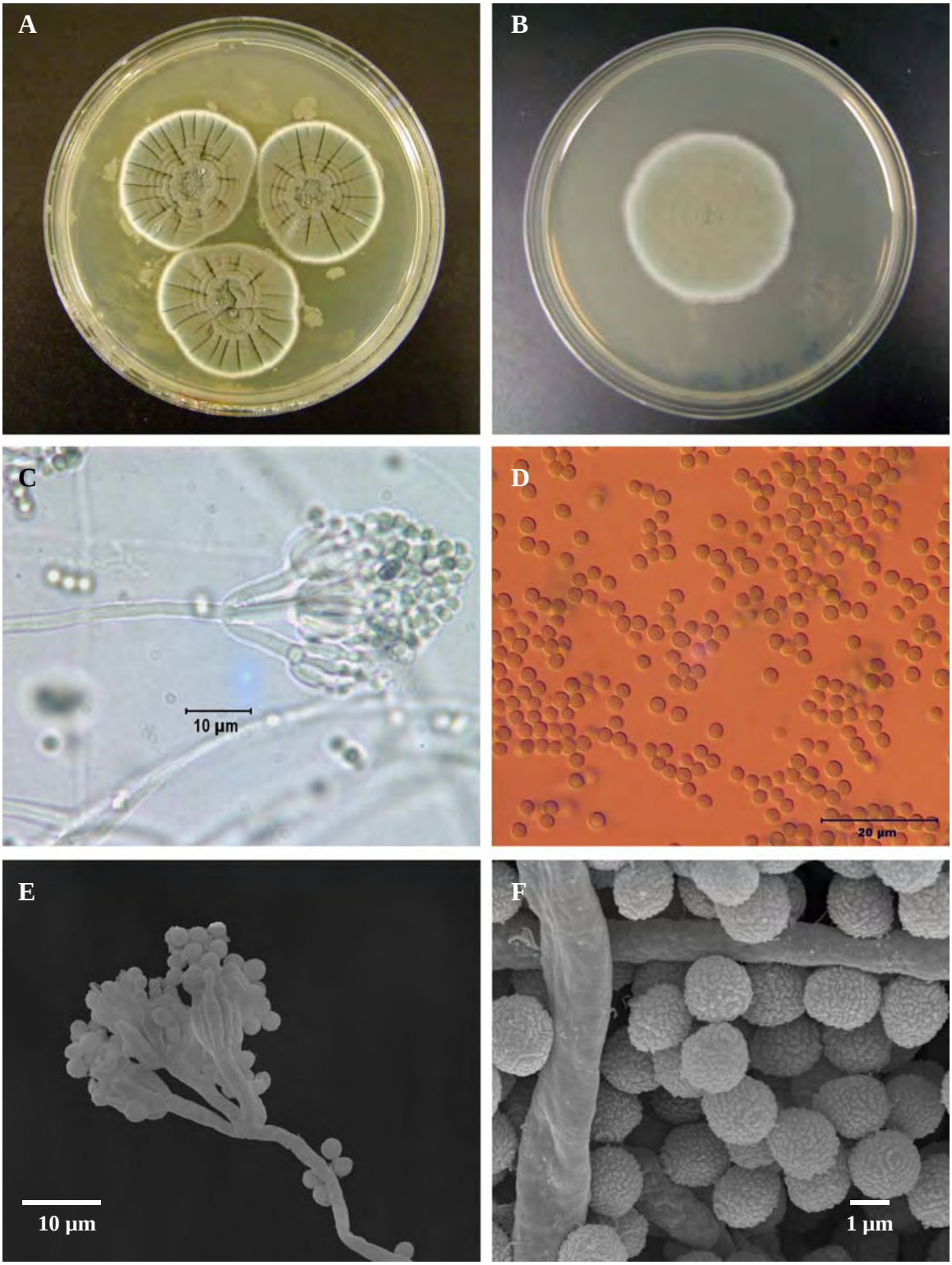

Figure 2.31 *Penicillium expansum* colonies on CYA (A) and ME-5 (B). Bright field microscopy (C) showing conidiophore. Nomarski microscopy (D) showing spores and SEM (E-F) of penicilli and spores.

Morphotype SC24

Figure 2.32- On SG and PDA, the colony white and cottony. Colonies grew very deep in the agar. Reverse cream. Conidiophore large with a conidial cluster. Conidia smooth. Frequently, a short conidiophore appeared with a single spore.

***Stemphylium sp*.- (Wallr)**

Figure 2.33- Dark conidiophore and conidia. Conidiophore proliferated through all conidial scars. Conidia had longitudinal septa, and variable in shape (globose to ellipsoidal). In PCA the colony SC28 had a tan colored mycelium. In V8 medium, colony was cream.

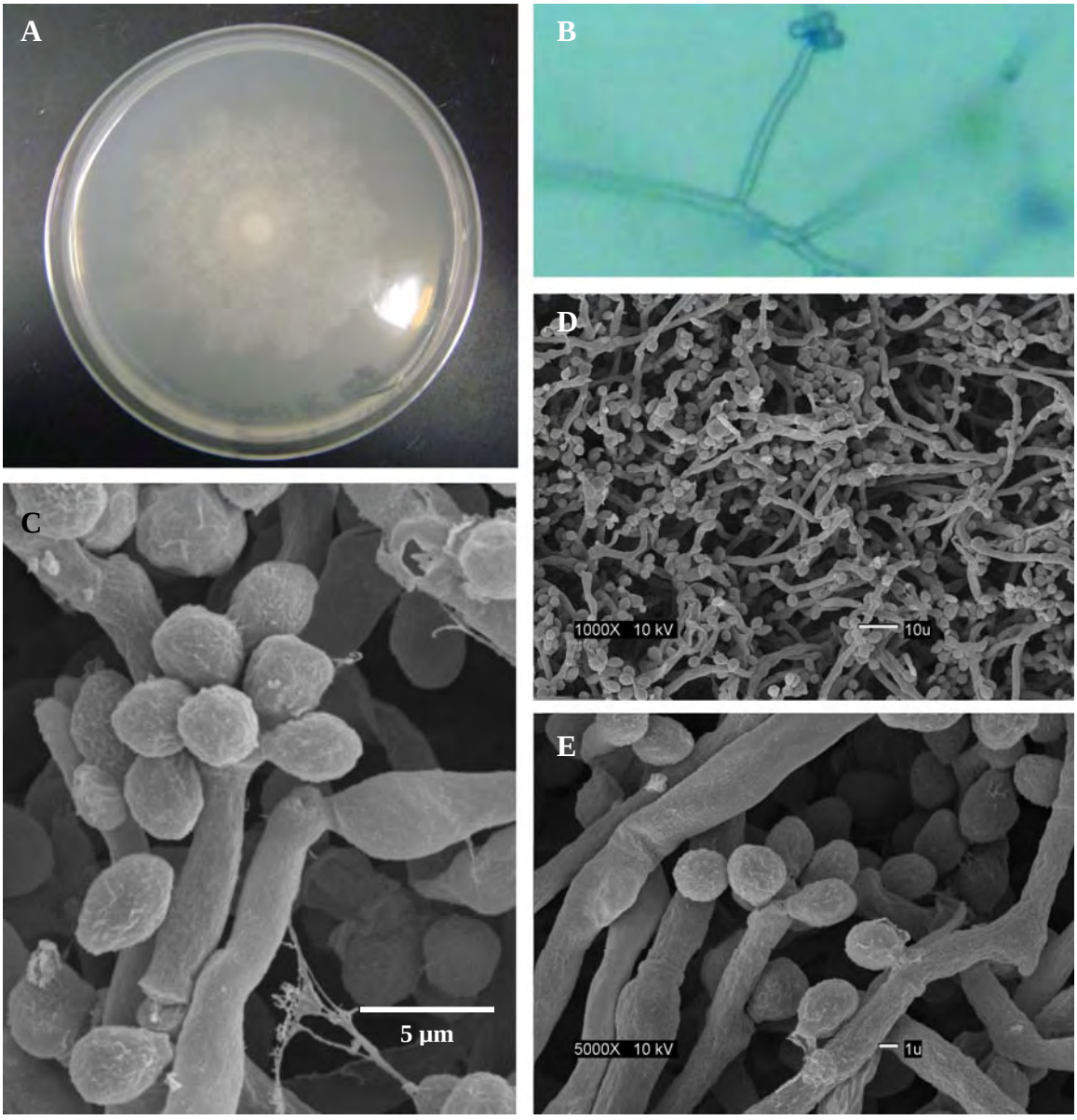

Figure 2.32 *SC24* colonies on SG-10 (A). Bright field microscopy showing the conidiophore (B). SEM (C-D-E) of the somatic structures.

