Chapter 2
Uncultivated Fungi from Antarctica

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2.1 Introduction

Antarctica is a remote and inhospitable continent that harbours the coldest and driest climate known on Earth (Rogers et al. 2007). This continent holds ~90% of the ice on Earth, which is equivalent to ~60–70% of the available freshwater on the planet. Only 1–3% of the Antarctic surface is free of ice in summer months, and most of these areas are rocky deserts and rocky ice-covered mountains (Yergeau et al. 2007; Singh et al. 2018).

Antarctica is located below parallel 60°S. Antarctic regions can be differentiated according to parameters that include soil type, geology, and glaciology (Rignota et al. 2019). From a biogeographic point of view, the most accepted classification scheme divides Antarctica into two regions—continental Antarctica and maritime Antarctica (Singh et al. 2015). The former region is located in the innermost part of the continent, characterized by high latitude and a layer covered with ice reaching more than 4250 m, which results in the most rigorous living conditions on the continent. The latter region includes a clearly defined maritime region that encompasses the Antarctic Peninsula and has longer and warmer summers than the mainland, as well as marine influences (Bolter et al. 2002).

One of the most inhospitable characteristics of the Antarctic environment is low temperatures, which can drop to −50 °C to −20 °C in the winter in the McMurdo Dry Valleys, and the monthly average temperature is below 0 °C. Additionally, frequent

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freeze-thaw cycles in marine Antarctica, strong winds, oligotrophy, aridity, high sublimation and evaporation, and periods of high incidence of UV radiation followed by prolonged dark periods represent major factors that limit the development of any form of life in the harsh Antarctic environment (Bolter et al. 2002; Onofri et al. 2007). These environmental conditions are not homogeneous and vary in different niches of the Antarctic continent, which include diversity in the types of soils, sediments, rocks, snow, and ice that can each vary in thermal properties, nutrient availability, hydration, and salinity (Ruisi et al. 2007; Koo et al. 2018).

2.2 Antarctic Microbial Diversity: Cultivation-Dependent and Cultivation-Independent Approaches

For many years, assessments of the biological diversity in Antarctica have focussed on organisms such as fish, birds, and marine mammals, while microorganisms were rarely considered (Vicent 2000). Recently, as a consequence of marked advances in molecular techniques for the study of microbial diversity in nature, knowledge about evolution, endemism, invasion, and microbial selection in the Antarctic environment has accumulated (Richter et al. 2014; Bendia et al. 2018; Coleine et al. 2018a).

Biogeochemical cycles and food chains in environments with restrictive characteristics, such as the ones found in Antarctica, often neglect the role of microorganisms, which play fundamental roles in energy transport, organic matter recycling, and mineralization of nutrients that are necessary for different ecological niches to function (Ruisi et al. 2007; Yergeau and Kowalchuk 2008; Bridge and Spooner 2012; Selbmann et al. 2017; Koo et al. 2018). Antarctic fungi are decomposers of organic matter and also represent a major proportion of the microbial biomass in samples introduced to Antarctica, such as historic woods and surrounding soils (Ludley and Robinson 2008; Blanchette et al. 2010; Arenz et al. 2006, 2011; Farrell et al. 2011; Held and Blanchette 2017). Cultivation-independent studies of fungal diversity have been carried out with both exotic samples, such as wood, and indigenous samples from natural Antarctic environments (Fig. 2.1).

Fungi that inhabit Antarctica have developed a huge metabolic diversity that has allowed them to colonize different available niches, including different types of desert, oligotrophic and ornithogenic soils, sediments, rocks of different typologies (such as sandstone, granite, dolerite, quartz, and lava-dike), snow, ice, permafrost, and vegetation (such as mosses, lichens, bryophyte, and flowering plants, including *Colobanthus quitensis* and *Deschampsia antarctica*) (Ruisi et al. 2007; Coleine et al. 2018a).

The majority of studies of Antarctic fungi have been described using cultivation-dependent methods (Rosa et al. 2009, 2010; Arenz and Blanchette 2011; Godinho et al. 2013; Connell et al. 2018; Duarte et al. 2018). In this sense, Antarctic fungal strains have been studied with a focus on taxonomy and biotechnology and have been reported in literature as producers of a wide range of biomolecules, including

