A SURVEY OF HAWAIIAN MARINE FUNGI AND YEAST

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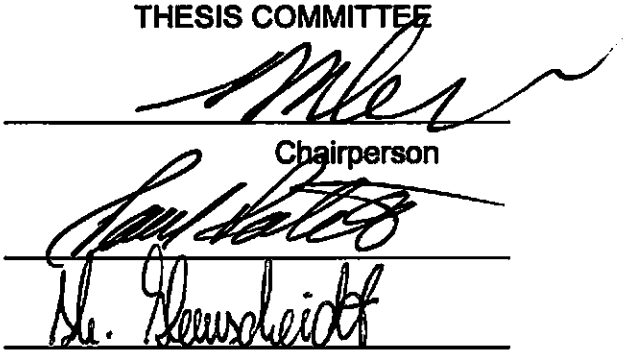
We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Microbiology.

THESIS COMMITTEE



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LIST OF TABLES

LIST OF FIGURES

ABSTRACT

A fundamental gap exists in our understanding of the phylogenetic diversity and distribution of marine fungi and yeasts off the Hawaiian Islands and Palmyra Atoll (Line Islands). During this 2 year study, 689 pure cultures of fungi and yeasts were prepared from seawater, sand, algae, wood, and other marine samples collected from 118 Hawaiian coastal sites and Palmyra Atoll, and water from the ~5000 m water column at the open ocean Station ALOHA north of O'ahu. The approach was innovative because it combined traditional cultivation techniques with 'new' molecular methods to facilitate rapid identification of cultured isolates, including those which may represent new species. Cultivated fungi and yeast abundance did not vary significantly in seawater collected off six of the high Hawaiian islands. Phylogenetic diversity was high, however, and included previously uncultivated strains. Potentially novel LM418^T isolated from wood on a Lana'i beach is tentatively described here as *Sympodiomycopsis mahdii*.

TABLE OF CONTENTS

CHAPTER I. LITERATURE REVIEW

A. Introduction to Marine Microbiology

1. History

Marine microbiology is generally considered to be the study of organisms smaller than 1 mm in the ocean. While these organisms are now known to be abundant, they were historically ignored since they were assumed to have little or no impact on marine biogeochemical cycles. Although marine biology as a field did grow rapidly during the nineteenth century, marine microbiology was still largely overlooked because methods to cultivate and even count marine microbes were still in their infancy. It wasn't until the Challenger expedition (1873–1876) completed the first deep sea studies, however, that the existence of bacteria in the sea was confirmed (Jannash, 1984). In addition to the widely used cultivation approaches, microscopy also became a method through which bacteria in aquatic samples were enumerated. The advent of microscopy methods that coupled DNA stains with flat polycarbonate filters led to the conclusion that bacteria were much more abundant in the ocean than anyone had believed possible (Hobbie, 1977). Such observations also began a debate on the usefulness of cultivation

methods to count bacterial cells in environmental samples (*e.g.*, Staley & Konopka, 1985). For example, it was argued that cultivation approaches yielded such a small percentage of cells compared to the number determined by microscopy and DNA stains that they had little value in environmental microbiology. The realization that such abundant bacteria in the ocean might actually play important roles in nutrient cycling and thus global biogeochemical cycles, however, is at the heart of what became known as the microbial loop (Pomeroy, 1974; Azam *et al.*, 1983). Soon after, what are now termed 'molecular methods' redefined what we knew of bacterial diversity in the ocean by allowing us to determine the nucleotide sequence of 16S rRNA genes in DNA extracted from an environmental sample (Stackebrandt & Woese, 1981). This approach revealed unexpected and largely uncultivated phylogenetic diversity among the marine bacteria (Giovannoni, 1990). Little attention was paid to the presence or diversity of picoeukaryotes in the ocean, and work on fungi and yeasts continued to focus largely on those that could be cultivated on enrichment media, especially from coastal areas.

Marine mycology has been relatively overlooked, despite early findings of the pathogenic marine Ascomycete *Sphaeria posidonia* dating to 1846 by C. Durieu de Maisonneuve and Montagne. It wasn't until the early 1900s that

studies focusing on marine fungi or yeast were conducted (Hyde, 2000). Zobell and Feltham (1934) observed yeasts in the open ocean and from other marine materials, while several other workers observed similar findings elsewhere (Johnson, 1961). Initial findings of marine fungi and yeast primarily described them as pathogens or parasites of fish, clams or oysters, but soon after, reports of parasitic relationships between algae were also described (Reed, 1902). By the late 1950's scientists began to describe different relationships between marine fungi and marine animals. Nevertheless, some scientists still believe marine fungi and yeast are neither important nor worthy of further discussion (Sherr *et al*, 2001). The first published Hawaiian mycology reports were of freshwater samples (Anastasiou 1964; Sparrow 1965), but the first to describe the mycoflora in marine samples was Kohlmeyer (1969). Thus, Hawaiian marine mycology has a rather short history compared with marine microbiology in general, but also of marine mycology.

The 1970s brought about a 'social awareness' of the ocean and its potential source of products for human use beyond fishing. The 'biosaline concept' was promoted, suggesting that material needs could be met by using the ocean in a variety of ways which benefit the environment and man (Aller & Zaborsky, 1979). Such views of the ocean led to significant developments in what

became the field of marine biotechnology and the search for new antibiotics and anti-tumor compounds from marine sources. In recent years marine mycology has attracted some interest as researchers consider the potential of marine fungi and yeasts as sources of novel byproducts.

2. Nature of the Ocean

The ocean covers ~70% of the Earth's surface. The Hawaiian Islands are located in the center of the Pacific Ocean, stretching from 18° 55' N (tropical) to 29° N (sub tropical), and longitude 154° 40' W to 162° W. The entire archipelago covers some one thousand five hundred twenty miles. Seawater surface temperature ranges from 71 °F to 81 °F, with an average of 78.3 °F (O'ahu -NOAA), with a salinity of 34 to 35. The average pH of Hawaiian surface waters is 8.2. Continental shelves to a depth of 200 m are considered part of the island topography and are part of the littoral zone, while depths between 200 and 400 m are considered the sublittoral zone.

B. Marine Mycology

1. Definition

As recently as 40 years ago, fungi isolated from marine habitats were not always recognized as marine, *sensu strictu*, despite several marine species

having been described in the previous one hundred years. It was only in the last 50 years that marine mycology was established as a distinct field (Johnson & Sparrow, 1961). Unlike other taxonomic groups, however, marine fungi cannot be defined exclusively by physiological or nutritional requirements. In this respect, Kohlmeyer (1965) defined marine Fungi as those that can reproduce or grow submerged in seawater or on intertidal substrates such as wood, sand or algae and are permanently or intermittently submerged. Facultative marine fungi are those that typically occupy freshwater or terrestrial milieus but are able to grow and sporulate in the marine environment (Kohlmeyer, 1974).

Marine fungi living part of their life cycle as single cells, primarily reproducing by fission or budding are considered marine yeasts. They are divided into two categories, obligate and facultative marine yeast. Obligate marine yeasts are those that have been collected exclusively from the marine environment, while facultative marine yeasts have also been collected from terrestrial sites (Kohlmeyer, 1979).

2. Geographic Distribution and Abundance

Marine Fungi and yeast are ubiquitous in the world's oceans, so to define a pattern of distribution the marine environment must be divided into habitats. The open ocean comprises benthic and pelagic regions, while coastal waters include 'littoral regions', those spanning the low tide mark toward the continental shelf, which typically reaches an average depth of 200-400 m (Wood, 1963). Within the littoral biotic zone three subdivisions exist: 1) the intertidal zone, 2) the eulittoral zone, and 3) the sublittoral zone. The pelagic zone can be divided horizontally into the oceanic and neritic regions. The oceanic region consists of open ocean over depths greater than 200 m and the latter includes open water from the shoreline down to a depth of 200 m. Vertically, the pelagic zone can be divided into the epipelagic zone from the surface to 200 m, and the mesopelagic zone extends down to the bathypelagic zone which receives no light penetration. These depths vary greatly depending on location. The abyssal pelagic zone refers to depths greater than 2,000 m.

The distribution of fungal habitats is determined by several factors such as the location of hosts, availability of nutrients, competition or availability of substrates. The most abundant marine populations occur on substrates in the intertidal zone, but indigenous fungi and yeast do occur in the deep sea.

Indeed, fungi have been collected from the Mariana Trench at 10,897 meters (Takamia, 1997). The availability of dissolved oxygen is an important factor in the distribution of fungi in marine habitats. Fungal growth is inhibited by low levels of dissolved oxygen in the water column or sediment. Dissolved oxygen concentrations of ~0.30 ml/liter or less typify an "oxygen minimum zone", and can inhibit growth of fungi, while an oxygen concentration of 1.26 ml/liter allows "ample fungal growth" (Kohlmeyer, 1969a).

3. Environmental Role

The role of fungi in marine food webs might on the one hand be considered significant, given that they take up dissolved organic substances, act as a food source for some benthic animals, can be a source of CO_2 to phytoplankton, and mediate decomposition of plant material such as leaves, wood and algae due to their ability to depolymerize cellulose, xylans, and pectin (Rheinheimer, 1992). In addition, the mycelia of Ascomycetous and Deuteromycetous fungi growing on cellulosic debris play an important role in the reproductive success of a species of nematode, since the fungi provide sufficient nutritional needs for the nematode by attracting animal predators. In another case, the fungus attracts pregnant females of another nematode species (Sieburth, 1979).

4. Physiological Adaptations

There have been several adaptations to life in the sea, many of which include the utilization of different nutrients. Barghoorn (1944) carried out a series of experiments that determined the most important adaptations were the ability to survive on media with sodium chloride concentrations three times those in seawater, as well as an elevated pH. Ascomycetes have adapted to the marine environment through evolution of appendages on their spores which aid flotation, dispersal, and attachment (Fig. 1).

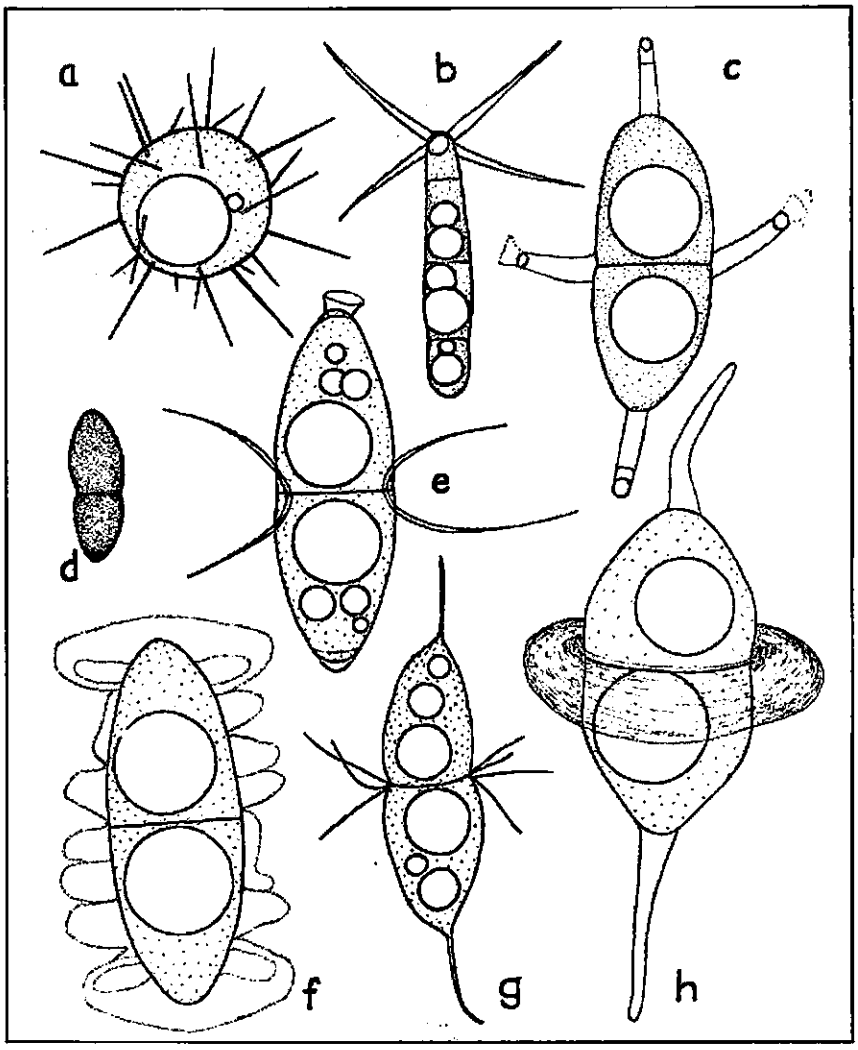


Figure 1a. Ascospores of marine Ascomycetes with different shaped appendages (except 1.d). a) *Amylocarpus encephaloides* Currey, b) *Torpedospora radiate* Meyers, c) *Ceriosporopsis calyptrate* Kohlm., d) *Microthelia martima* (Linder) Kohlm., e) *Halosphaeria mediosetigera* Cribb et Cribb, f) *Remispora maritma* Linder, g) *Peritrichospora integra* Linder, h) *Halosphaeria torquata* Kohl. Magnification ~2000x. Figures by E. Kohlmeyer (Kohlmeyer, 1963).

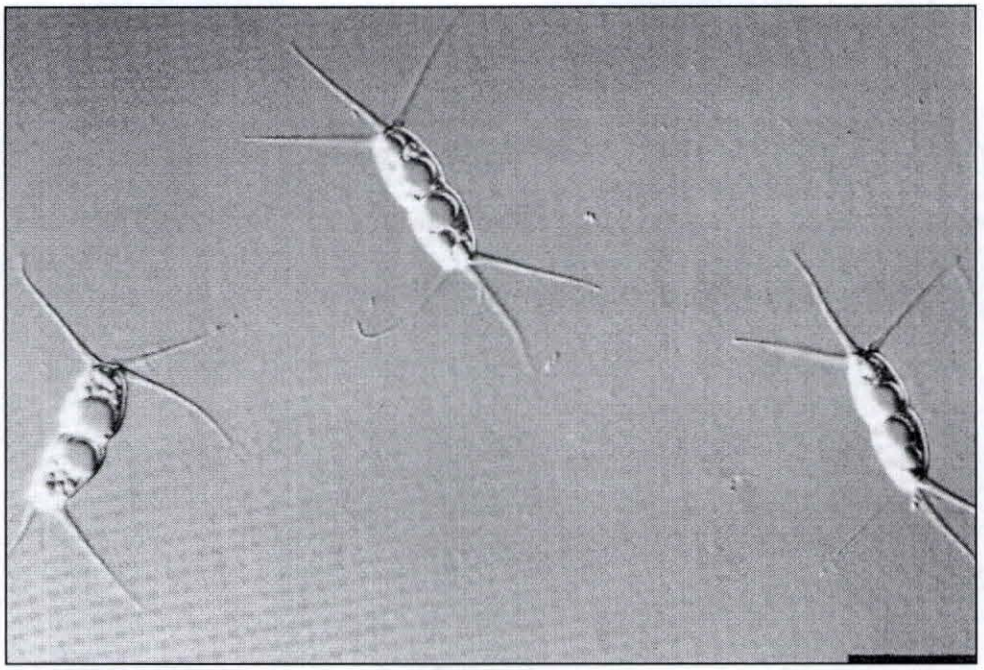


Figure 1b. *Corollospora trifurcate*, ascospores with three elongated appendages at each apex; scale bar = 25 µm. (Kohlmeyer, 1979).

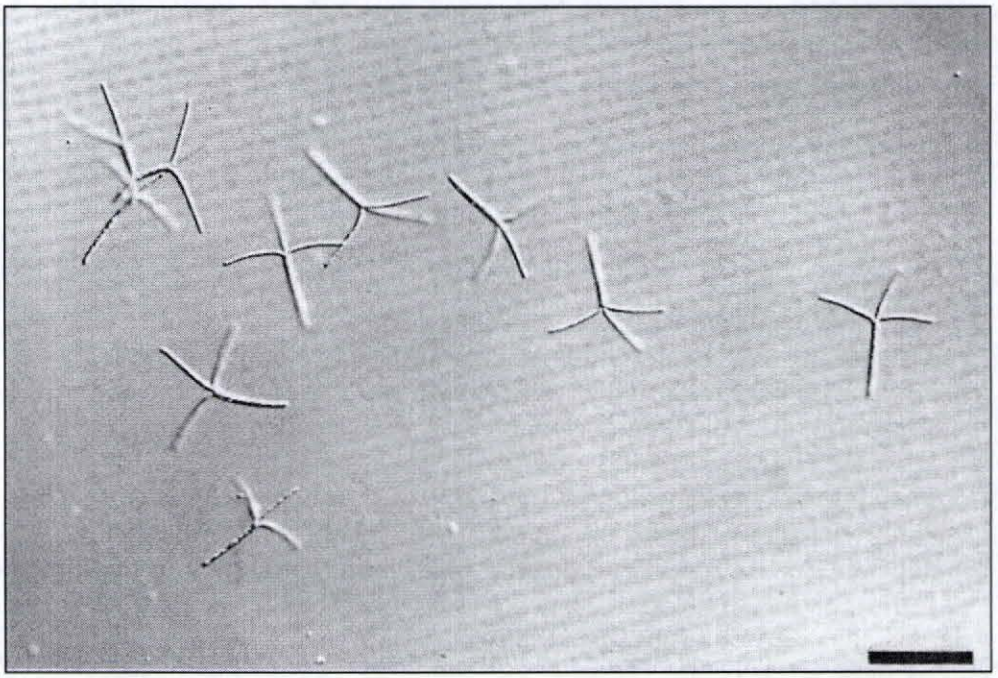


Figure 1c. *Digitatispora marina*, tetraradiate basidiospores; scale bar = 50 µm. (Kohlmeyer, 1979)

The structure of the cell wall as either unitunicate (single cell wall), or bitunicate (double cell wall) is also an adaptation to deliquescing or fissitunicate asci, respectively (Figs. 2 - 4). Other adaptations include the shape of spores, and their method of dispersal into the water or host, the presence of protective slime sheaths, or oil droplets within the cells for nutrients during germination (Kohlmeyer, 1965).

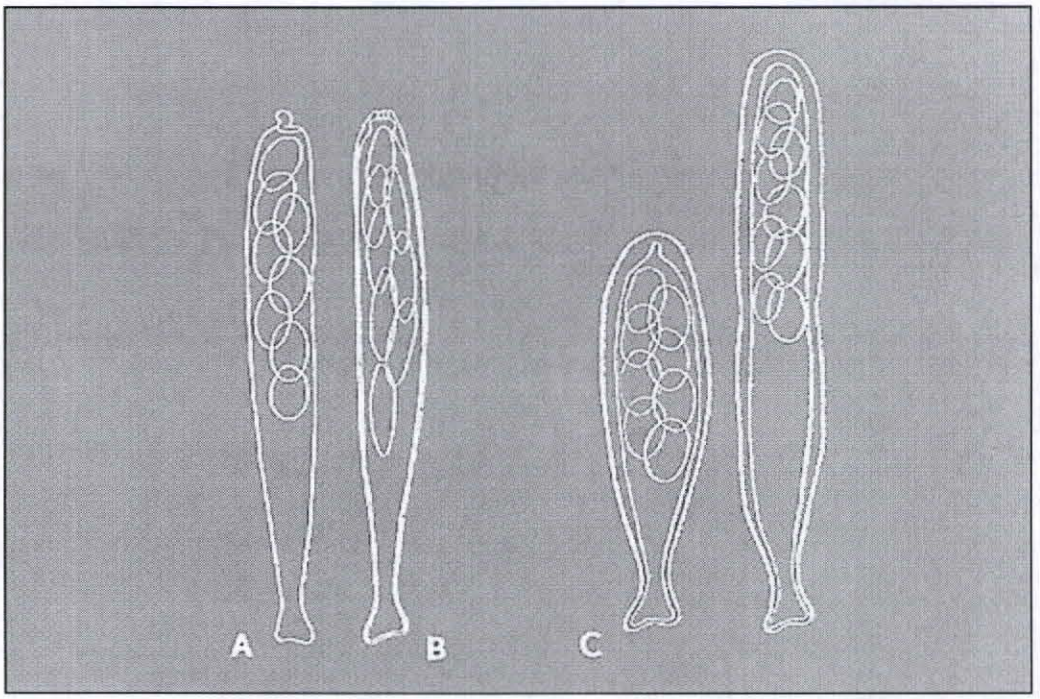


Figure 2. A, B) Unitunicate Ascus, C) Bitunicate Ascus (Kendrick, 2001)

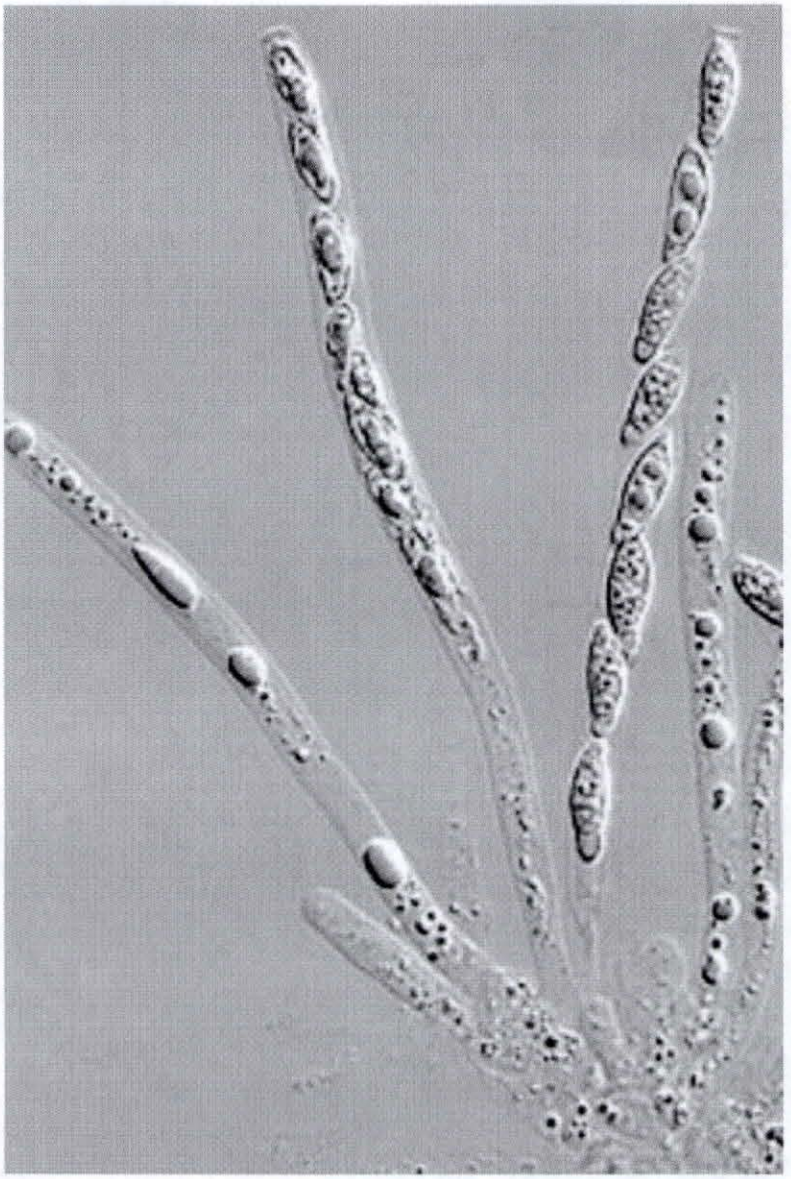


Figure 3. Unitunicate deliquescing asci *of Hypomyces chrysopermus.* (Wikipedia).

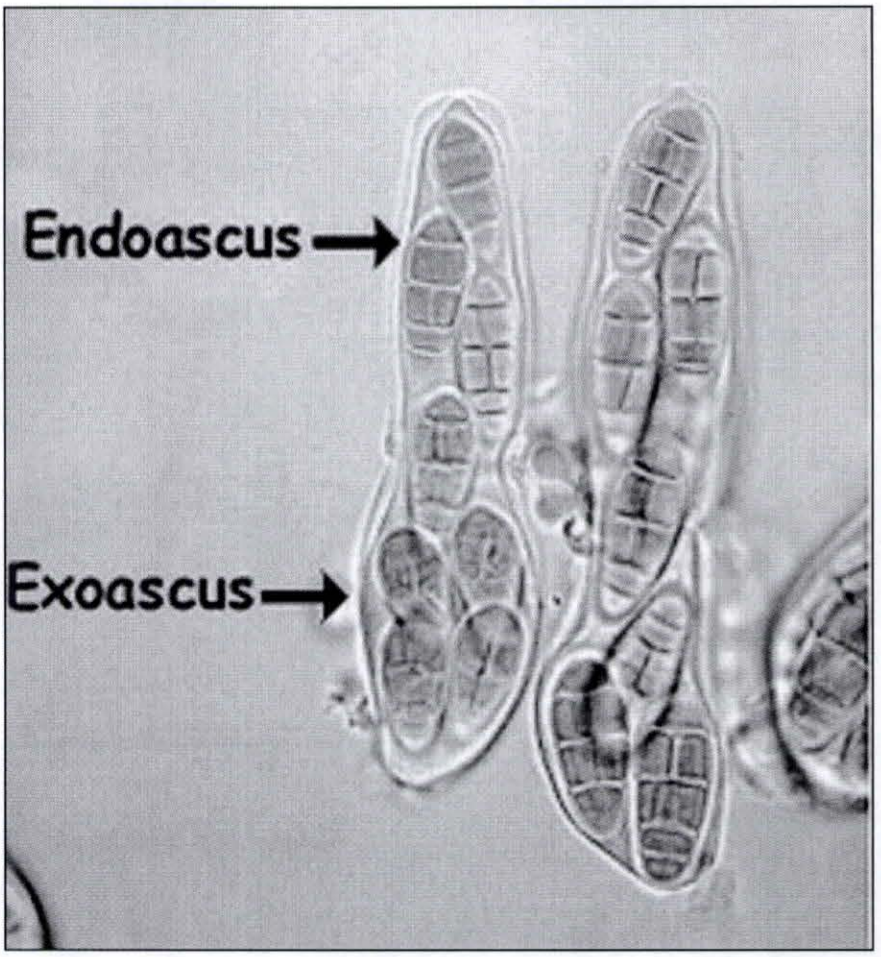


Figure 4a. Bitunicate Ascus of *Leptosphaerulina*. The flexible endoascus has grown through the rigid exoascus (UHM, Department of Botany)

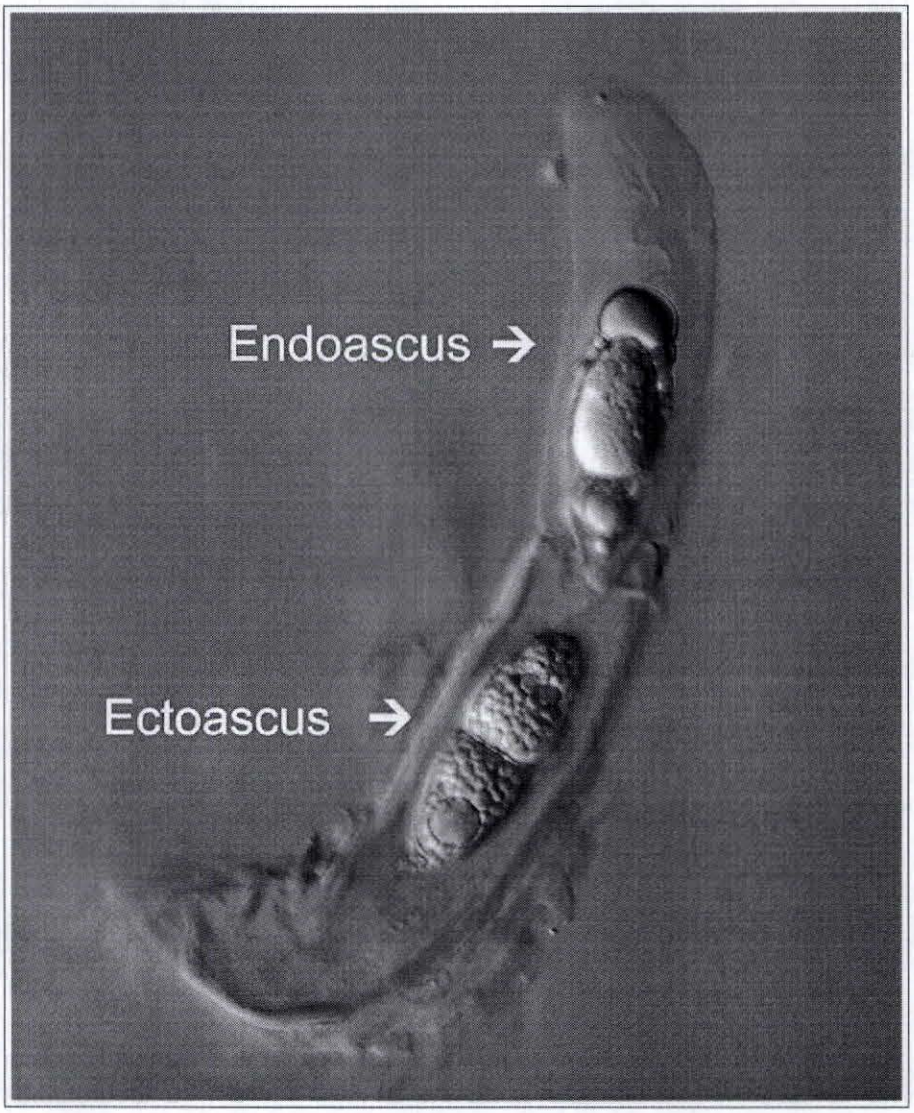


Figure 4b. Fissitunicate ascus showing separation between endoascus and ectoascus. (University of Illinois at Urbana-Champaign)

C. *Marine Fungi*

1. Colony Morphology

Filamentous fungi generally grow in a radiating circular pattern. The hyphal cells can be of varying length, and may grow vertically as well as horizontally.

The colony can have one or more colors in rings, and visible spores. Spore density can vary from single spores on hyphal tips to a powdery layer of spores across the entire colony surface.

2. Ultrastructure

Filamentous fungi are eukaryotic cells composed of long, thread like filaments called hyphae. Connected end to end, these hyphae compose the body of the fungus known as the mycelia. Typically, each mycelium acquires nutrients by absorption or penetration of a substrate. Hyphae that penetrate host cells for nutrient absorption are known as haustoria. The hyphae have rigid walls that are responsible for the stability of their vegetative and reproductive structures. The rigid cell wall is comprised of a matrix that includes mannans, glucans, and polyuronides which also embeds microfibrils. The microfibrils are made either of chitin or cellulose, and provide structure and rigidity. Chitin is a long carbohydrate polymer that is also found in the exoskeleton of arthropods, insects, and spiders. The plasmalemma is the space between the cell wall and the cell membrane, and is where lomasomes characteristic of fungal structures are found. Lomasomes are membrane-bound tubules that can occur singly or in groups, and while their function is not entirely known, they

may be involved in excretion as well as increasing the surface area at the periphery of the cell.

The cytoplasmic organelles are similar to those in other eukaryotic cells and include the nucleus, mitochondria, storage vacuoles, and ribosomes. Golgi bodies are rare in fungi, and the endoplasmic reticulum forms a bubble like vesicle in young cells but is generally infrequent in mature cells. The nuclei are well defined and often multiple nuclei are present. When there is no distinction between individual cells they are referred to as coenocytic, but otherwise they are normally separated by septa with or without pores. Typically Ascomycetes and Basidiomycetes have septa, while Zygomycetes are coenocytic.

Most fungi lack flagella, with the exception of the chytrids. The chytrids are the oldest fungal lineage known and contain flagellated gametes. Since chytrids are aquatic in nature it is believed that fungi evolved in water. The absence of flagella is then a synapomorphy which unites the remaining groups of fungi. The lack of flagella means two organisms must come into direct contact for reproduction. In fungi, there is a division of labor where some mycelia participate in conjugation with specific structures, and/or the dissemination of spores and others with the assimilation of nutrients.

3. Reproduction

Fungi are able to reproduce both sexually and asexually. The biological act of spore formation is a direct response at the DNA level to a physiochemical environmental change. This results in the alteration from a vegetative state to spherical reproductive state by an internal stimulus that regulates cellular activity.

The formation of asexual spores in large numbers occurs under stressful conditions by fission, fragmentation, cleavage and extrusion (Smith, 1977). Asexual spores are important for a build up in biomass, and normally occur at the end of a growth cycle. It is a means of survival, dispersal, and propagation. Fission occurs when cells within the mycelium separate at double walled septa forming two genetically identical cells; formation via fragmentation occurs when the cytoplasm becomes concentrated in certain cells within a filament, leaving the remaining cells without cytoplasmic contents. Formation by cleavage is when the protoplasmic material of a cell divides into fragments, each of which becomes surrounded by a new cell wall, and finally, spore formation by extrusion describes the process of spores being produced as extensions from the sides or ends of sporogenous cells. The requirements of spore formation are a mixture of biological events that

are distinguished by low water content, a lack of cytoplasmic movement, and a low metabolic turnover.

Spores formed by sexual reproduction in fungi and yeasts are often resistant to environmental stressors; they are thick-walled structures that develop through the simple union of hyphae between haploid nuclei, or the conjugation of differentiated multinucleated female and male gametangia. The specific details of spore formation and methods of spore dispersal will be detailed in the following chapters. In general however, spore dispersal is by sacrifice of a supporting cell, fracture, or fission of a double septum.

D. Marine Yeast

Yeasts are considered a polyphyletic group of Ascomycetous and Basidiomycetous fungi characterized by unicellular growth phases, and reproduction by budding, fission, or fragmentation. They can build up self-perpetuating populations in marine environments (Uden, 1968). They are also able to sexually produce spores and are in fact ubiquitous in the ocean. They occur on substrates such as sediment, wood, and algae. Many yeasts are obligate aerobes, occurring no deeper than the upper few centimeters of marine sediments (Uden, 1968; Kurtzman, 2004). Approximately 100 genera

and more than 700 species have been described from the ocean (Kurtzman & Fell, 2004). Yeast populations decrease with distance from land, but they remain the dominant fungi in the open ocean (Fell, 1986).