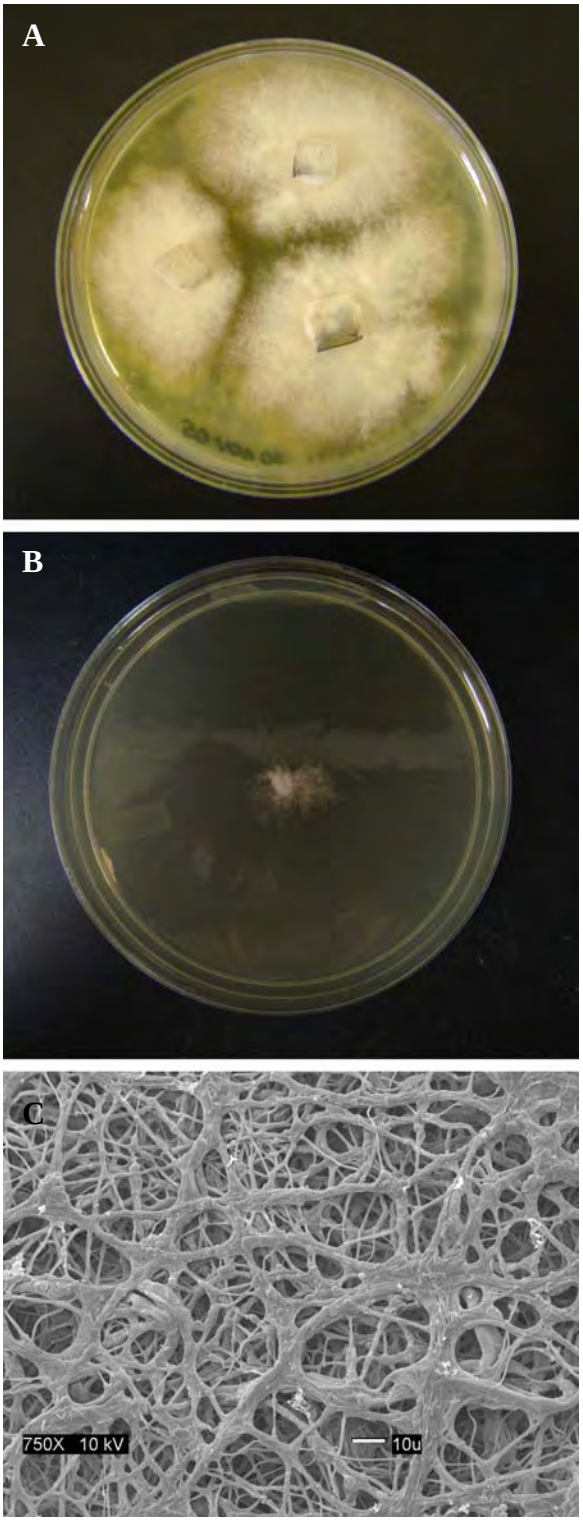

Figure 2.33 *Stemphylium* sp. colonies on PCA (A) and PD-15 (B). SEM microscopy (C) of mycelium.

Discussion

This study was focused on the isolation of filamentous fungi from the solar saltern of Cabo Rojo, Puerto Rico. This study represents the first attempt to determine the filamentous mycobiota present at a hypersaline environment in the Caribbean. A large number of isolates were obtained growing on different media used in this study. The taxonomical analysis performed to the selected isolates led to the classification of seven different genera, a total of seventeen species and an unknown morphospecies preliminary classified as part of Sordariales group. Their characterization was based on morphological features, secondary metabolites profiles and molecular characterization using ITS or 18S regions of the genomic rDNA.

The black yeast *Hortaea werneckii* had the highest frequency of isolation among all the fungi obtained from the solar salterns. This demonstrates the halophilic nature of this dimorphic yeast. Its natural ecological niche is the hypersaline waters in salterns (Gunde-Cimerman et al., 2000; Díaz-Muñoz and Montalvo-Rodríguez, 2005). Although our study was focused in the isolation of filamentous fungi, the *Hortaea* strains were selected due to their occurrence through all media and their morphological diversity. They were isolated as filamentous form at the conditions tested in this study. *Hortaea* isolates showed dark septate mycelia and anellidic budding. The morphological characteristics observed on these strains revealed features corresponding to those described in the literature for the genus *Hortaea* (de Hoog and Gerrits van den Ende 1992). Restriction fragment length polymorphism analysis of a PCR amplicon containing the 5.8S rDNA and ITS1-ITS2 regions was used to molecularly classify the strains into groups. The restriction pattern obtained with enzymes *BsaHI* and *HaeIII* revealed that the

isolates could not be divided in different groups and might represent the same *Hortaea* species. Therefore, strain SC29 was selected randomly as representatives of this group for further analyses. Physiological experiments on strain SC29 revealed that eventhough growth was recorded at 0% NaCl, it can be classified as halophilic by the ability to grow close to NaCl saturation conditions (30%). The optimum salt concentration was around 10% NaCl, the pH at 5 and the optimal temperature was around 29°C since this combination showed the largest colony diameter under the conditions tested. Growth at NaCl saturation conditions was previously reported for *Hortaea werneckii* isolated from a solar saltern in Slovenia (Gunde-Cimerman et al. 2000). Even though it has been reported that *Hortaea werneckii* has a preference for lower temperatures (de Hoog and Gerrits van den Ende 1992; Zalar et al. 1999b; Gunde-Cimerman et al. 2000), our results indicate that the strains might prefer higher temperatures to grow optimally. This might show an adaptation of the genus to the usual high temperatures of tropical environments. A PCR amplicon consisting of the ITS1-5.8SrDNA-ITS2 for each isolate was submitted for sequencing to determine the taxonomical position of the strain molecularly. *In silico* analysis of the sequence revealed similarities to strains of *Hortaea werneckii*. This information was used to construct a phylogenetic tree using the neighbor-joining method. The consensus distance tree places SC29 in the *H. werneckii* monophyletic cluster. All the results presented here strongly suggest that isolate SC29 is a strain of *H. werneckii*. This constitutes the first report of this organism from a hypersaline niche in the Caribbean (Díaz-Muñoz and Montalvo-Rodríguez, 2005). In the solar saltern ponds from Slovenia, several black yeast-like fungi species from the genera *Phaeotheca and Trimmatostroma* and *Aureobasidium pullulans* were also found (Gunde-Cimerman et al.

2000). The halophilic yeast *Trimmatostroma salinum* was reported before as new species from the same Slovenian solar saltern (Zalar et al. 1999a). Other yeast species were reported in hypersaline waters around the world (Butinar et al., 2005b). The isolates include the genera *Candida, Debaryomyces, Pichia, Trichosporon, Metschnikowia, Rhodosporidium, Yarrowia* and *Rhodotorula.*

The second most frequent and the most diverse genus was *Aspergillus*. The morphological characteristics observed on these strains revealed features corresponding to those described in the literature for the genus. Restriction fragment length polymorphism analysis of the ITS regions was used to classify the strains into groups. The restriction pattern obtained with enzymes *BsaHI* and *HaeIII* revealed that these strains were very heterogeneous and can be separated into various groups. The genus was represented by eight anamorphic species and one perfect form (*Eurotium*).

The most common isolated species was *Aspergillus sydowii*. This fungus appeared through all media used and in all ponds sampled. Several strains similar to *A. sydowii* were also isolated. Secondary metabolite profiles confirm their classification as a single species. However, some of those strains showed morphological and molecular differences that will be considered for further studies to elucidate if they represent varieties into the same species. Marine and terrestrial strains of this species had been isolated before. Marine strains are pathogens of sea fans and terrestrial strains apparently are not pathogenic (Geiser et al., 1998b). This suggests means that this species is composed by strains with different physiological properties. In spite of the isolation of *Aspergillus* species from the ocean, they were not considered normal inhabitants of marine environments. Strain SC20 was selected as representative of this species for

further characterization. Phylogenetic analysis using the neighbor-joining method places this strain in the *A. sydowii* cluster. Physiological experiments on these strains revealed growth at 0% NaCl. *A. sydowii* can not be classified as halophilic but probably is part of the indigenous community of the salterns as suggested by its occurrence in all ponds. The fungus can be classified as extremely halotolerant by their ability to grow at high salinity (up to 30% NaCl). Growth at NaCl saturation conditions was not previously reported for *A. sydowii*. It was previously reported that the minimal water activity required for germination is 0.806 (Wheeler and Hocking, 1988). In salterns of Cabo Rojo the a_w was around 0.773. The optimum salt concentration for SC20 was around 5% NaCl, the pH around 5, and the optimal temperature was around 29°C. These results strongly suggest that SC20 probably is a marine strain of *A. sydowii*. The water origin in the ponds (marine) can explain its presence in the hypersaline environments of the salterns.