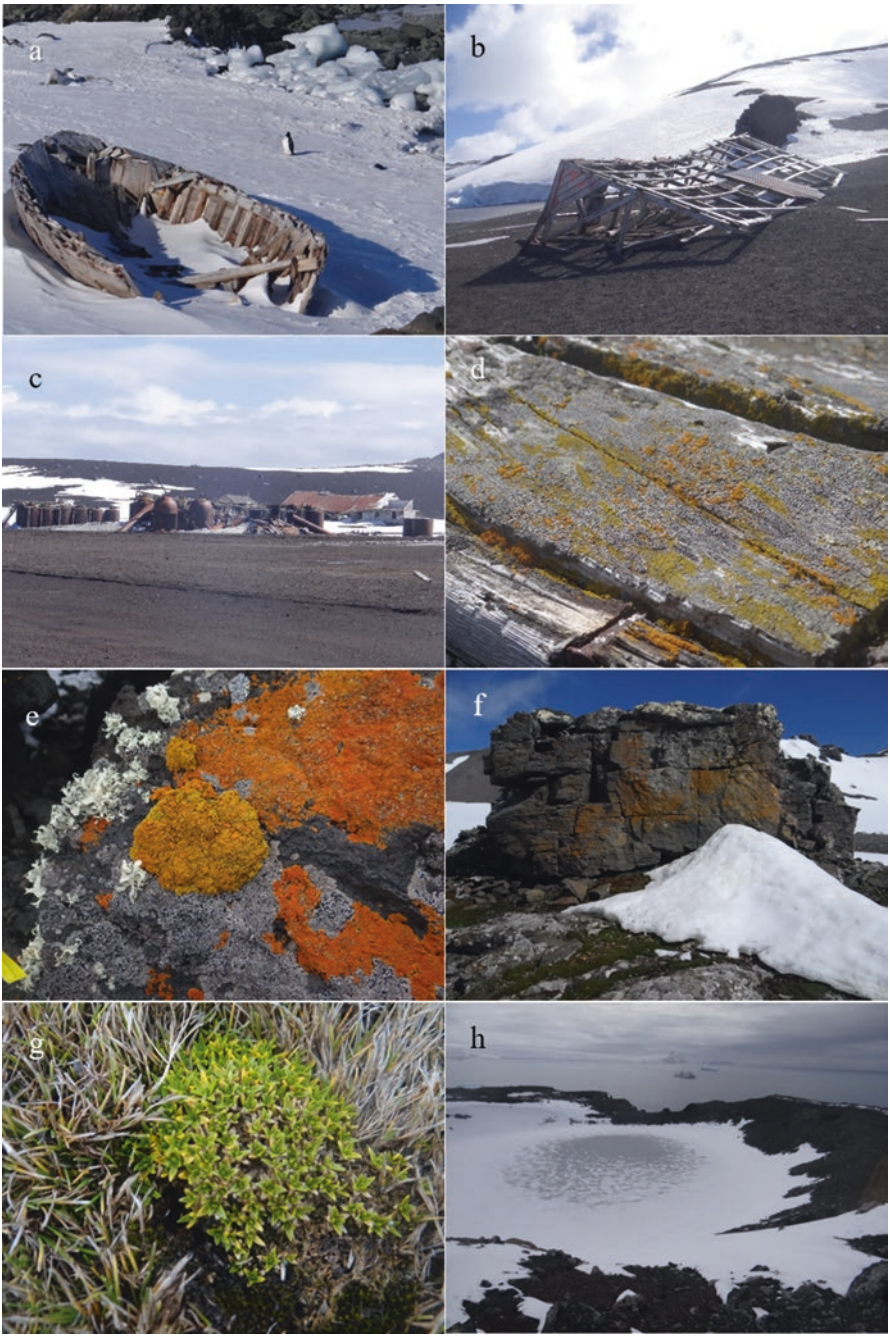

Fig. 2.1 Exotic (**a**–**d**) and natural (**e**–**h**) substrates in Antarctic environments. (**a**) Wood from boats on point *Hennequin*, King George Island; (**b**–**d**) Wood and metal structures from historic expedition huts on Deception Island; (**e**) Crustaceous lichens from Penguin Island; (**f**) Rocks from Nelson Island; (**g**) *Colobanthus quitensis* in Arctowski station, King George Island; and (**h**) an Antarctic lake under ice in Penguin Island. (Photos Credits: AWF Duarte)

those with antibacterial and antifungal (Gonçalves et al. 2015; Svahn et al. 2015), antiviral, and antituberculosis (Wang et al. 2015), antioxidant (Abrashev et al. 2016), photoprotector (Barahona et al. 2016), antiprotozoal (Gonçalves et al. 2015), insecticidal (Edgington et al. 2014), and cytotoxic (Brunati et al. 2009) activities. This promising biotechnological arsenal recovered by cultivation-dependent methods highlights the astonishing potential of Antarctic fungi for industrial applications and suggests an uncultured diversity that is possibly even greater.