1. Cell and Colony Morphology

The morphology of yeast cells can be described as spherical, ogival, triangular, elongated, flask shaped, ovoid, apiculate, globose, or cylindrical and are often specialized to a specific species (Fig. 5). It is possible for a single species to be polymorphic during its ontogenetic development. A typical yeast cell size is between 2 and 50 µm in length, and up to 10 µm across (Phaff, 1966). Colony morphology has taxonomic significance when describing a species, whether novel or extant, and is described in a similar manner as for bacteria. For example, elevations are described as slightly raised, crateriform, umbonate, flat, convex, or subaerial. It is also important to note the form of the colony, as rhizoid, filamentous, irregular, or circular. Finally, the colony margin is described as curled, lobate, filamentous, undulate, or entire. Any pigmentation as well as surface texture of the colony, *e.g.*, glistening, dry, dull, curled further are also noted and aid identification (Fig. 6).

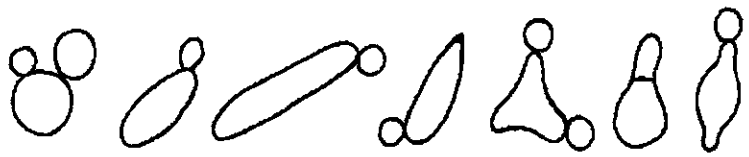


Figure 5. Yeast cell shapes and modes of reproduction. L to R: Spheroidal, ovoidal, cylindroidal, ogival, triangular, flask-shaped, and apiculate (Phaff, 1966)

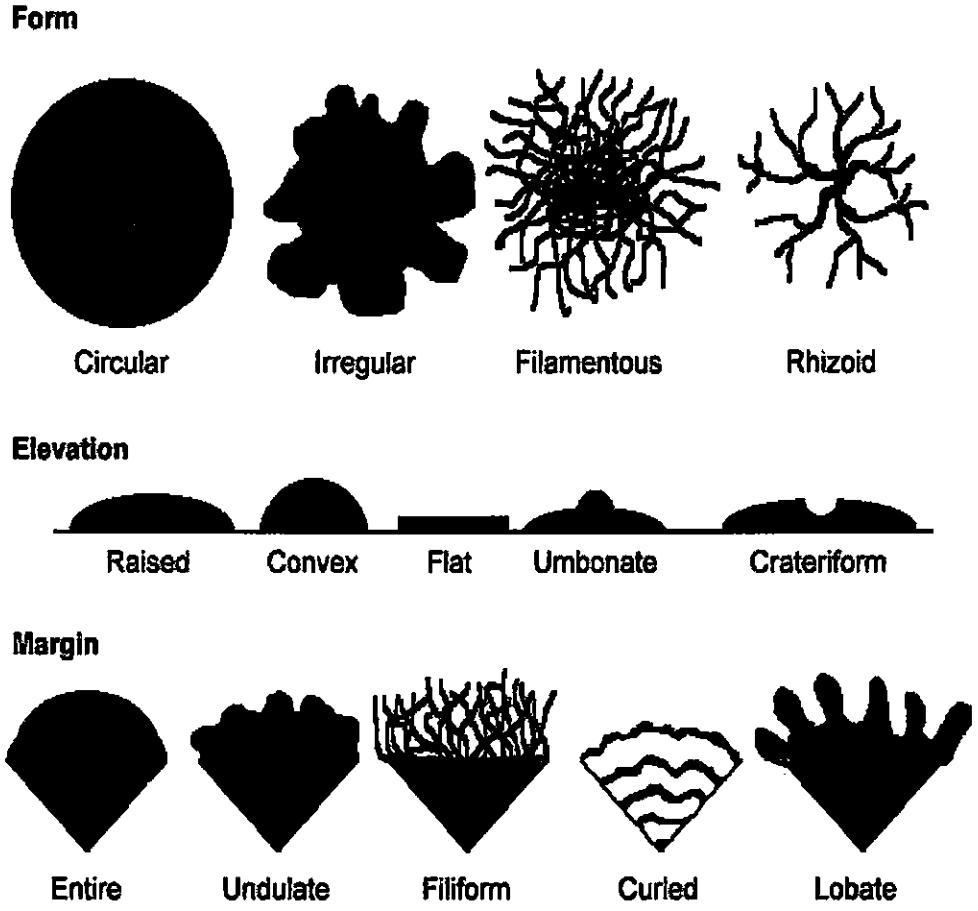


Figure 6. Colony Morphology descriptors. (Washington State University, Department of Microbiology)

Yeast morphology is generally determined by the vegetative reproduction of a cell, or in some cases the formation of spores. The vegetative body of yeast is known as the thallus or soma, and can also be a determining characteristic of a species. For example, the thallus may be seen alone, or with one or more budding cells still attached to it. This may result in the formation of chains or small clusters which form a structure called a pseduomycelium (Phaff, 1966).

2. Ultrastructure

The rigid cell wall accounts for 25% of the dry weight of the yeast cell and is approximately 25 nm thick (Berry, 1982). It comprises a matrix that includes mannans, glucans, microfibrils, and polyuronides which also embeds microfibrils. Mannans provide an upper layer of rigidity, and are water-soluble polysaccharides of the sugar D-mannose that are linked by α-1, 6, α-1, 2 or sometimes α-1, 3 bonds between residues. Some yeasts such as *Sporobolomyces* and *Rhodotorula* do not contain this type of mannan in their cell walls. The highly insoluble polysaccharide, glucan, is what gives the cell its basic shape and rigidity (Phaff, 1966). Glucan has been shown to contain varying proportions of β-1, 3 linkages of glucose as well as β-1, 6 linkages between units, providing a stable branched structure (Rose, 1969). The microfibrils are either chitin or cellulose, and provide structure and rigidity.

Chitin is a long carbohydrate polymer of β-1, 4 linked *N*, acetyl-glucosamine that is associated with the production of bud scars. The concentration of chitin can vary with species, *e.g.*, *Sporobolomyces* and *Rhodotorula* both have a much higher content than other genera, while in some species in the same genus chitin may be absent (Phaff, 1966; Berry, 1982).

The plasmalemma is the space between the cell wall and the cell membrane. It contains receptors for mating hormones which trigger a chain reaction leading to the production of a diploid cell (Spencer & Spencer, 1997). During budding, the plasmalemma forms a closing aperture to separate mother and daughter cells during the 'pinching off' process, resulting in a bud scar. Bud scars are the most distinct feature visible on the cell wall, and can be indicative of the cell's age. The birth scar also remains on the cell surface, but is usually less visible than bud scars (Rose, 1969). Other functions of the plasmalemma include the intake of nutrients from the media into the cell, as well as the release of compounds from fermentation such as ethanol out of the cell into the medium (Rose, 1969; Berry, 1982).

Certain genera of yeast such as *Hansenula*, *Rhodortorula*, *Cryptococcus*, *Candida*, and *Trichosporon* may excrete capsular materials or other extracelluar substances that surround the cells. These substances may be

phosphomannans, which form a viscous slimy layer on the surface of cells, heteropolysaccharides, or hydrophobic substances which allows the formation of a pellicle in liquid media (Phaff, 1966). The function of these compounds is not yet known (Sieburth, 1979).

Similar to the cytoplasmic organelles found in other eukaryotic cells, yeast also have a membrane bound nucleus, mitochondria, endoplasmic reticulum, Golgi bodies, perixosomes, storage vacuoles, lipid granules (sphaerosomes), and ribosomes. The nucleus of yeast is protected in a double membrane which contains several nuclear pores. Mitochondria are found throughout the cell, have a double layered outer wall and are important in the respiratory activity of the yeast-aerobic energy conversion, the synthesis of proteins and RNA (Rose 1969; Phaff, 1966). The endoplasmic reticulum is important in proteins synthesis and secretion, budding yeast cells, and the formation of distinct organelles (Rose, 1969). Golgi bodies are controversial in yeast, but sort and package proteins into secretory vesicles, and carry these vesicles to the plasmalemma for cell wall synthesis (Rose, 1966; Spencer & Spencer, 1997). Storage vacuoles are easily identified usually by as single large sac under the microscope. They are surrounded by a single membrane and hold small particles such as ribosomes, lipids, volutin, enzymes, and esterases.

The vacuoles act as a reservoir for the parent cell, and fragments into smaller vacuoles during budding such that the daughter cell ends up with several small vacuoles (Rose, 1969; Berry, 1982).

3. Reproduction

Yeast are capable of asexual and sexual reproduction. Species that are considered 'perfect' form spores, and alternate between the diplophase and haplophase, while species referred to as 'imperfect' may be haploid, and the diplophase is suppressed (Spencer & Spencer, 1997). Asexual reproduction occurs primarily in the formation of spores by budding, and fission. Multilateral budding is the most common form of reproduction in most genera, and is characterized by buds forming on the ends of long axes and shoulders of vegetative cells. Yeast that are spherical can form buds anywhere on the surface, while in some apiculate yeast, budding is restricted to opposite poles of the cell and is known as bipolar budding. The latter results in a lemon shaped yeast cell (Phaff, 1966; Rose, 1969).

The average number of daughter cells produced by a single cell is twenty four but can range from nine to forty three (Phaff, 1966). Depending on the genus, these asexual spores are called conidia, blastospores, arthrospores,

ballistospore, or chlamydospores, and are taxonomically important (Phaff, 1966). Reproduction by fission is another distinguishing characteristic of some genera, and occurs when one cell forms cross-walls, or septa without any constriction of the original cell wall, and essentially splits forming two separate cells (Phaff, 1966). Bud scars are not produced as a result of fission (Rose, 1969; Phaff 1966).

Sexual reproduction can occur by conjugation, a process initiated by the production of α-mating factor from a haploid cell. This chemical induces other potential mates to release a-mating hormone to signal availability. The cell that produces the highest concentration of the hormone is selected as the mate. Both cells then begin to form copulation tubes known as "protuberances" towards each other, which, upon contact fuse and become contiguous. Karyogamy takes place causing transition from a haploid stage to a diploid one, eventually leading to the production of buds or promycelium (Spencer, 1997) Yeasts can produce sexual spores of various shapes such as "fat ovals", bean or kidney bean shaped, round, needle-shaped, or crescent (Sieburth, 1979). One adaptation to yeast sporulation was discovered in the injection of needle-shaped ascospores into its brine shrimp host by the

pathogenic yeast *Metschnikowia bicuspidates* var. *australis* (Kohlmeyer, 1979).

E. Ascomycetes

Of the at least 400,000 filamentous fungal species believed to exist, the largest group is the Ascomycetes, yet fewer than one thousand are marine in origin. Ascomycetes can produce both asexual spores called conidia, and sexual spores termed ascospores. Ascospores occur within an ascus, a highly specialized structure, as a result of karyogamy and meiosis. The ultrastructure of the spores and ascus vary depending on the Ascomycetes order in question (Sieburth, 1979). Unlike their terrestrial counterparts who are often darkly pigmented to protect against desiccation and UV radiation, marine ascospores are generally hyaline (clear) in appearance. Spore dispersal in marine species is also often unlike that in terrestrial species which show a "jack-in-the-box" mechanism for effective dispersal. The spores or ascus of marine species are passively released into the water and quickly dispersed without the need for active expulsion. Active sporulation can take place in species that may be exposed at low tide, and which exploit the wind to disperse the spores.

Adaptations in marine species for spore dispersal include a unitunicate (single cell wall), or bitunicate (double cell wall) that either deliquesce or fissitunicate asci (Sieburth, 1979). In unitunicate and bitunicate asci, maturity of the cells causes an increase in osmotic pressure which elongates the ascus (Kohlmeyer, 1979). Unitunicate asci are enclosed in a single wall and readily deliquesce the asci, which is the release of spores through the breakdown or liquefaction of the cell wall upon maturity. Fissitunicate asci describe a method of active spore discharge that requires the presence of two clearly identifiable cell walls known as the endoascus, and the ectoascus. The outer ectoascus breaks open liberating the inner endoascus, which then releases the spores through a single pore called the ostiole (Kohlmeyer, 1979; Kohlmeyer, 1986). Another method of passive spore dispersal is by oozing of the spores from the perithecium (Sieburth, 1979). Marine Ascomycetes have adapted to life in the sea with appendages on the spores, which aid in flotation, dispersal and settlement on suitable substrates (Wood, 1963). Such appendages may be mucilaginous, gelatinous, or tough veil-, thorn-, tube-, cap-, spine- or fiber-like structures protruding from the body of the cell. These are often dry, and sticky. A germ tube penetrates the substrate once attachment is successful. These appendages can appear identical in different

genera, but are often produced in varying manners during ontogeny, and thus have some value during taxonomic descriptions (Kohlmeyer, 1979).

The two largest taxonomic groups that filamentous marine fungi fall into are the Pyrenomycetes, and Loculoascomycetes, while the marine yeasts are typically Hemiascomycetes (Sieburth, 1979; Kreger, 1984). Pyrenomycetes have hyaline, unitunicate deliquescing asci which mature within the perithecuim and eventually seep out. There are usually eight septate ascospores per ascus. The perithecium is the fruiting body, or ascocarp, and occurs in varying shapes from spherical to flask. They can also have different colors and textures, and may occur alone, on a mat of hyphae, or aggregated. The Loculoascomycetes can have dark to brightly colored ascocarps, and differ from Pyrenomycetes by having a distinct bitunicate ascus (Sieburth, 1979) (Fig. 7). Both the Pyrenomycetes, and Loculoascomycetes are often found on leaves, wood, pine cones, and sometimes algae and sea grasses.

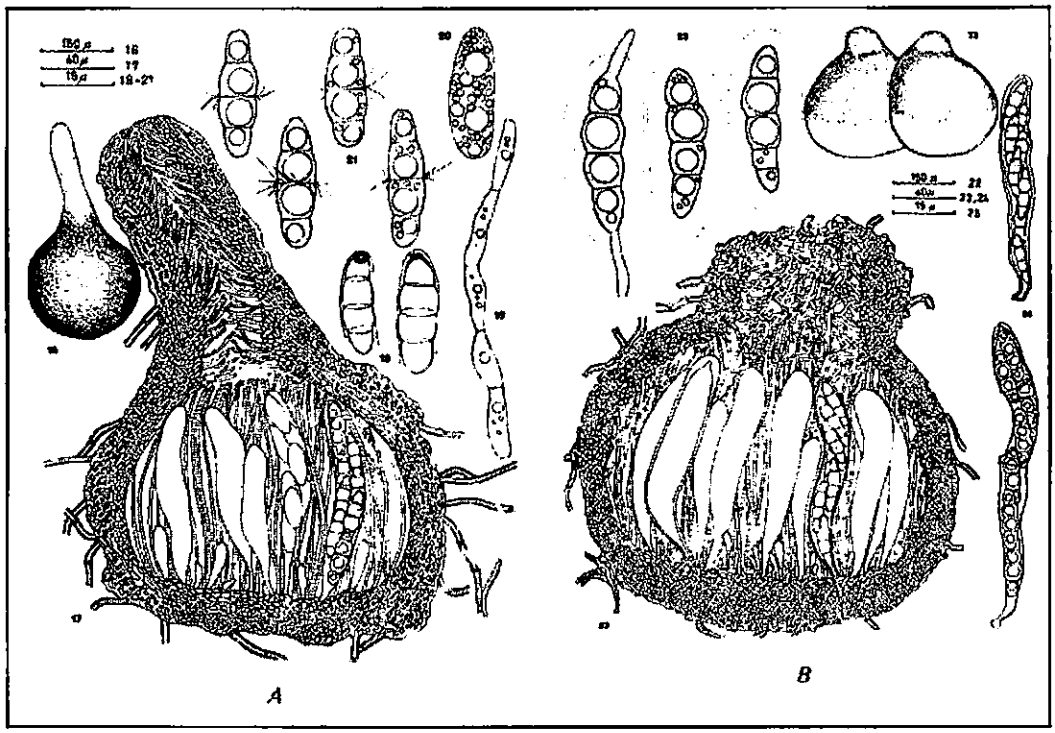


Figure 7. A comparison of a Pyrenomycete *(Chaetosphaersa cbaetosa)* A) with its unitunicate ascus, and a Loculoascomycete (*Leptosphaeria contecta*) (B) with its bitunicate ascus. The ascocarps (perithecia) are shown in external view (16 and 22) and in cross section (17 and 23); the asci with their ascospores are shown within the ascocarp and in greater detail outside the ascocarp (Kohlmeyer, 1963).

Many Ascomycetous yeasts can also reproduce via spores created by "free cell formation". Spores are formed in the protoplasm of the spore sac, also known as the ascus, and are not attached to each other or the ascus wall, unlike those found in the Basidiomycetes group. The ascus forms as a result of sexual fusion between two specialized gamates or cells. Cross-mating can occur between two types, **a** and **α**, during conjugation with the aid of specific

hormones. Both heterothallic and homothallic cells can exist, depending on life cycles and nutrient conditions (Fell, 1976) (Fig. 8).

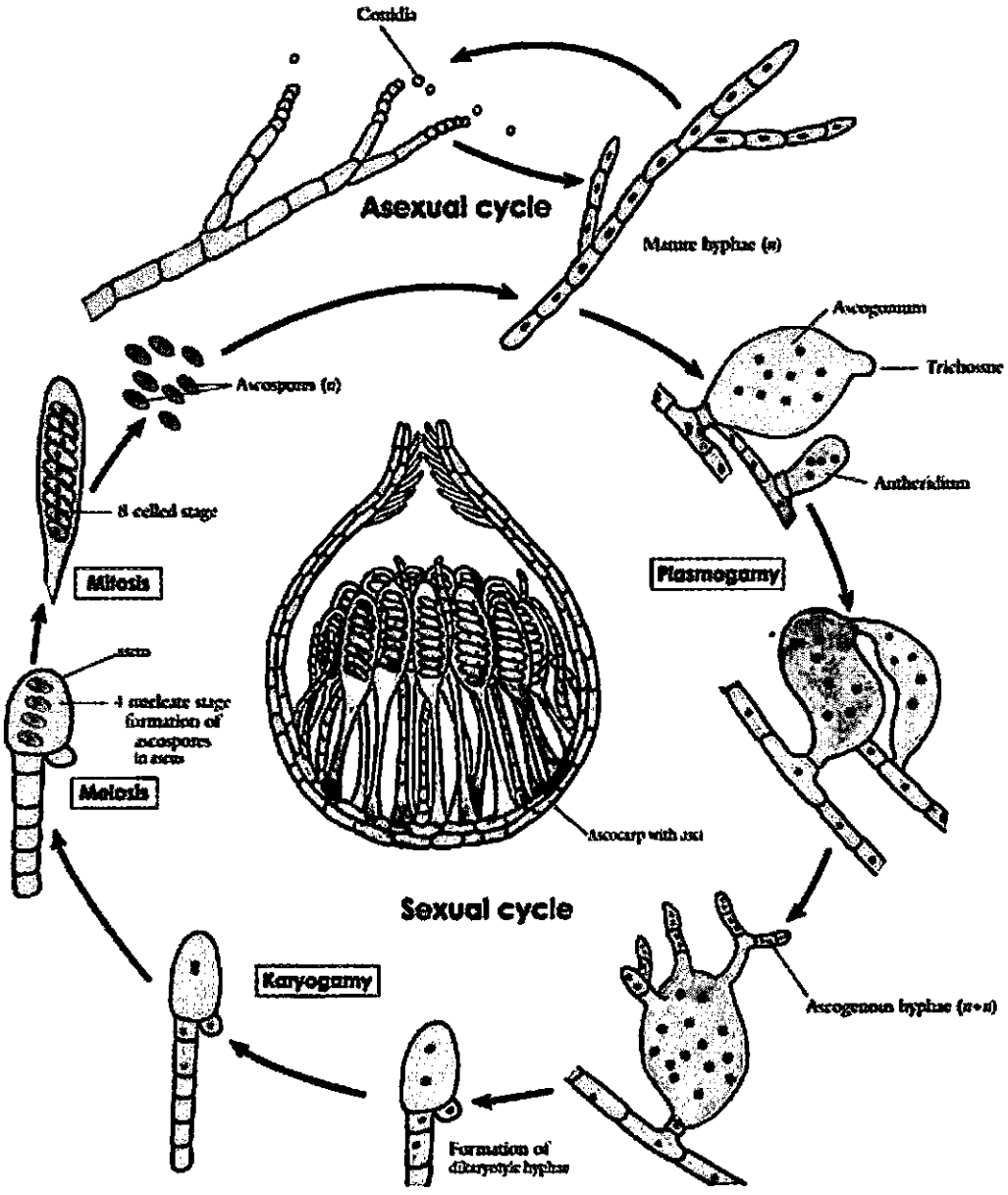


Figure. 8. Life cycle of Ascomycetes (University of Winnipeg, Canada).

Ascomycetous yeast are part of the class Hemiascomycetes, whose members characteristically lack ascocarps and ascogenous hyphae. Marine yeast genera fall into two major families, the Spermopthora and Saccharomycetaceae. Needle- or spindle-like ascospores are what determines the Spermophthoraceae family, while the Saccharomycetaceae family have asci that vary in shape from oval to hat shaped, but they also reproduce by fission or budding of single cells (Sieburth, 1979). Early studies revealed that most Ascomycetous yeast belonged in the class Cryptococcaceae with the perfect stages of the species *Rhodotorula, Candida,* and *Cryptococcus*. It was later discovered, however, that the most common species in the ocean belonged in the genus *Metschnikowia* (Sieburth, 1979).

F. Basidiomycetes

Of the at least 30,000 Basidiomycetes believed to exist, less than two dozen are obligately marine Basidiomycetes, although they have been found in all three classes, the Uredinomycotina, Ustilaginomycetes, and Hymenomycetes. The Basidiomycetes are the most highly evolved class of fungi and are distinguished by external basidiospores on structures called the basidium, which is where karyogamy and meiosis take place as part of the life cycle

(Sieburth, 1979) (Fig. 9). Basidiospores germinate to form septate hyphae or yeast cells which are uninucleate and haploid (Sieburth, 1979). Most likely, these spores are passively dispersed by wave action immediately after maturity (Kohlmeyer, 1979).

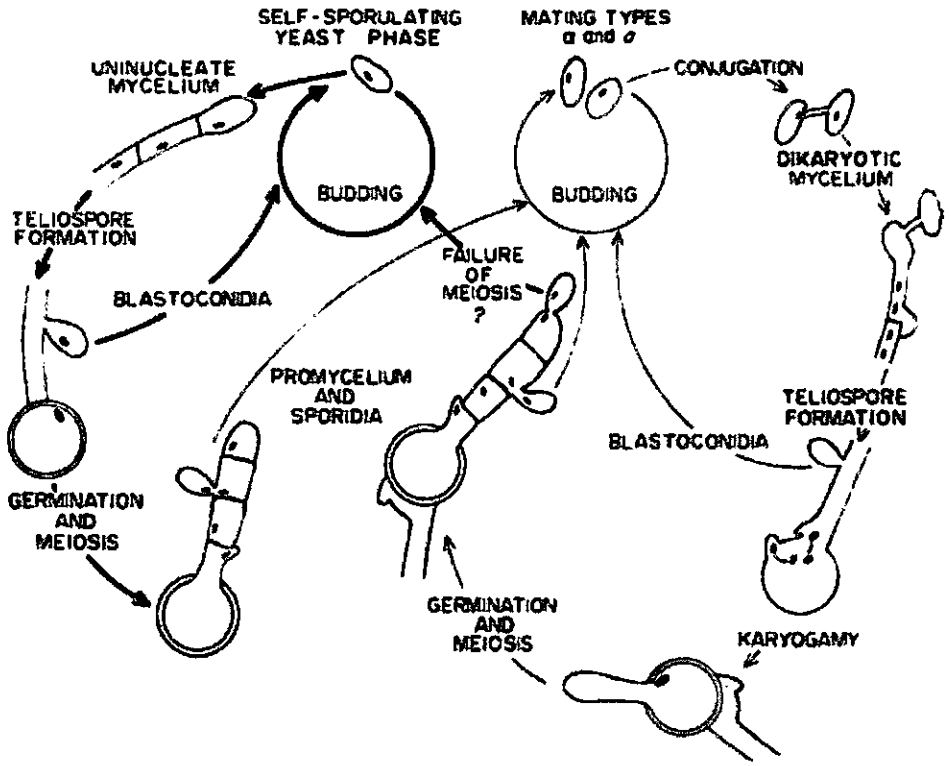


Figure 9. Life cycle of heterobasidiomycetous yeast (Newell and Fell, 1970).

Another method of sporulation is the production of ballistospores. This is more common in terrestrial species, but is also found in the yeast *Sporobolomyces* life cycle (Sieburth, 1979). Ballistosprores are created in the sterigma, and are forcibly discharged with the aid of hilar droplet found internally, resulting in a

surface tension catapult. They are asexual, and created by a number of different cells such as yeast cells, hyphae, basidia, or even other ballistospores (Kurtzman, 2000). Many marine species have lost this ability over evolutionary time. Terrestrial species in this the Basidiomycetes include mushrooms, rusts, and smuts. The few recognized (obligately) marine fungal species include *Melanotaenium ruppiae*, *Digitatisporea marina*, and *Nia vibrissa*. *Digitatisporea marina* has an adaptation to the marine environment with the presence of tetraradiate basidiospores which is believed to increase the efficiency of spore impaction and rate of attachment to substrate (Webster, 1959). *Nia vibrissa* has also become uniquely adapted to optimize maximum spore dispersal distance. It traps air within several superficial hairs on its exterior allowing it to float on the surface of the water for up to seven days, after detachment from the substrate (Kohlmeyer, 1979).

Basidiomycetous marine yeast were discovered in 1949 after evidence of a heterobasidiomycete-like sexual cycle for *Sporidobolus*, and soon after the sexual life cycle of certain strains of *Rhodotorula* was discovered. The new genus was named *Rhodosporidium* and placed in the Basidiomycetes. This red yeast is the most common type of yeast found in the open ocean (Sieburth, 1979). Sexual reproduction in Basidiomycetous yeasts is either

heterothallic or homothallic, although neither are well understood (Kurtzman, 2000). Conjugation of opposite mating types of haploid uninucleate cells leads to the formation of binucleate mycelial formation with clamp connections, and the development of teliospores. The clamp connections act as a link between cells during cell division, allowing the movement of nuclear products to adjacent cells, maintaining dikaryotic conditions (Sieburth, 1979; Kurtzman, 2000). The mycelium then forms teliospores which are thick walled spores much larger than the vegetative cells (Fig. 10). They eventually germinate to form a promycelium after karyogamy has taken place (Sieburth, 1979; Kurtzman, 2000). The result is a four-celled promycelium with transverse septa. Uninucleate sporidia then bud from the tip of the cell as well as at the septa. From the sides of the mycelium, chains of blastoconidia are formed which are also uninucleate (Sieburth, 1979). All fungi and yeast that produce clamp connections are Basidiomycetes, but not all Basidiomycetes produce clamp connections (Kurtzman, 2000).

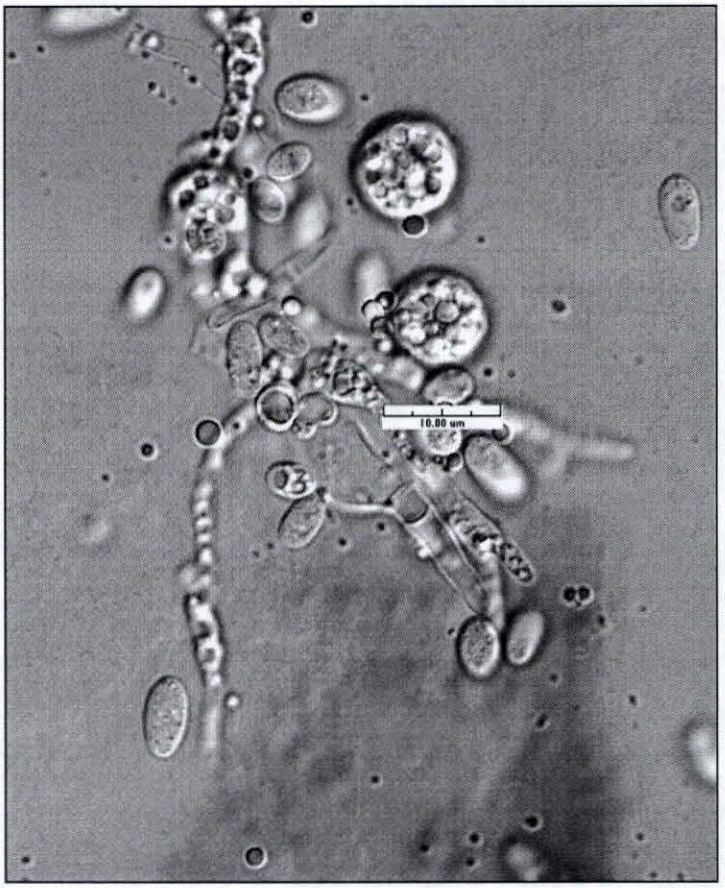


Figure 21. Teliospore formation in LM530 after 7 days on Dalmau plate

Several genera within the Basidiomycetes share similar life cycles, but have been given different names based on their ability to produce pigments. This is the case between the red to orange colored *Rhodotorula*, and the cream or white colored *Candida*, who have nearly indistinguishable life cycles. Few of the 1,000 known yeast species are obligately marine, but at least 50% of those cultivated from marine samples today are probably novel (Jack Fell, pers. comm.).

G. Deuteromycetes

The 25,000 species classified as Deuteromycetes are also known as Fungi Imperfecti, a subdivision that hosts fungi which produce only conidial states, meaning they are unable to form sexual spores of any type. This subdivision includes the imperfect, asexual, or conidial states of Ascomycetes, Basidiomycetes, and even some Zygomycetes. Deuteromyces produce asexual spores that are known as conidia. If the conidia arise on hyphae (conidiophores) they belong to the class Hyphomycetes and are usually passively released. If the spores are inside fruiting bodies, the species belongs in the class Coelomycetes. Spore release has not yet been observed in marine Coelomycetes (Kohlmeyer, 1979; Sieburth, 1979). The conidiophores can be highly differentiated or simple, but function to assist in positioning the developing conidium away from the parent mycelium. They are not developed by free cell formations or by cytoplasmic cleavage. Other forms of reproduction include hyphal fragmentation resulting in arthrospores, budding, formation of true mycelium by fission, or the formation of pseudomycelium. The pseudo-mycelium is often accompanied by blastospores created by budding, or pseudo-hyphae (Sieburth, 1979).

Deuteromycetous yeast from marine environments are found in the Cryptococcaceae which lack ballistospores, or in the Sporobolomycetaceae, which produce ballistospores. Ballistospores are actively projected upon maturity (Sieburth, 1979).

H. Zygomycetes

Marine-related species of the Zygomycetes are rarely mentioned in the literature and will be considered only briefly here. The Zygomycetes consists of roughly one percent of all described fungi and yeast species, amounting to less than one thousand species (Kirk, 2001). Zygomycetes species range from being pathogens in humans, plants, or animals to being saprophytes, or mutualists living on plants.

The terrestrial Zygomycetes are characterized by asexual reproductive structures called zygomycota, chlamydoconidia, conidia, or sporangiospores contained on simple or branched hyphae known as sporangiophores. They can be uni-to multi-spored sporangia. They are also distinguished by the thick walled sexual reproductive structure that results from gametes fusing, called zygospores. When the zygosporangium germinates it produces a mitosporangium (Fig. 11). The spores are often actively dispersed (Kendrick, 2001). The hyphae of Zygomycetes are coenocytic, thin-walled, and wide.

They can only assimilate starch and sugar substrates, unlike the Basidiomycetes and Ascomycetes which use a wider range of substrates (Madison, 2006).

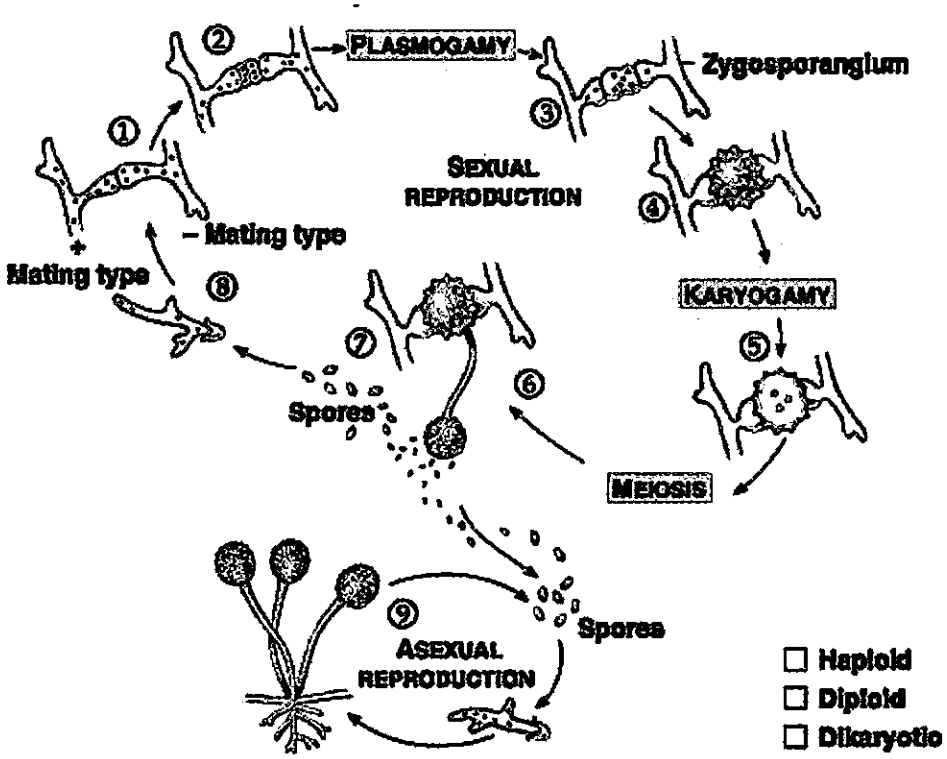


Figure 10. Life cycle of the Zygomycete, *Rhizopus* (Campbell, 2002).