Another species of the *Aspergillus* group isolates was *A. terreus*. This species was very frequent but was only isolated in MEA-15. Therefore, strain SC34 was selected as representative of this species. A PCR amplicon consisting of the ITS regions was submitted for sequencing to determine the taxonomical position of SC34 molecularly. *In silico* analysis revealed that this strain was phylogenetically related to *A. terreus* strains sequences deposited in the GenBank. Secondary metabolite profiles were in agreement to our morphological and molecular results. All the results suggested that isolate SC34 belongs to *A. terreus*. Physiological experiments demonstrated growth at 0% NaCl. The optimum salt concentration was around 5% NaCl and the optimal temperature was around 35°C. This strain can be classified as extremely halotolerant like *A. sydowii* (SC20) since it also has the ability to grow at saturation conditions (up to 30%). Growth

at NaCl saturation conditions was not previously reported for *A. terreus* strains but it has been found before as a cellulose-decomposing fungus of salt marshes in Egypt (Abdel-Hafez et al., 1978). The optimal pH value for SC34 was 8. This value is considered high for fungi, but it is known that species of *Aspergillus* can tolerate alkaline conditions. This strain can tolerate high temperature, salinity and pH. The data suggest that this fungus may be part of the fungal community of the salterns by the ability to tolerate a combination of hostile conditions as mentioned before.

Aspergillus versicolor was reported previously from samples near the Dead Sea. Genomic diversity of this species in saline, non saline and hypersaline Dead Sea environments was determined (Kis-Papo et al., 2003b). Several strains belonging to *A. versicolor* were isolated. The strains were very similar except for SC40 and SC31. They had some morphological differences and the most important are the molecular differences in the ITS region. Secondary metabolite profiles classified them as a single species but it is recommended to do further studies in order to make sure that they do not represent different species or varieties of the same species. Strain SC40 was selected as representative of the group. Growth was observed at 0% NaCl for this strain. The isolate can grow up to 25% of NaCl. This result suggests that the strain could be classified as extremely halotolerant. The optimum salt concentration for SC40 was around 5% NaCl, the optimal pH at 5, and the optimal temperature was around 29°C. Growth at NaCl saturation conditions was previously reported for *A. versicolor* (Kis-Papo et al., 2003b). This study constitutes the first report of this fungus from hypersaline waters in the Caribbean.

Aspergillus oryzae was also isolated from Cabo Rojo. It is commonly used in oriental food fermentation processes. Strain SC46 was selected to be further analyzed. Physiological experiments on this strain showed that the optimum salt concentration for SC46 was around 5% NaCl and the optimal temperature was around 29°C. Since growth was observed at 0 and 30% NaCl, it can be classified as extremely halotolerant. Growth at NaCl saturation conditions was not previously reported for this fungus. The consensus distance tree places SC46 into the *A. oryzae* and *A. flavus* cluster. Therefore, this species was included in the *Aspergillus* Section *Flavi*. Some evidence suggests that *A. oryzae* is a morphological variant of *A. flavus*. *A. oryzae* is a species that evolved by domestication from one group of *A. flavus* (Geiser et al., 1998a). Secondary metabolite profiles reveal that this strain belongs to *A. oryzae*. Strain SC58 was identified as the same species based in metabolite profiles but morphological observations suggests that additional analysis should be performed to confirm this result. SC46 is a sclerotium forming strain but SC58 did not showed the development of this structure at same conditions. The growth at 15% of NaCl was very different between strains. SC58 grew more rapidly and formed a more robust colony. Some physiological differences were observed too (no ITS sequence is available for SC58). This strain should be included in the phylogenetic analysis of SC46 in order to establish possible molecular differences between the two strains. For the first time, *A. oryzae* was reported associated with solar saltern systems. This fungus can tolerate high salinity and their enzymes were previously studied in response to high NaCl concentrations (Hashimoto and Nakata, 2003).

The black aspergilli are classified into the Section *Nigri*. They were divided in two main groups: uniseriate and biseriate. The uniseriate includes the species *A.*

japonicus and *A. aculeatus*. The biseriate includes *A. carbonarius* and the members of *A. niger* aggregate. The aggregate includes the species *A. niger, A. tubingensis* and *A. foetidus*. Another species, "*A. brasiliensis*", was proposed to be include in the aggregate. The species on this group were very difficult to distinguish using morphological properties only. RFLP analysis was used to solve the classification of this group (Kusters-Van Someren et al., 1993; Mégnégneau et al., 1993; Parenicova et al., 1997, 2001). Three morphotypes belonging to the Section *Nigri* were isolated from the saltern waters. SC35, SC41 and SC60 were selected according to their morphological differences. Secondary metabolite profiles indicated that all three strains belonged to the *A. niger* aggregate. The strain SC60 was specifically classified as *A. niger*. Analysis of ITS regions revealed differences between these strains. The consensus distance tree places these isolates SC35 apart from SC41 and SC60. Morphological data showed that strain SC35 was uniseriate. The spores are different from those of SC41 and SC60 that are very similar in size and shape. Strain SC35 should be classified as uniseriate (*A. japonicus* or *A. aculeatus*) but the specific species is uncertain. Some authors identified these two species as a single species (All-Musallam, 1980) and others separated them (Kozakiewicz, 1989). These two species could be differentiated by RFLP analysis of mtDNA (Hamari et al., 1997). The other strains (SC41 and SC60) were placed in the same cluster of the phylogram with species of the *A. niger* aggregate. These two strains were classified as *A. niger* aggregate morphotype 1 and morphotype 2, respectively. Physiological experiments on strains revealed that they grow at 0% NaCl. They had the same values for optimal NaCl percent (5%) and pH of 3. Strain SC35 can be classified as moderate halotolerant because it normally grows up to 15 % of NaCl. On the other hand,

strains SC41 and SC60 can grow up to 20% NaCl and they can be classified as extremely halotolerant. Temperature values are variable for the uniseriate and biseriate species. SC35 has an optimal of 35˚C, while strains SC41 and SC60 had an optimal temperature of 29˚C. These data suggest that SC41 and SC60 may represent the same species, but due to the difficulty in separating the species included in the *A. niger* aggregate, and in accordance with the secondary metabolite profiles, we have maintained them as different morphotypes (1 and 2). Species in this group can be classified by the size of conidia in CYA, but no important differences were observed in this criterion. *Aspergillus niger* was reported before in several low water environments. They are considered as common food spoilage fungi (Raper and Fennell, 1965; Pitt and Hocking, 1997).

The *Aspergillus* species in section *Circumdati* includes organisms like *A. ochraceus, A. elegans* and *A. sclerotiorum*. Isolate SC17 belongs to this section. The secondary metabolite profile indicates that it is a member of *A. elegans*. The phylogenetic analysis of ITS regions reveals that the strain was placed into a cluster that includes *A. sclerotiorum* (bootstrap value of 100). However, the strain was closely related with another strain of *A. elegans*. The morphological data suggest that SC17 was more similar to *A. sclerotiorum*. For SC17, growth was recorded at 0% NaCl. It can be classified as extremely halotolerant by their ability to grow close to NaCl saturation conditions (up to 30%).The optimum salt concentration was around 5% NaCl, the optimal pH at 5, and the optimal temperature was around 29°C. Growth at NaCl saturation conditions was not previously reported for this species.

Eurotium is a perfect form of *Aspergillus*. *Eurotium amstelodami* had been isolated from saltern waters. Strain SC3 was selected as representative of this genus.

Physiological experiments revealed growth at 0% NaCl. SC3 can be classified as extremely halotolerant by their ability to grow close to NaCl saturation conditions (30%).The optimum salt concentration was around 10% NaCl and the optimal temperature was around 29°C. Optimal pH value was around 3. Growth at NaCl saturation conditions was previously reported for *Eurotium* (Butinar et al., 2005a). A complete description of *Eurotium* species isolated from saltern water was performed during this study. They are described as member of indigenous fungal community in hypersaline waters. According to their results, some species are contaminants and others probably are real inhabitants. This group includes *Eurotium amstelodami* (Butinar et al., 2005a).