The Antarctic fungi that have been characterized by cultivation-dependent methods are cosmopolitan, possibly because the region continuously receives microbial propagules from outside Antarctic regions by routes that include oceanic and wind currents. The dispersal of fungi may occur through a combination of favourable climatic factors such as winds and storms, in addition to dissemination by oceanic currents and vectors, such as dust, plant seeds, and birds (Margesin and Miteva 2011). Alternatively, some authors have argued that the great fungal diversity in Antarctica remains poorly understood, and there is evidence of a large number of endemic fungi (Bridge and Spooner 2012), a viewpoint that has been more recently reinforced by studies employing cultivation-independent methods (Pudasaini et al. 2017; Coleine et al. 2018a, b).

Although microbial isolation and cultivation techniques have improved in recent years, such as by the combined use of medium with high or low concentrations of nutrients (Ferrari et al. 2011) and cultivation with long-term incubation periods (Pulschen et al. 2017), our understanding of microbial ecology remains insufficient to recover and cultivate most microbes (Vester et al. 2015; Pulschen et al. 2017). Only 0.01–1% of the microorganisms in an environmental sample can be recovered through cultivation (Amann et al. 1995; Rappe and Giovannoni 2003). By fluorescence microscopy using DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride), a fluorescent marker that binds DNA, direct counting of bacteria in a soil sample was estimated at 4×10^{10} cells per gram of soil. However, after the enrichment and cultivation of this soil, only 1×10^{6} colony-forming units, or 0.01% of bacteria, were recovered (Handelsman 2004).

Although values for cultivable fungi are different, they are still low. Approximately 70–90% of fungi in different soil environments are uncultivable (Magnuson and Lasure 2002). Some years ago, the entire Fungi kingdom on Earth was estimated to include 5.1 million species based on high-throughput molecular sequencing analysis (Blackwell 2011), and this estimate was recently increased to at least six million species (Taylor et al. 2014). Based on these estimates, only 2% of fungi species are currently known (Vitorino and Bessa 2018). These points raise the question, "where do most of these uncharacterized fungi exist?" In Antarctica, the relationship between fungi existing in the environment and those that are known is even more pronounced, considering the extreme conditions of that continent and difficulty in faithfully cultivating them.

Among the main limitations imposed by cultivation-dependent methods is the fact that most microorganisms occur as symbiotic consortia, performing their own functions while often contributing to the activities or survival of other microorgan-

isms, making their isolation and cultivation a virtually impossible task (Woyke et al. 2006).

By contrast, cultivation-independent analysis of an environmental sample is considered to be an alternative way to understand more precisely the diversity and function of a microbial community, including the mycobiota, as well as allowing the full exploitation of its metabolic potential. This approach does not involve any selection step, such as the cultivation or enrichment of samples (Pearce et al. 2012; Su et al. 2012; Newsham et al. 2015; Baeza et al. 2017; Borruso et al. 2018).

Antarctic fungal diversity has been accessed by various cultivation-independent methods, including in situ direct observations using magnifying lens (Coleine et al. 2018a), scanning electron microscopy of pieces of rocks (Yung et al. 2014), scanning electron microscopy with backscattered electron imaging of rocks (Archer et al. 2017), fluorescence microscopy (Wierzchos et al. 2004; D'elia et al. 2009; Marfenina et al. 2016), and microarray analysis (Chan et al. 2013; Wei et al. 2016). Additionally, ergosterol, quantified using HPLC (UV detection at 282 nm), has been used as biochemical marker of higher fungal active biomass in Antarctic permafrost samples, as described by Velázquez et al. (2016).

Microscopy techniques have been used to assess Antarctic fungal diversity, mainly using samples that include rocks colonized by endolithic microorganisms (de los Rios et al. 2004; Wierzchos et al. 2004), historic wood samples such as pine and birch (Blanchette et al. 2004, 2010), accretion ice (D'elia et al. 2009), and soil (Marfenina et al. 2016). The FUN-1 fluorescence viability probe that targets yeasts and filamentous fungi was used to observe metabolically active microorganisms by confocal laser scanning microscopy in granite rocks collected in McMurdo Dry Valleys (Wierzchos et al. 2004) and through fluorescence microscopy of soil samples for the detection of spores, mycelium, live cells, and dead cells (Marfenina et al. 2016). In addition to detecting fungi in Antarctic environmental samples, some studies have evaluated the impact of fungal growth in wood samples by light microscopy to detect soft rot cavities (Blanchette et al. 2004).

In recent decades, the development of new molecular techniques in clinical microbiology, such as the PCR amplification of rRNA gene regions combined with fingerprint methods (Lawley et al. 2004; Yergeau et al. 2007; Rao et al. 2012; Kochkina et al. 2012; Dreesens et al. 2014; Selbmann et al. 2017), clone libraries (Lawley et al. 2004; Antony et al. 2016), quantitative PCR (Ji et al. 2016), and RNA extraction followed by cDNA sequencing (Rao et al. 2012), has been shown to be more informative, reproducible, and faster compared with previous methods. Thus, these approaches have been widely employed to describe fungal communities (Fig. 2.2).