I. Fungi In the Environment

In general, marine Fungi are considered to be saprotrophs, symbionts, or parasites on plants or animals. Saprobic fungi derive their nutrition from non-living organic material and participate in the recycling of nutrients. Decomposition of cellulose by saprobic fungi is an important process on

driftwood, mangrove roots, algae, sea grass, and leaves. Symbiotic relationships have been determined between fungi and algae, snails and tubeworms. Parasitic relationships exist between fungi and fish, invertebrates, plants (mangroves), and marine mammals, but primarily with algae (Polglase, 1986; Porter, 1986; Rheinheimer, 1992; Kohlmeyer, 2004). Most of the 40 species of parasitic fungi belong in the Ascomycetes. Fungi as pathogens are known to infect fish (eggs and larvae), crustaceans, and shellfish, among other marine animals. These relationships were determined through reports of fungi on economically important stocks such as oysters, herring, and mackerel.

Ascomycetes, Basidiomycetes, and Deuteromycetes are often associated with plants, animals, guano and sand in saprobic, symbiotic, or parasitic relationships. Saprobes use dead organic material as a source of nutrients, such as that trapped in the interstitial species of the sediments. Symbiotic, commensal and mutualistic relationships on the other hand involve differing degrees of dependence or interdependence between fungi and/or yeasts and another organism; a parasitic fungus or yeast is usually an ectoparasite, living externally on the host and causing damage to the host in the form of discoloration or tissue damage.

1. Sand/ Foam

Arenicolous fungi are those living among or on grains of sand. They do not obtain nutrients from the sand, but instead break down organic material in the interstitial space. Organic material here can be derived from algae, sea grasses, leaves, animal remains, feces, or driftwood. Arenicolous fungi are able to break down cellulose, alginate, laminarin, or agar from algae (Koch, 1974; Kohlmeyer, 1979). The ability to degrade cellulose likely makes arenicolous fungi important in the marine nutrient cycling, at least in coastal waters. Sand grains are commonly covered with bacteria, diatoms, algae and sometimes fungi, but studies have primarily been taxonomic and morphological, with a few exceptions (Kohlmeyer, 1966). Fungi can usually be found in the upper few centimeters of sand, but up to seven species of fungi, including *Corollospora* and *Carbosphaerella* has been found below 15 cm on untreated buried wood panels (Fell, 1960; Koch, 1974; Johnson, 1974) Yeast were reported in the upper 2 cm of sediments at 540 m depth in the Gulf Stream (Fell & Uden 1963; Fell, 1968) but up to 9 cm in areas with higher wave impact. These observations led to the conclusion that distribution of yeast populations in sand is limited by the availability of oxygen (Kohlmeyer, 1979).

During sporulation events, spores released by fungi can become trapped by air bubbles in the foam. Foam is the accumulation of marine microorganisms generated by wave action that traps bubbles, and washes ashore. Foam is an excellent source of marine adapted conidia, ascospores, and basidiospores; all of which are non-germinated due to constant wave action. Upon removal from foam, or cessation of wave action, these spores germinate within hours. Ascospores have adapted to survive in a dried state for several days, and if left at the high tide line will survive until the next high tide. Fruiting bodies of Ascomycetes can also be found attached to shell fragments or grains of sand (Kohlmeyer, 1979).

There is no method to accurately quantify fungi or yeast in sand or foam, or the frequency of a species within this habitat. The presence and abundance of foam depends on tides, precipitation, wind direction and velocity. The fact that foam is present, however, is an unreliable indicator of both fungi and yeast abundance, and their phylogeny. Studies have confirmed that on a daily basis the ability of foam to trap spores varies even over a period of hours. Probably the only accurate study of fungi and yeast associated with foam is that conducted by Kohlmeyer (1979), who reported significant differences in abundance of thee taxa in foam over periods of just hours.

2. Algae

Fungi and yeast living on algae are known as algicolous, and have been found in all groups except Basidiomycetes (Kohlmeyer, 1979). Most marine fungi found on algae are Ascomycetes, while several are Deuteromycetes. These species are parasitic, symbiotic, or saprobes but are not consistently found on all types of algae. Growth is often inhibited by antibiotic substances produced by healthy algae or competing bacteria (Sieburth, 1968). The relationship between parasitic fungi and their algal host is not well understood as some of them have little or no effect on the host's appearance, while others can cause light or dark patches to appear. There does appear to be a correlation between specific hosts and colonizers (Kohlmeyer, 1979).

3. Plant Material-Wood, Leaves, Mangroves

Fungi or yeast on wood or other cellulosic materials are termed lignicolous. Untreated wood is the easiest substrate to test for fungal colonization although it should be borne in mind that low levels of dissolved oxygen limited colonization degradation of wood by higher marine fungi (Kohlmeyer, 1969a).Such a phenomenon may be observed in buried or partially buried wood. 'Driftwood', defined as pieces of wood that are found floating along the shore, and 'intertidal wood' (fragments of wood or structures partially buried in

the sand or wedged between rocks) are generally excellent sources of fungi and yeasts. The most common colonizers on wood are the Ascomycetes, followed by the Deuteromycetes, and finally the Basidiomycetes. It appears that the fungi are not host specific. Marine fungi or yeast associated with leaves are termed foliicolous. They are also considered saprobes on dead loose leaves.

4. Animals

Associations between animals and marine fungi are generally limited to saprobic relationships with exoskeletons, protective tubes, or shells of crustaceans or invertebrates. There are also well defined relationships between wood boring mollusks, crustaceans, amphipods, and nematodes (Johnson & Sparrow, 1961) Marine yeasts are more frequently isolated from surfaces or within animals, including invertebrates, fish and marine mammals (Fell & Uden, 1963). This association is likely due to the incidental ingestion of yeast since they are ubiquitous in the ocean. Yeasts have been isolated from surface swabs and gut samples of shellfish, lobsters, conch, fiddler crabs, amphipods, copepods, mollusks, and oysters (Johnson & Sparrow, 1961). Yeast may also be associated with the guts, skin, gills, mouth, and feces of fish. Few studies have been conducted on yeast collected from marine

mammals. On two occasions, however, yeasts were isolated from the stomach and intestines of a dolphin and porpoises (Fell, 1970; Mori, 1973). A true relationship has never been established, however, and it was thought the yeast were associated with recently consumed food. For example, intestinal samples taken from eight California Sea Lions lacked yeasts, most likely because conditions in the gut were unsuitable for survival of the yeast (Fell & Uden, 1963).

5. Avian Guano

Marine yeasts are common in the guts of invertebrates, fishes, marine mammals and seabirds, and are therefore present in these animals' feces or guano. The intestines and rectal regions of free-living gulls and terns from Baja, California hosted high densities of yeasts. The most common species isolated from birds in the Pacific and Atlantic oceans were *Candida tropicalis* and *Torulopsis glabrata* (Fell & Uden, 1968). It has been suggested that yeast cells proliferate in birds who then disperse them into bodies of water worldwide (Kawakita & Uden, 1965). This theory has been disputed by evidence that yeast found in gulls were not always found in the surrounding seawater (Uden & Castelo-Branco, 1963). This cannot be conclusive evidence that seabirds do not disperse these cells, however, since rapid

dispersion of guano in seawater might require an inordinately large water sample be processed to actually detect these yeasts.

6. Impact on Humans

The impact on humans of Fungi and yeast ranges from diseases, to beneficial applications in the food industry, *e.g.*, brewing and baking. A positive example is of course the discovery and subsequent application of Penicillin as an antibiotic by Alexander Fleming in the early 1900's. On the other hand, several species of fungi and yeast can be detrimental to human health. Infections can be minor such as Athletes Foot, to severe such as Aspergillosis which can be fatal. The use of fungi and yeast species in the production of beverages and food is a common practice, most commonly in the production of alcohol, bread and cheeses.

Marine fungi and yeast are also slowly making their mark on humans. In 1999 it was reported that two species, *Corollospara lacera* and *Corollospara maritima* were being used in the in bioremediation of oil spills (Cooney, 1993). Negative publicity has come from marine fungi and yeast contaminating seafood for human consumption, such as oysters infected with *Dermocystidium marinum* on the Gulf coast and southeastern states (Sieburth, 1979). Marine fungi are also known to colonize marine

infrastructure, damage to which may be costly to repair. Marine fungi have been implicated in attracting wood boring organisms such as the crustacean *Limmoria*, and larvae of the boring mollusk, *Toredo pedicellata* to preferentially colonize pre-digested wood rather than fresh wood. Colonization of terrestrial wood by many Ascomycetes and Deuteromycetes results in 'soft rot', or decomposition of the wood.

J. Deep Sea Fungi and Yeast

In the context of this research the deep sea will be considered as that below 500 m, where hydrostatic pressure exceeds 50 atm. This is based upon the observation that fungi found below 500 m differ from those found in the epipelagic zone. Adaptations in deep sea fungi to high pressure and low temperatures are expected (Kohlmeyer, 1979) although none have thus far been proven.

Meyers *et al.* (1967) noted that the Black Sea contained the largest yeast populations in its upper 1000 m, while below this point only 25% of the cells cultivated were yeast. This distribution is consistent with the distribution of dissolved oxygen in the Black Sea, currents, and high concentrations of hydrogen sulfide at greater depths (Meyers *et al.* 1967). It was also noted that

a seasonal variation of yeast populations existed, with greatest abundances during the summer likely being related to a bloom of a large marine dinoflagellate, *Noctiluca miliaris*.

CHAPTER II. BACKGROUND OF THIS STUDY

A. Motivation for the study

The Hawaiian Archipelago is a 'biodiversity hotspot' and home to thousands of unique plant and animal species, but one in which novel microorganisms are rarely reported (Amadon, 1947; Kohlmeyer, 1969, 1985; Dring *et al.*, 1971; Barr & Hodges, 1987; Carr *et al.*, 1989; Kohlmeyer & Volkmann-Kohlmeyer, 1989; Myers *et al.*, 2000; Donachie *et al.*, 2003; 2004a, b, 2005). It is reasonable to assume, however, that many microorganisms are yet to be discovered in Hawai'i's diverse and isolated habitats (Donachie *et al.*, 2004a). Each of the six major Hawaiian Islands is unique in age and human population. Spatially constrained habitats over small vertical scales and across well defined microclimates on each island can host unique plant and animal species (cf. Carr *et al.*, 1989).

The marine mycoflora of the six main Hawaiian Islands, Station ALOHA, and Palmyra Atoll has never been extensively studied. The few studies that have been conducted in marine waters of the Hawaiian Islands primarily utilized traditional culture techniques rather than new molecular methods, were of limited duration, and also of limited geographic extent. In the study described

here I exploited advances in methods in the last 20 years, specifically that while using 'traditional' techniques to bring fungi and yeasts into culture, I also employed molecular methods to rapidly sort cultures phylogenetically. This combined approach has not been used in any study of the Hawaiian marine mycoflora to date.

B. Defining the problem and proposed solution

Since no region wide study of Hawai'i's marine fungi and yeast has been conducted, the primary goal of this research was to establish the first locally based collection of fungi and yeast from Hawaiian coastal waters and station ALOHA; The opportunity to collect samples from Palmyra Atoll some 950 miles south of Hawai'i came late in the course of this research, but enabled the project's geographic coverage to be significantly expanded. Other goals included determining phylogenetic relationships among the fungi and yeasts isolated from diverse habitats, isolating and describing novel species.

The value of cultivating microorganisms *versus* using a solely molecular approach to describe phylogenetic diversity by amplifying and cloning ribosomal genes from community DNA is that cultures *in vitro* provide a collection that can be accessed by for years to come; researchers thus have access to cultures that can be screened for secondary metabolite production

including antibiotics, cytotoxins and nutraceuticals. Previous workers have approached marine fungi and yeast by only one of cultivation, molecular or chemical methods. Studies of the Hawaiian marine mycoflora, however, have to date only employed cultivation techniques. The significant novelty and value of the work described here, is that I combined traditional cultivation on different enrichment media with molecular methods, specifically DNA sequencing which facilitated rapid phylogenetic placement of the isolates. I thus applied this combined approach to conduct the most extensive investigation to date of marine fungi and yeast seawater and other marine-associated samples around the six main Hawaiian Islands, Station ALOHA, and Palmyra Atoll. This is in fact the first such work in Hawai'i since Kohlmeyer's studies 30 years ago, during which he collected relatively few samples that were subsequently incubated on cultivation media. Moreover, Kohlmeyer's descriptive work was to some extent limited by the lack of rapid DNA sequencing technologies and absence of ribosomal DNA nucleotide sequence databases.

C. Thesis goals and objectives

The specific aim of the research described here was to determine the abundance and phylogenetic diversity of fungi and yeast in marine samples

collected from around the six high Hawaiian islands (O'ahu, Hawai'i, Kaua'i, Maui, Lana'i, and Moloka'i, Palmyra Atoll (5° 52' N, 162° 06' W), and from different depths at Station ALOHA (22° 45' N, 158° 00' W) (Karl & Lukas, 1996). Samples included seawater, sediment, algae, terrestrial plant material (wood, leaves, mangrove pods) found on beaches and in the ocean, plus invertebrates, and avian guano. Station ALOHA presents a ~5000 m water column in the oligotrophic Subtropical North Pacific Gyre. Although the deep sea mycoflora has been described in different parts of the ocean around the world, that at Station ALOHA has never been considered (Fell 1963; Uden, 1968; Sieburth, 1979; Fell, 1986; Kurtzman, 2004). The data presented here describe marine fungi and yeast abundance and phylogenetic diversity around the high Hawaiian islands, at Station ALOHA, and at Palmyra Atoll.

Objectives

1. To enumerate fungi and yeasts in marine habitats of six of the high Hawaiian Islands, Station ALOHA, and Palmyra Atoll.
2. To establish a locally-based collection of marine fungi and yeast collected from sites described in objective 1.

3. To assign cultivated strains to taxonomic groups using a combined molecular and physiological approach.

4. To describe at least one novel species.

5. To determine which material or sites host the most abundant novel species, *i.e.*, island and source.

6. To compare the contribution of phylogenetic Classes to the fungal community at each location.

7. To determine whether fungi and yeast abundance varies in water samples collected around each island.

8. To determine if the abundance of fungi and yeast differ with depth at Station ALOHA.

9. To determine if phylogenetic differences exist in the mycoflora isolated from different depths at Station ALOHA.

D. Experimental Design

Fungi and yeasts were enumerated on different enrichment media inoculated with a range of samples collected from the coasts of each of the six high Hawaiian Islands and Palmyra Atoll. The number of visits to each island

varied as a function of site accessibility. Station ALOHA collections were based on ship availability. Standard microbiological procedures described below were used to prepare pure cultures of representative strains from each sample and medium, after which DNA sequencing and in selected cases other descriptive criteria were used to assign isolates to taxonomic groups.

CHAPTER III. MATERIALS AND METHODS

A. Sample sites

The high Hawaiian Islands are those in which the original volcanic feature remains above sea level (Fig. 12), as opposed to the low islands in the north of the archipelago which comprise limestone caps (atolls) on eroded or subsided volcanic bases.

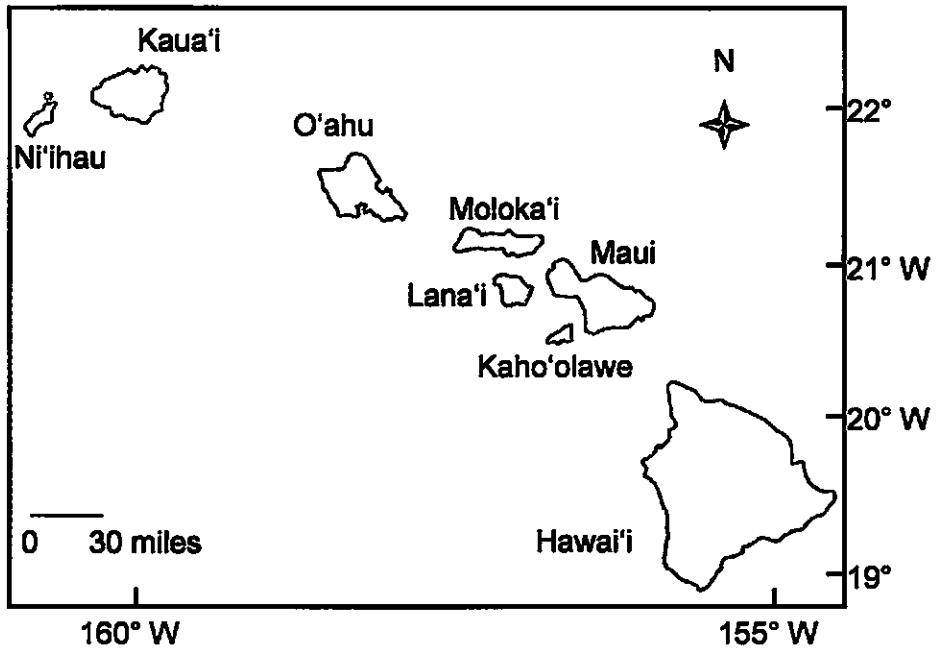


Figure 12. Map of the high Hawaiian Islands.

Kaua'i

The fourth largest and the northernmost of the populated high islands, Kaua'i covers 550 sq. miles and has 111 miles of coastline. The island is geologically the most mature of the main Hawaiian Islands and there are abundant fringing coral/algal reefs and sandy beaches (Fig. 13).

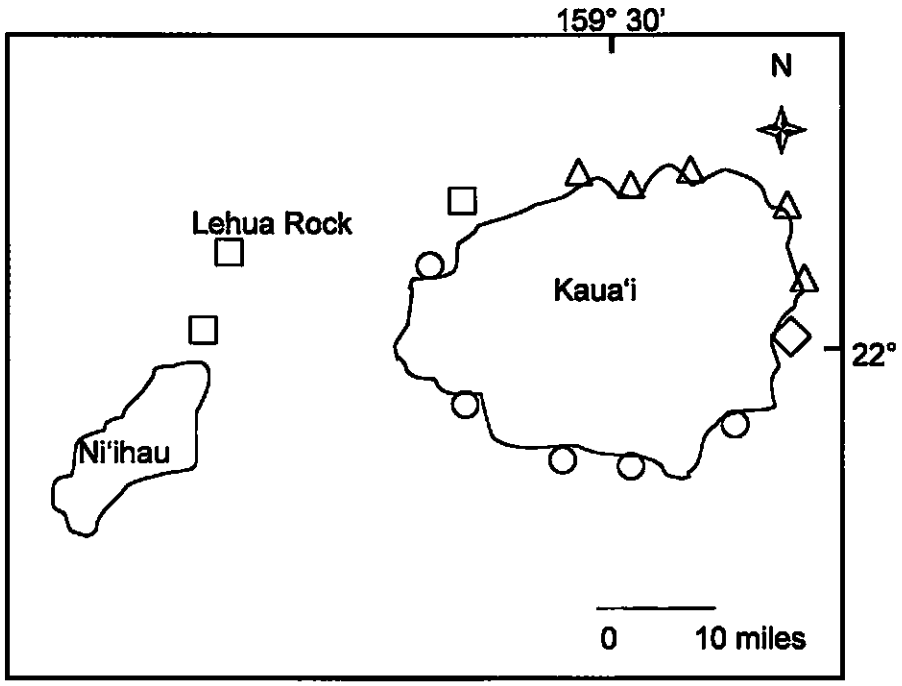


Figure 13. Sample collection sites on Kaua'i.

O'ahu

The third largest of the Hawaiian islands, covering 608 sq. miles, with 112 miles of coastline. The most populous island with almost one million residents, O'ahu also hosts approximately five million tourists annually. The island has abundant fringing coral/algal reefs and sandy beaches (Fig. 14).

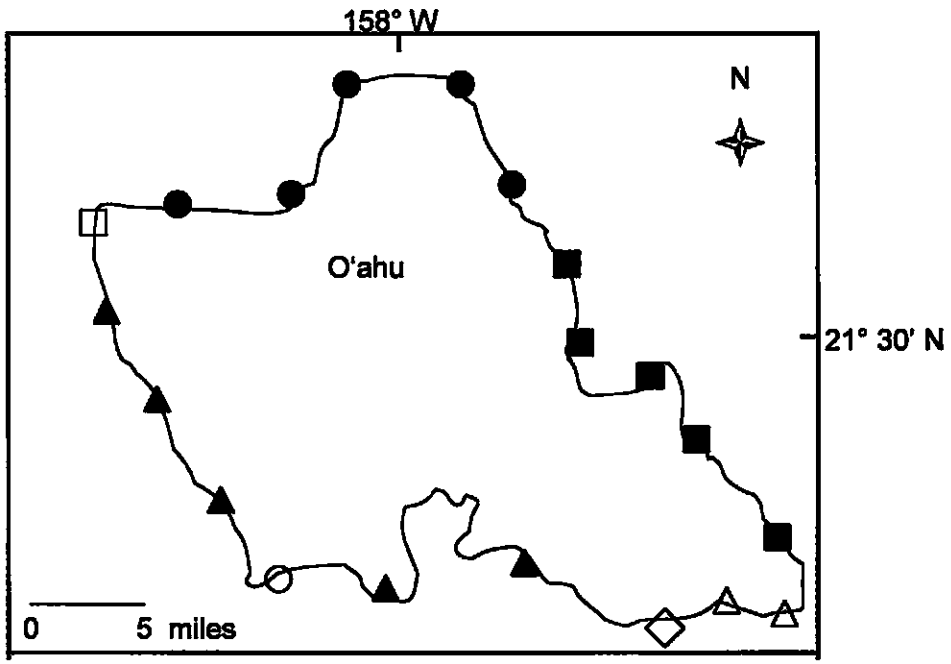


Figure 14. Sample collection sites on O'ahu

Maui

The second largest Hawaiian island, covering 728 sq. miles and with 120 miles of coastline. Maui hosts over 117,000 residents and some 2.2 million visitors annually (Fig. 15).

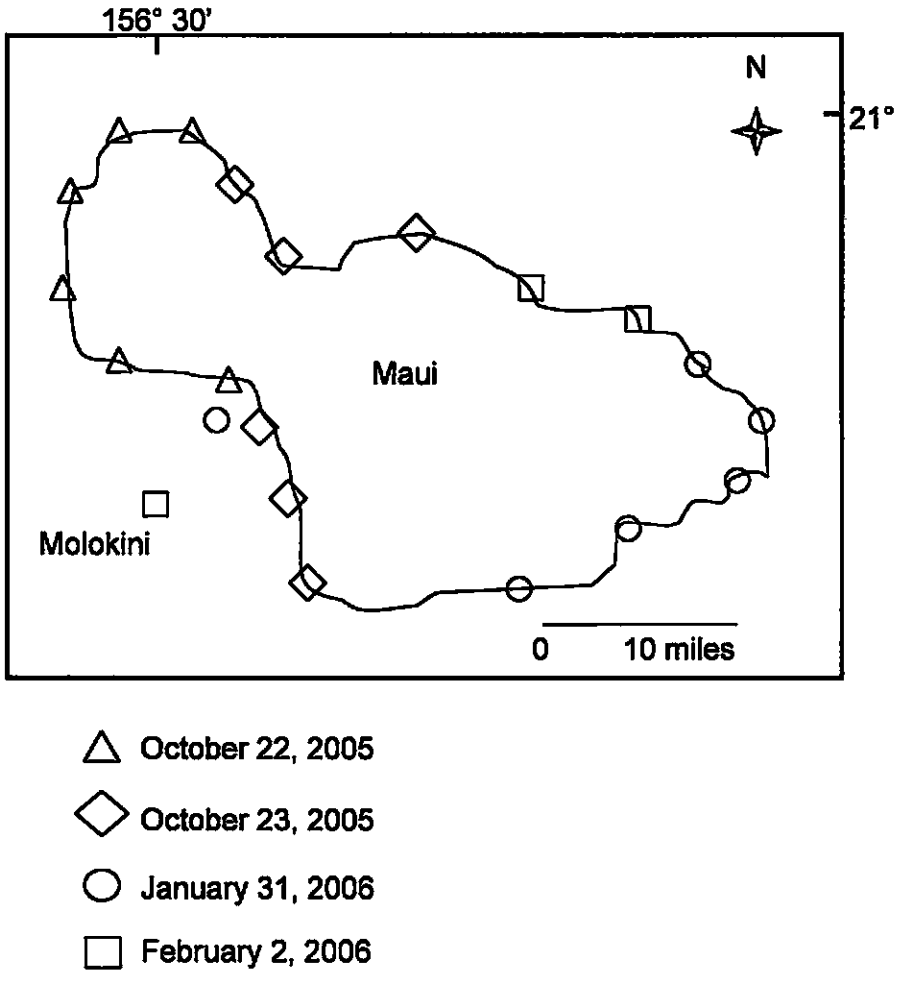
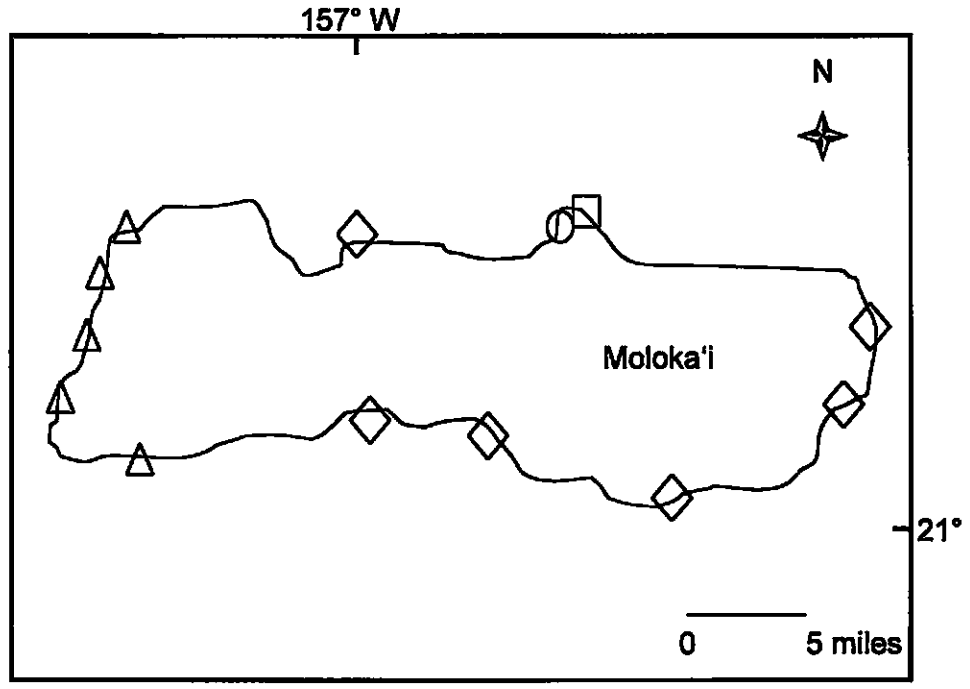


Figure 15. Sample collection sites on Maui

Moloka'i

The fourth largest island, covering 260 sq. and with 88 miles of coastline, this island hosts 7,400 residents (Fig. 16).



△ September 3, 2005

◇ September 4, 2005

○ May 21, 2006

□ May 22 , 2006

Figure 16. Sample collection sites on Moloka'i

Lana'i

The smallest of the main islands covers 141 sq. miles and has 47 miles of coastline. Lana'i hosts just ~3,100 residents (Fig. 17).

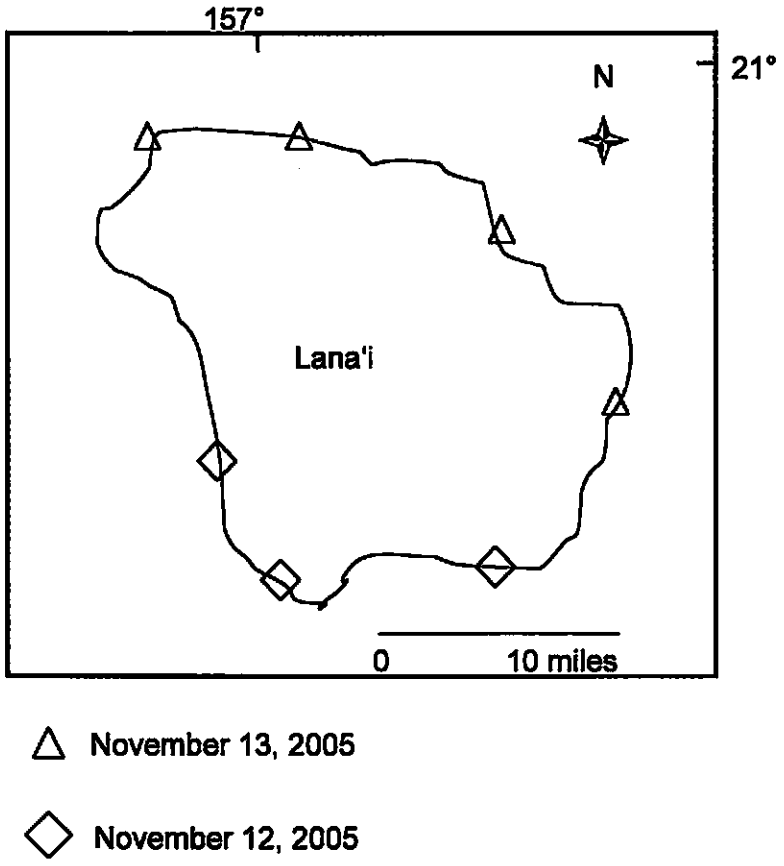


Figure 17. Sample collection sites on Lana'i

Hawai'i

The largest of the main islands, covering 4,038 sq. miles and with 266 miles of coastline. The islands population is 149,000 residents who are divided between two main population centers, Hilo and Kona, on opposite sides of the island (Fig. 18).

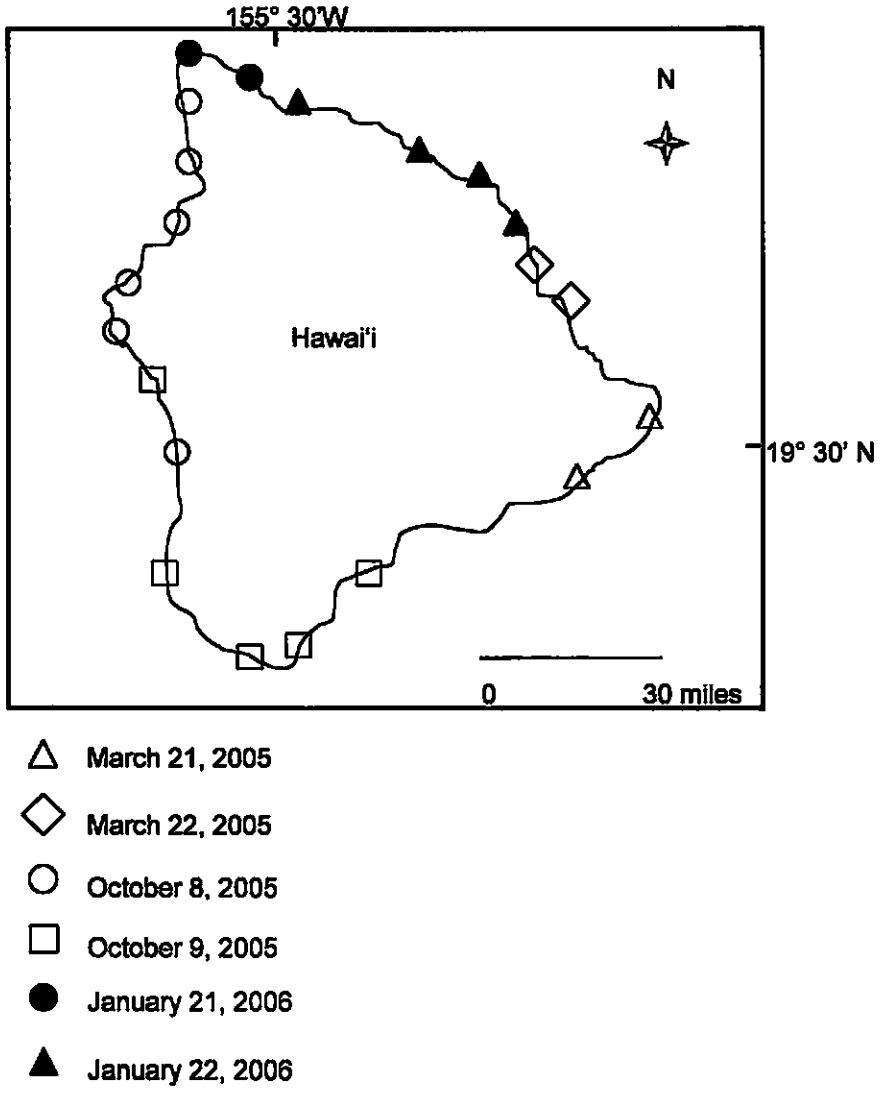
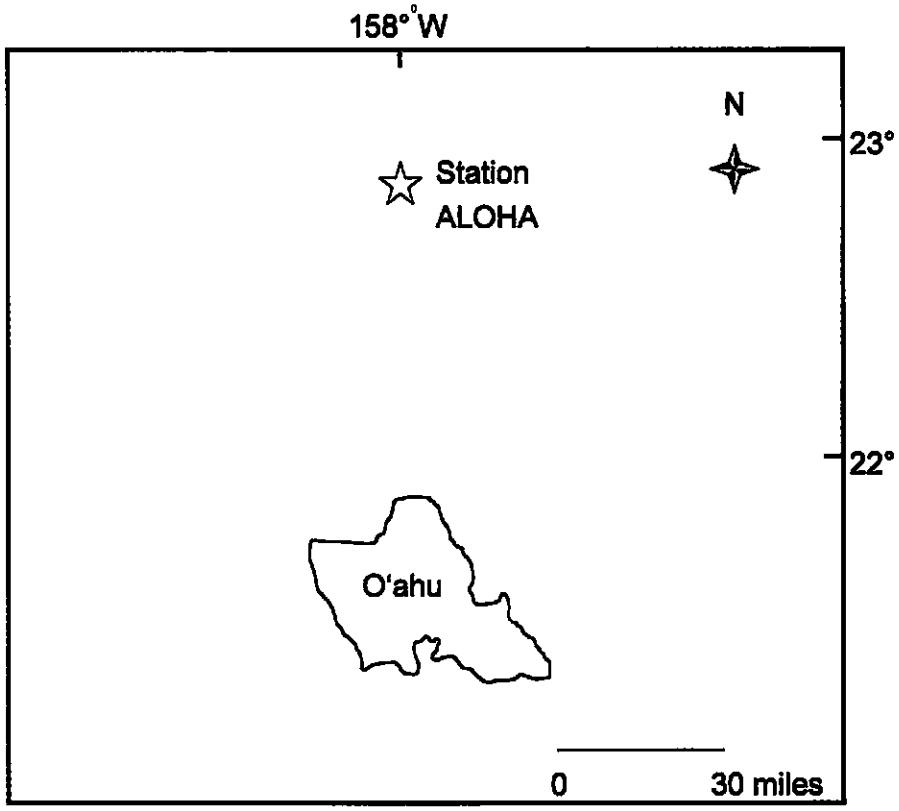


Figure 18. Sample collection sites on Hawai'i.