Cladosporium is a genus that had been isolated before from hypersaline waters (Gunde-Cimerman et al., 2000). Two strains were further analyzed. The first, SC33, was selected as a representation of the species *C. sphaerospermum*. Physiological experiments showed that SC33 can be classified as extremely halotolerant by their ability to grow up to 25% of NaCl. Also, it can grow at 0% NaCl. The optimum salt concentration was around 5% of NaCl, the optimal temperature was around 25°C, and optimal pH at 10. This strain showed preference for lower temperatures. Growth at 25% NaCl appears after 8-10 days. *In silico* analysis of these sequences revealed similarities to strains of *Cladosporium sphaerospermum* and other species of the genus. The consensus distance tree places these isolate in one cluster together with strain SC51. This other strain (SC51) belongs to *C. cladosporioides*. The optimum salt concentration was around 5% NaCl and the optimal temperature was around 29°C. Optimal pH was around 5. This strain prefers lower temperature as SC33. However, growth at 0% of NaCl was very limited. Members

of this genus were very closely related as suggested by ITS analysis. The two representatives of the genus are related to the cluster of *C. sphaerospermum* but more closely to the *Lacazia loboi* branch. Species in database corresponding to *C. cladosporioides* are more distanced. Many sequences of this genus are unavailable in GenBank and this makes the molecular analysis of our strains very difficult.

The genus *Penicillium* was represented by two species. The first was *P. chrysogenum*. The strain SC48 was selected for further analysis and as a representative of this species. Based in physiological experiments, it can be classified as extremely halotolerant because it can grow up to 20% of NaCl. However, the optimum salt concentration was around 5% NaCl at 29°C. Optimal pH was 8. The consensus distance tree places these isolates in the *P. chrysogenum* cluster. Secondary metabolite profiles confirmed that this strain belongs to this species. Growth at NaCl saturation conditions was previously reported for this genus. The other *Penicillium* strain was SC30. Based on morphological characteristics this strain was preliminarily classified as *P. citrinum*. However, secondary metabolite profiles indicate that maybe SC30 might belong to *P. expansum*. The phylogentic analysis of ITS regions demonstrates that SC30 was more related to *P. citrinum*, forming a cluster that had a bootstrap value of 100.

Alternaria strains were reported to be present at saltern waters in Slovenia. The strains isolated from salterns of Cabo Rojo apparently represent the same species based on RFLP patterns and morphological characteristics. The catenulation of this strain suggested that is a member of the *Brevicatenatae* group like *A. tenuissima* but the classification of this strain need more studies. Physiological tests revealed that strain SC59 (strain selected as representative of *Alternaria* sp.) grows best at 0% NaCl. It was

one of the strains that tolerate lower salinities. Due to the physiological features observed and the low frequency of isolation this species might be a contaminant and can be classified as moderate halotolerant. The ITS sequences of the strain and the sequences in NCBI databank are practically identical. The phylogentic analysis was not performed because they did not showed differences. This region may be highly conserved for the genus and may not allow discrimination between species using this type of analysis.

An unknown morphotype was isolated as mycelia sterilia. The fungus developed the reproduction structures when it was transferred to a lower salt medium. Strain SC24 was further analyzed. This fungus was isolated from SG-15. The best growth conditions were at 10 % NaCl and 29˚C. The morphology of this fungus is similar to members of the *Sordariales*. Phylogentic analysis using ITS region suggests that SC24 was closely related to uncultured marine ascomycetes. The strain forms a distant branch apart from the other sequences. Phylogenetic analysis of the 18S rDNA partial sequence showed that it is related to the genus *Meliola*, a black mildew. The morphological data did not show a relationship with the data obtained from 18S phylogeny. These data suggest that this strain may represent a fungus whose sequence is not available in the database, or maybe it is an unknown organism.

All species described in this study had the physiological properties that allow their survival in extreme environments, like the hypersaline waters of the solar salterns. Many of them probably can not survive for long terms. This group includes common contaminants. Air dispersion of their spores may contribute to their presence in water. However, several strains isolated demonstrated to have halotolerant nature. They appeared frequently through all ponds. They can grow at extremely salinity for long time

and their optimal NaCl percent for growth ranged between 5-10 %. Normally, fungi grow at low pH values. Some isolates have a preference for alkaline pH for growth. The pH of the salterns is neutral. The results presented here may indicate a preference for saline environments.

Part III

Fungal Diversity at the Salterns Determined by Phylogenetic Analysis of 18S rDNA Clone Libraries

Materials and Methods

I. Sample Processing

Four samples, each containing 250 ml of saltern water were collected from three ponds in the solar saltern of Cabo Rojo using sterile plastic bags. Fifty ml of water sample were filtered through 0.45µm nitrocellulose membranes (Millipore).

II. DNA Extraction from Environmental Samples

Filters were cut into small pieces and used for total genomic DNA extraction with the modified method of Saghai-Maroof and others (1980). The DNA extraction product was resuspended in 1X TE, pH 8. The DNA quality was checked on 0.8% agarose gels after staining with ethidum bromide.

III. PCR Amplification and Gel Electrophoresis

PCR amplification of rDNA [762 bp according to *S. cereviseae*, GenBank accession no. J01353] including V4 (partial), V5, V7 and V8 (partial) variable regions of the 18S gene (Figure 3.1) was performed using primers nu-SSU-0817-5' 5'-TTAGCATGGAATAATRRAATAGGA-3' and nu-SSU-1536-3' 5'-ATTGCAATGCYCTATCCCCA-3' (Borneman et al., 2003). PCR reactions (small and large scale volume) were performed using ≈ 10 ng of template, 1X PCR buffer, 300 µM dNTP's, 3 mM $MgCl_2$, 0.66 pmol/µl of each primer and 0.05U of Taq DNA Polymerase in Storage Buffer B (Promega ®). Thirty five cycles of amplification were performed after initial denaturation of DNA at 95ºC for 3 minutes. Each cycle consisted of a denaturation step at 95ºC for 30 seconds,

annealing at 56ºC for 1 minute, extension step at 72ºC for 2:30 minutes and a final extension at 72ºC for 10 minutes. PCR products were cleaned by using the MinElute PCR purification Kit (USA QIAGEN Inc.) according to the manufacture's protocol. PCR products were verified on 1% agarose gels after staining with ethidium bromide. PCR product concentration was determined using a spectrophotometer at 260 nm.

IV. Cloning of PCR Products

The products were ligated and transformed using the pGEM T Vector System II (Promega ®) according to the manufacturer's protocol.

V. Screening and Plasmid Extraction

The colony PCR technique (Gussow and Clackson, 1989) with the same 18S primers was performed to check for inserts. The Promega ® PCR Mix was used to perform reactions with a final volume of 30 µl. Positive clones were selected for sequencing. Plasmid extraction was performed using QIAprep Spin Miniprep Kit (QIAGEN) according to manufacturer's instructions.

VI. DNA Sequencing

Plasmids of selected clones containing the proper inserts were sent to Iowa and Macrogen DNA facilities. They were sequenced using the core primer T7 promoter. Plasmid concentration for sample preparation was measured using a spectrophotometer at 260 nm.

VII. Phylogenetic Analysis

To check for possible chimeric sequences all clones were analyzed using the Chimera Check program of the RDP database (Cole et al., 2003) version 2.7. The DNA sequences obtained were deposited in Gen Bank from the National Center for Biotechnology Information (NCBI) (Table 3.1). The sequences were used for *in silico* similarity analysis using the NCBI BLAST program (Altschul et al., 1997) Selected sequences were used to determine the isolates phylogenetic position with respect to similar strains. Sequences were aligned using Clustal W and the Bioedit program. Sequence similarity values were calculated by pairwise comparison of the sequences within the alignment. Seqboot was used to generate 100 bootstrapped data sets. Distance matrices were calculated with dnadist. One hundred trees were inferred by using neighbor joining analysis. Any bias introduced by the order of sequence addition was minimized by randomizing the input order. Consense was used to determine the most frequent branching order. The final tree was drawn using treeview program (Page, 1996).

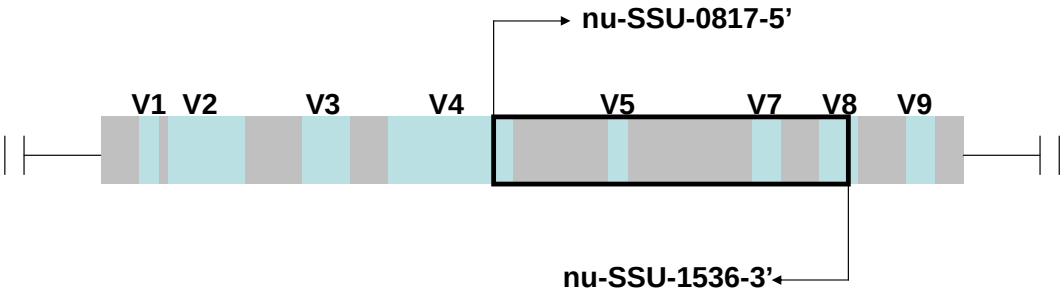

Figure 3.1 Schematic representation of 18S rDNA gene organization present in most fungi. Targets areas in the amplification of ITS and 5.8S rDNA regions are in bold.