More recently, next-generation sequencing (NGS) approaches have become more affordable and widely used in studies of Antarctic fungal communities (Dreesens et al. 2014; Newsham et al. 2015; Baeza et al. 2017; Borruso et al. 2018). NGS-based studies of fungal diversity have been performed using different sequencing platforms, such as the 454 FLX titanium (Dreesens et al. 2014; Newsham et al. 2015; Park et al. 2015; Ji et al. 2016; Pudasaini et al. 2017; Brady et al. 2018),

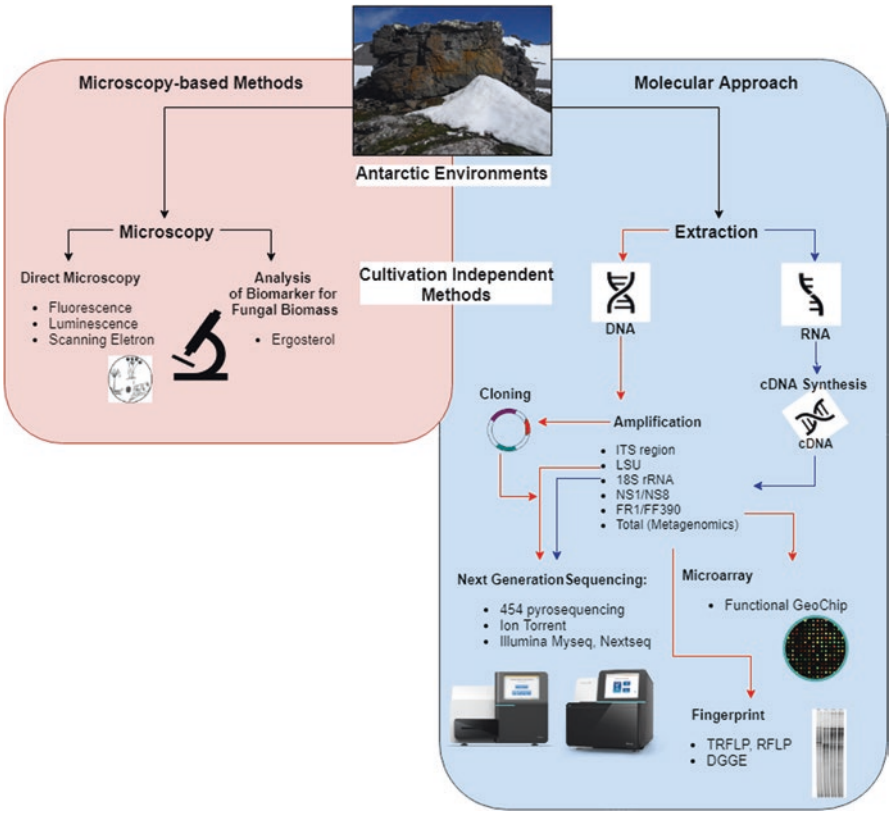

Fig. 2.2 Diversity analysis of Antarctic fungi by cultivation-independent methods

Illumina MiSeq (Czechowski et al. 2016; Rojas-Jimenez et al. 2017; Borruso et al. 2018; Coleine et al. 2018a, b), and Ion Torrent (Baeza et al. 2017) platforms.

The concept of "metagenomics" was initially proposed by Handelsman et al. (1998), which was defined as the application of modern genomic techniques to the study of microbial communities obtained directly from the natural environment, without the need for isolation or cultivation of individual species. This technique consists of extracting DNA from all organisms present in a given sample, such as snow or soil. This DNA can be directly evaluated by whole-genome shotgun metagenome (WGSM) sequencing to understand the structure, composition, and interactions of microbial communities, which represents an ecological approach (Schloss and Handelsman 2003). Alternatively, environmental DNA can be used to construct metagenomic libraries, followed by screening the resulting clones to search for a specific biological activity. This approach is often known as functional metagenomics and allows the metabolic potential of the microbial community in a given environment to be explored.

In these types of analyses, the starting point is a DNA of good quality for both purity and fragment size (Ekkers et al. 2012). Then, large fragments of DNA are ligated into vectors, such as BACs (bacterial artificial chromosome), fosmids, or cosmids, depending on the size of the DNA fragment to be analysed. This material is inserted into laboratory-cultured host cells, yielding a collection of clones known as a metagenomic library. Finally, these clones are subjected to subsequent screening for a biological activity of interest (Gillespie et al. 2002). Metagenomics allows for the identification of functional genes and a picture of the complexity of microbial communities, unveiling the richness, diversity, and dynamics of a community and enabling the recovery of complete genomes of hitherto uncultivated organisms (Alves et al. 2018).