Station ALOHA

The National Science Foundation funded the Hawai'i Ocean Time Series (HOT) program 18 years ago. Approximately monthly measurements of a range of oceanographic (biological and chemical) parameters are conducted at this site 60 miles north of O'ahu, with Station ALOHA (22° 45' N, 158° W) being the focal point (Fig. 19). Water depth here is almost 5000 m, and the average seawater surface temperature is >23 °C. Diverse oceanographic studies have been conducted at Station ALOHA since 1988 but none has specifically targeted any aspect of deep sea fungi and yeast.



HOT 163 September 27 - October 1, 2004
HOT 171 July 15 - July 19, 2005
HOT 177 January 23 - January 27, 2006
HOT 178 February 13 - February 17, 2006
HOT 180 March 31 - April 4, 2006
HOT 181 May 25 - May 29, 2006

Figure 19. Station ALOHA, 60 miles north of O'ahu

Palmyra Atoll

No marine mycological studies on or within the extensive atoll and lagoons of Palmyra have been reported. The atoll hosts thousands of nesting seabirds, numerous fish species, and is home to one of the healthiest reefs in the United States and its territories. The opportunity to collect samples at Palmyra was only presented late in this study, but it presented an excellent study site given the goals of this project.

Palmyra is located at 5° 52' N, 162° 06' W, or ~1,000 miles south of the Hawaiian Islands. Just 680 acres of land emerge to a maximum of 6 ft. elevation, while reefs and lagoon cover 15,512 acres. The atoll is situated in the intertropical convergence zone just north of the equator (Fig. 20), where trade winds from the northern and southern hemispheres meet. The atoll hosts 29 bird and 125 coral species. Several seabird species found throughout the Hawaiian Islands, some rarely observed, exist in immense nesting colonies on the atoll. Such birds include Red Footed Boobies (*'Ā*), Brown Boobies (*'Ā*), Masked Boobies (*'Ā*), Great Frigate Bird (*'Iwa*), Black Noddy (*Noio*), White Tailed Tropicbird (*Koa'e kea*), Red Tailed Tropicbird (*Koa'e'ula*), White Tern (*Manu-o-ku*), and Sooty Tern (*'Ewa'ewa*).

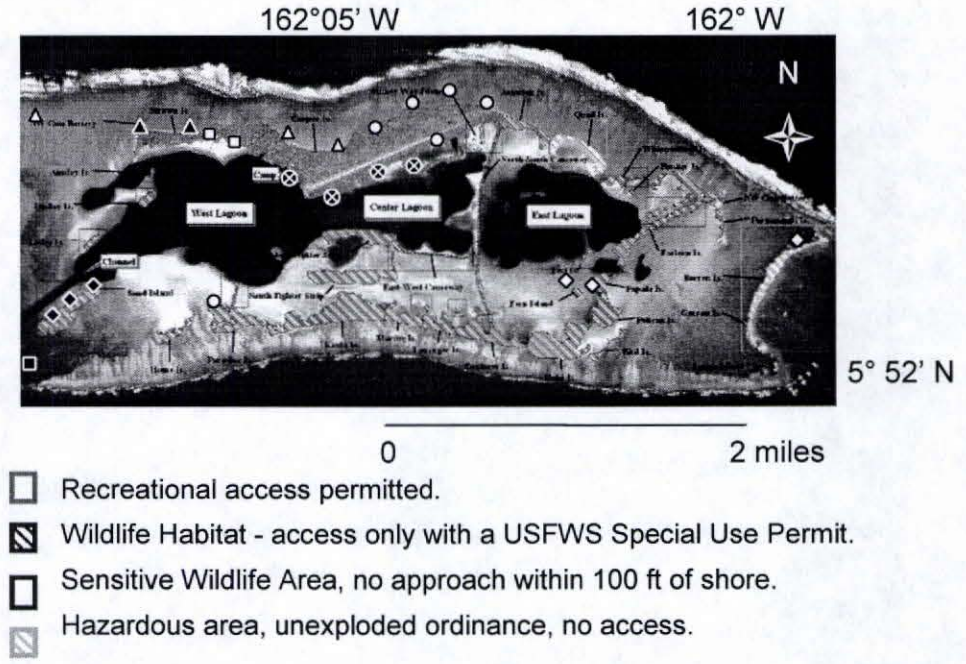


Figure 20. Sample collection sites on Palmyra Atoll, ~1000 miles south of Hawai'i

Other points of interest when considering sampling at Palmyra include the current minimal human impact, and unique plant and animal biodiversity. Since its discovery in 1798 A.D., ownership of the atoll transferred hands many times, but it was acquired by the U.S. in 1961 and most recently by The

Nature Conservancy in 2000. The atoll's human population varies between 8 and 25, but the greatest population of ~5,000 was reached during the 1940's when the atoll was 'occupied' by the United States Navy. The Navy dredged channels in the lagoon and dumped the 'spoil' to form causeways for vehicles and landing strips for aircraft. Several islets have seen little human impact since the 1940's because of the presence of unexploded ordinance, but they have become popular nesting sites for seabirds. The high density of seabirds likely impacts the abundance and phylogenetic diversity of culturable fungi and yeast in the surrounding waters.

B. Sample collection

Sampling began on August 20, 2004 on the island of O'ahu and ended in July 2006 at Palmyra Atoll (Tabs. 1 - 2). Each of the six high Hawaiian Islands was sampled, covering as much of the circumference as geographically possible in the time available (Fig. 12). Samples were also collected at various depths during six Hawai'i Ocean Time (HOT) series cruises to Station ALOHA, beginning with HOT-163 (September 27 - October 1, 2004), and ending with HOT-181 (May 25 – May 29, 2006). Palmyra Atoll samples were collected in July 2006.

Table 1. Sample sites and materials collected at each location

Kaua'i	Date	Water	Sand	Algae	Wood	Misc.	Misc. Item
Anahola Beach	8/11/05	X	X		X		
Secret Beach	8/11/05	X	X	XX	X	XXX	3, 9, 11
Hanalei Bay	8/11/05	X	X	X	X	XX	3, 11, 12
Kee Beach	8/11/05	X	X	X	XX	X	
Wailua River	10/14/05	X					
Wailua Bay	10/14/05	X					
Polihale Beach	2/14/06	X	X				
Waimea Pier	2/14/06	X	X		X		
Port Allen	2/14/06	X	X	X	X		
Poipu Beach	2/14/06	X	X	X			
Kalepaki Bay	2/14/06	X	X				
Na Pali	2/15/06	X					
Lehua Rock	2/15/06	X					
Ni'ihau	2/15/06	X					

Moloka'i	Date	Water	Sand	Algae	Wood	Misc.	Misc. Item
Hale O Lono Harbor	9/3/05	X	XX	X	X		
Halawa Valley Beach	9/4/05	X	X	X	X		
Rocky Point Beach	9/4/05					X	11
Old Kamela Wharf	9/4/05		X				
Dixie Manu Beach	9/3/05	X	XX			X	4
Kaula Ili Way Rocky Bh	9/3/05	X	X	XXX		X	13
Moomomi Beach	9/4/05	X		XX	X	X	14
Kapaa Rocky Beach	9/3/05		X	XX			
Papahoku Sandy Bh	9/3/05	X	X		X		
Kaunakakai Wharf	9/4/05	X			X		
Coconut Grove	9/4/05		X	XX	X		
Kalaupapa, Oceanview	5/20/06	X		XX			
Kalaupapa, Bayview	5/20/06	X		XX			
Kalaupapa, KeoKeo	5/21/06	X	X				
Kalaupapa, Kahili	5/21/06	X	X	X	X		
Kalaupapa, Kahiu Point	5/21/06	X				XX	2, 8
Kalaupapa, Hoolehua	5/21/06	X	X	X	X		
Kalaupapa, Junkyard	5/21/06	X	X	X	X		
Kalaupapa, The Landing	5/21/06	X		X		X	2
Kalaupapa, Mormon Pond	5/21/06	X	X	X			
Kalaupapa, The Point	5/21/06	X	X	X			
Kalaupapa, Kauhako Crater	5/21/06	X			X		

Table 1. (Continued) Sample sites and materials collected at each location

O'ahu	Date	Water	Sand	Algae	Wood	Misc.	Misc. Item
Laie Point	8/17/05	X		X			
Turtle Bay	8/17/05	X	X	X		X	4
Pupukea Beach	8/17/05	X	X		X		
Haleiwa Alii Beach	8/17/05	X	X	X			
Mokuleia	8/17/05	X	X	X		X	13
Kaena Point	7/1/05					XXXX	3: Albatross
Hananuma Bay	9/3/06	X	X	X	XXX		
Barbers Point	9/7/04	X	X				
Makapuu Point	9/5/04		X				
Blowhole	8/20/04	X	X	XXXX	X		
Ewa Beach	2/6/06	X	X	X			
Electric Beach	2/6/06	X	X				
Yokohama	2/6/06	X	X	X			
Waianae	2/6/06	X		X			
Kahana Valley	2/13/06	X	X				
Kahaluu Boat Ramp	2/13/06	X					
Kailua Beach	2/13/06	X	X				
Kaneohe Bay	2/13/06	X					
Baby Makapuu	2/13/06	X	X				

Hawai'i	Date	Water	Sand	Algae	Wood	Misc.	Misc. Item
Opihaku	3/21/05						
Kapoho 1	3/21/05	X	X				
Kapoho 2	3/21/05	X		X			
Kapoho 3	3/21/05	X		XXX			
Kapoho 4	3/21/05	X	X	XX			
Kapoho 5	3/21/05					X	3: unknown
Hilo Harbor	3/22/05	X	X				
Hilo Bay 1	3/22/05	X					
Hilo Bay 2	3/22/05	X					
Mahukona Beach Park	10/8/05	X		X			
Spencer Beach	10/8/05	X	X		X		
Waikaloa Beach	10/8/05	X	X	X	X		
Puako Beach	10/8/05	X	X				
Kealakekua Bay	10/8/05	X	X	X			
Natural Energy	10/8/05	X	X	X			
Milolii Beach	10/9/05	X	X	X			
Kailua Kona	10/9/05	X		X			
Green Sand Beach	10/9/05	X	XX	X	XX		
Kaulana South Point	10/9/05	X	X				
Punaluu Bl. Sand Beach	10/9/05	X	X	X			
Waipio Valley	1/20/06	X	X		X		
Pololu Valey	1/20/06	X	X		X	X	2
Onomea Bay	1/21/06	X	X	X	X		
Bl-Laupahoehoe	1/21/06	X		X			
Bl-Kolekole	1/21/06	X		X	X		
Keokea	1/21/06	X					

Table 1. (Continued) Sample sites and materials collected at each location

Maui	Date	Water	Sand	Algae	Wood	Misc.	Misc. Item
Honokohau	10/22/05	X	X		X		
Blowhole	10/22/05	X				XX	9, 11
DT Fleming	10/22/05	X	X		X	X	11
Kaanapali	10/22/05	X	X		X	X	11
Olowalu	10/22/05	X	X	XXX	X	X	5, 10
Maalaea Harbor	10/22/05	X	X	XX			
Waihee	10/23/05	X	X		X		
Kahalui Harbor	10/23/05	X	X		X		
Hookipa Beach	10/23/05	X	X		X		
Kalama Kihei	10/23/05	X		XX			
Makena Big	10/23/05	X	X				
Ahihi-Kinau	10/23/05	X	X	X		X	8
Maalaea Bay	1/31/06	X					
Natural Arch	1/31/06	X		X			
Ch.Lindberg Grave	1/31/06	X	X	X			
Koki Beach	1/31/06	X	X		X		
Red Sand Beach	1/31/06	X	X	X	X		
Waianapanapa	1/31/06	X	X		X		
Nahiku	2/1/06	X				X	11
Keanae	2/1/06	X		X			
Molokini	2/1/06	X					

Lana'i	Date	Water	Sand	Algae	Wood	Misc.	Misc. Item
Polihua	11/12/05	X	X	X			
Shipwreck	11/12/05	X	X	X	X		
Keomoku Village	11/12/05	X	X	X	X		
Naha	11/12/05	X	X	X	X		
Hulopoe Bay	11/13/05	X					
Kaunalo	11/13/05	X		XX			
Kaumalapau Harbor	11/13/05	X		XX			

Palmyra Atoll							
Strawn Island Tip	7/8, 11/2006	X	XXXX	X	XX	XXXX	1, 2, 3:RFB, 11
North Beach	7/8,10/2006	X	XXXX	XXX	XX	XXX	2, 3:RFB, 11
Papala Island	7/9/06	XX	X	X		X	6
North Coral Gardens	7/9/06	X		X			
Paradise Island	7/10/06	X					
Penglun Spit	7/10,14/2006	X		X		XX	3:RFB, 4
Airstrip, Cooper Island	7/12/06	X				XX	2, 3: J. RFB
Sand Island	7/15,16/2006	X	X		X	XXX	3: Chick RFB(2), 7
Tortogonis	7/8/06	X	X				

Miscellaneous sample key: 1) Crab Exoskeleton; 2) Foam; 3) Guano; 4) Hermit Crab; 5) Mucus; 6) Black Sponge (*Axinyssa* sp.); 7) 'Oily' Film on seawater; 8) Cyanobacteria Mat; 9) Fecal Pellets (origins unknown, and from *Calcinus elegans* (Hermit crab)); 10) 'Scum' (on rocks); 11) Leaf; 12) Flower (unidentified); 13) Sea urchin shell (species unknown); 14) Pencil urchin spine.

Table 2. Water samples collected at Station ALOHA.

	HOT cruise no.					
Depth (m)	163	171	177	178	180	181
5	X	X	X	X	X	X
75		X	X	X	X	X
125		X	X	X	X	X
250	X	X	X	X	X	X
520	X	X	X	X	X	X
1000	X	X	X	X	X	X
2000	X	X		X	X	X
3000	X	X		X	X	X
4000	X	X		X	X	X
4740	X	X		X	X	X

Dates: HOT-163, 9/27 - 10/1/04; 171, 7/15 - 7/19/05; 177, 1/23 - 1/27/06; 178, 2/13 - 2/17/06; 180, 3/31 - 4/4/06; 181, 5/25 - 5/29/06

Seawater samples were collected from the shore or tidal pools directly into sterile Nalgene bottles or polypropylene tubes. Subsurface water samples at Station ALOHA were collected into 20 L Go-Flo bottles on a 24 bottle rosette. Upon return to the surface, sub-samples were transferred from each Go-Flo to sterile 1 L or 4 L bottles. Sediments in tidal pools were collected with sterile plastic scoops or by 'coring' with sterile 50 ml centrifuge tubes. Marine invertebrates, driftwood, macroalgae, or other plant material were collected directly into sterile 50 ml centrifuge tubes or 500 ml Nalgene bottles, depending on their size. Seabird guano was collected opportunistically into

sterile 50 ml centrifuge tubes. Sample sites on each island were selected on the basis of distance between them and their accessibility, such that an island's entire circumference could be sampled at approximately regular spatial intervals. Among the species of algae collected during this study are *Dictyota acutiloba, Dictyosphaeria cavernosa, Dictyopteris plagirgramma, Curstose coralline, Entermorpha flexuosa, Gracilaria coronopifolia, Halimeda incrassate, Sargassum echinocarpum, Spyridium filimentosa, Turbinaria ornata, Ulva fasciata and Padina* sp.

C. Sample Processing and Cultivations

Upon return to the lab (land- or ship-based), 150 ml of each water sample was filtered (0.2 µm Millipore HA) and placed onto media designed to enrich fungi and yeasts (§ III. D). Homogenized or sectioned invertebrate and plant tissues, and guano were serially diluted in 0.2 µm filtered and autoclaved seawater and spread (200 µl each dilution) on the same enrichment media. Plates were incubated at 25 °C for up to four weeks, and inspected daily for growth. A duplicate set of media inoculated with seawater from below 1,000 m collected at Station ALOHA was also incubated at 4 °C to cater to potentially psychrophilic strains. Hyphal growth and yeast colonies considered representative of growth on a medium in terms of morphology, including

shape, color, opacity, *etc.*, were streaked for purification on the same isolation medium.

Each isolate was assigned an identification number that began with the prefix "LM", commencing with LM1. Each culture was assigned a unique descriptor, which describes the isolation medium, the island of origin, and type of sample. For example, colony number 4 from a plate of filtered water collected from Halawa Valley on Moloka'i grown on Potato Dextrose would be called: PD Mo HV W4. O'ahu was represented by "O", Kaua'i = "K", Maui = "Ma", Moloka'i = "Mo", Lana'i = "L", Big Island="Bi", Station ALOHA HOT cruise = "HOT#", Palmyra Atoll = "PA". In the event an island was visited multiple times, the number of the visit was listed after the location code, for example the second trip to Maui is recorded as "Ma2", or the third trip to the Big Island is recorded as "Bi3". The sample source was identified with a similar code, water was recorded with a "W", and sand, algae, wood were assigned numerical values.

D. Isolation Media

Three media were used for primary cultivation of organisms, specifically Sabouraud Dextrose (SD), Potato Dextrose (PD), and Wickerham's YM. The first two are manufactured by Difco, while the latter is made in house. YM, or Yeast Extract-Malt Extract, comprises by weight 1% glucose, 0.5% peptone,

0.3% yeast extract, and 0.3% malt extract. Each medium was supplemented with 20 grams of sodium chloride per liter. Prior to pouring to Petri plates, 300 mg of Penicillin G, and 250 mg of Streptomycin Sulfate per liter were added to inhibit bacterial growth.

E. Enumeration of colonies

Fungi and yeast colonies were counted manually using a colony counter pen (Bel-Art Products) and the average of three counts per plate was considered the result. Filters that contained too many colonies to count were recorded thus and assigned a numerical value of 500. Fungi were distinguished from yeast by colony morphology, *i.e.*, the presence of hyphal cells indicated fungi, while a lack thereof was considered to indicate the presence of yeast. Colonies were counted after a minimum of four days of incubation, and a maximum of twenty one days based on the rate of overgrowth of neighboring colonies, or time. Plates that did not yield any colonies after one week of incubation at 25 °C were transferred to 4°C for one week to encourage slow growing organisms or psychrophiles.

F. Preservation and Archiving Samples

Colony morphology, size, color and texture were recorded along with digital photographs of the original sample, original culture plates, and isolated

culture. Pure cultures were transferred to agar slants for storage at 4 °C, and into 10 ml of liquid medium in a 15 ml Falcon tube to an OD_{600} >1.5. After 48 hours, ~1 ml of culture was transferred to three 1.8 ml Nunc cryovial tubes containing sterile glycerol (60% final concentration) for long term storage at -20 °C. Approximately 1 ml of culture was also transferred to a microfuge tube and spun down (5000 rpm, 30 seconds) to create a pellet of cells for DNA extraction; pellets were stored at -20°C, as was as the remainder of the liquid culture.

G. Identification

Each culture was provisionally identified through sequencing the internal transcribed spacer (ITS) 1 and 2 regions between the 18S and 28S rDNA genes (Fell, 2001). No single DNA extraction technique suited all the cultures, so a range of methods was utilized, including the classic phenol-chloroform extraction, and modified commercial systems (Marmur, 1961; *e.g.*, Fell, 2001). Initial phylogenetic assignment was based on a polymerase chain reaction (PCR) with ~1 µl of the loose pellet, and 1 µl of each of the forward NS7 (5'-GAGGCAATAACAGGTCTGTGATGC-3') and reverse LR6 (5'-CGCCAGTTCTGCTTACC-3') primers. PCR products were cleaned in the MoBio Ultraclean PCR Clean-Up Kit and sequenced in a core laboratory at

the University of Hawai'i. Sequence chromatograms were manually checked for quality and edited where required. A consensus sequence was compared with other sequences in the public domain through a BLAST*n* search of the GenBank database (Altschul *et al.*, 1997) through which the nearest neighbors were determined. This was also used as a selection process for potentially novel species, with novelty defined as there being <97% nucleotide identity with the nearest validly described neighbor. For phylogenetic placement, sequencing provided approximately 320 base pairs of high quality sequence from the 3' end of the 18S rRNA along with full ITS 1, 5S rRNA, and partial ITS2 sequence. The 18S rRNA gene portion was subsequently used in phylogenetic analyses. Sequences were aligned in BioEdit (Hall, 1997) initially using Clustal W followed by manual editing. Trees inferring sequence relationships were generated in the PHYLIP software package (Felsenstein, 2004) using the program DNAdist with Jukes-Cantor correction to construct distance matrices and the program Neighbor to carry out Neighbor-Joining analysis. Phylogenetic trees were visualized and annotated in the program Adobe Illustrator 9.0 (Adobe Systems Incorporated).

I collaborated with Dr. Jack W. Fell at the Rosenstiel School of Marine and Atmospheric Science at the University of Miami in order to describe novel

yeast species. (Vandamme *et al.*, 1996; Kurtzman & Robnet, 1998; Yarrow, 1998; Fell *et al.*, 2000). The ITS4 region of the 28S gene, also known as the D1/D2 region, was amplified to provide further identity confirmation. The D1/D2 region was amplified in a subsequent PCR with the forward F63 (5'-TAAGCGGAGGAAAAG-3') and reverse primer R635 (5'CCCGTCTTGAAACACG-3'). Nearest neighbors were determined as described above.

H. Description of Sympodiomycopsis mahdii, LM418^T, sp. nov.

1. Materials and Methods

Determining an isolate's ability to assimilate single carbon and nitrogen sources is an integral part of describing a novel species. The ability to assimilate any of fifty two carbohydrates presented as sole carbon sources was tested as described by Kurtzman & Fell (1997) (Tab.3) 1X concentrations of each sugar, where 1X was equivalent to 5 g of glucose per 100 ml, were diluted in either Yeast Nitrogen Base (Difco), or a Yeast Carbon Base (Difco) medium. 1 ml of each 0.2 µm filter-sterilized sugar was transferred to separate 2 ml microfuge tubes and was inoculated with 100 µl of the pure culture. Tubes were then incubated on a Rollerdrum (New Brunswick Scientific Co.) at 22 °C. The turbidity in each tube was recorded after three days, then weekly

for up to one month. Growth was assessed visually using a white index card with three lines 0.75 mm thick and 5 mm apart drawn with India ink. A score of 3+ indicated the three lines were completely obscured, 2+ indicated the three lines had diffused edges, 1+ indicated the three lines were distinct but had fuzzy edges, +/- indicated a swirl of cells lifted upon vortexing or inverting the tube, - indicated no growth and the three lines appeared with clear edges. Latent positives (l) were characterized by a 2+ or 3+ reading that developed rapidly after two weeks or longer; weak positives (w) had a 1+ reading, and slow positives (s) are designated a 2+ or 3+ if the turbidity developed slowly over a period exceeding two weeks.

Table 3. Assimilation of single carbon sources by LM418^T

Substrate		Substrate/test	
YNB Plain (neg. control)	-	α-methyl-D-glucoside	+
D-Glucose	w	Salicin	-
D-Galactose	-	D-gluconate	l
L-Sorbose	+	2-keto-D-gluconate	-
Maltose	w	5-keto-D-gluconate	-
Sucrose	-	Vitamin free	+
Cellobiose	-	D, L-lactic acid	w
Trehalose	-	Sodium succinate	+
Lactose	-	Sodium citrate	+
Melibiose	+	Methanol	-
Raffinose	+	Inositol	-
Melezitose	-	Nitrate	+
Inulin	-	Starch formation	-
Starch hydrolysis	+	Hexadecane	-
D-xylose	-	10% NaCl + 5% gluc	w
L-arabinose	-	50% Glucose	w
D-arabinose	-	DBB*	+

D-ribose	-	Gelatin liquefaction	-
L-rhamnose	-	Growth at 37 °C	-
D-glucosamine	-	K D saccharate	-
Ethanol	-	Sodium D-glucouronate	-
Glycerol	w	D-gluconate lactone	+
Erythritol	-	Creatinine	-
Adonitol	-	Urease	+
Dulcitol	-	Creatine	-
D-mannitol	+	Glucose fermentation	-
D-glucitol	+		

+, positive
-, negative
l, latent positive
w, weak positive
*, DBB distinguishes Ascomycetes from Basidiomycetes

Fermentation tests were conducted in Durham tubes containing 2 - 4% of a single sugar in a yeast extract, peptone base. Durham tubes consisted of a screw top 16 mm test tube with an inverted Durham insert. Fermentation media also contained a pH indicator dye, Brom Cresol purple. Fermentation was indicated by accumulation of gas in the inner test tube, and a color change in the medium which indicates acidification. Results were read after 3 days, and then weekly for up to one month. Results were read as 'positive' if gas filled the tube within seven days, 'delayed positive' if the tube was filled rapidly after seven days, 'slow positive' if the tube was filled slowly after seven days, 'weakly positive' if the tube filled to less than one third full, or negative if

no gas accumulated in the tube. Glucose is often used as an indicator of the ability to ferment, and if this test is negative no further fermentation tests are considered necessary. Literature also states that other members of *Sympodiomycopsis* do not ferment sugars (Kurtzman & Fell, 2000). Other tests included starch formation, growth at 37 °C, gelatin liquefaction (for proteolytic activity), Diazonium Blue B color reaction (DBB) to determine whether anasexual yeast is an Ascomycete or Basidiomycete, high osmotic pressure, hydrolysis of urea, and cyclohexamide resistance.

Colony morphology was described on Malt Extract plates with and without NaCl supplement for three days at 22 °C. The elevation, shape, size, color, texture, and margin of the colony were all recorded. Variations or preference in growth between Malt Extract with 2% NaCl, and without NaCl were also recorded. Cell shapes and dimensions were observed on a microscope slide in a three day culture in liquid Malt Extract medium without salt. The lengths and widths of twenty cells were measured and averaged. Observations of cell shape and if they occurred singly, in pairs, or in clusters were conducted.

The presence or absence of sexual structures or mycelial formation was determined on cornmeal Dalmau plates after seven days and thirty days (Kurtzman & Fell, 1998). Dalmau plates are created by taking a loop or needle

of cells and creating a split in the cornmeal plate, being careful not to cut through to the bottom of the plate. The line is approximately one inch long. Two small adjacent dots are made near the cut. A sterile cover slip is placed aseptically over part of the line, and one of the dots (Fig. 21). This provides aerobic and anaerobic growth conditions on media with little essential nutrients, forcing the culture to form sexual structures such as teliospores (Fig. 21). Placement of the cover slip also allows microscope access directly to the agar. Most observations of this type, however, were done by viewing through the bottom of the plate.

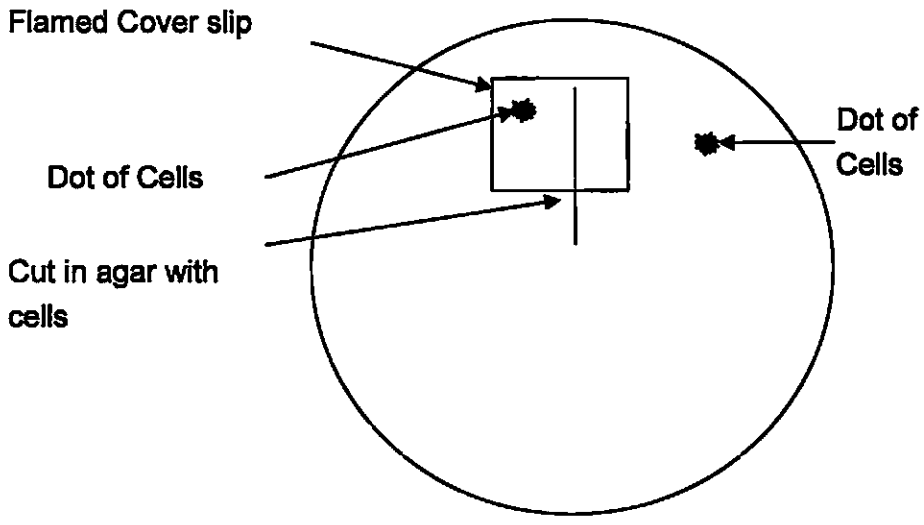


Figure 21. Diagram of Cornmeal Dalmau Plate

2. Sympodiomycopsis mahdii sp. nov., a basidiomycetous yeast from the Hawaiian Islands. To be submitted to FEMS Yeast Research

Abstract

A new basidiomycetous yeast is described for which the name *Sympodiomycopsis mahdii* is proposed. The type strain LM418^T, isolated from driftwood collected on a beach in Lana'i (Hawai'i), shares prominent characteristics of each of the genera *Sympodiomycopsis, Rhodotorula*, *Microstroma*, and *Quambalaria*, which make up three different families in the Microstromatales. The species was determined based on morphological, physiological, and molecular data. Sexual reproduction was not observed. GenBank accession numbers for nucleotide sequences of regions of the 18S and 28S ribosomal RNA genes in LM418^T are DQ990016, DQ990017 and DQ990018.

Introduction

The Hawaiian Archipelago hosts thousands of unique plant and animal species (Amadon, 1947; Carr *et al.*, 1989). Although the region is widely acknowledged as a 'biodiversity hotspot', few novel cultivated microorganisms from the archipelago have been described (Kohlmeyer, 1969, 1985; Dring *et al.*, 1971; Barr & Hodges, 1987; Kohlmeyer & Volkmann-Kohlmeyer, 1989;

Myers *et al.*, 2000;). In this respect, one-third of *Bacteria* species (each 'species' shared ≤97% 16S rDNA nucleotide sequence identity) cultivated from Hawaiian lakes and at the submarine Lō'ihi volcano were potentially new species or genera, just four of which have thus far been described (Donachie *et al.*, 2003; 2004a, 2005, 2006). Since just a fraction of the effort devoted globally to cultivating marine bacteria has been applied to cultivating marine fungi and yeasts, the latter two are poorly represented in culture collections and more so in the literature. Although fungi and yeasts are ubiquitous in the ocean, those off Hawai'i have rarely been considered (Fell, 1976, 2001; Donachie & Zdanowski, 1998; Nagahama *et al.*, 2001; Liu *et al.*, 2003; Tsukamoto *et al.*, 2004). Over thirty years ago, however, Kohlmeyer (1969) stated that, "...the marine fungal flora of Hawai'i is rich in species and deserves further thorough investigation." Kohlmeyer (1985) also made an excellent case for novelty in Hawai'i by isolating *Nimbospora octonae* during two collections in the state 15 years apart, but nowhere else in the world in 4000 collections over a 25 year period. Considering Kohlmeyer's work here, and that phylogenetically novel taxa appears abundant among cultivated Hawaiian *Bacteria*, we believe that a dedicated cultivation effort directed towards Hawaii's marine fungi and yeasts will yield new species. In this

respect, fewer than one thousand of the known ~400,000 Ascomycetes are marine in origin, less than thirty obligately marine Basidiomycetes have been described, and few of the 1000 known yeast species originate from the ocean. Thus, a large fraction of the marine fungi and yeasts cultivated from marine samples today are likely to be novel species. As part of a project to describe phylogenetic diversity and secondary metabolite production in Hawaiian marine fungi and yeasts, we sample diverse marine habitats throughout the Hawaiian Archipelago, supporting cultivation techniques with DNA sequencing methods that quickly enable tentative phylogenetic assignment of cultures. Molecular evidence of phylogenetic novelty is then tested through morphological and physiological observations. We present here a description of *Sympodiomycopsis mahdii* LM418^T, a new basidiomycetous yeast isolated from driftwood collected on a beach in Lana'i, Hawai'i.

Materials and Methods

Sample collection. In November 2005 we collected a piece of driftwood in the intertidal zone of 'Shipwreck Beach' on the island of Lana'i, Hawai'i (20° 55' 15" N, 156° 54' 30 W). The sample was collected directly into a 50 ml sterile polypropylene tube with a few milliliters of seawater, and processed in the laboratory at the University of Hawai'i at Mānoa as described below for

cultivation of yeast and fungi (Fig.17).

Yeast Isolation. The sample was vortexed vigorously for 30 seconds, after which 200 µl sub-samples of the 'diluent' were spread on Potato Dextrose Agar (PDA) (Difco) containing sodium chloride (20 g/L), 300 mg/L of penicillin G, and 250 mg/L of streptomycin sulfate. Plates were incubated at 25 °C. Representatives of colonies that arose after seven days of incubation were streaked for isolation on PDA and incubated at 25 °C. The purity of isolated strains was checked by consistency of colony morphology and uniformity of cells in wet mount preparations. Strain LM418^T was characterized by standard methods (Yarrow, 1998). Cells were archived on Potato Dextrose slants, and in Potato Dextrose broth containing 2% NaCl and 60% glycerol, and stored at -20 °C. Non-frozen cultures were retained for preliminary phylogenetic assignment through DNA sequencing.

Identification. Cells of LM418^T grown in 10 ml Potato Dextrose broth (with 2% NaCl) for 72 hr in an orbital shaker at 120 rpm and room temperature were collected by gentle centrifugation (30 sec). Initial phylogenetic assignment was based on a polymerase chain reaction (PCR) with 1 µl of the loose pellet, and 1 µl of each of the forward NS7 (5'-

GAGGCAATAACAGGTCTGTGATGC-3') and reverse LR6 (5'-CGCCAGTTCTGCTTACC-3') primers. The D1/D2 region of the 28S large sub-unit rDNA gene was amplified in a subsequent PCR with the forward F63 (5'-TAAGCGGAGGAAAAG-3') and reverse R635 (5'CCCGTCTTGAAACACG-3') primers. PCR products were cleaned in the MoBio Ultraclean PCR Clean-Up Kit and sequenced in a core laboratory at the University of Hawai'i. Sequence chromatograms were manually checked for quality and edited. Consensus sequences were compared with other sequences in the public domain (GenBank) through BLAST*n* searches (Altschul *et al.*, 1997). Sequencing provided approximately 545 base pairs from the 3' end of the 28S rRNA gene, including the D1/D2 region (Fell, 2001). Sequences were aligned in BioEdit followed by manual editing, and phylogenetic trees were generated in the PHYLIP software package (Felsenstein, 2004) using the program DNAdist with Jukes-Cantor correction to construct distance matrices and the program 'Neighbour' to carry out Neighbour-Joining analysis. Maximum likelihood analysis was conducted in the PHYLIP software package (Felsenstein, *ibid*) using the program DNAml. Phylogenetic trees were annotated and refined for publication in Adobe Illustrator 9.0 (Adobe Systems Incorporated).