Results

After performing several methods for total genomic DNA extraction from the saltern waters, the Sahai-Maroof method was selected. We obtained total genomic DNA of PCR quality (Figure 3.2). These products were used as template for PCR amplification of variable regions of the 18S rDNA gene including V4 (partial), V5, V7 and V8 (partial). It was necessary to perform several optimizations of the PCR parameters to finally obtain the desired PCR product. Taq DNA polymerase from Promega® was the best enzyme for amplification of these samples. The PCR products had a size of approximately 750 bp. (Figure 3.3). Several genomic libraries were constructed with

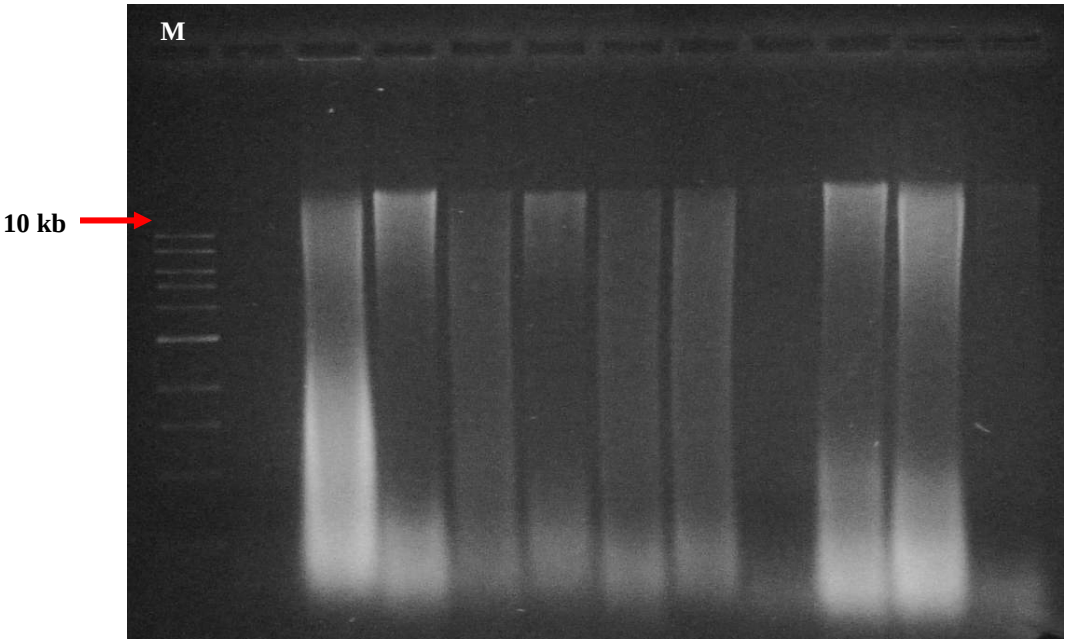

Figure 3.2 Total DNA extraction using the modified Saghai-Marrof method. 1 Kb ladder was used as DNA marker. M= 1 kb ladder (New England).

these samples. A total of 300 clones were obtained. Colony PCR revealed that a total of 44 clones had inserts of the expected size. Positive clones were selected for sequencing.

Many sequences belonged to the green algae *Dunaliella*. The selected primers (nu-SSU-0817 and nu-SSU-1536) showed lower specificity than expected.

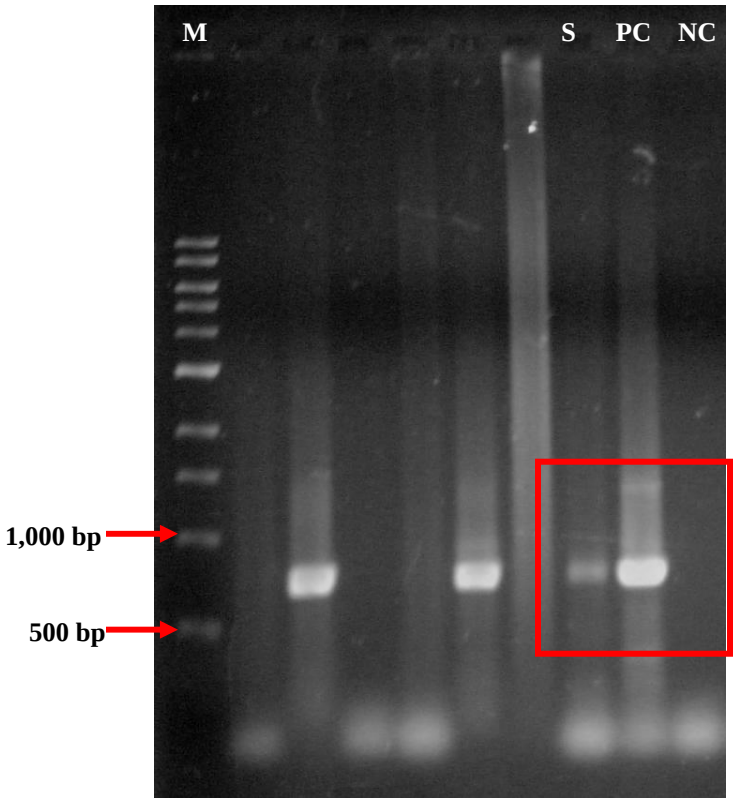

Figure 3.3 PCR product of partial 18S of rDNA amplified from total DNA water samples (red square). M= 1 kb ladder (New England). S = water sample, PC = positive control and NC= negative control

The 18S clones analyzed were separated into three principal groups. After *in silico* analysis to compare sequences a total of 20 Operational Taxonomical Units (OTU) were selected. The accession numbers of fungal clones are listed in table 3.1. The first group had the most frequent and the most diverse clones. They belonged to the genus *Aspergillus* (Figure 3.4, 3.5 and 3.6). A set of fungal clones were related to uncultured ascomycetes. The Blast analysis revealed that many sequences related to these clones are lichenized fungi. However, in the phylogenetic tree (Figure 3.5) the clones appeared to be more related with non lichenized fungi like *Cladosporium* or marine ascomycetes, but

they form a completely separate cluster. Clone CLS09 was related to an uncultured soil fungus as seen in Figure 3.6. The numbers on the branches in the tree indicate the number of times the partition of the species into the two sets which are separated by that branch occurred among the trees, out of 100 trees. An analysis for the possibility of chimeric sequences was performed. This analysis suggested that no chimeric sequences were present in our clones.

Table 3.1. Gen Bank accession numbers of environmental 18S rDNA clones. N/A= not available

Clone Number	Accession Number
CLS1	DQ346767
CLS3	DQ346766
CLS5	DQ346768
CLS43	DQ346772
CLS44	DQ346773
CLS51	DQ346774
CLS138	DQ346745
CLS157	DQ346746
CLS158	DQ346747
CLS159	DQ346748
CLS166	DQ346744
CLS175	DQ346751
CLS177	DQ346753
CLS178	DQ346754
CLS182	DQ346757
CLS304	DQ346759
CLS308	DQ346775
CLS309	DQ346776
CLS312	DQ346760
CLS314	DQ346761
CLS504	DQ346763
CLS506	DQ346764
CLS508	N/A
CLS009	N/A