Essentially, all metagenomic studies of Antarctic fungi are based on WGSM sequencing, and only one functional metagenomics-based study was found in literature (Ferrés et al. 2015). In that study, a metagenomic fosmid library was constructed from DNA extracted from glacial meltwater collected in the Antarctic Peninsula, yielding 52,000 clones (35–40 kb average insert size and almost 2 Gb of genetic information). This library was screened for bacterial genes that encoded cold-adapted enzymes, such as lipase/esterase, cellulase, and manganese peroxidase, and bioinformatics analysis revealed that eukaryotic DNA was less than 0.1% of total sequences.

In most studies of general microbial diversity based on cultivation-independent analysis from an Antarctic environment, bacteria are the most abundant group, followed by fungi or archaea depending on the environmental sample that is evaluated (Rogers et al. 2013; Pudasaini et al. 2017). Since recent advances in molecular methods, cultivation-independent methods have been broadly applied to study microbial communities of different environments including Antarctica.

Analyses of Antarctic fungal diversity based on cultivation-independent methods have been mainly performed on soil samples, including cold arid soil (Rao et al. 2012; Goordial et al. 2016), soil covered with vegetation (such as the mosses *Chorisodontium aciphyllum* and *Sanionia uncinata* and the lichen *Usnea antarctica*) (Yergeau et al. 2007), and maritime Antarctic soil (Lawley et al. 2004; Bridge and Newsham 2009). In addition to soils, other samples have been studied from samples that include wooden structures at historic sites (Blanchette et al. 2004, 2010), rocks colonized by endolithic microorganisms (de los Rios et al. 2004, 2005; Wierzchos et al. 2004; Selbmann et al. 2017; Coleine et al. 2018a, b), lichens (Park et al. 2015), permafrost (Kochkina et al. 2012, 2014), accretion ice from lakes (Rogers et al. 2013), hypoliths (Wei et al. 2016), ice-covered lakes (Rojas-Jimenez et al. 2017; Connell et al. 2018), hypersaline unfrozen brines found within a perennial frozen lake (Borruso et al. 2018), rock surface niches (Chan et al. 2013), and snow (Antony et al. 2016).

In general, the Antarctic samples analysed in fungal diversity studies can be divided into two main groups: (1) samples obtained from Antarctic natural environments, namely, natural or autochthonous samples, such as permafrost, lakes, desert rocks, or stones, and (2) samples introduced in Antarctica by anthropic activity, namely, exotic or allochthonous samples, such as woods and other structures left

behind by Antarctic explorers during expeditions, as shown in Fig. 2.1. In this chapter, we will cover the main types of samples that have been evaluated in cultivation-independent studies of fungal diversity (Table 2.1).

Table 2.1 Samples and methods used in studies of Antarctic fungal diversity by cultivation-independent approaches

Methods	Antarctic sample	Origin of sample	Technique	References
Microscopy-based				
	Accretion ice	Lake Vostok	Fluorescence microscopy with live/dead BacLightT	D'elia et al. (2009)
	Granite rocks colonized by endolithic microorganisms	Taylor Valley, Canadian Glacier (McMurdo Dry Valleys)	Confocal microscopy and fluorescent probe (FUN-1)	Wierzchos et al. (2004)
	Granite rocks colonized by endolithic microorganisms	McMurdo Dry Valleys	Confocal microscopy and fluorescent probe (FUN-1)	de los Rios et al. (2004)
	Granite rocks colonized by endolithic microorganisms	Canadian Glacier, Taylor Valley (Dry Valleys) and Cape Geology, Granite Harbour (Ross Sea)	Confocal laser scanning microscopy and fluorescence microscopy	de los Rios et al. (2005)
	Granite rocks colonized by endolithic and chasmoendolithic microorganisms	McMurdo Dry Valley	Scanning electron microscopy with backscattered electron imaging	Archer et al. (2017)
	Microbial mats from blighted and unaffected mats	Byers Peninsula (Livingston Island)	Scanning electronic microscopy and biomarker quantification (ergosterol)	Velázquez et al. (2016)
	Soil	Progress Station (valleys of the Larsemann Hills oasis) and West Russkaya Station (Marie Byrd Land)	Luminescence microscopy using calcofluor white, ethidium bromide, and fluorescein diacetate	Marfenina et al. (2016)
	Surface of rocks	Miers Valley (McMurdo Dry Valley)	Scanning electron microscopy	Yung et al. (2014)
	Wood samples including pine, spruce, and birch	Cape Royds and Cape Evans huts (Ross Island)	Scanning electron microscopy	Blanchette et al. (2004)
	Wood samples as pine and birch	Ross Sea Region	Scanning electron microscopy	Blanchette et al. (2010)
Molecular-based				