Morphological and physiological analyses. The morphology of LM418^T colonies was determined on Malt Extract (ME) plates containing NaCl (2% w/w) after incubation at 22 °C for three days and one month. Cell size and shape were recorded after seven days and one month in ME broth with NaCl (2% w/v). The assimilation of single carbon and nitrogen sources by LM418^T was determined in liquid media in 2 ml microfuge tubes after Kurtzman & Fell (2000). Fermentation of glucose was investigated in Durham tubes with a 2% sugar mixture and Brom Cresol purple, read after 3 days and then weekly for up to one month. The presence or absence of sexual structures was determined on commeal Dalmau plates after seven days and thirty days. The presence or absence of ballistospores was determined on inverted malt extract plates observed for one month (Kurtzman & Fell, 2000).

Results

Colony morphology

LM418 colonies on ME after three days at 22 °C are opaque white with a slight pink hue. They are also smooth, convex, and have entire margins. Surface texture is creamy with a sticky consistency. A preference for 2% NaCl in ME was determined as colonies on this medium were larger than those

grown on ME without NaCl. Cells ranged from 1.34 to 9.38 μm in length, and 1.34 to 4.02 μm in width. Cells were circular to ovoid, and occurred singly or in pairs. Vegetative reproduction occurs by bipolar budding (Fig. 22).

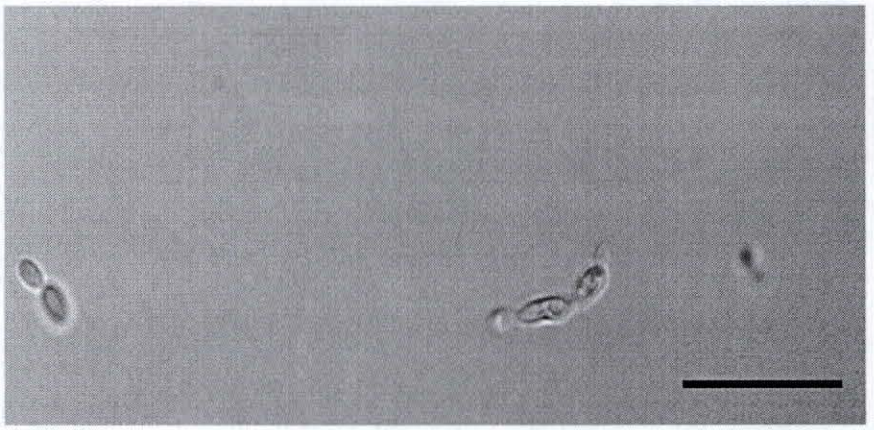


Figure 22. Bipolar budding shown by LM418 in Malt Extract broth after 3 days. Scale bar = 10μm

Sexual structures were not observed after thirty days on Dalmau plates, although true hyphae were observed (Fig. 23). Ballistospore formation was not observed after thirty days on inverted ME plates.

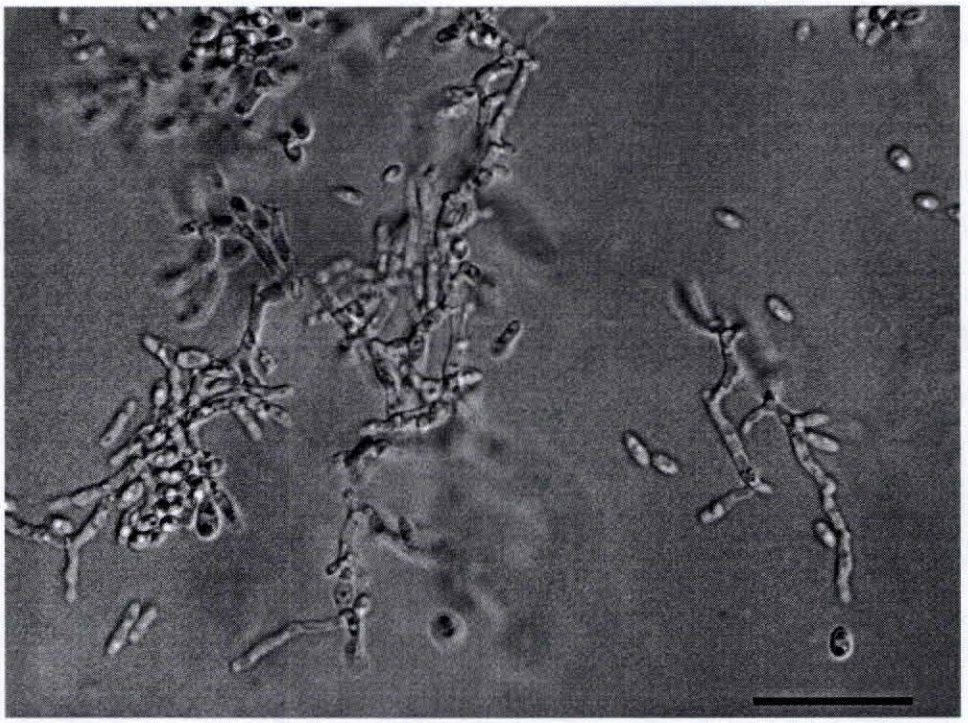


Figure 23. True mycelium formation on Dalmau plates after seven days. Scale bar = 10µm

A range of carbohydrates presented as single carbon sources was assimilated by LM418^T (Tab. 4). Since glucose was not fermented, other fermentation tests were deemed unnecessary. Neighbor-joining and maximum parsimony analyses show that LM418^T comprises part of the Microstromatales (Fig. 24) however the use of various alignment methods indicated differing nearest neighbors. Consensus sequences generated in PCRs with the NS7/LR6, 18S forward/reverse, and F63/R635 primer pairs were submitted to GenBank and assigned the accession numbers DQ990017, DQ990018 and DQ990016, respectively.

Table 4. Physiological properties of LM418, type species *Sympodiomycopsis mahdii* and related species†.

	Strain/species*					
	1	2	3	4	5	6
Pigmentation						
Cell length µm	1.34-9.38	4.0-11.0	1.47-4.7	1.53-3.0	2.4-4	2.3-3.3
Cell Width µm	1.34-4.02	2.0-6.0	2.7-5.4	7.0-24.0	5.5-10	4.7-9.4
Assimilation						
D-Galactose	-	vw	-	+	-	-
L-Sorbose	+	+	-	+	v	-
Sucrose	-	+	+	-	+	+
Cellobiose	-	+	+	+	s	-
Trehalose	-	+	+	+	+	-
Melibiose	+	+	-	+	-	-
Melezitose	-	+	+	-	+	+
Soluble starch	+	w	-	+	+	-
D-xylose	-	+	-	+	+	-
L-arabinose	-	+	+	+	+	+
D-arabinose	-	+	-	+	+	-
D-ribose	-	+	-	s	+	-
D-glucosamine	-	n	-	-	-	-
Ethanol	-	n	v	+	-	+
Erythritol	-	+	+	-	+	+
Adonitol	-	-	-	+	s	-
Dulcitol	-	-	-	+	-	-
α–methyl-D-glucoside	+	+	s	-	+	+
Salicin	-	-	-	+	s	-
D-gluconate	l	n	-	+	-	-
2 keto D-gluconate	-		n	n	n	n
5 keto D-gluconate	-		n	n	n	n
Vitamin free	+	+	+		-	+
N-acetyl D glucosamine		n	-	s	-	-
D,L lactic acid	w	w	-	-	-	-
Sodium succinate	+	+	w	+	+	+
Sodium citrate	+	-	w	+	s	-
Methanol	-	n	-	-	-	-
Inositol	-	+	+	-	-	s
Nitrate	+	+	-	+	+	+
Hexadecane	-	n	-	-	-	-
10% NaCl + 5% glucose	w		-	s	-	-
50% Glucose	w	-	-	-	-	-
DBB	+	+	+			
Gelatin hydrolysis	-		-	-	-	-
Na D-glucoronate	-		-	+	-	-
D-gluconate lactone	+					
Creatine	-		-			
True or pseudomycelia	+	+	-	-	w+	-

*1) LM418^T 2) *S. paphiopedili* 3) *R. hinnulea* 4) *R. javanica* 5) *R. bacarum* 6) *R. phylloplana.*

vw – very weak; w – weak; l – latent; n – not done; s – slow; v – variable. No strain

assimilates L-rhamnose, forms starch, ferments glucose, or grows at 37 °C; only *S. paphiopedilli* assimilates lactose and inulin, and in both cases it is weak. LM418^T does not assimilate K D-saccharate or creatinine. All strains assimilate raffinose, D-glucitol and urea; all assimilate D-glucose, maltose and glycerol, but these are weak in LM418^T. All strains assimilate D-mannitol, but it is weak in *R. bacarum*.

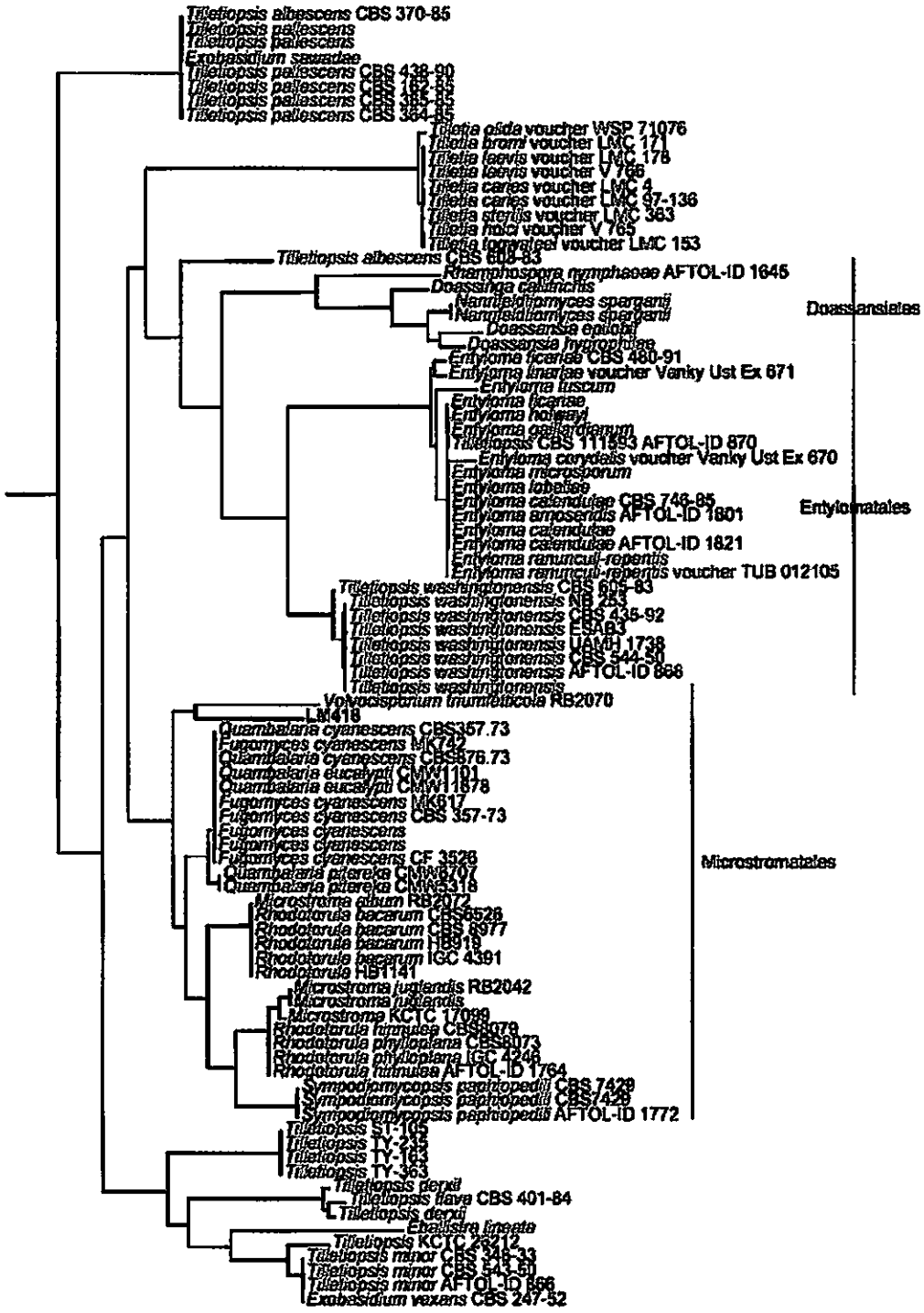


Figure 24. Neighbor-joining analysis of ribosomal RNA region in LM418, neighboring yeasts and representative taxa. Only bootstrap above 50% are included.

Discussion

Characterization of yeast strain LM418^T isolated from driftwood collected on Shipwreck Beach, Lana'i showed it could not be assigned to an extant species. Although molecular data place LM418^T firmly in the Microstromatales, morphological and physiological data support establishment of the strain as a new species within the *Sympodiomycopsis*, for which the name *S. mahdii* is proposed. *S. mahdii* LM418^T appears to be the anamorphic haploid yeast phase of an unknown telomorph. Different alignment methods of the D1/D2 and ITS1 and 2 regions of the 18S and 28S rRNA genes show that LM418^T is part of the Microstromatales but is not specifically associated with an existing genus (Fig.24). Genus placement could not be determined through molecular data alone, and creating a new genus was rejected on the basis of the lack of definitive tests used to define such terms, the lack of relevant data for species in neighboring genera, and the fact that we have only a single strain.

Placing LM418^T in the *Sympodiomycopsis* was governed by other factors, specifically the results of assimilation and fermentation tests. This course of action was determined by the fact that DNA sequence analysis could not consistently place the strain in just one genus. For example, the nucleotide

sequence of the D1/D2 region shared 96.0% identity with that in *Quambalaria cyanescens* (DQ317623), although taxa also related on the basis of this region include *Sympodiomycposis paphiopedili* (94.8%), *Rhodotorula bacarum* (94.4%), *Rhodotorula hinnulea*, *Rhodotorula javanica*, and *Rhodotorula phylloplana*. The inclusion of *Rhodotorula javanica* as a related species was determined by keying the species based on assimilation results (Kurtzman, & Fell, 2000).The nucleotide sequence of the ITS1 and ITS4 regions indicated that *Sympodiomycposis paphiopedili* (DQ317631) is the nearest described strain in this respect, sharing 96.9% sequence identity, while *Microstroma juglandis* (DQ317632) is the nearest neighbor (95.6% identity) on the basis of 18S DNA sequence comparison. Assimilation data for LM418^T distinguishes it from the *Rhodotorula* and *Sympodiomycopsis*, although limited data on *Sympodiomycopsis* are available since only one species in this genus has been described (Sugiyama *et al.*, 1991). Furthermore, insufficient physiological data have been published for *Quambalaria* spp. and *Fugomyces cyanescens* (Begerow *et al*, 2001; De Beer *et al*, 2006). It is known, however, that *Quambalaria* spp. and *Fugomyces cyanescens* both belong in the ascomycota, while LM418^T was determined to belong in the basidiomycota by the Diazonium Blue B test (Kurtzman & Fell,

2000).

Phylogenetic placement of LM418^T is also complicated by the fact that some of the species with which it aligns are likely synonyms, *e.g.*, *Fugomyces* spp., and the apparent relationship with *Volvocisporium* is probably due to long branch attraction (Fig.24). This group comprises mostly plant-associated filamentous basidiomycetes, with a handful of *Rhodotorula* and *Sympodiomycopsis* yeasts, and this is the most likely reason that *Quambalaria* and *Volvocisporium* species do not occur in the genus. Describing LM418^T as a new species in the group will validate the strain until the teleomophic state is found, and preclude subsequent loss of an anamorphic generic name should we opt to describe a new genus at this point. Addition of a new anamorphic genus based on a single strain is also unwarranted until the sexual state is determined.

Rhodotorula species do not form pseudomycelia or true mycelia on Dalmau plates, whereas this is a characteristic of *Sympodiomycopsis*. In this respect, LM418^T did form true mycelia after seven days on Dalmau plates, and also displayed sympodial budding as seen in *Sympodiomycopsis paphiopedilli*. LM418^T also exhibited a slight pink pigmentation when grown on ME, another shared characteristic with *Rhodotorula* species, while the lack of pigmentation

in *Sympodiomycopsis paphiopedili* distinguishes it from LM418^T (Sugiyama *et al.*, 1991).

We thus propose that LM418^{T}is the type strain of a novel species, termed *Sympodiomycopsis mahdii* based on both molecular and morphological data. Although the nearest neighbors of *S. mahdii* on the basis of nucleotide sequences are *Quambalaria cyanescens*, *Sympodiomycopsis paphiopedili*, and *Microstroma juglandis*, LM418^T is morphologically similar to *Sympodiomycopsis paphiopedili*. Both LM418^T and *Sympodiomycopsis paphiopedili* assimilate glucose, maltose, raffinose, D-mannitol, D-glucitol, and Na Succinate, and do not assimilate L-rhamnose, hexadecane, nor produce starch. Neither ferments glucose. It should be reiterated that comparative data for *Q. cyancescens* and *M. juglandis* are unavailable, while the DBB test indicated LM418^T is a basidiomycota. In addition to differences observed in assimilation results, *S. mahdii* differs from *S. paphiopedili* on the basis of 32 nucleotides in the D1/D2 regions of the 28S gene and the production of a pale pink pigment, while both exhibit sympodial budding in their formation of true mycelia.

I. Statistical Analyses

The average number of CFU from water samples collected from the same islands and Palmyra Atoll were compared with the non-parametric Kruskal-Wallis test in Minitab (Minitab, 2004). This test was also used to compare the CFU of fungi and yeast at different depths at Station ALOHA. This non-parametric test is ideal for samples which are not normally distributed, and where sample sizes may be unequal. The calculation converts observations to ranks, and uses the following formula:

$$K_1 = n_1 n_2 + \frac{n_2(n_2+1)}{2} - R_2$$

$$K_2 = n_1 n_2 + \frac{n_1(n_1+1)}{2} - R_1$$

Where n = number of observations, R = sum of ranks of observations in each sample

CHAPTER IV. RESULTS

Between August 20, 2004 and July 17, 2006, 379 samples were collected from coastal waters and other marine-associated materials around six of the high Hawaiian Islands and Palmyra Atoll. Water samples (n = 169) only were collected at standard depths between the surface and ~5000 m during six cruises to Station ALOHA. This is the most extensive study of marine mycology ever conducted in the Hawaiian Islands, and the first of such at Station ALOHA. Fungi and yeasts in all water samples were enumerated on three different enrichment media, and representative colonies and hyphal growth (based on colony characteristics) transferred to the same enrichment media for purification. Enumerating fungi and yeasts in other samples was a secondary consideration since the diversity of materials tested makes comparisons difficult and likely unreliable, at least on the basis of CFU per gram of material; the number of CFU arising from the different diluents used to wash and serially dilute sand cannot be compared with the number of CFU arising from a piece of driftwood settled on the same media (Kohlmeyer, 1979). A significant product of this work is the first locally housed collection of fungi and yeasts isolated from Hawaiian marine habitats. In addition to using traditional cultivation methods to enumerate and bring these taxa into culture,

DNA-based molecular methods enabled the tentative identification of isolated strains by relatively rapid sequencing of fragments of their ribosomal genes. A second advantage of this combined 'traditional' and molecular approach is that it minimizes redundancy in the collection, an important consideration given the effort required to maintain a large collection, and difficulties microbiologists have historically had in differentiating taxonomically distant microorganisms that may share similar physiological profiles, pigmentation, and even colony size and shape.

By August 2006, 689 pure fungi and yeast cultures had been prepared from all samples. The majority of these cultures were sequenced in terms of their ITS1 and 2 or D1/D2 regions. Indeed, of the 592 cultures in which these sequences were determined, 272 (46%) share less than 97% nucleotide identity over one or both these regions with the nearest described species, *i.e.*, one for which a type strain has been published in the peer reviewed literature. The implication of these data is that significant phylogenetic novelty exists in both the collection established here, and by definition in the Hawaiian marine environment. Several non-fungal taxa were also cultivated, including five viridiplantae (algae), and two antibiotic resistant bacteria determined to be *Serratia marcesens* and *Pseudomonas putida*. The former is commonly

cultivated from environmental samples. *Ps. putida* has a more cosmopolitan distribution, having been implicated in nosocomial infections and showing promise in bioremediation through its ability to degrade organic solvents, and even to convert styrofoam to biodegradable polyhydroxyalkanoate plastics (Ward *et al.*, 2006). Two protozoans and one stramenopile were also brought into pure culture. These non-fungal taxa will not be discussed further here.

Taken as a whole, the phylogenetic affiliations of all cultures in the collection reflect that which would be expected for samples of marine origin. On the basis of only BLAST*n* results, for which nearest neighbors of the test sequence are listed, 398 Ascomycete cultures were defined, and these comprise 68% the collection, the 175 Basidiomycetes are 30%, and the remainder comprises just 10 (2%) Zygomycetes (Fig. 25).

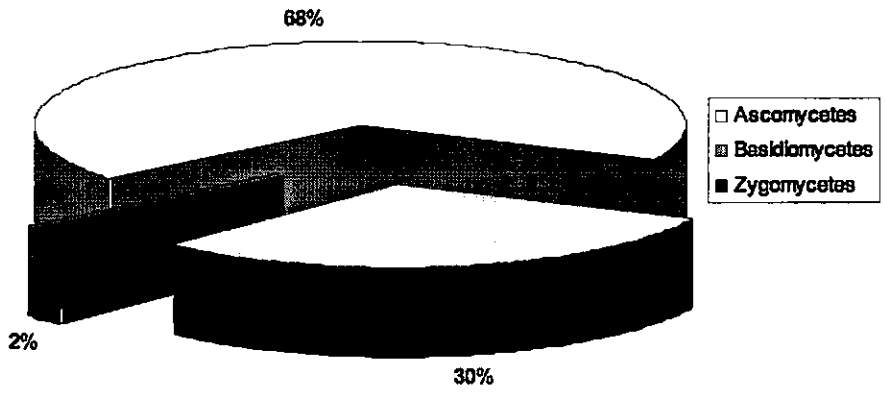


Figure 25. Phylogenetic affiliations of entire collection

This ratio of major taxa is consistent with previously published reports of such work (Kohlmeyer 1979; Sieburth, 1979). In terms of each island's contributions to the collection, the majority of isolates sequenced to date were isolated from O'ahu (141 pure cultures), of which 82% were Ascomycetes (116), 16% Basidiomycetes (23), and 2% Zygomycetes (2) (Fig. 26). 59 isolated cultures came from Kaua'i, 73% Ascomycetes (43), 22% Basidiomycetes (13), and 5% Zygomycetes (3) (Fig. 26). Moloka'i yielded 66 sequenced isolated cultures with 78% Ascomycetes (52), 17% Basidiomycetes (22), and 5% Zygomycetes (3) (Fig. 26); Maui had 55 sequenced cultures which were 73% Ascomycetes (40), 25% Basidiomycetes (14), and 2% Zygomycetes (1) (Fig. 26); Lana'i represented 16 of the entire

collection and was comprised of 63% Ascomycetes (10), and 37% Basidiomycetes (Fig. 26); Hawai'i had 84 sequenced cultures, 74% Ascomycetes (62), and 26% Basidiomycetes (22) (Fig. 26).

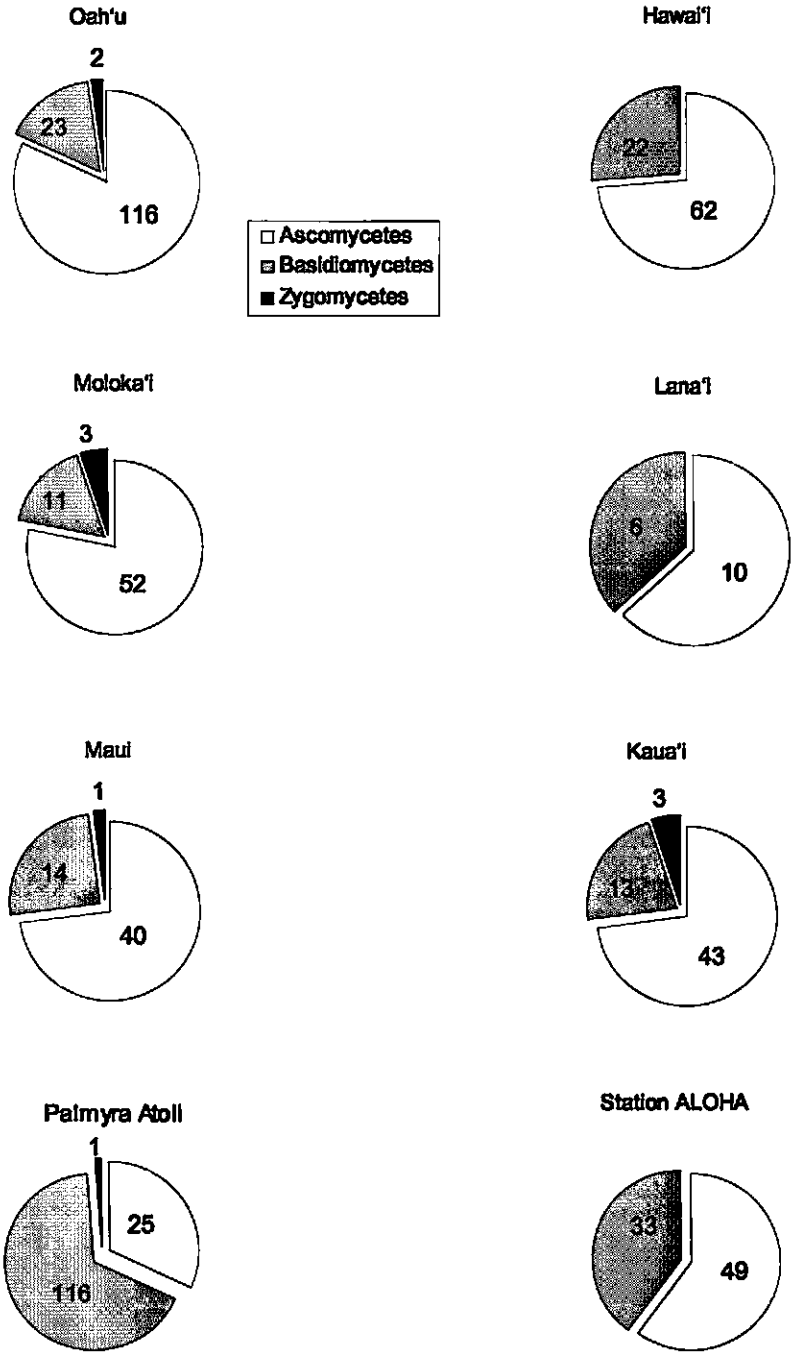


Figure 26. Phylum level phylogenetic affiliations of fungi and yeast cultivated from marine samples in the Hawaiian Islands and at Palmyra Atoll (Line Islands) between August 2004 and August 2006.

The phylogenetic composition of the mycoflora cultivated from Palmyra Atoll differs markedly from that of the six high Hawaiian islands. For example, of the 79 cultures from Palmyra sequenced to date, just 32% were Ascomycetes (25), 67% Basidiomycetes (53), and 1% Zygomycetes (1) (Fig. 26) No Zygomycetes were cultivated from Station ALOHA, but 81 other sequenced isolates comprised 60% Ascomycetes (49) and 40% Basidiomycetes (Fig. 26).

Zygomycetes were cultivated from all sites except Lana'i, Hawai'i and Station ALOHA. The latter in particular is consistent with the fact that currently described Zygomycetes are primarily of terrestrial origin. One would not expect predominantly, if not exclusively terrestrial taxa to be represented at an open-ocean location such as ALOHA and more so below the mixed layer at this location. That Zygomycetes were actually present in any sample is of interest given that this was a marine-centric study, but the fact is that with the exception of collections at Station ALOHA, all samples here were taken from coastal sites. Their relatively poor representation is thus consistent with reports that Zygomycetes in marine samples are rare (Kohlmeyer 1979; Sieburth, 1979; Kurtzman & Fell, 2000; Maddison, 2006). Only ten

Zygomycetes were cultivated overall, and two of these share a ~95% (which gene) sequence identity with their nearest described neighbor in the public domain. These collections and isolations represent the first of marine Zygomycetes around Hawai'i and Palmyra Atoll, and have the potential basis to establish marine Zygomycetes as representative class of marine fungi. The data described here for fungi and yeast abundance and phylogeny at Palmyra Atoll are significant because the marine mycoflora here has never been examined. Indeed, studies of fungi and yeasts in any isolated atoll lagoon are noticeably absent from the literature.

The diversity of species cultivated at Palmyra varies from those found in the Hawaiian Islands and at Station ALOHA, with the majority belonging in the Basidiomycetes (Fig. 26). According to BLAST results, most species were recorded in samples taken around O'ahu, while only 4 distinct species were collected around Lana'i.

The greatest number of distinct species, described here as a function of the different taxa listed in the BLAST*n* outputs, was derived from water samples, while animal sources provided the lowest number of taxa (Tab. 5).

Table 5. Number of different fungi and yeasts grouped by island or location.*

Source	No. of species*
O'ahu	79
Kaua'i	47
Maui	43
Moloka'i	47
Lana'i	14
Hawai'i	55
Station ALOHA	38
Palmyra Atoll	50

*Isolates (or cultures) are considered to belong to the same 'species' if they share ≥97% nucleotide identity over aligned ITS1 and ITS 2, or D1/D2 regions of the 18S to 28S ribosomal genes.

The 272 potentially novel species (those cultures sharing <97% sequence identity with their nearest neighbors in GenBank) were sorted on the basis of their origins, *i.e.*, type of material. This view showed that 70% (n = 13) of cultures from 'foam' were potentially novel; given that foam is considered an effective trap for marine ascospores, basidiospores and conidia (Kohlmeyer, 1979), this seems a reasonable observation. The 'lowest' rate of novelty for cultivated organisms came from sand, avian guano, and water, within each of which less than 40% of cultures were potentially novel (Fig. 27).

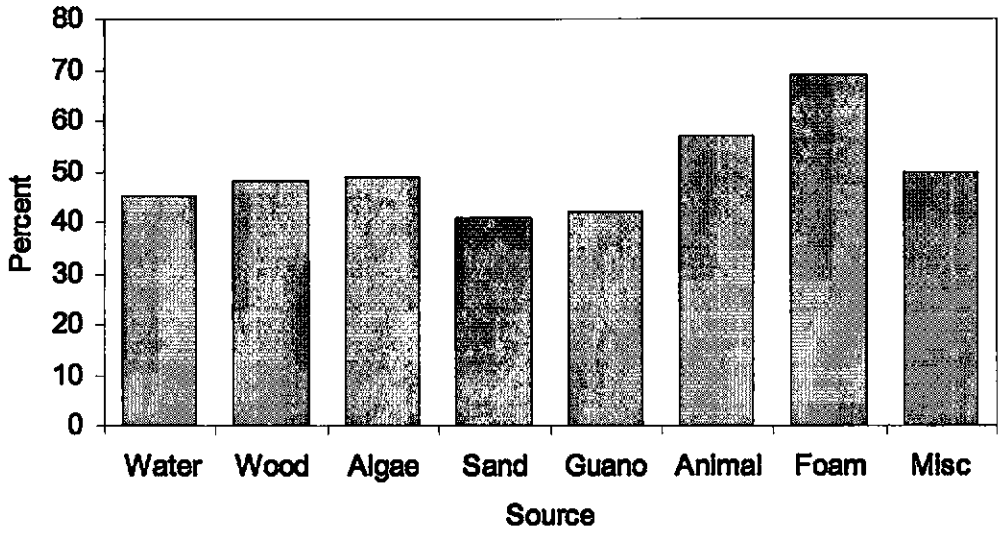


Figure 27. Percent novelty of cultivations by source

While even this rate can hardly be considered low, it was anticipated that avian guano would be a greater source of novelty considering the abundance of cultivate. Geographically, the highest *percentage* of novel cultures from one site came from Moloka'i (75%, n = 65) and Kalaupapa Peninsula in particular, followed by Palmyra Atoll, and Hawai'i where 50 - 60% of cultures are potentially novel (Fig. 28).

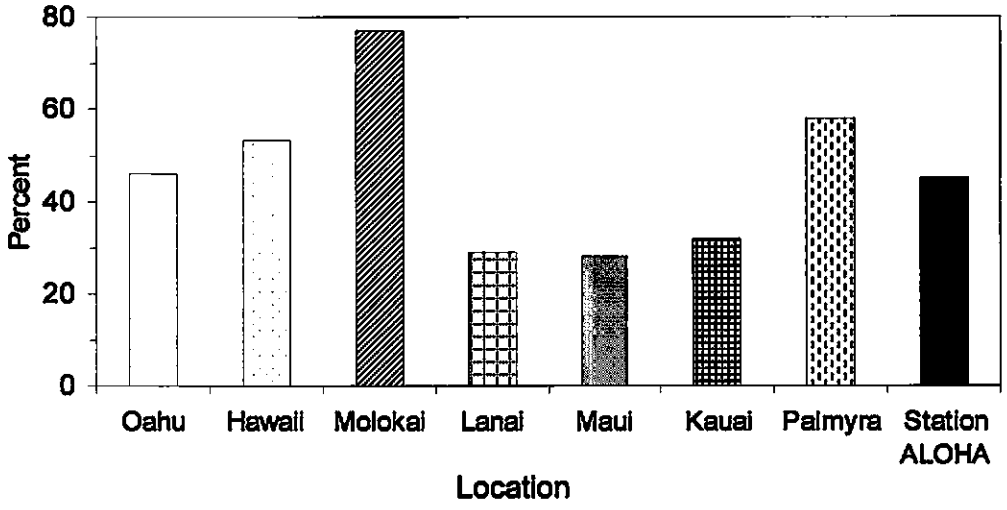


Figure 28. Percent novelty among cultures, by location

Kalaupapa is a National Park with few residents, and strict regulation of visitor numbers and activities. Indeed, NPS provided a guide and a 4-wheeled drive vehicle to assist collections around Kalaupapa, while some sites could only be reached after hours of rigorous hiking. Geographic isolation and little human interference may be instrumental in maintaining a high diversity in waters off Moloka'i and the Kalaupapa Peninsula in particular. The same factors and large populations of nesting seabirds may be invoked to explain the high percentage of novel yeast cultivated from Palmyra (cf. Fell & Uden, 1963).