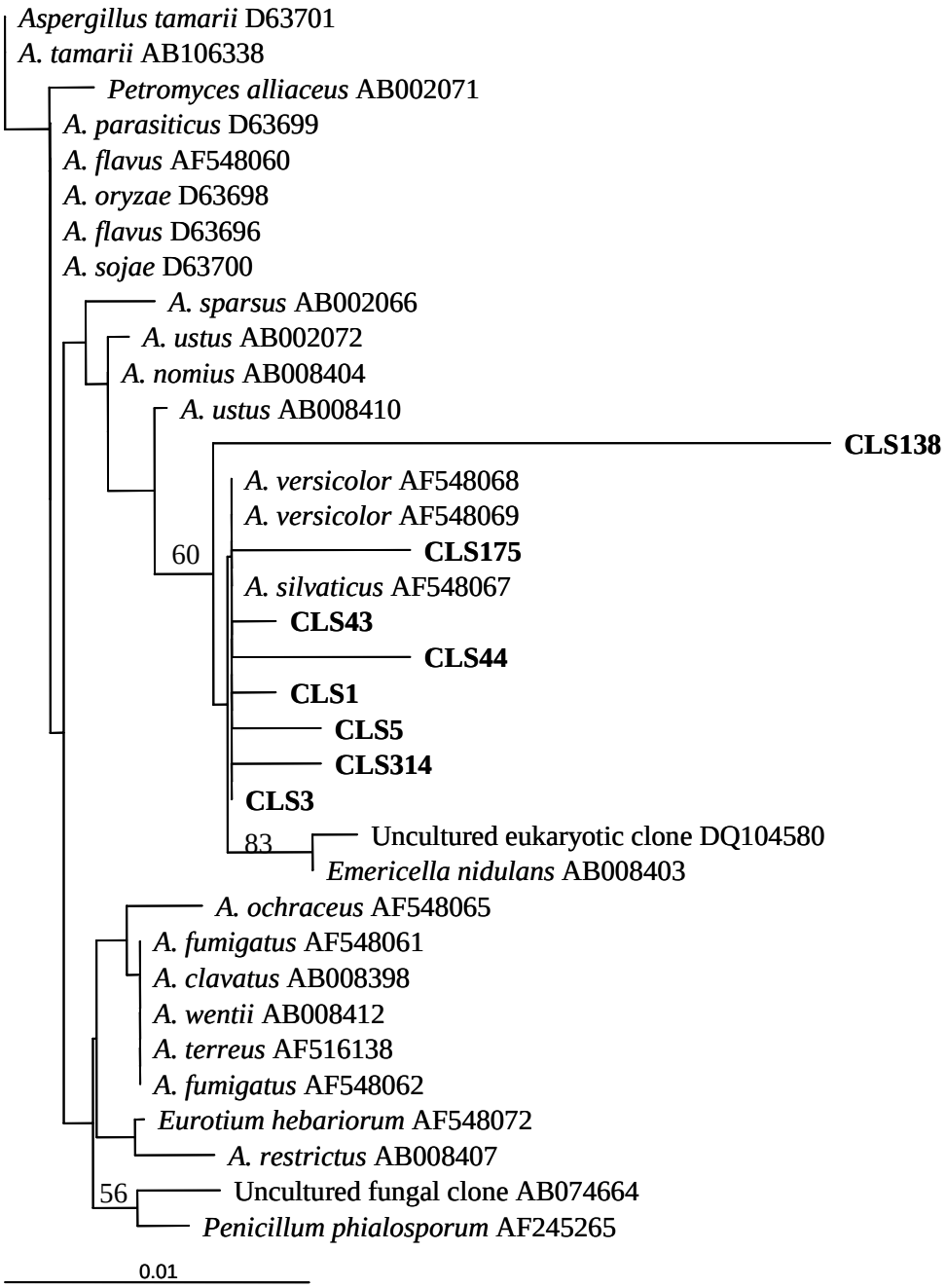

Fig. 3.4 Neighbor-joining distance tree of partial 18S sequence for *Aspergillus* clones Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated.

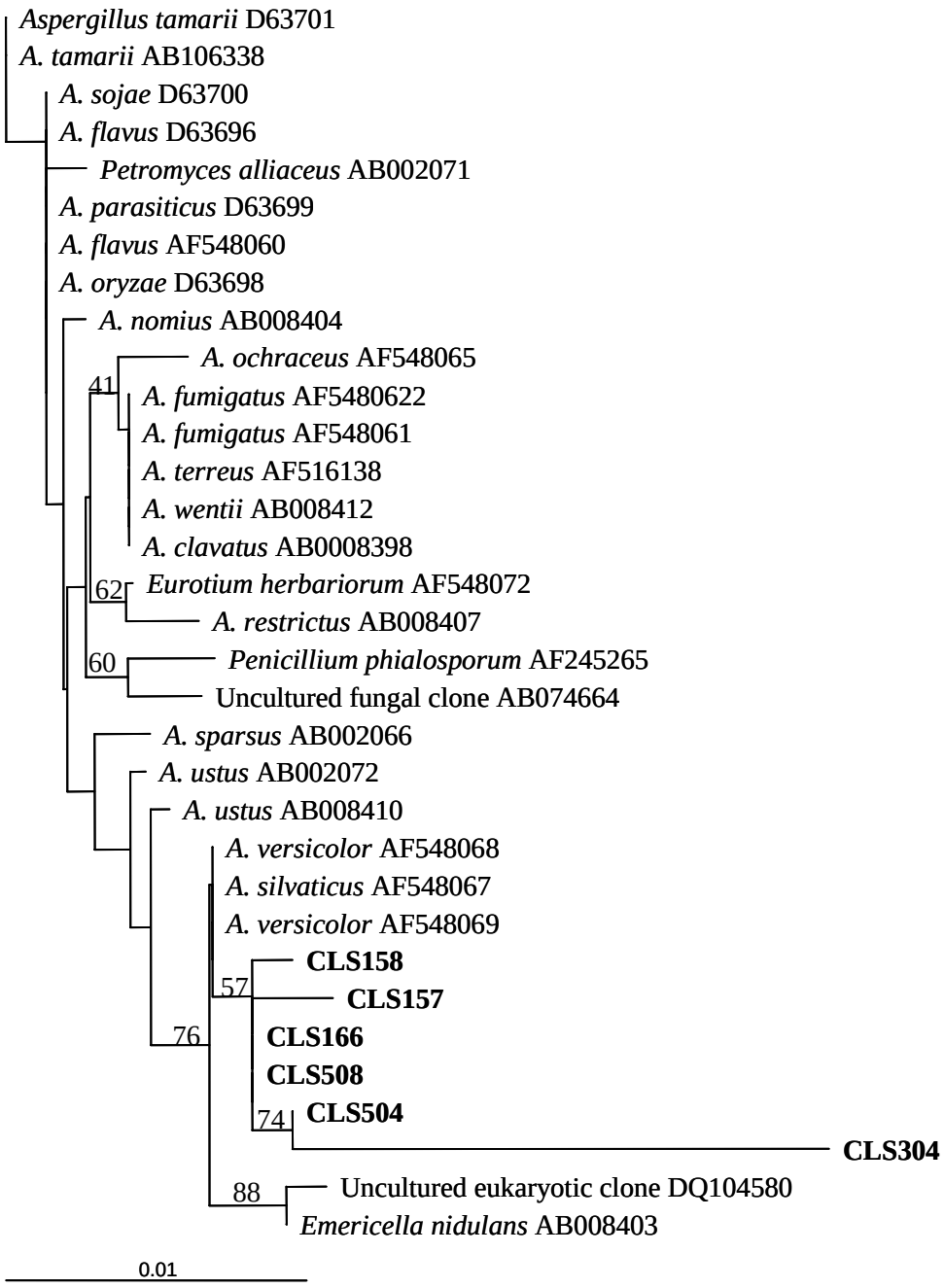

Fig. 3.5 Neighbor-joining distance tree of partial 18S sequence for *Aspergillus* clones Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated.