Table 2.1 (continued)

Methods	Antarctic sample	Origin of sample	Technique	References
	Accretion ice	Lake Vostok	DNA and RNA extraction followed by cDNA synthesis and 454 pyrosequencing	Rogers et al. (2013)
	Permafrost soil	Next to Bellingshausen, Novolazarevskaya, Progress, Russkaya stations and Banger Oasis and the Beacon Valley (Dry Valley)	DNA extraction, ITS region amplification, clone libraries, and sequencing	Kochkina et al. (2012)
	Permafrost (active layer)	Stations Novolazarevskaya (Schirmacher Oasis), Druzhnaya (Sandefjord Bay), Leningradskaya (Wilson Hills), and the Mirny observatory (Oates Coast).	DNA extraction, ITS region amplification, clone libraries, and sequencing	Kochkina et al. (2014)
	Permafrost soil	*University Valley*, McMurdo Dry *Valleys*	DNA extraction, ITS region-targeted pyrosequencing using the Roche 454 platform	Goordial et al. (2016)
	Surface of rocks	Miers Valley (McMurdo Dry Valley)	DNA extraction, PCR, T-RFLP and sequencing	Yung et al. (2014)
	Rocks of different typologies (sandstone, granite, dolerite, quartz, and lava-dike)	Along a latitudinal transect ranging from 73°S (Chisholm Hills, Cosmonaut Glacier) to 76°S (Battleship Promontory), and an altitudinal gradient from sea level (Kay Island)	DNA extraction, PCR and Denaturing Gradient Gel Electrophoresis (DGGE)	Selbmann et al. (2017)
	Sandstones with cryptoendoliths	University Valley (Upper Dry Valleys)	DNA extraction, ITS region amplification, and 454 pyrosequencing	Brady et al. (2018)
	Rocks of different typologies (sandstone, granite, dolerite, quartz, and lava-dike)	Along a latitudinal transect ranging from 73°S to 76°S (Victoria Land)	DNA extraction, ITS region amplification sequencing by Illumina MiSeq	Coleine et al. (2018a)

(continued)

Table 2.1 (continued)

Methods	Antarctic sample	Origin of sample	Technique	References
	Rocks (endolithic)	Along an altitudinal gradient from 834 to 3100 m a.s.l.—74°S–77°S (Victoria Land)	DNA extraction, ITS region amplification of ITS1 with ITS1F and ITS2 primers and sequence by Illumina MiSeq	Coleine et al. (2018b)
	Hypersaline unfrozen brines found within a perennial frozen lake	Tarn Flat (Northern Victoria Land)	DNA extraction, ITS region amplification and sequencing by Illumina MiSeq	Borruso et al. (2018)
	Lichens	Barton and Weaver Peninsulas (King George Island)	DNA extraction, amplification of LSU (eukaryotic large subunit), and 454 pyrosequencing	Park et al. (2015)
	Microbial mats from blighted and unaffected mats	Byers Peninsula (Livingston Island)	DNA extraction, amplification of 18S rRNA gene, and 454 pyrosequencing	Velázquez et al. (2016)
	Snow	Princess Elizabeth Land	DNA extraction, amplification with primers NS1/NS8, clone libraries, and sequencing	Antony et al. (2016)
	Soils covered or not with vegetation, such as mosses (*Chorisodontium aciphyllum* and *Sanionia uncinata*) and lichen (*Usnea antarctica*)	Anchorage, Signy, Fossil Bluff and Coal Nunatak	DGGE, real-time PCR, and microarray hybridization	Yergeau et al. (2007)
	Soil (maritime Antarctica)	Transect across Jane Col, Signy Island; Rothera Point, Adelaide Island; Mars Oasis and Coal Nunatak, Alexander Island, Sky-Hi Nunataks, Ellsworth Land and the LaGorce Mountains	DNA extraction, amplification, clone libraries, RFLP, and sequencing	Lawley et al. (2004)

(continued)

Table 2.1 (continued)