Hawai'i is also rugged, and a 4-wheel drive vehicle and considerable hiking were required to access the shoreline. By the same token, Palmyra Atoll is an isolated small island surrounded by deep ocean; most samples were easily collected along the shore or by accessing the lagoons by boat. Almost one-third (88) of potentially novel species from all habitats were Basidiomycetes, a significant finding given that only ~24 obligate marine Basidiomycetes species have been described (Kohlmeyer 1979; Sieburth, 1979; Kurtzman & Fell, 2000; Maddison, 2006). A reevaluation of this opinion in light of the data presented here could have significant repercussions for how Basidiomycetes are viewed in terms of their role in marine nutrient cycling.

A total of 581 representative ITS1 and ITS2, and D1/D2 nucleotide sequences submitted to GenBank at the NCBI have been assigned accession numbers EF060395 to EF060975.

The number of CFU recorded per liter of seawater from each site ranged from 55 to 356; the mean CFU for the Hawaiian Islands and Palmyra was 225/L (Fig. 29). CFU in the upper 500 m at Station ALOHA numbered 22/L, and 26/L below 500 m (Fig. 30).

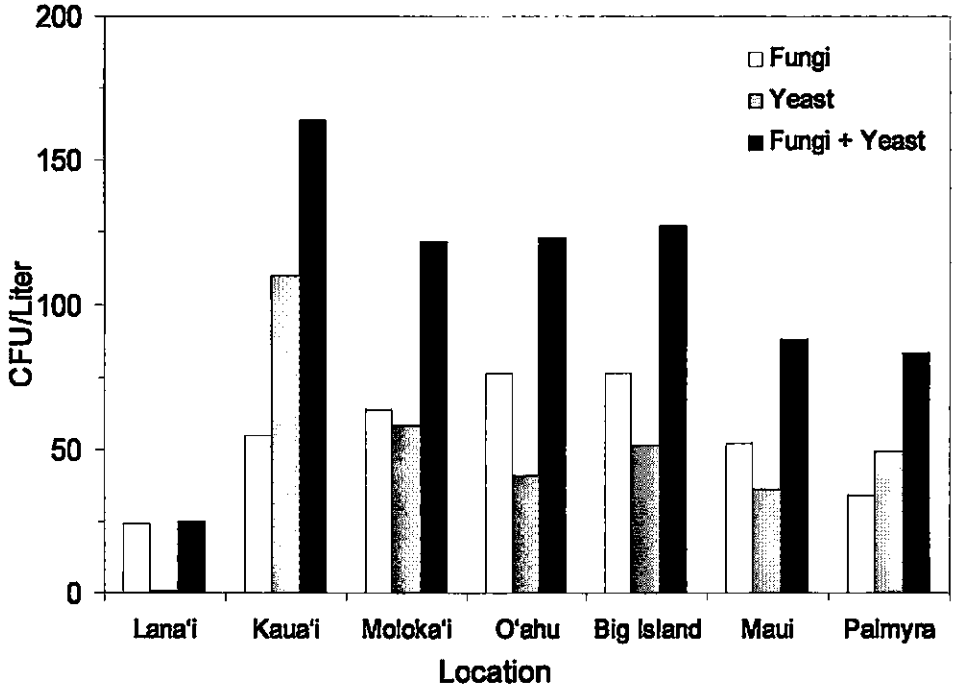


Figure 29. Average CFU per liter by location

These counts fall within previously published values of for fungi and yeast densities in tropical waters (Fell & Uden, 1963; Taysi, 1964). Others have recorded average counts from 95 CFU/L to nearly 2000, with this variability being attributed to factors such as the level of pollution, temperature, and nutrient availability (Taysi, 1964;Buck, 1965; Hagler, 1981). The shape of the depth profile for CFU distribution at Station ALOHA is typical of those for fungi and yeasts, and for heterotrophic microorganisms in general (Fell & Uden, 1963). Such profiles tend to reflect the availability of organic matter, the position of the oxygen minimum, and depth of mixing. Such factors vary

temporally at Station ALOHA; sampling here was conducted over a period of 20 months.

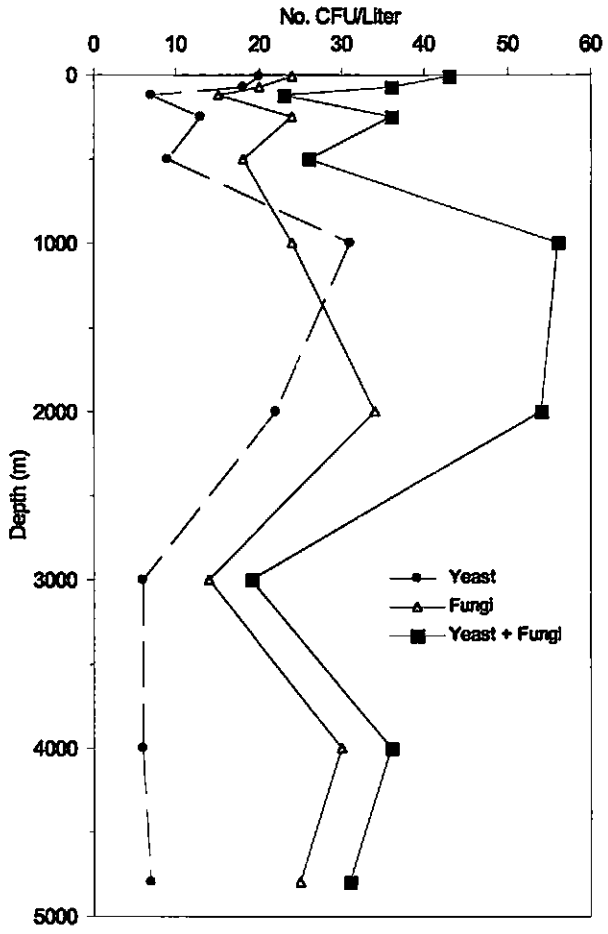


Figure 30. Depth profile of mean fungi and yeast CFU (six cruises) at Station ALOHA.

Statistical analysis determined there was no significant difference of CFU per liter between island locations (Tab. 6), or between depths at Station ALOHA including a comparison between shallow (<500 m) and deep (>500 m) waters. (Tab. 7)

Table 6. Kruskal-Wallis analysis of numbers of Fungi and yeast CFU determined at Hawai'i and Palmyra Atoll

Kruskal-Wallis Test: log CFU vs. Island (total fungi and yeast)

K-W H_0: The observed frequencies are homogeneous and the departure is due to sampling error or scatter.

K-W H_1: The observed frequencies depart from those expected of a homogeneous distribution by an amount that cannot be explained by sampling error.

Island	N	Median	Av. rank	Z
Big Island	24	4.041	53.9	-0.2
Kaua'i	14	4.429	64.3	1.18
Lana'i	7	2.485	19.7	-3.05
Maui	21	4.248	56	0.17
Moloka'i	19	4.511	61.6	1
O'ahu	14	3.8	53.8	-0.15
Palmyra	10	4.465	56.4	0.15
Overall	109		55	

Kruskal-Wallis test on log transformed CFU data

H = 10.86, df = 6, p = 0.093

H = 10.86, df = 6, p = 0.093 (adjusted for ties)

Accept H_0: There is no statistical difference in the means of the populations from the data set.

Kruskal-Wallis Test: log CFU vs. Island (fungi only)

K-W H_0: The observed frequencies are homogeneous and the departure is due to sampling error or scatter.

K-W H_1: The observed frequencies depart from those expected of a homogeneous distribution by an amount that cannot be explained by sampling error.

Island	N	Median	Av. Rank	Z
Big Island	24	3.569	54.3	-0.13
Kaua'i	14	3.884	58.1	0.4
Lana'i	7	2.398	26.8	-2.44
Maui	21	3.761	57.5	0.41
Moloka'i	19	3.951	63.7	1.32
O'ahu	14	3.713	61.4	0.81
Palmyra	10	3.146	41.3	-1.44
Overall	109		55	

Kruskal-Wallis test on log transformed CFU data
H = 9.76, df = 6, p = 0.135
H = 9.76, df = 6, p = 0.135 (adjusted for ties)
Accept H_0: There is no statistical difference in the means of the populations from the data set.

Kruskal-Wallis Test: log CFU* vs. *Island (yeast only)
K-W H_0: The observed frequencies are homogeneous and the departure is merely due to sampling error or scatter.
K-W H_1: The observed frequencies depart from those expected of a homogeneous distribution by an amount that cannot be explained by sampling error.
104 cases were used
5 cases contained missing values

Island	N	Median	Av. Rank	Z
Big Island	23	2.773	54.3	0.32
Kaua'i	14	2.850	59.9	0.99
Lana'i	5	0.000	11.4	-3.12
Maui	20	2.835	55.5	0.49
Moloka'i	19	2.944	54.6	0.33
O'ahu	13	1.792	42.9	-1.23
Palmyra	10	3.598	61.3	0.96
Overall	104		52.5	

Kruskal-Wallis Test on log transformed CFU data
H = 12.65, df = 6, p = 0.049
H = 12.67, df = 6, p = 0.048 (adjusted for ties)
Accept H_0: There is no statistical difference in the means of the populations from the data set

Table 7. Kruskal-Wallis analysis of numbers of Fungi and yeast CFU determined at different depths at Station ALOHA.

Kruskal-Wallis Test: log_{10} (CFU)* vs. *depth (m) (total fungi and yeast)
K-W H_0: The observed frequencies are homogeneous and the departure is merely due to sampling error or scatter.
K-W H_1: the observed frequencies depart from those expected of a homogeneous distribution by an amount that cannot be explained by sampling error.
149 cases were used

12 cases contained missing values

Depth (m)	N	Median	Av. Rank	Z
5	20	2.398	75.8	0.08
75	16	2.565	75.8	0.08
125	15	1.792	60.9	-1.33
250	18	2.697	72.3	-0.28
500	20	2.562	69.5	-0.62
1000	23	3.135	87.6	1.52
2000	10	3.803	93.8	1.42
3000	9	2.89	68.9	-0.44
4000	9	1.792	63.8	-0.8
4750	9	3.664	77.2	0.16
Overall	149		75	

H = 6.67, df = 9, p = 0.671

H = 6.68, df = 9, p = 0.671 (adjusted for ties)

Accept H_0: There is no statistical difference in the means of the populations from the data set.

Kruskal-Wallis Test: log_{10} (CFU) vs. depth (m) (fungi only)

144 cases were used

17 cases contained missing values

Depth (m)	N	Median	Av. Rank	Z
5	20	2.35	72.6	0.01
75	16	2.525	76.4	0.4
125	16	1.869	62	-1.06
250	16	2.072	65.2	-0.74
500	19	2.485	70.9	-0.17
1000	21	2.565	77.4	0.58
2000	10	2.909	85.5	1.02
3000	9	2.485	64.1	0.62
4000	9	1.792	72.4	0
4750	8	3.22	84	0.8
Overall	144		72.5	

H = 3.89, df = 9, p = 0.918

H = 3.90, df = 9, p = 0.918 (adjusted for ties)

Accept H_0: There is no statistical difference in the means of the populations from the data set.

Kruskal-Wallis Test: log_{10} (CFU) vs. depth (m) (yeast only)

93 cases were used

68 cases contained missing values

Depth (m)	N	Median	Ave Rank	Z
5	11	2.398	55.4	1.09
75	9	1.386	40.7	-0.73
125	9	1.946	39.4	-0.89
250	13	2,197	46.5	-0.07
500	12	1.386	40	-0.96
1000	16	3.006	53.3	1.03
2000	9	3.091	53.4	0.75
3000	5	1.609	42.4	-0.39
4000	4	2.282	50.1	0.24
4750	5	2.565	42	-0.43
Overall	93		47	

H = 4.83, df = 9, $p = 0.849$

H = 4.86, df = 9, $p = 0.846$ (adjusted for ties)

NOTE One or more small samples

Accept H_0: There is no statistical difference in the means of the populations from the data set.

Phylogenetic trees constructed using all the sequenced data collected during this study, and sequence strains within trees were color coded (Fig. 31), demonstrating the diversity of marine fungi and yeast cultivated. While most species were cultivated by one or more islands, some locations appeared to have a correlation with a specific species or vice versa. For example, all *Rhodosporidium spaerocarpum* species, and *Sporidiobolus johnsonii* were collected from Station ALOHA and are known to be open ocean species (Fell & Uden, 1963, Kohlmeyer, 1979; Nagahama *et.al* 2001a). On two separate occasions the deep sea red yeast *Rhodotorula lamellibrachii* was cultivated,

once from Station ALOHA sharing less than 97% sequence identity with described taxa, and once from Palmyra Atoll, with a 95% sequence identity with described taxa (Nagahama *et.al* 2001a, b).

Clustering of one group of 9 cultivated isolates from Palmyra Atoll is closely related to *Bullera* sp. and *Cryptococcus luteolus* species, possibly indicating a new genus. Another cluster of 6 cultivated organisms from Moloka'i is closely related to *Pichia mexicana* and *Candidia tenuis*. These patterns could be an indication of island specific endemic species. A duplicate phylogenetic tree using all the data has also been constructed to illustrate the influence of source material on specific species cultivated (Fig. 32). As mentioned previously all *Rhodosporidium spaerocarpum* species were cultivated from water samples collected at Station ALOHA, but nearly all the *Rhodosporidium kratochvilovae* and *Rhodotorula mucilaginosa* species were also cultivated from water samples but coming from each island location with the exception of Palmyra Atoll. The other non-water source, were all from avian guano indicating the possibility open ocean water fungi and yeast are correlated to those which birds disperse through guano (Fell & Uden, 1963). Individual phylogenetic trees have been created for each location with color coding for

source material (Figs. 33-40). Among individual locations, there are no large groupings based on source material of any particular species indicating that most species are ubiquitous in the marine environment (Fell & Uden, 1963, Fell 1967, Kohlmeyer, 1979).

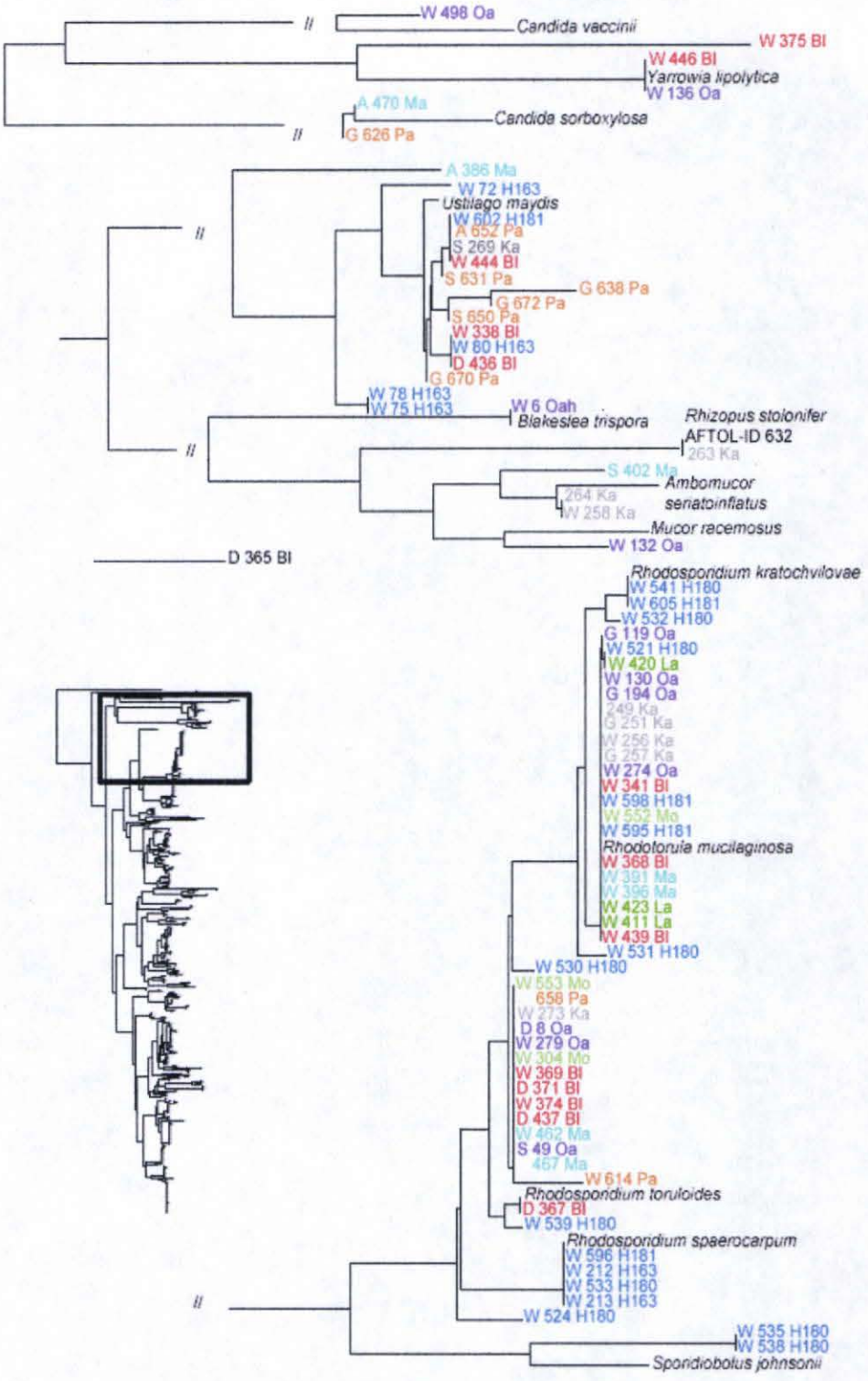


Figure 31. Phylogenetic Tree for entire collection indicating location by color. (Purple: O'ahu, Red: Hawai'i, light blue: Maui, Gray: Kaua'i, Green: Lana'i, light green: Moloka'i, Orange: Palmyra Atoll, Blue: Station ALOHA; W: water, D: wood, S: sand, A: algae, G: guano, F: foam).

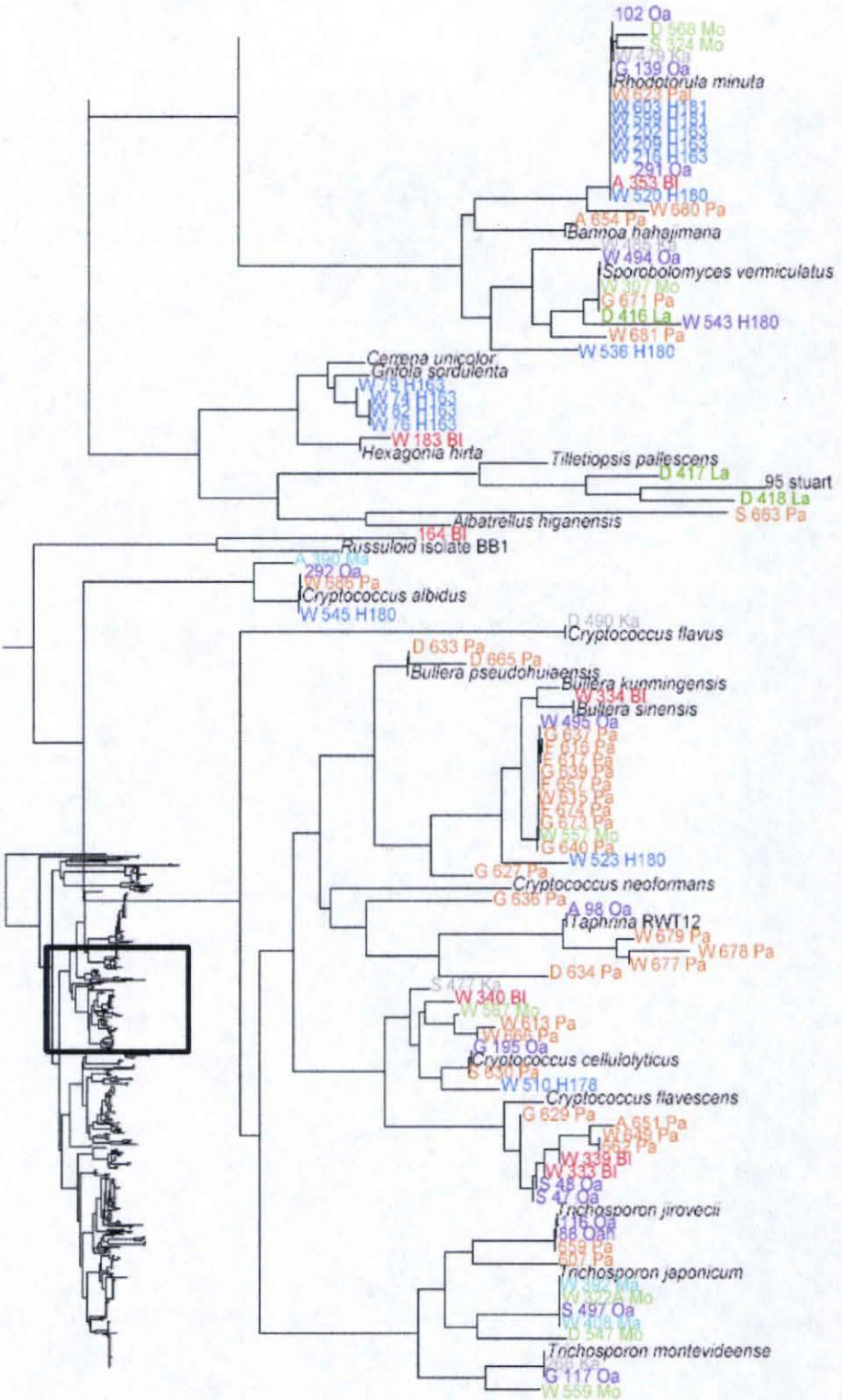


Figure 31. (Continued) Phylogenetic Tree for entire collection indicating location by color. (Purple: O'ahu, Red: Hawai'i, light blue: Maui, Gray: Kaua'i, Green: Lana'i, light green: Moloka'i, Orange: Palmyra Atoll, Blue: Station ALOHA; W: water, D: wood, S: sand, A: algae, G: guano, F: foam)

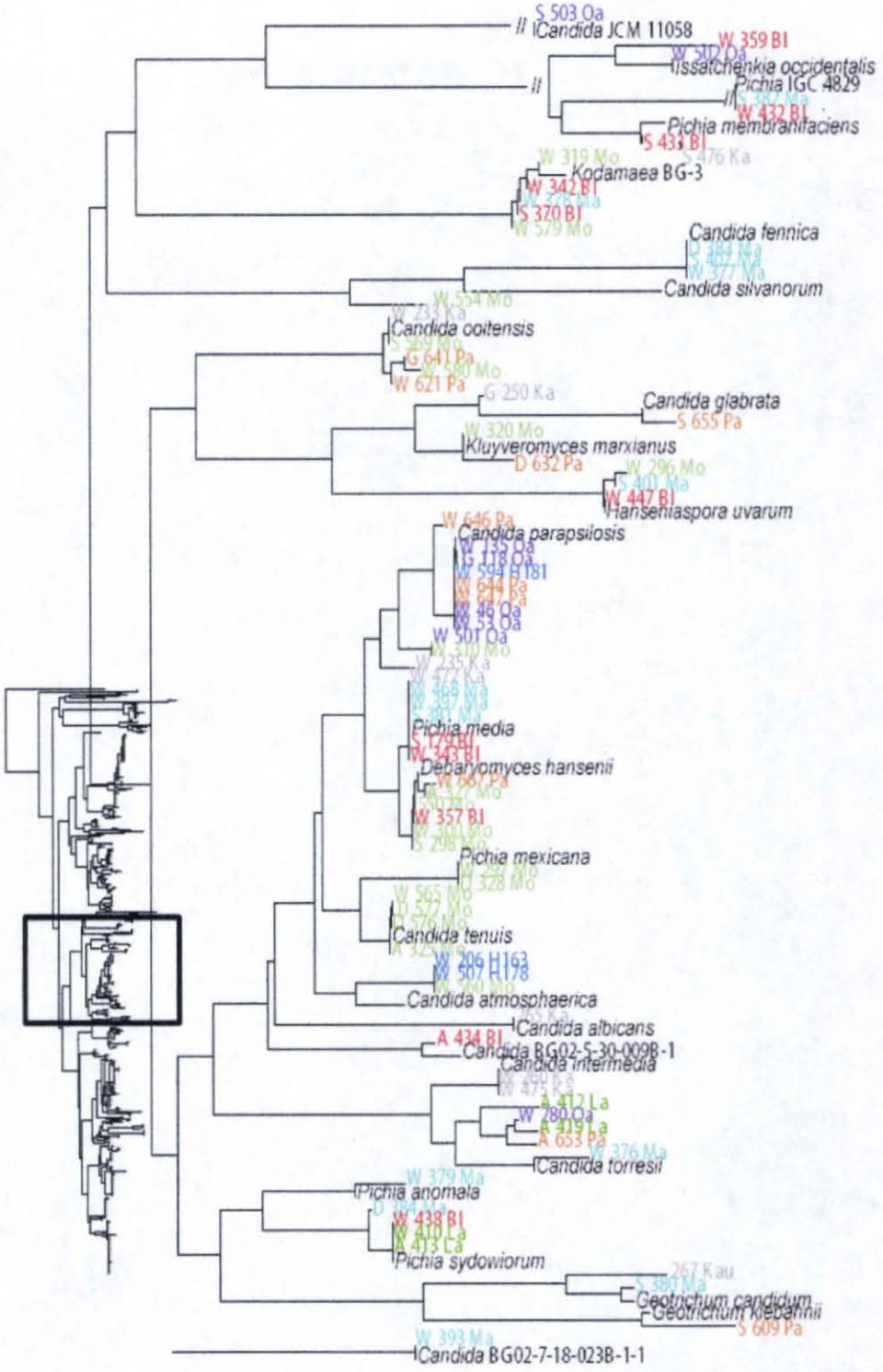


Figure 31. (Continued) Phylogenetic Tree for entire collection indicating location by color. (Purple: O'ahu, Red: Hawai'i, light blue: Maui, Gray: Kaua'i, Green: Lana'i, light green: Moloka'i, Orange: Palmyra Atoll, Blue: Station ALOHA; W: water, D: wood, S: sand, A: algae, G: guano, F: foam)

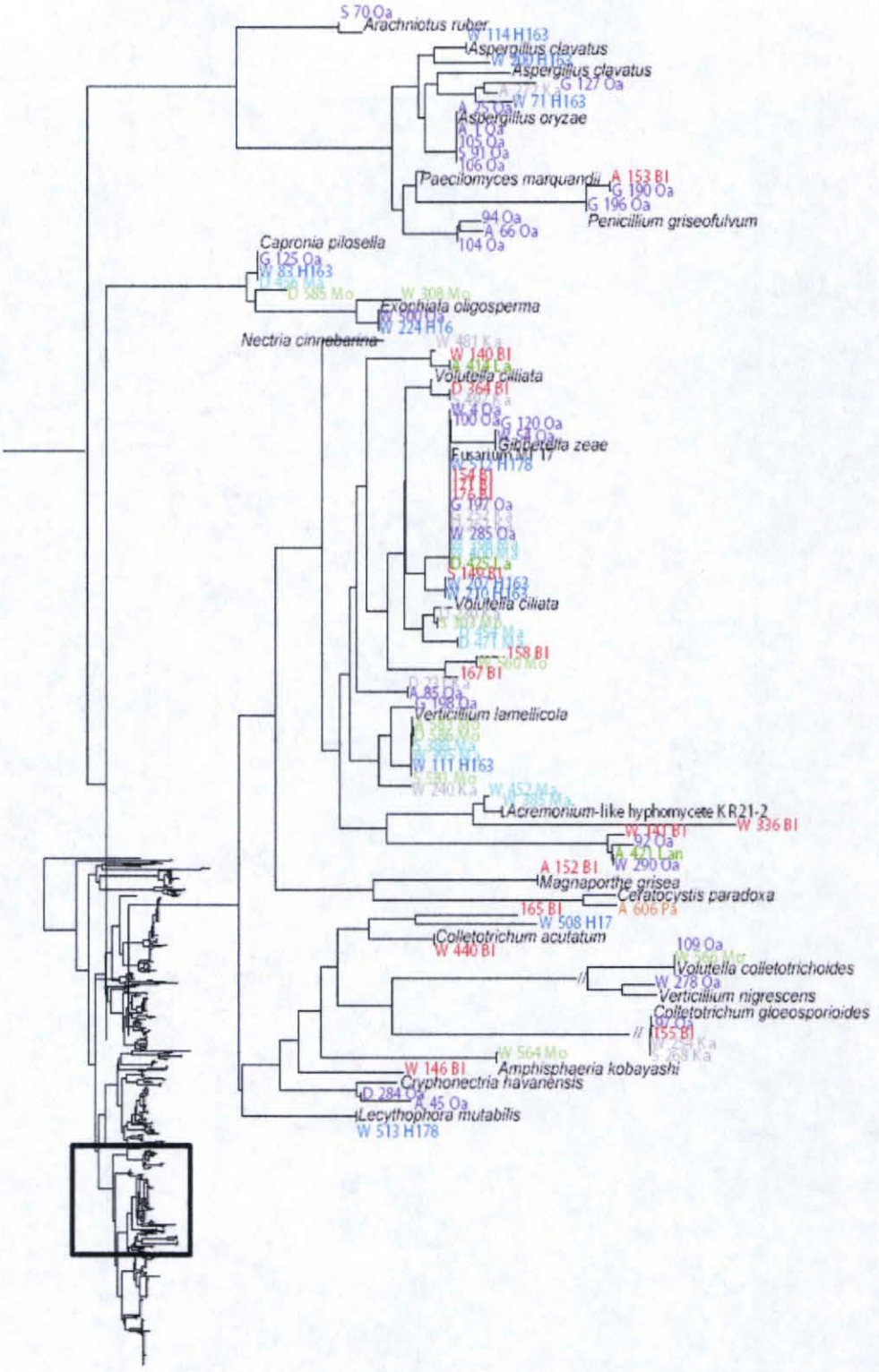


Figure 31. (Continued) Phylogenetic Tree for entire collection indicating location by color. (Purple: O'ahu, Red: Hawai'i, light blue: Maui, Gray: Kaua'i, Green: Lana'i, light green: Moloka'i, Orange: Palmyra Atoll, Blue: Station ALOHA; W: water, D: wood, S: sand, A: algae, G: guano, F: foam)

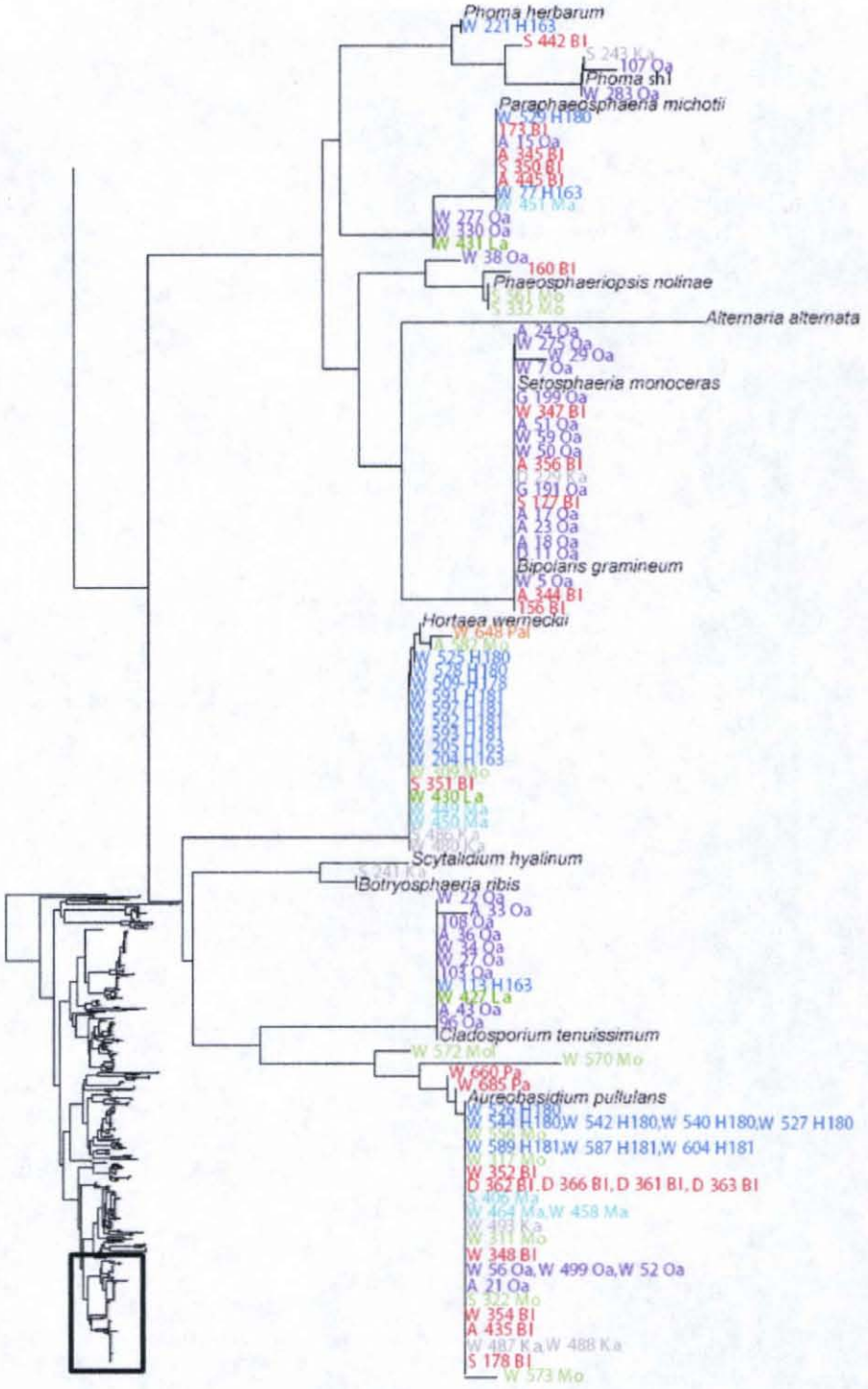


Figure 31. (Continued) Phylogenetic Tree for entire collection indicating location by color. (Purple: O'ahu, Red: Hawai'i, light blue: Maui, Gray: Kaua'i, Green: Lana'i, light green: Moloka'i, Orange: Palmyra Atoll, Blue: Station ALOHA; W: water, D: wood, S: sand, A: algae, G: guano, F: foam)