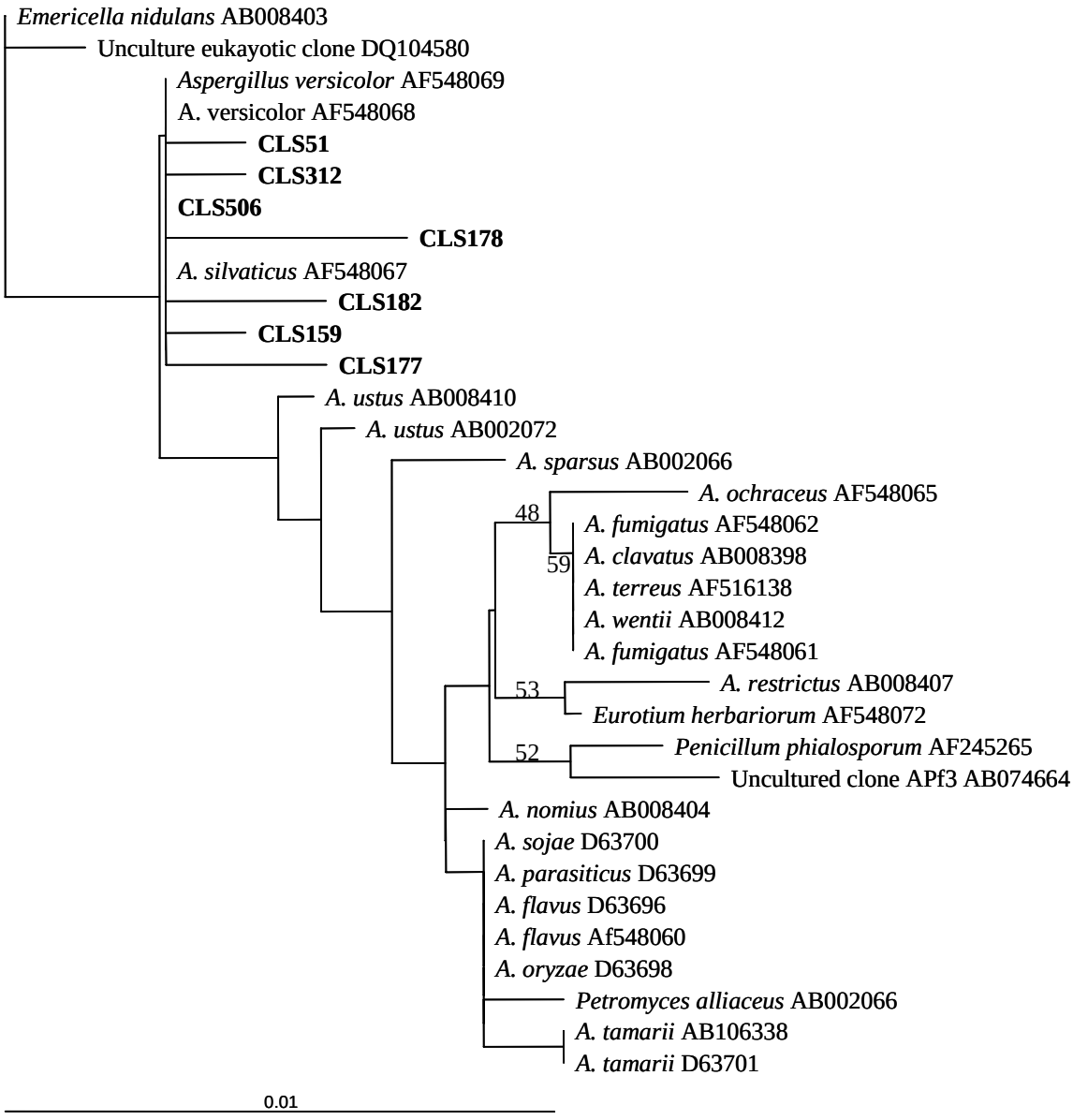

Fig. 3.6 Neighbor-joining distance tree of partial 18S sequence for *Aspergillus* clones Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated.

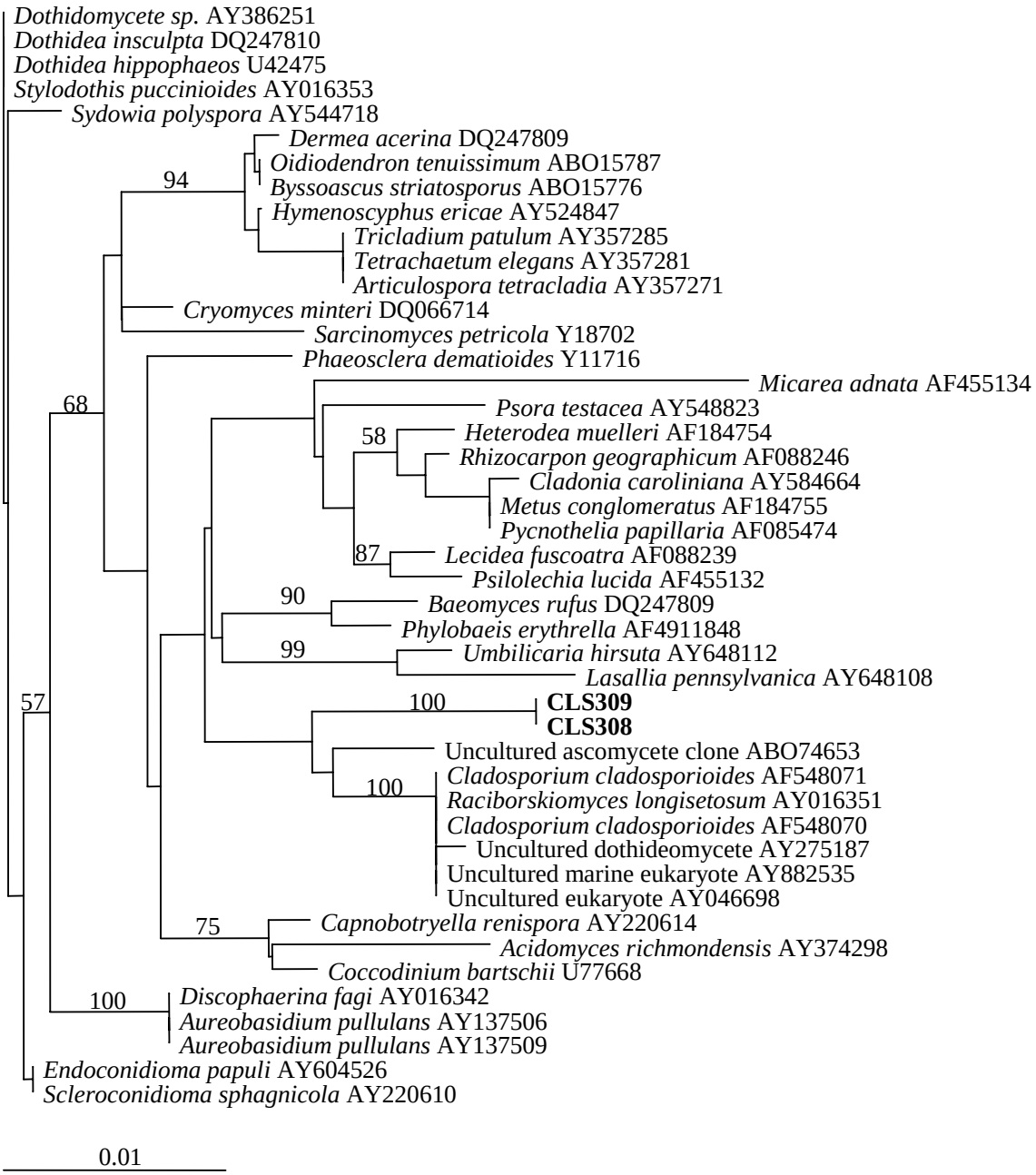

Fig. 3.7 Neighbor-joining distance tree of partial 18S sequences for clones CLS308 and CLS309. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated.