Methods	Antarctic sample	Origin of sample	Technique	References
	Soil (maritime Antarctica)	Mars Oasis (Alexander Island)	DNA extraction, amplification, clone libraries, and sequencing	Bridge and Newsham (2009)
	Soil (continental Antarctica)	McKelvey Valley (McMurdo Dry Valley)	DNA and RNA total extraction, followed by cDNA synthesis, amplification, clone libraries, T-RFLP, and sequencing	Rao et al. (2012)
	Soil	Miers Valley, Beacon Valley, Battleship Valley, Wright Valley, Alatna Valley, University Valley (McMurdo Dry Valleys)	DNA extraction, T-RFLP, and 454 pyrosequencing	Dreesens et al. (2014)
	Soil	Along a 1650 km gradient between 72 °S and 60 °S (Alexander, Jenny, Lagoon, Adelaide, Blaiklock, Detaille, Wiencke, Goudier, Seymour, Alectoria, Spert, James Ross, Deception, Livingston, Robert, Greenwish, King George and Signy Islands and Cape Evensen)	DNA extraction and 454 pyrosequencing	Newsham et al. (2015)
	Soil	Mitchell Peninsula (Windmill Islands)	DNA extraction, ITS region amplification, 454 pyrosequencing, and quantitative PCR (qPCR)	Ji et al. (2016)
	Soil	Mount Menzies, Mawson Escarpment and Lake Terrasovoje	DNA extraction, 18S rRNA amplification, and sequencing by Illumina MiSeq	Czechowski et al. (2016)
	Soil	King George, Deception, Snow, Dee, Livingstone, Greenwich, Robert, Nelson, Litchfield and Lagotellerie Islands, and Union Glacier	DNA extraction and sequencing using Ion Torrent technologies	Baeza et al. (2017)

(continued)

Table 2.1 (continued)

Methods	Antarctic sample	Origin of sample	Technique	References
	Soil	Browning Peninsula (Windmill Islands)	DNA extraction, ITS region amplification, and 454 pyrosequencing	Pudasaini et al. (2017)
	Soil under of *Colobanthus quitensis* and *Deschampsia antarctica* (vascular plants that occur in Antarctica)	Bird, Signy, and Léonie Islands	DNA extraction, ITS region amplification, and 454 pyrosequencing	Cox et al. (2016)
	Soil and hypoliths	Miers Valley (McMurdo Dry)	DNA extraction, GeoChip functional microarray	Wei et al. (2016)
	Soil and rock surface niches	McKelvey Valley	Microarray with GeoChip-based functional genes	Chan et al. (2013)
	Water from ice-covered lakes	Five lakes in the Taylor and Miers Valleys (McMurdo Dry)	DNA and RNA extraction, cDNA synthesis, 18S rRNA amplification, and sequencing by Illumina MiSeq	Rojas-Jimenez et al. (2017)
	Wooden structures and surrounding soils	South Pole, Robert F. Scott and Ernest Shackleton (Ross Island)	DNA extraction and DGGE using the internal transcribed spacer (ITS) regions	Arenz et al. (2006)

Although fungal biomass has been found to be relatively low in the various samples collected in Antarctica, there is a considerable fungal richness, with a higher abundance of fungi of the phylum Ascomycota, followed by Basidiomycota; this pattern is similar to that observed in cultivation-dependent studies. Additionally, the occurrence of *Zygomycota* and *Chytridiomycota* in soils (Cox et al. 2016) and *Zygomycota*, *Cryptomycota*, and *Blastocladiomycota* in ice-covered lakes (Rojas-Jimenez et al. 2017) has been reported. Some authors have observed that Antarctica is characterized by a low frequency of endemic fungi, with the majority of them belonging to the class *Eurotiomycetes* (Cox et al. 2016).

Despite the high fungal richness in Antarctica, a limited literature addresses the functional diversity of these microorganisms by a cultivation-independent approach. However, it is possible to predict that these organisms are crucial for the maintenance of the Antarctic ecosystem, as demonstrated by Wei et al. (2016) who observed

via microarray hybridizations to a functional GeoChip that a high frequency of fungal organisms is related to the carbon and nitrogen cycle, in addition to expressing genes related to environmental stress responses.

2.3 Antarctic Samples Analysed by Cultivation-Independent Approaches

2.3.1 Soil

Antarctic soil is distinct from other biomes as consequence of its long-term persistence under harsh environmental conditions, in addition to its long history of isolation, resulting in a high degree of endemism (Convey 2010). Today, anthropic influences increasingly threaten the unique Antarctic soil communities through human-mediated climate change, increasing pollution, and the introduction of exogenous organisms by exploration activities, which may outcompete the endemic diversity in some environmental sites.

Antarctic soils have distinct characteristics, ranging from continental arid soils that are characterized by the low availability of nutrients and low moisture content to ornithogenic soils from penguin regions that receive a high content of faeces and are consequently characterized by high levels of carbon, nitrogen, phosphorus, silicon, and moisture (Rao et al. 2012; Guo et al. 2018). Recently, Guo et al. (2018) observed that the expansion of penguin activity in maritime Antarctica has been associated with a change in the soil geochemistry, which consequently affects the composition of the soil microbiome.