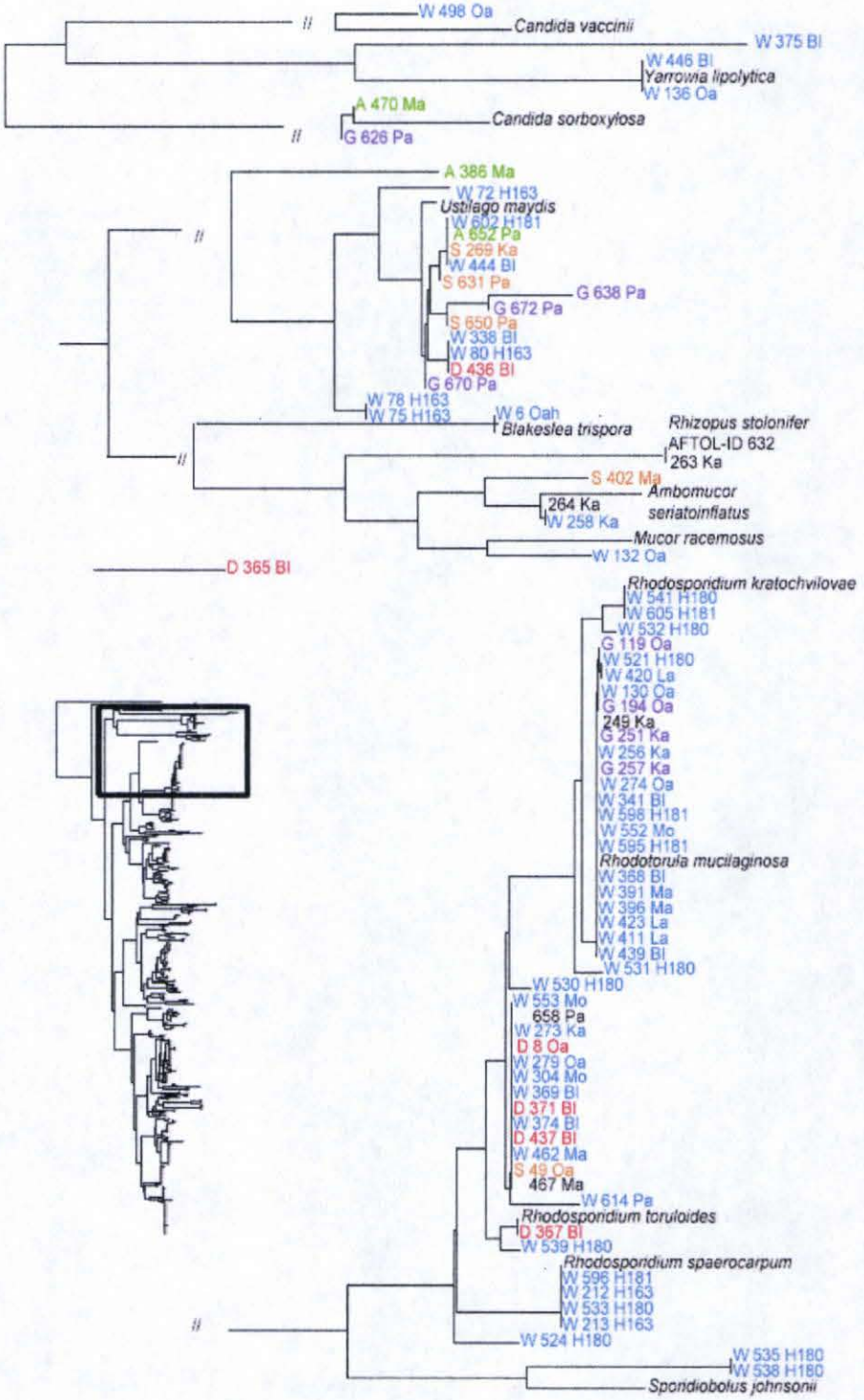


Figure 32. Phylogenetic Tree for entire collection indicating source origin by color. (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam; O'ahu : Oa, Kaua'i: Ka, Maui: Ma, Moloka'i: Mo, Hawai'i:Bl, Lana'i: La, Station ALOHA: H#, Palmyra Atoll:Pa)

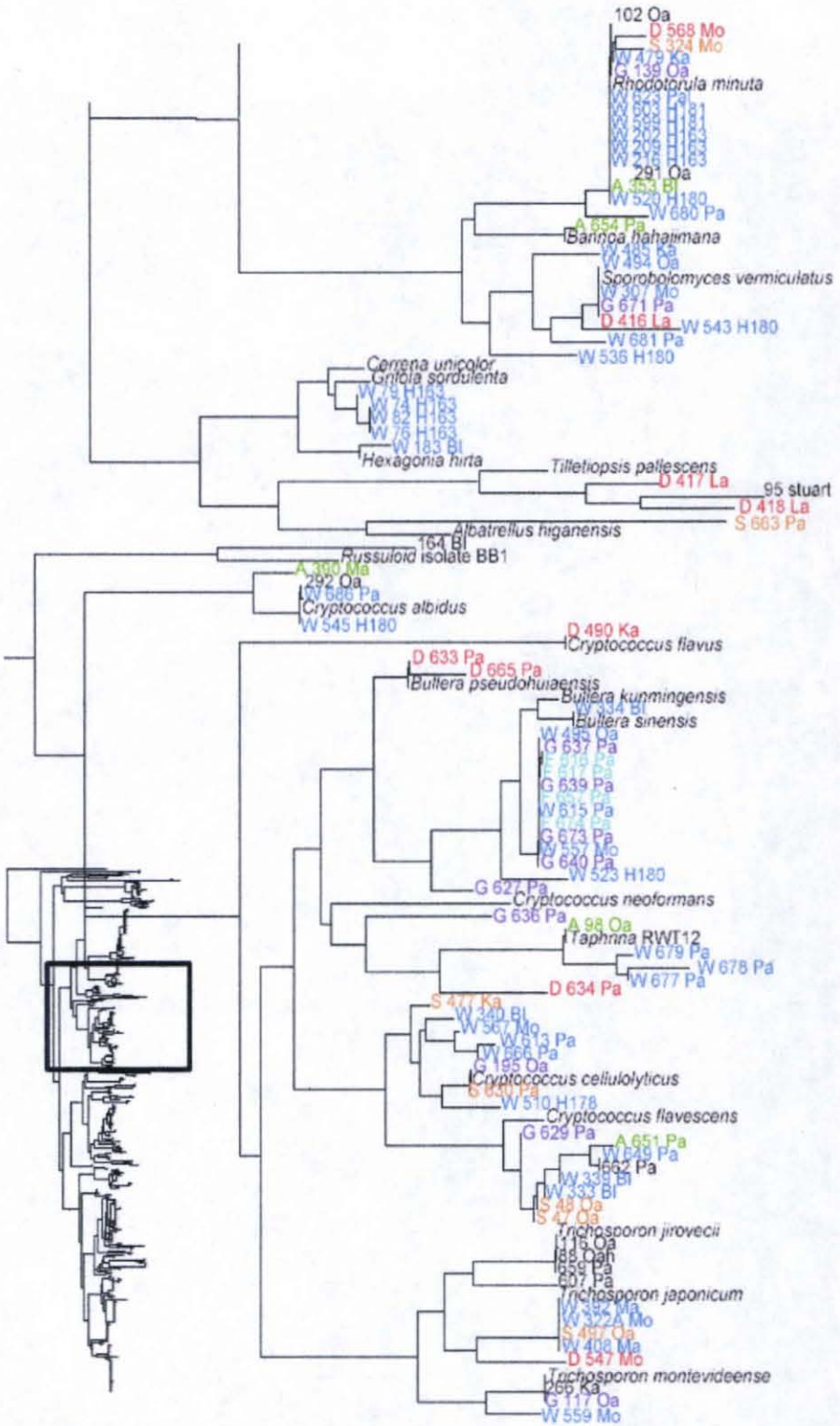


Figure 32. (Continued) Phylogenetic Tree for entire collection indicating source origin by color. (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam; O'ahu: Oa, Kaua'i: Ka, Maui : Ma, Moloka'i : Mo, Hawai'i :Bl, Lana'i : La, Station ALOHA: H#, Palmyra Atoll :Pa)

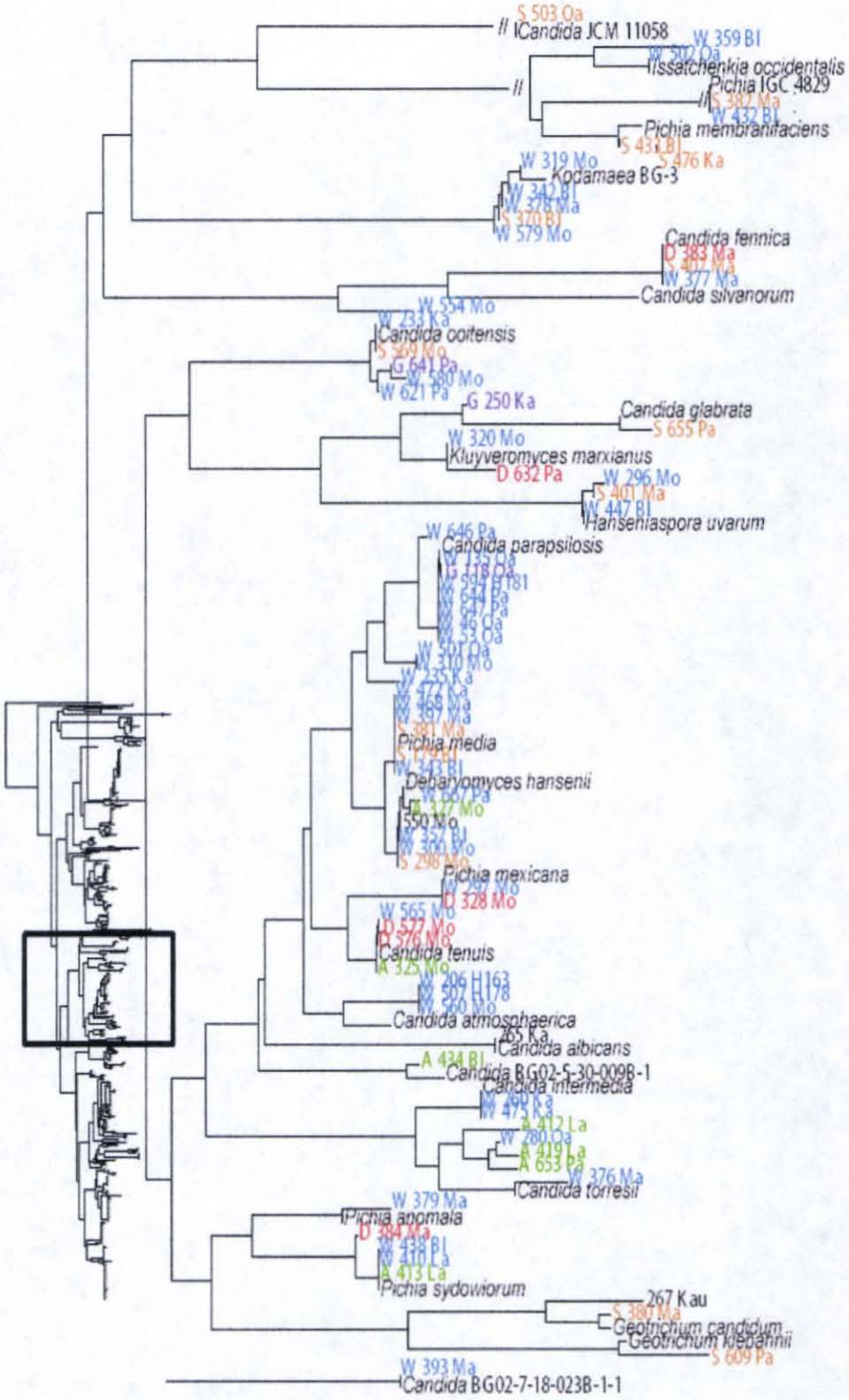


Figure 32. (Continued) Phylogenetic Tree for entire collection indicating source origin by color. (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam; O'ahu : Oa, Kaua'i : Ka, Maui : Ma, Moloka'i : Mo, Hawai'i :Bl, Lana'i : La, Station ALOHA : H#, Palmyra Atoll :Pa)

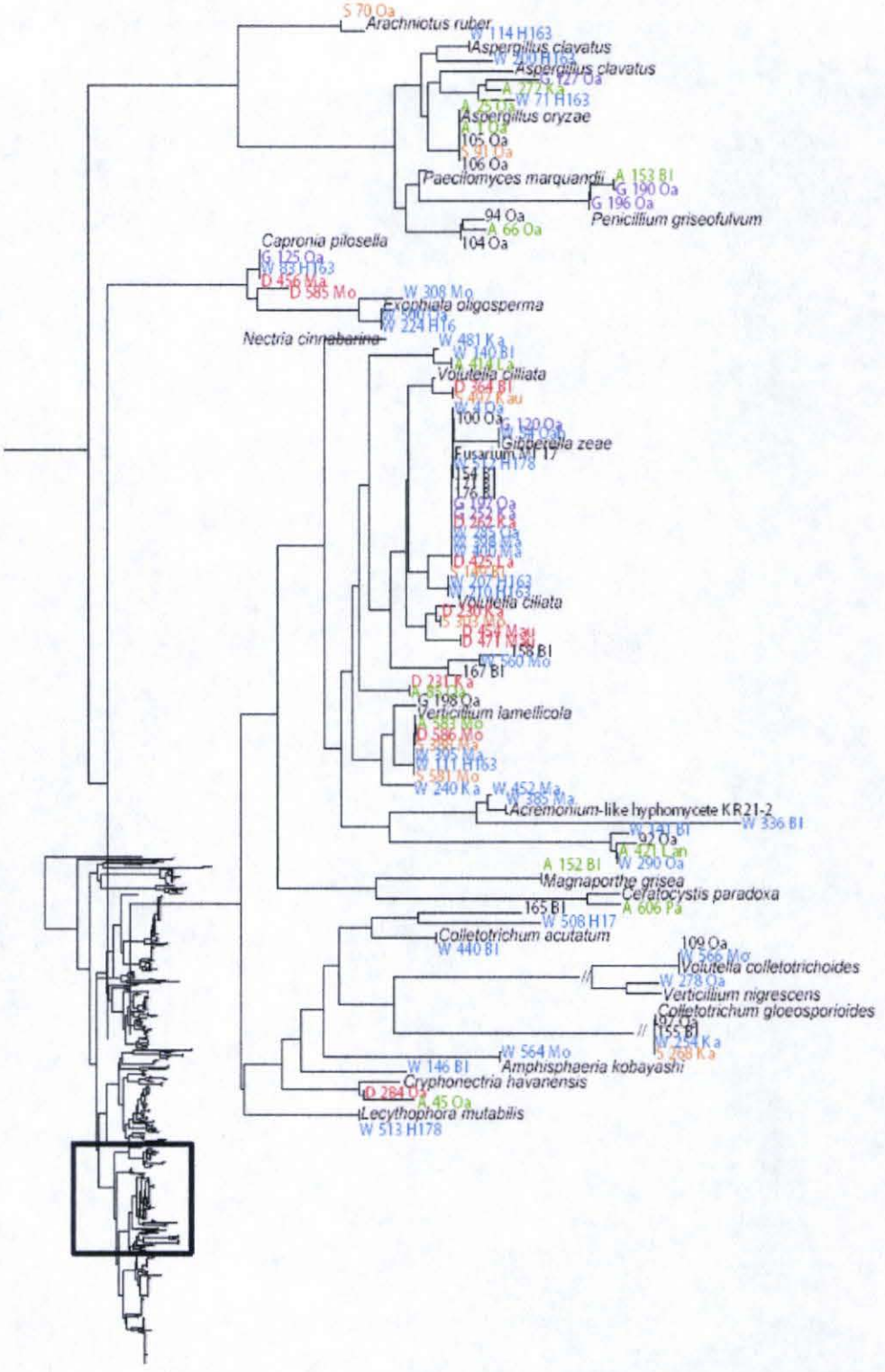


Figure 32. (Continued) Phylogenetic Tree for entire collection indicating source origin by color. (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam; O'ahu : Oa, Kaua'i : Ka, Maui : Ma, Moloka'i : Mo, Hawai'i :Bl, Lana'i : La, Station ALOHA : H#, Palmyra Atoll :Pa)

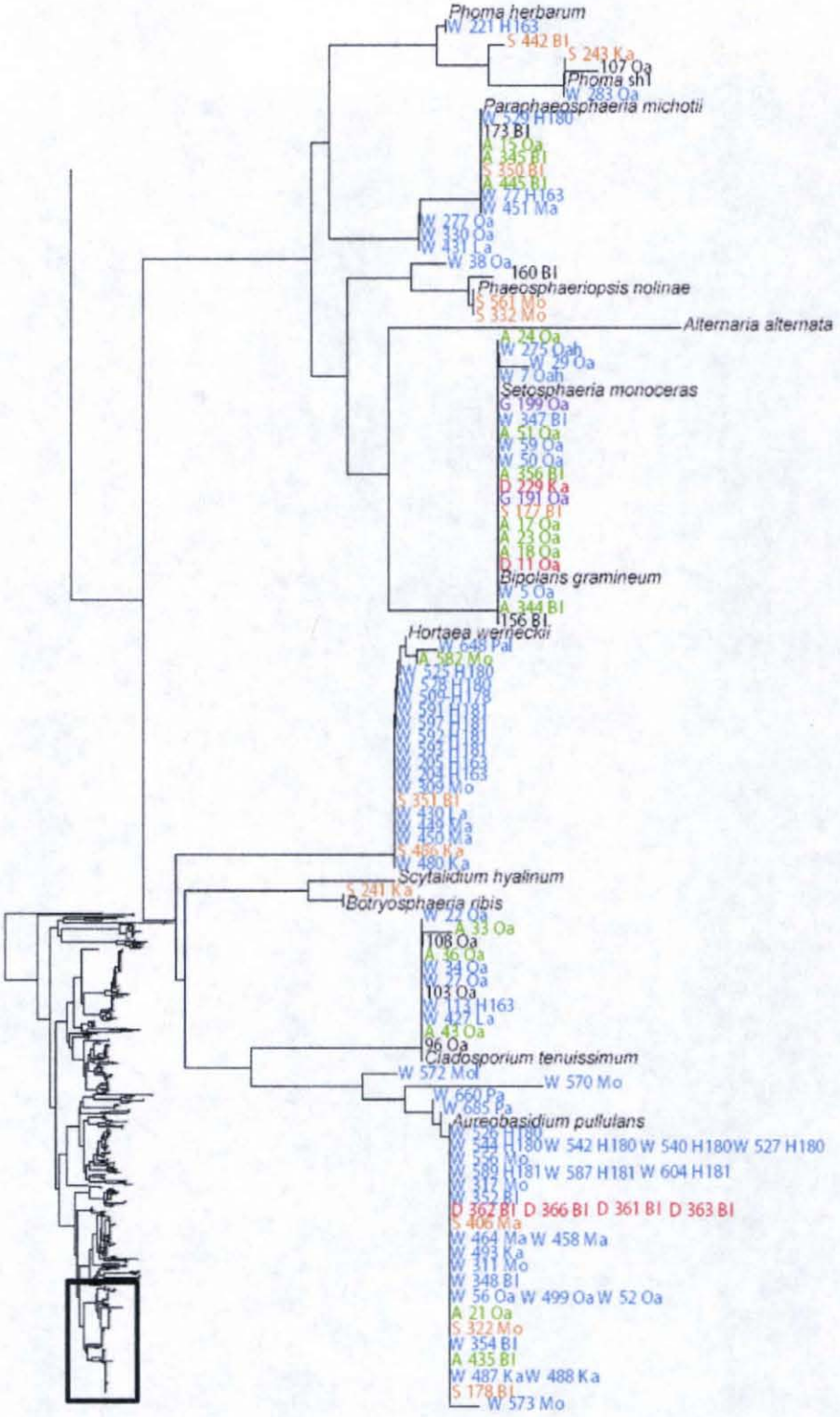


Figure 32. (Continued) Phylogenetic Tree for entire collection indicating source origin by color. (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam; O'ahu : Oa, Kaua'i : Ka, Maui : Ma, Moloka'i : Mo, Hawai'i :Bl, Lana'i : La, Station ALOHA : H#, Palmyra Atoll :Pa)

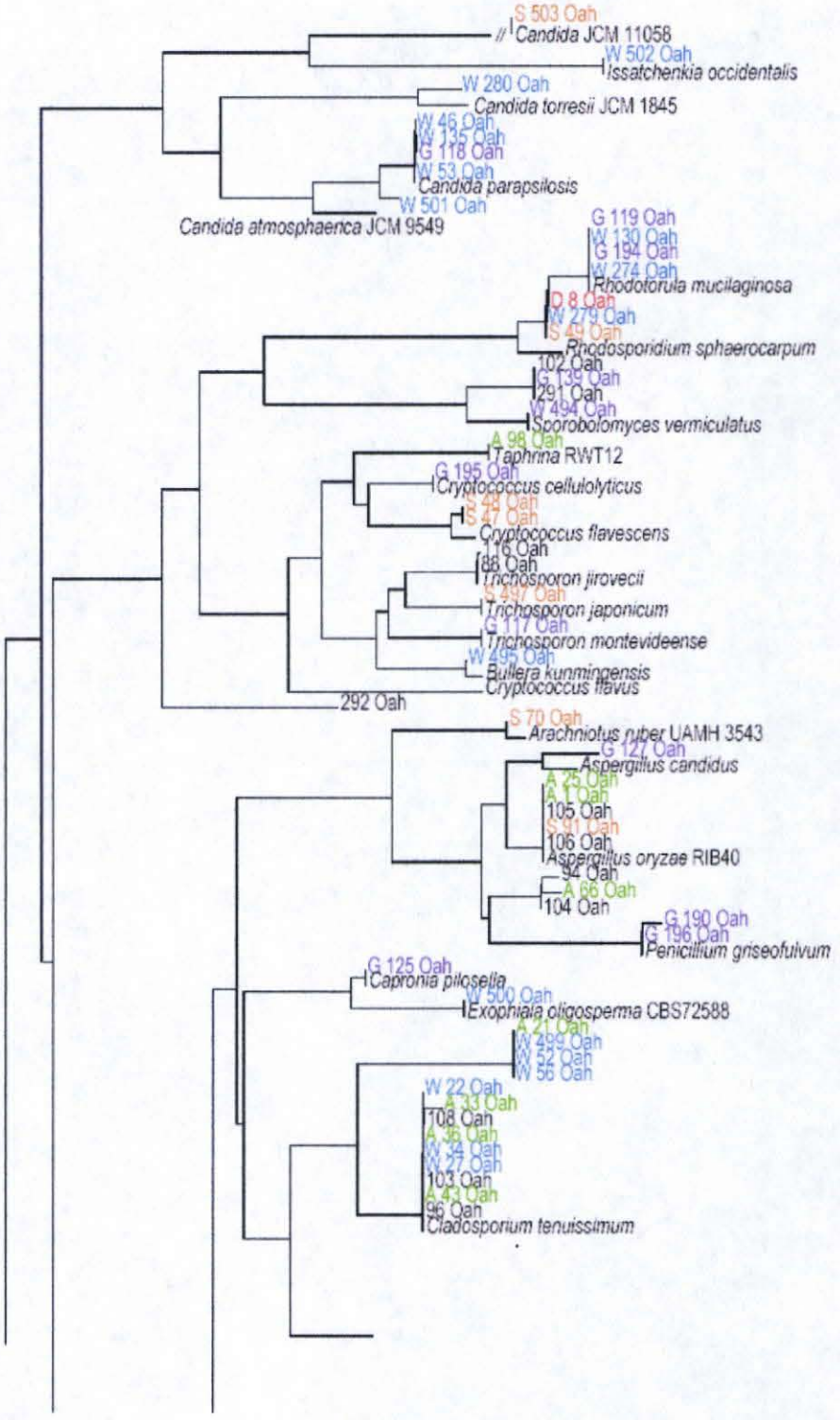


Figure 33. Phylogenetic Tree for O'ahu (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam)

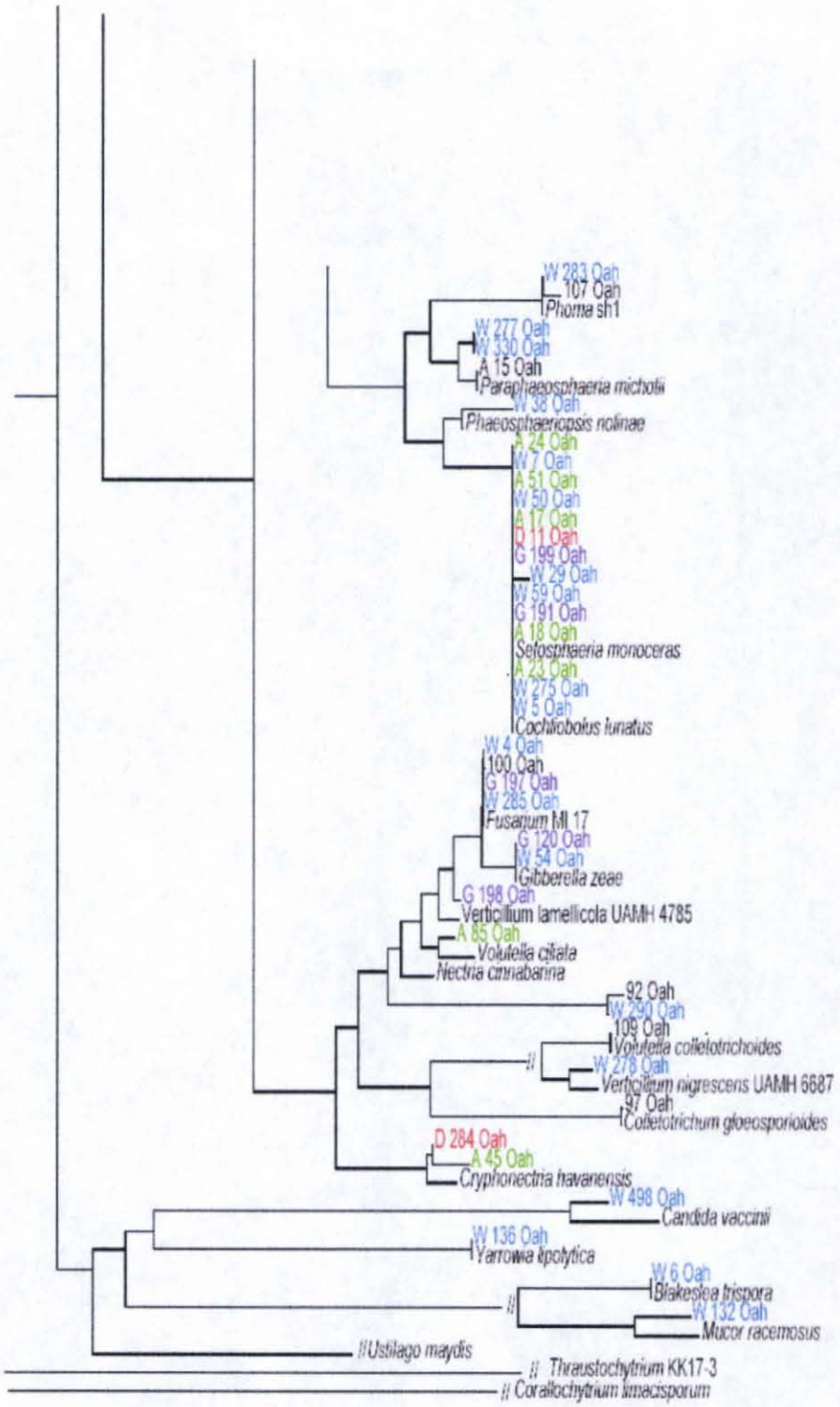


Figure 33. (Continued) Phylogenetic Tree for O'ahu (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam)

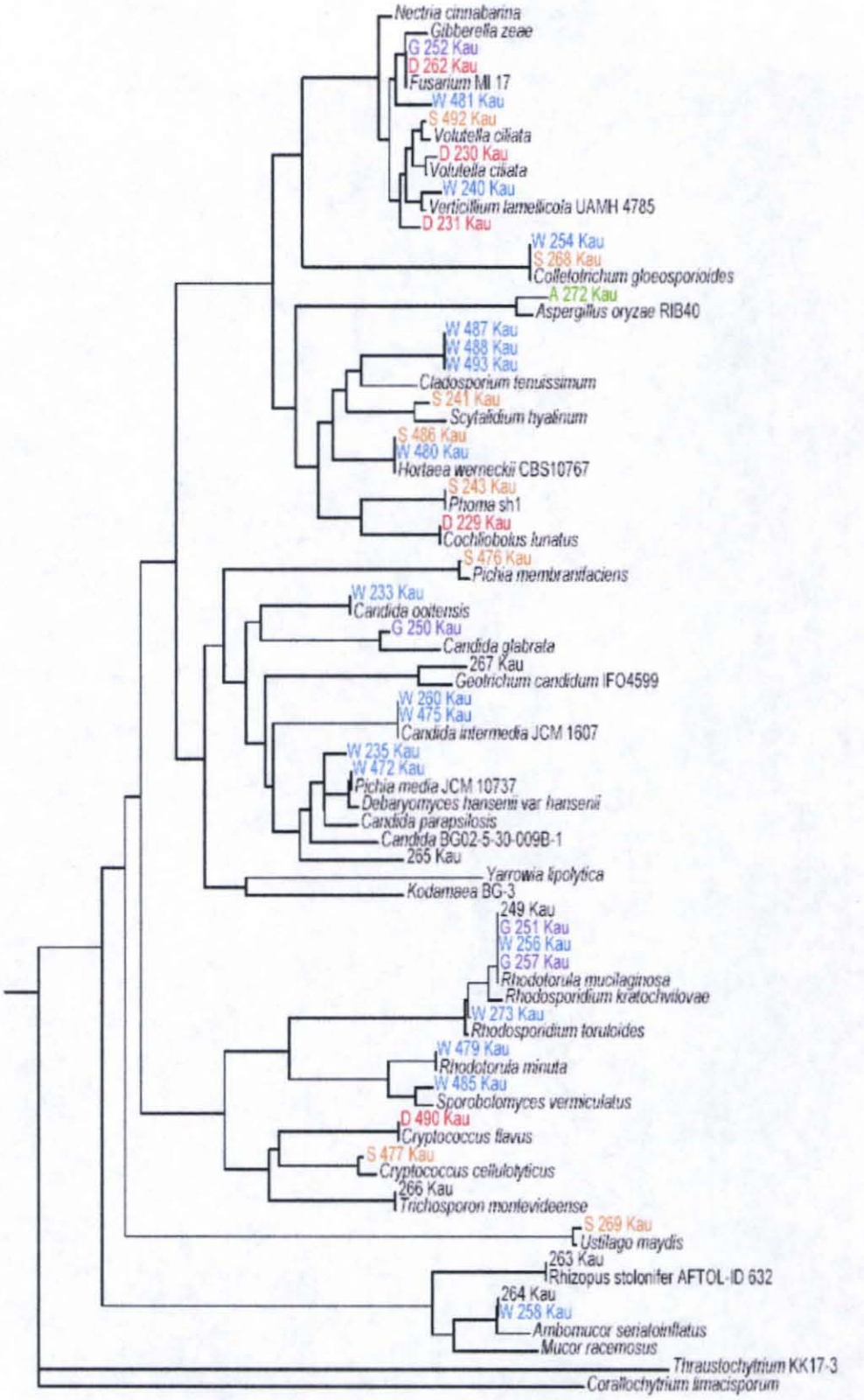


Figure 34. Phylogenetic Tree for Kaua'i (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam)

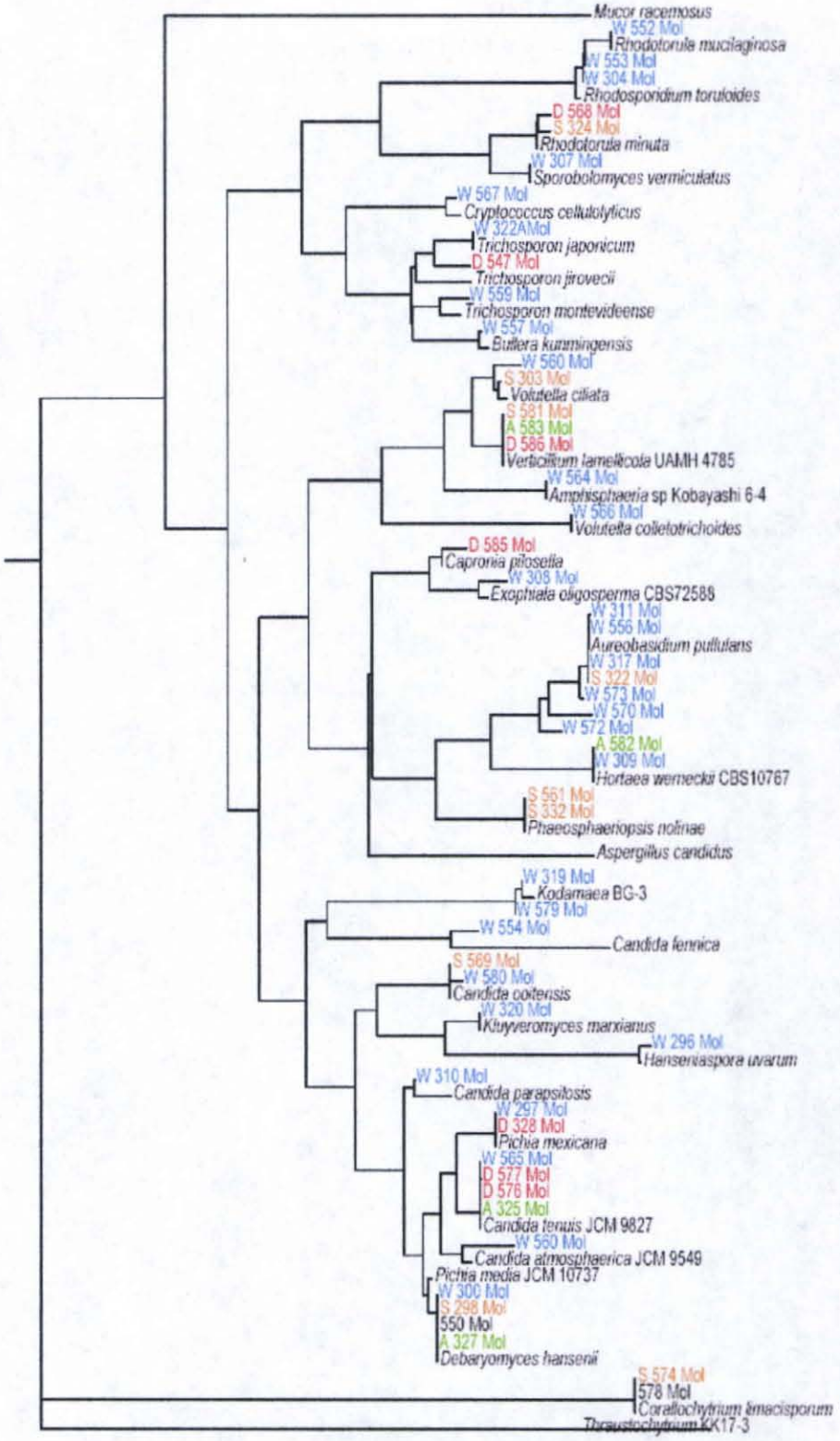


Figure 35. Phylogenetic Tree for Moloka'i (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam)