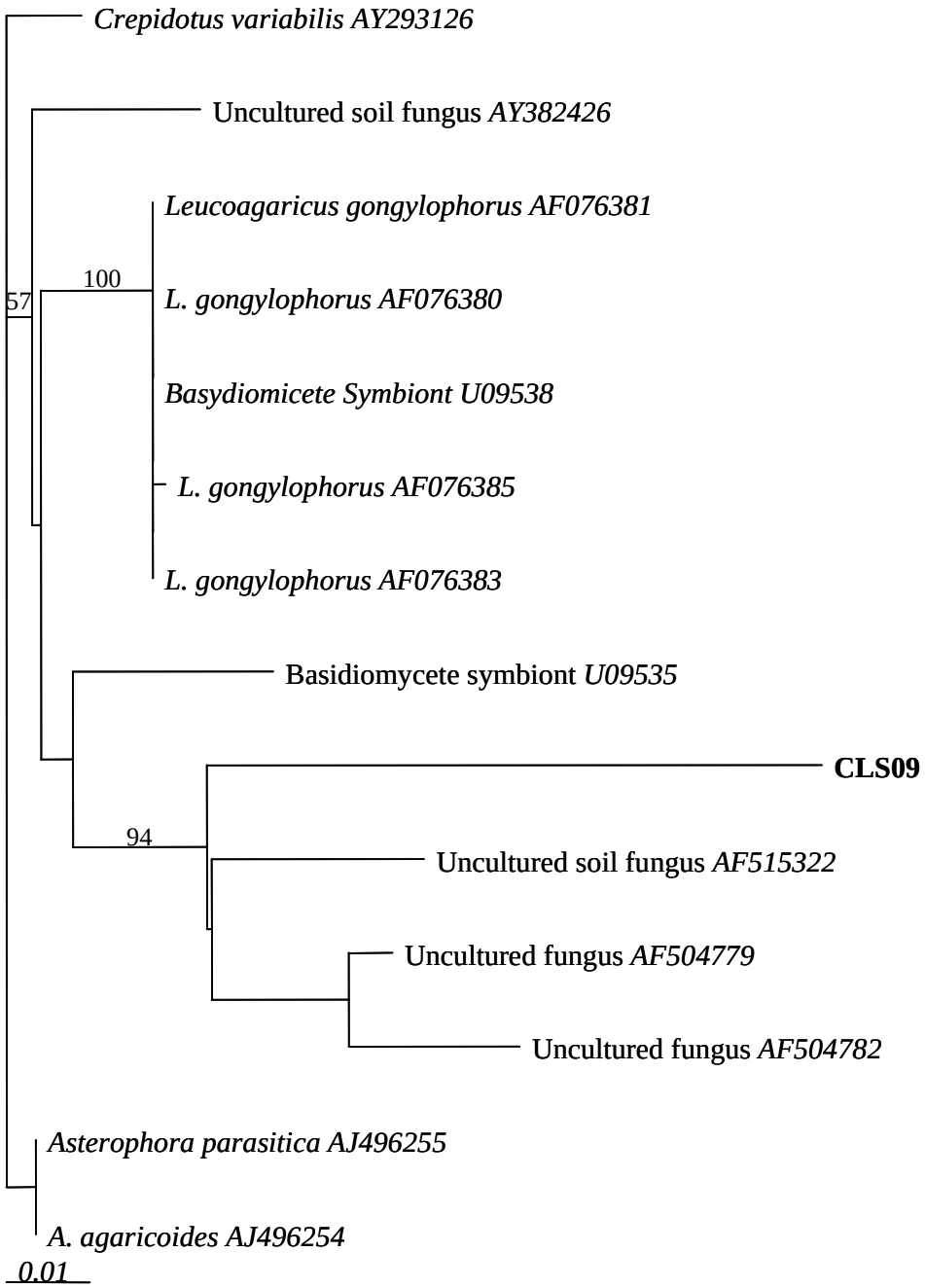

Fig. 3.8 Neighbor-joining distance tree of partial 18S sequences for clone CLS09. Bar represents 1 substitution per 100 nucleotides. Significant bootstrap values are indicated.

Discussion

The principal aim of this part of the study was to estimate fungal diversity in environmental samples of the solar saltern of Cabo Rojo, Puerto Rico, by using non-culturable methods like 18S rDNA genomic libraries. Total DNA from water samples were extracted. A method for DNA extraction of isolates was used and a good yield was obtained. This method was very effective for all isolates. In order to extract all possible DNA, the method was used for water samples. Other methods were previously used but they were not effective to extract fungal DNA.

Due to the diversity of microorganisms present in the saltern waters, the amplification of fungal DNA was more difficult. PCR reactions were optimized in order to achieve the best amplification product. Several polymerases were tested. The Taq polymerase, in storage buffer B (Promega ®), was the best for our samples. Borneman and Hartin, (2000) designed two sets of primers to amplify a partial region of the18S rDNA gene. The set used for this study was nu-SSU-0817 and nu-SSU-1536. This set was selected because was proved to be highly specific for fungi (Borneman and Hartin, 2000; Anderson et al., 2003). The other set designed in 2000, showed to be non highly specific for fungi as a result of the investigation of Anderson and others. The majority of their amplified sequences using these primers were related to soil invertebrates.

A clone library was constructed for each pond. In our case, many sequences belonged to the green algae *Dunaliella*. This demonstrated the low specificity of the primer set in our samples. A big problem in the study of fungal diversity from environmental samples is in the primers and their possibility of co-amplification. The 18S of rDNA is highly conserved and this complicates the analysis (Bruns et al., 1991;

Buchan et al., 2002). An important thing is that primer specificity can be different as a result of the types of environments that they were tested on. In the salterns the population of *Dunaliella* might dominate over fungal populations. When the PCR was performed, the high quantity of algae DNA interfered and this allowed the co-amplification in our samples. However, in spite of obtaining many algae clones, different fungal clones were also obtained.

The selected OUT's were classified in few groups. The most frequent belonged to the genus *Aspergillus*. When the Blast analysis was performed they were apparently the same clone, but in the phylogenetic analysis important differences were detected. Some *Aspergillus* clones (CLS138 and CLS304) form a completely separate branch in the tree and they might represent a different group (Figure 3.4 and 3.5). Some OTU's are related to *Aspergillus versicolor* and *A. sylvaticus*. *Aspergillus versicolor* was isolated from the saltern waters. *Aspergillus* strains were the most diverse between our isolates. An OTU, CLS138, had a significant distance from the other clones (Figure 3.4).

Hortaea werneckii was described as a natural member of the saltern's community (Gunde-Cimerman et al., 2000). Surprisingly, *Hortaea* clones were not detected. The bias might be related to the primers used or the DNA extraction procedure. The isolated *Hortaea* strains presented some difficulty in their DNA extractions. Other clones, CLS308 and CLS309 were related to ascomycete fungi. Species like *Cladosporium* and marine ascomycetes were more closed to these clones. *Cladosporium* strains were also isolated in this study as well as others studies in Slovenia and Spain (Gunde-Cimerman et al., 2000; Méjanelle et al., 2001). Clones CLS308 and CLS309 were also related to sequences belonging to meristematic fungi including black yeast *Endoconidioma*, the

genus *Capnobotryella, Acidomyces* (dothideomycetes), *Aureobasidium pullulans* and members of *Mycosphaerellaceae* group (*Discosphaerina*). Several species belonging to black yeast and other meristematic fungi were previously isolated from salt pans (Zalar et al., 1999a-b; Gunde-Cimerman et al., 2000). However, many sequences that appeared during the Blast analysis belonged to lichenized fungi. Lichens are known to live in saline habitats (coastal rocks), but there are no reports of these organisms thriving in artificial salt ponds like the salterns of Cabo Rojo.

An OTU (CLS009) was closely related to uncultured soil fungus. The other related species are basidiomycetes. No members of this group were found using culturing methods. The media used for isolation might not be suitable for the isolation of this type of fungi. The 15% of NaCl can also be a limitating factor for basidiomycetes growth.

The sequences more related to the clones belonged to groups that include some of our isolates. For example, black yeasts are members of *Dothideales*. A large number of sequences belonged to *Aspergillus*, but many species isolated did not appear as an OTU.

This is the first attempt to study the fungal diversity in saltern waters from the Caribbean using molecular approaches like genomic libraries. It is necessary to optimize the procedures. Our results suggest the necessity of more testing of primers, DNA extraction, and PCR parameters in order to improve the resolution of this type of analysis.

Conclusions

- Halotolerant and halophilic fungal strains can be isolated from the waters of the solar salterns of Cabo Rojo, Puerto Rico.

- The combination of morphological, physiological and molecular approaches was very useful in the characterization of the isolates.

- A total of eight genera and seventeen species were reported. These include the filamentous fungi *Alternaria* sp. *Aspergillus elegans, A. sydowii, A. terreus, A. versicolor, A oryzae, Aspergillus* sp. (Section Nigri), *A. niger aggregate, Cladosporium cladosporioides, C. sphaerospermum, Eurotium amstelodami, Penicillium chrysogenum, P. expansum, Stemphylium* sp. and an unknown species.

- Dimorphic yeast, *Hortaea werneckii,* was isolated with high frequency and it represents the first report of this yeast in an extreme environment in the Caribbean.

- The halotolerant nature of several strains suggests that they probably are part of the indigenous community of the salterns.

- Other isolates (*Alternaria* sp. and *Stemphylium* sp.) survive the hostile conditions of the salterns but they probably are transitional members of this ecosystem. Air dispersion of their spores may contribute to their presence in water samples.

- The 18S clones analyzed were separated into three principal groups. The most frequent clones belonged to the genus *Aspergillus.* A set of fungal clones were related to uncultured ascomycetes and one clone was related to an uncultured soil fungus.

- This was the first attempt to study filamentous fungal diversity in artificial, hypersaline environments at Puerto Rico and the Caribbean (Díaz-Muñoz and Montalvo-Rodríguez, 2005).

Recommendations

- The design of different sampling methods specific for fungi that can allow the isolation of more species. Other isolation media should be used.

- A study through all the year can provide detailed information about the fungal occurrence as a function of time. The study of yeast diversity of the salterns is strongly recommended.

- Further studies of strains SC17, SC22, SC30, SC31 and SC58 were recommended in order to establish if they are the same species as proposed.

- Experiments of survival of mycelium and spores at different salinities can provide more information about their halotolerant or halophilic nature.

- It is necessary to design specific primers to amplify fungi from this environment. The analysis of the community composition will be very important to determine the primer design.

- The use of clone libraries to elucidate fungal diversity in this environment needs more optimization due to the high eukaryotic population in the salterns which can interfere with the detection of fungi from the water samples.

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