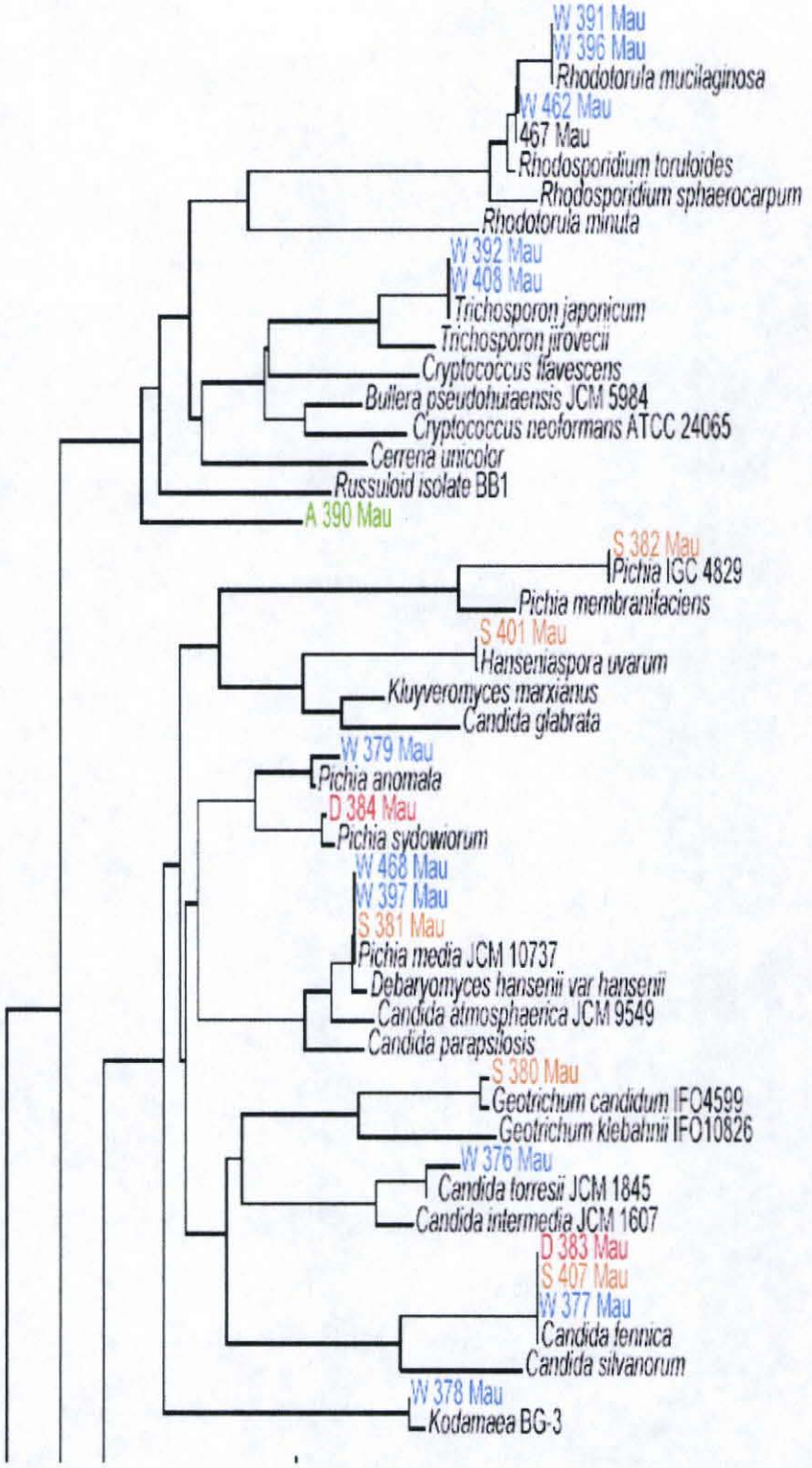


Figure 36. Phylogenetic Tree for Maui (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam)

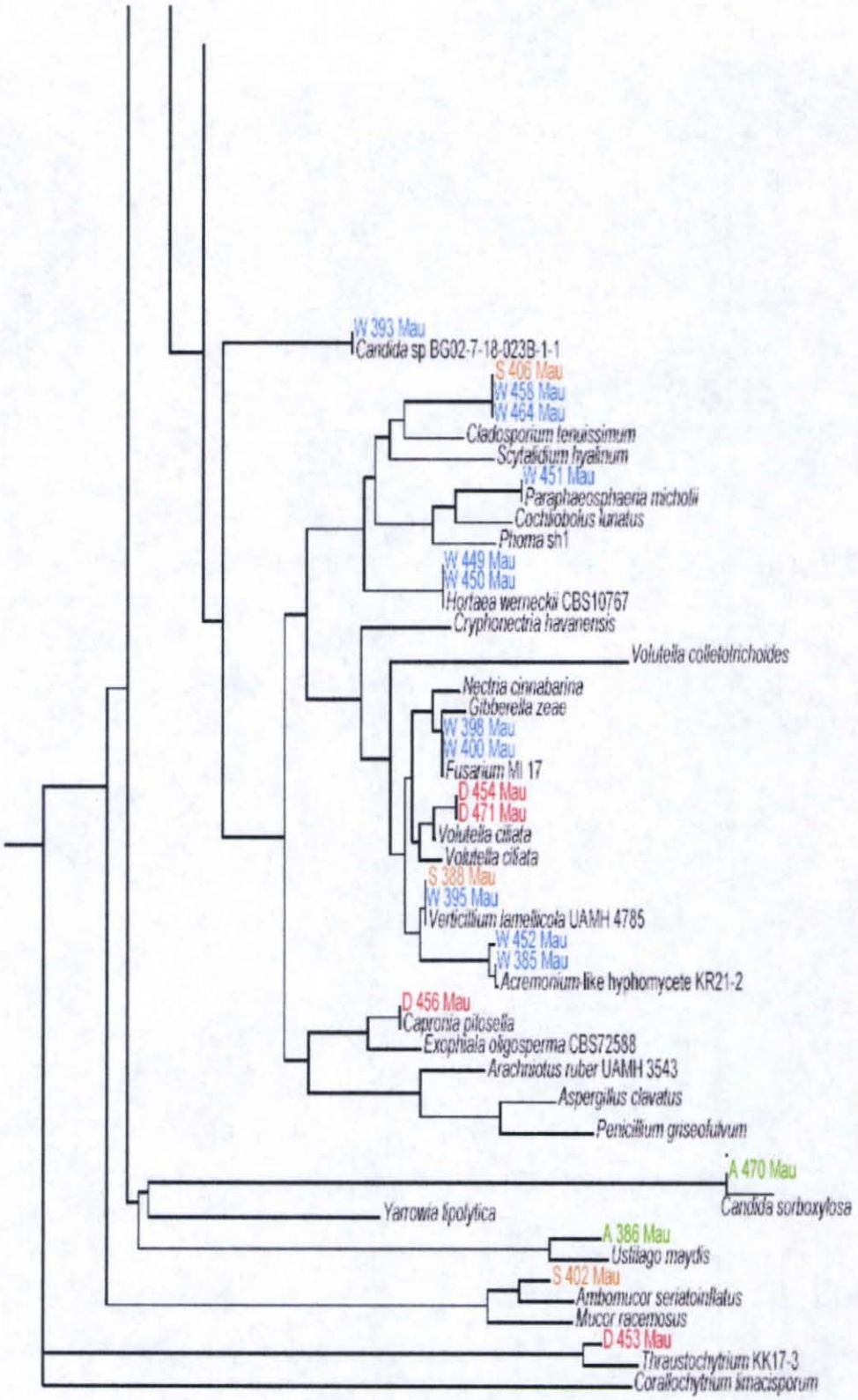


Figure 36. (Continued) Phylogenetic Tree for Maui (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam)

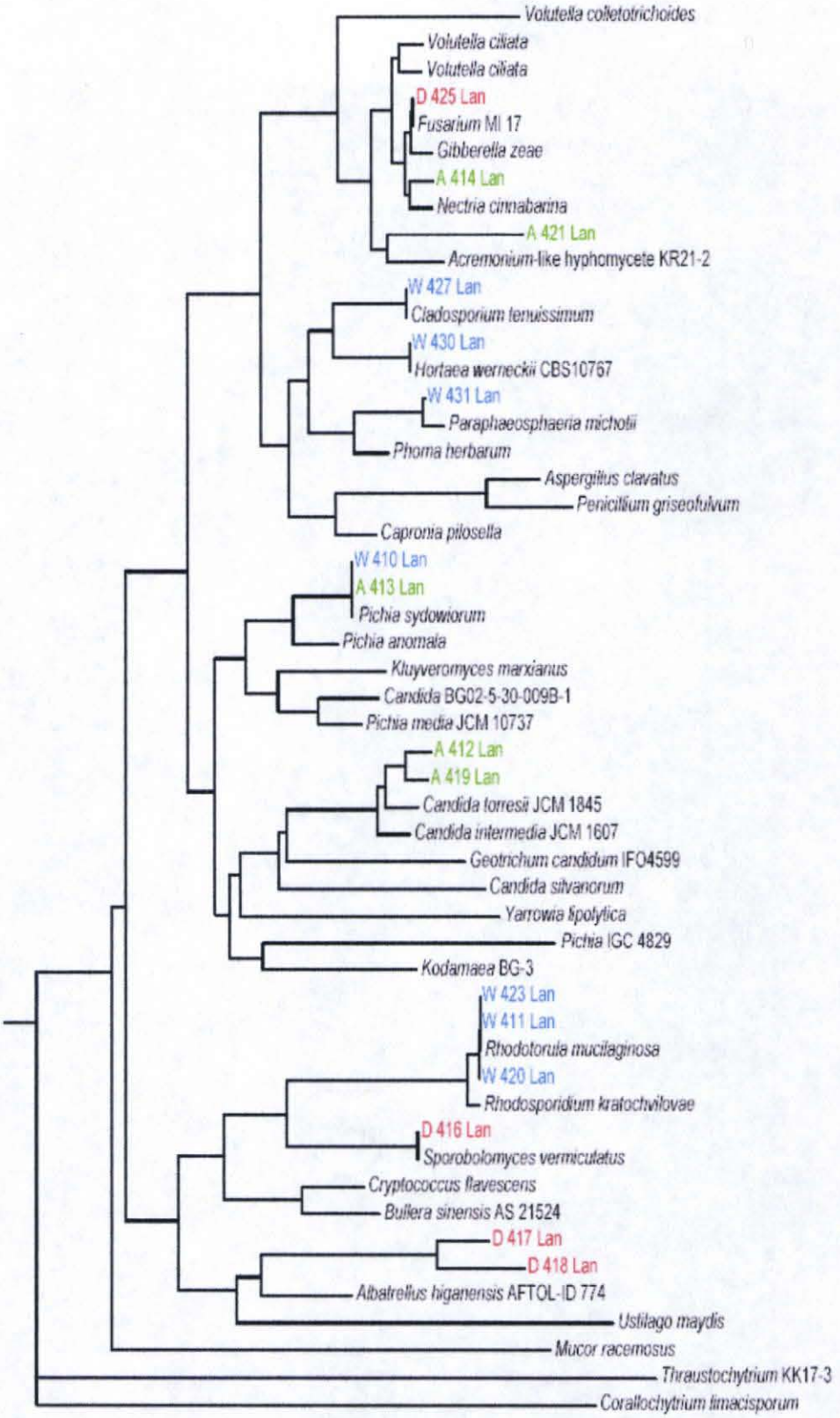


Figure 37. Phylogenetic Tree for Lana'i (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam)

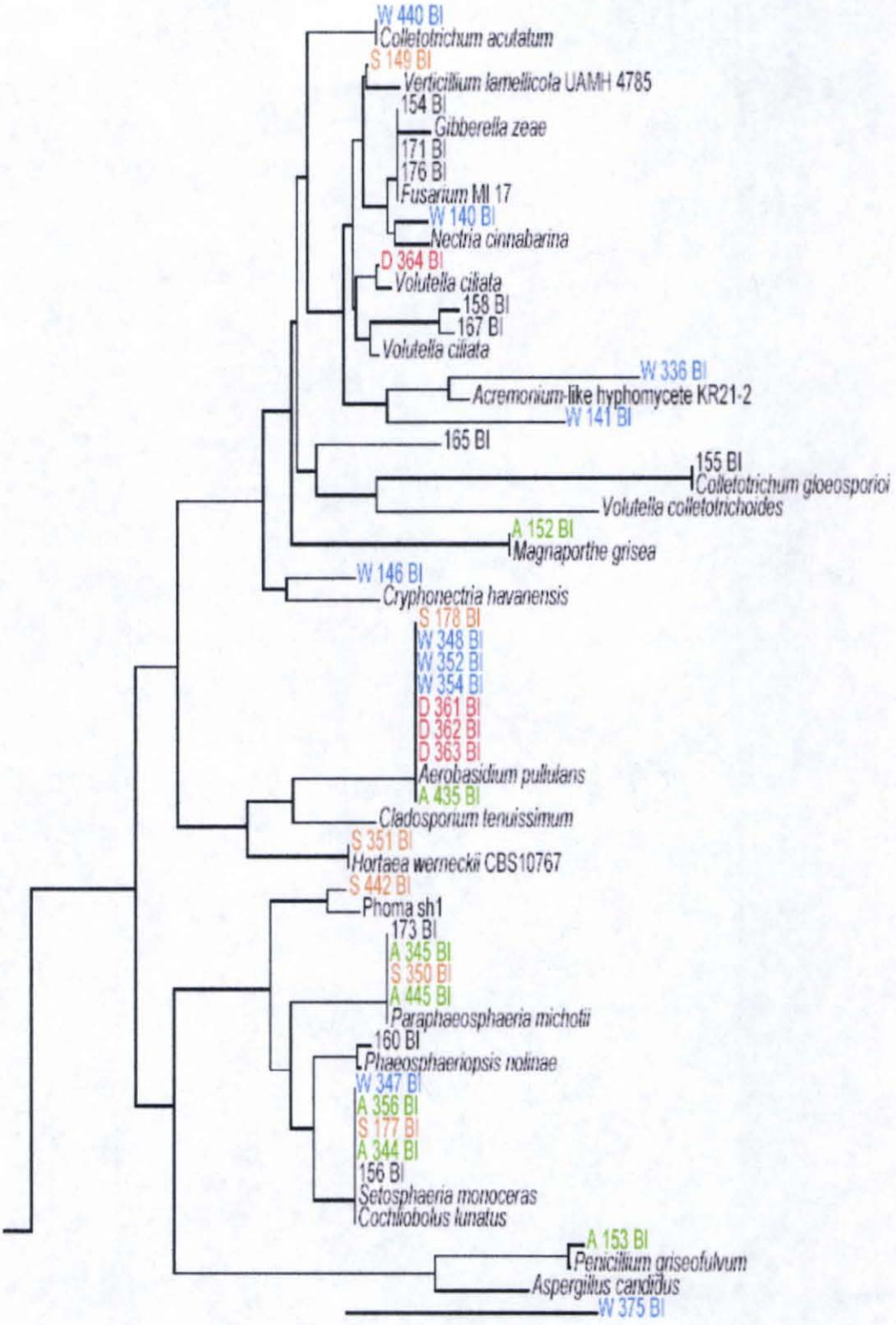


Figure 38. Phylogenetic Tree for Hawai'i (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam)

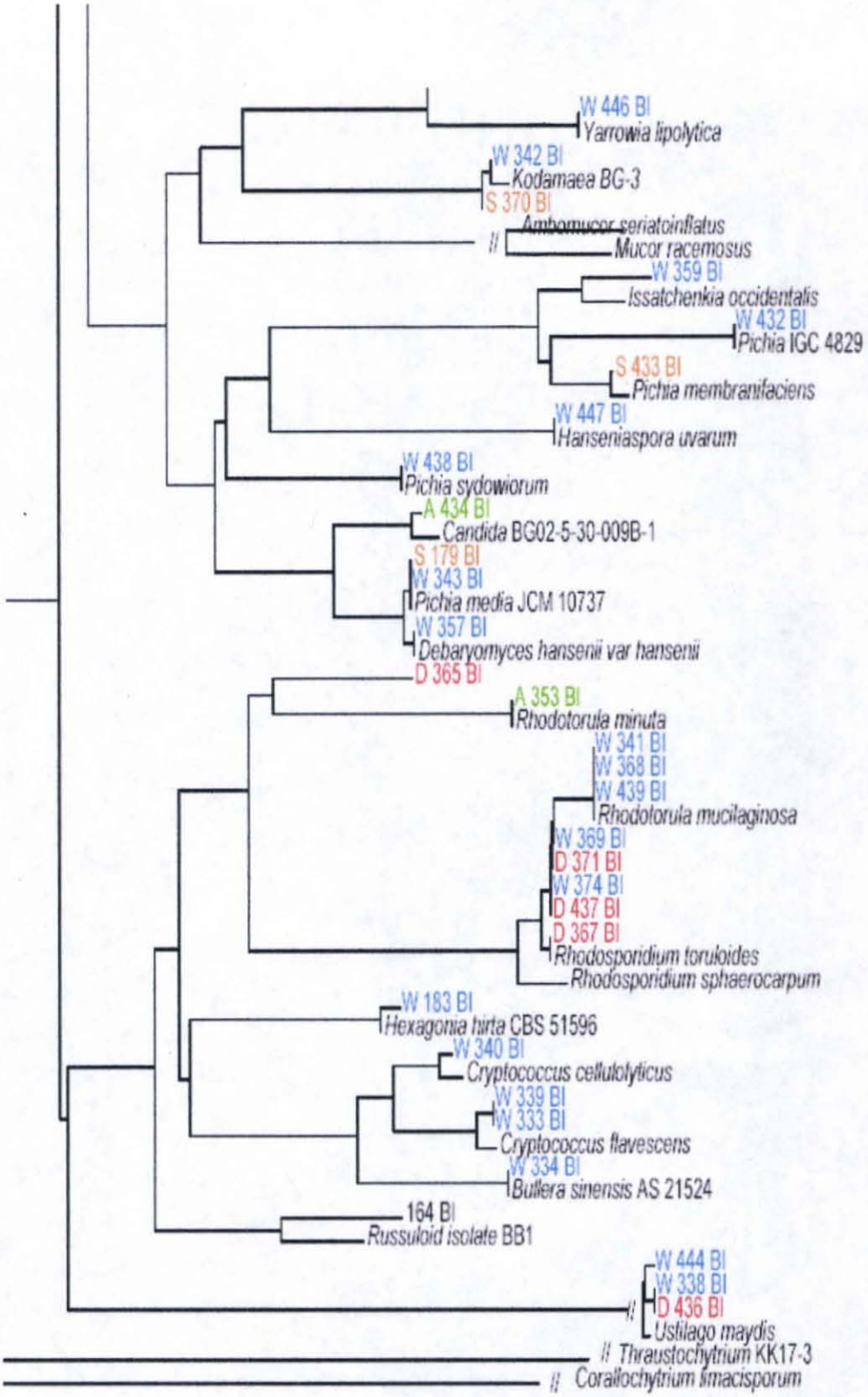


Figure 38. (Continued) Phylogenetic Tree for Hawai'i (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam)

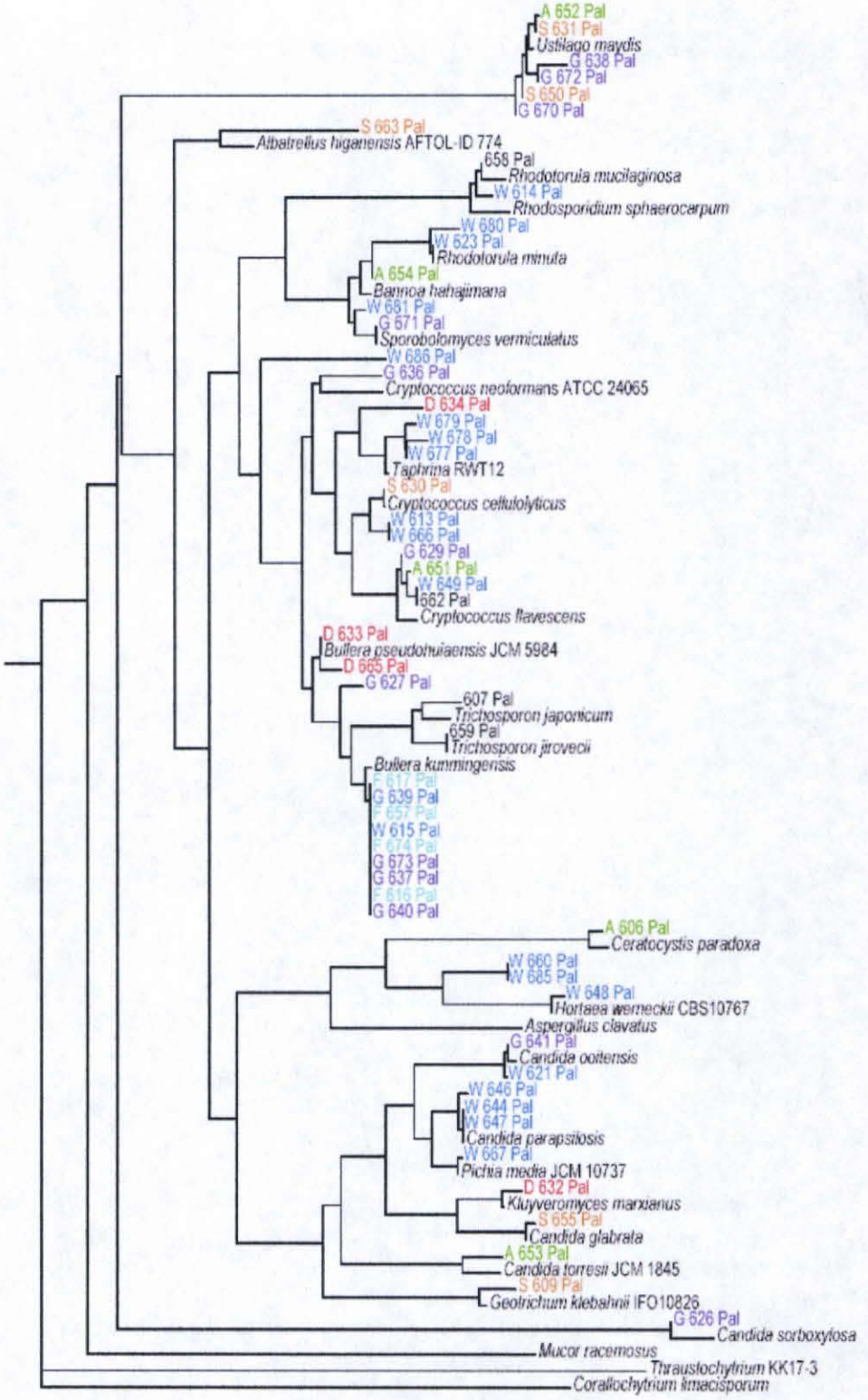


Figure 39. Phylogenetic Tree for Palmyra Atoll (Blue: water, Green: algae, Orange: sand, Red: wood, Purple: guano, light blue: foam)

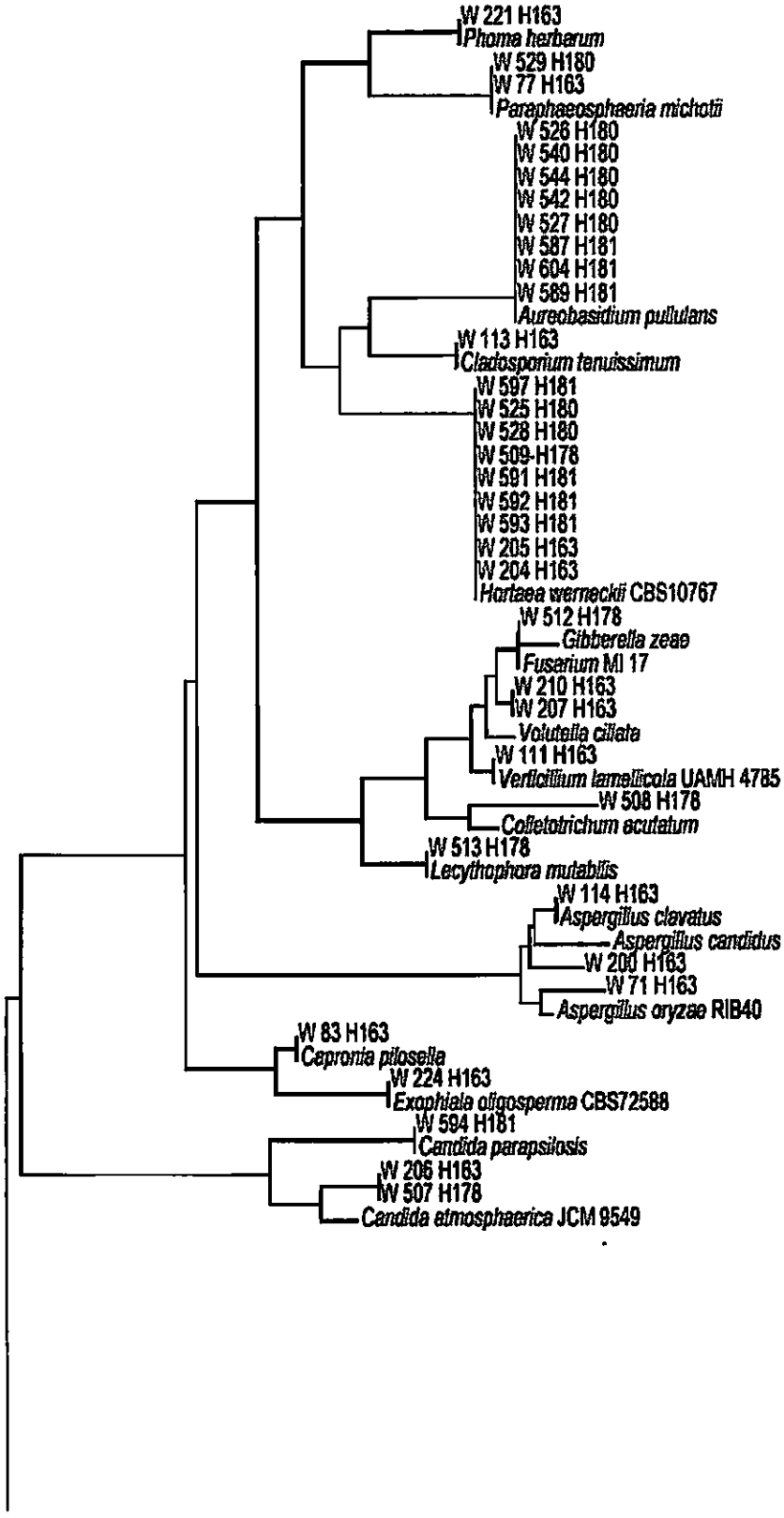


Figure 40. Phylogenetic Tree for Station ALOHA

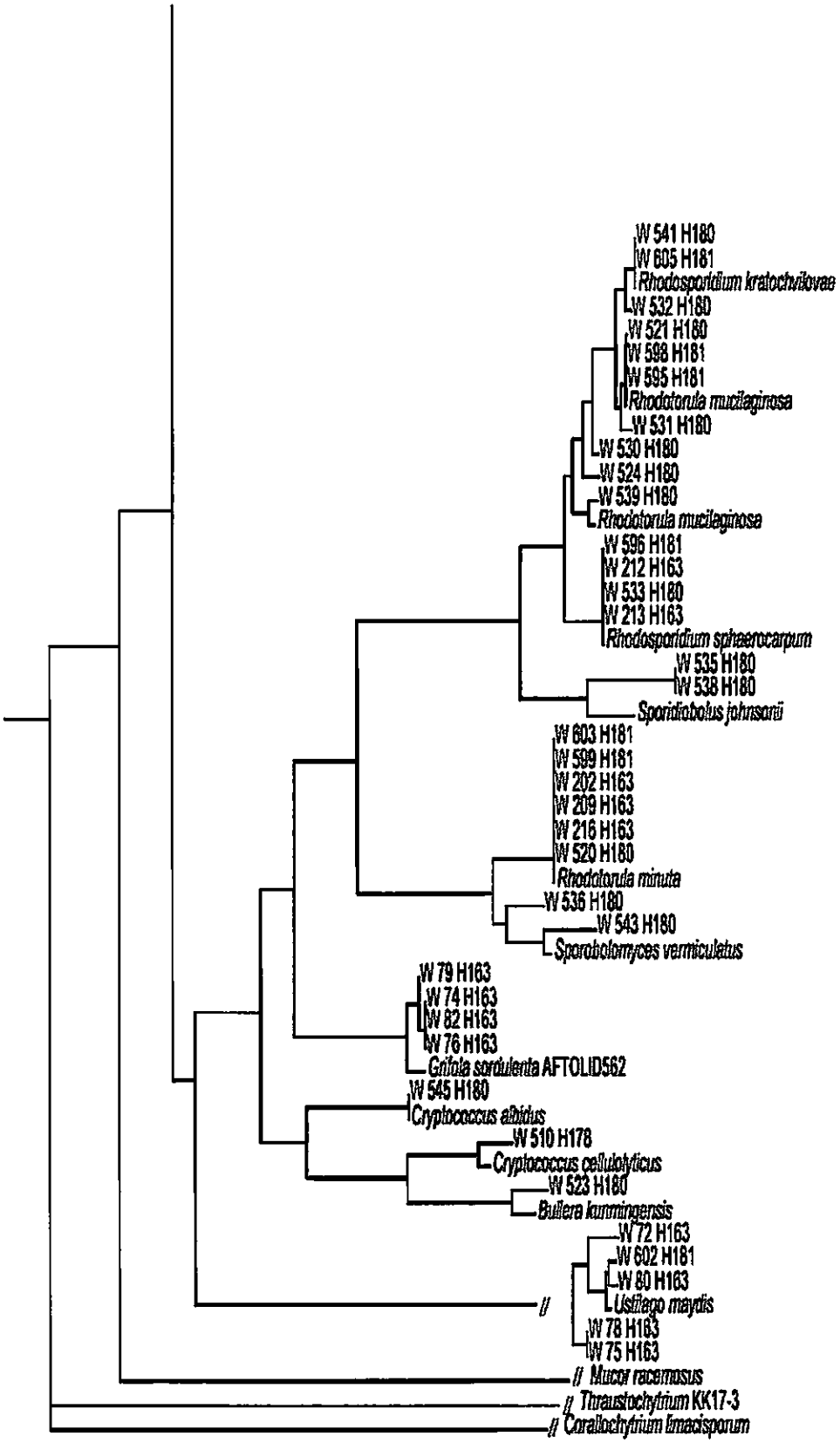


Figure 40. (Continued) Phylogenetic Tree for Station ALOHA

CHAPTER V. SUMMARY AND CONCLUSION

A. Summary of Results

A total of 689 isolates were purified from over 250 samples of water, sand, macroalgae, wood, and avian guano from the six high Hawaiian Islands, Palmyra Atoll, and Station ALOHA. Yeasts cultivated from Station ALOHA and Palmyra Atoll differs phylogenetically from those cultivated from Hawaiian littoral and sub-littoral sites. Those from the latter two habitats tend to be most closely related to, or appear to comprise, genera widely considered terrestrial in origin. Open ocean strains are more often related to those reported from the deep sea and marine sediments around the world, such as *Rhodotorula* spp.

The greatest number of potentially novel species was derived from Moloka'i, followed by Palmyra. The islands with the fewest potentially novel cultivated species were Lana'i, Maui, and Kaua'i.

The number of CFU isolated from water samples did not vary significantly across the six high Hawaiian Islands. Only the numbers of yeasts cultivated from Lana'i showed some indication of being lower than at any other island site, but there were insufficient data to support such a conclusion (Tab. 6).

The numbers of yeast only, fungi only, and yeast and fungi combined did not vary significantly over the ~5000 m water column at Station ALOHA; data were compared between individual depths, and shallow *versus* deep water samples. The Kruskall-Wallis test did suggest there was a higher number of CFU in the shallow (<500 m) water *versus* those in deep water (>500 m), but again, insufficient data based on just six cruises were unable to resolve this (Tab. 7).

One culture, LM418 isolated from wood on Shipwreck Beach, Lana'i is being described as a new *Sympodiomycopsis*, specifically *Sympodiomycopsis mahdii*. A manuscript is being prepared for submission to FEMS Yeast Research, but is summarized here in §H2.

B. Conclusions and Recommendations

This study indicates significant phylogenetic diversity among the marine mycoflora exists around the high Hawaiian Islands, at Station ALOHA and Palmyra Atoll, both in water *per se* and on many other materials. Cultures generated in this study will serve as a foundation for future research on secondary metabolites in Hawaiian marine fungi and yeast.

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