In a study of eukaryotic diversity in Antarctic soil from the Prince Charles Mountains (East Antarctica) that used an 18S rDNA amplicon sequencing and metataxonomic approach, the main phylotypes were fungal species, followed by nonalgal protists (Czechowski et al. 2016). This study revealed that sequences related to the extremophilic fungus Ascomycota *Cryomyces antarcticus* were among the most abundant. Other sequences were mainly related to lichenized fungi (a mycobiont), highlighting the importance of lichen diversity, even for the microbial composition present in the Antarctic soil (Czechowski et al. 2016).

Marfenina et al. (2016) reported that the fungal biomass in Antarctic soils was 0.3 and 0.6 mg/g of soil in arid soil and soil covered with vegetation (i.e., mosses and lichens), respectively. These were both mainly composed of small spores (2.5 μm), as observed by luminescence microscopy. The same study recovered 38 species of cultivated microscopic fungi, with *Penicillium* and *Phoma* being the most abundant genera in soil (Marfenina et al. 2016). The presence of vegetation and birds are two parameters that were found to strongly influence microbial diversity in Antarctic soils (Teixeira et al. 2013).

The McMurdo Dry Valleys are characterized by arid desert and mineral soil; different studies have reported that this environment harbours a restricted fungal diver-

sity (Rao et al. 2012; Pudasaini et al. 2017). Environmental DNA and RNA (cDNA) from soil collected in the McMurdo Dry Valleys have been used to infer total fungal diversity and putative metabolically active assemblages, respectively (Rao et al. 2012). Additionally, the authors employed cultivation-based approaches using a variety of laboratory growth conditions. ITS sequence analysis revealed a highly restricted diversity, with sequences affiliated with to two known genera, *Helicodendron* and *Zalerion*. Moulds and mitosporic genera commonly found in marine Antarctic samples were not found in this study, and a comparison of diversity estimates by DNA and RNA (cDNA) sequences suggested that taxa active metabolically can be present in different abundances than indicated by DNA libraries. *Debaryomyces hansenii* was recovered using plating techniques, but it was not detected by the cultivation-independent approach, which might reflect the inherently low abundance of this yeast in soil samples when compared to other yeasts (Rao et al. 2012). These findings indicate that these two approaches are complementary, and each accesses a distinct microbial fraction of total diversity in nature (Arenz et al. 2006; Rao et al. 2012; Antony et al. 2016).

In another study, Dreesens et al. (2014) observed that soil samples from the McMurdo Dry Valleys were quite heterogeneous in physicochemical properties, with salinity ranging from 107 to 3920 μS and moisture content ranging from 1% to 3%, which was correlated with the fungal diversity associated with individual samples. Fingerprinting by T-RFLP and 454 pyrosequencing analysis revealed different levels of heterogeneity in fungal diversity, with a significant number of OTUs annotated as derived from *Chytridiomycota* species, especially in the Miers Valley sampling point. Similar to the other two sampling points (Alatna Valley and Battleship Promontory), most OTUs were annotated as Ascomycota, although *Chytridiomycota* was also highly abundant. Notably, these findings are quite interesting because previous studies suggested that Antarctic Dry Valley soils were dominated by Ascomycota and *Basidiomycota* based on both cultivation-dependent (Arenz and Blanchette 2011) and -independent methods (Rao et al. 2012).

In a study that used pyrosequencing to assess fungal diversity in 29 soils sampled from a 1650 km-transect in maritime Antarctica, Newsham et al. (2015) showed that the most abundant fungal OTUs were related to *Ascomycota*, followed by *Basidiomycota*. Moreover, redundancy analysis based on presence/absence data indicated that changes in the frequency of the lichenized fungi *Verrucaria* largely accounted for increased fungal diversity in warmer soils (Newsham et al. 2015). These authors reported that surface air temperature is an important factor that shapes fungal diversity in Antarctic soil. Moreover, warmer soil in maritime Antarctica showed more water availability and enhanced metabolic activity, which extended the period for which fungi are active and enabling a switch from survival to growth and dispersal strategies.

One of the distinguishing characteristics of maritime Antarctic soils is frequent freezing and thawing cycles. Besides being a limiting factor to microbial development, this environmental phenomenon also affects the physical properties of such soils, as fine mineral particles are surrounded by other elements, such as coarser material, stones, and rocks (Lawley et al. 2004).

A reduction in microbial richness and abundance correlated with increases in latitude was proposed by Yergeau et al. (2007). However, some authors have shown that lower diversity in Antarctic soil samples was not related to increases in latitude, but it was instead associated with the local environmental conditions of the sampling site (Lawley et al. 2004; Ji et al. 2016).

Denaturing gradient gel electrophoresis (DGGE) and real-time PCR analyses revealed that fungal 18S rRNA gene abundance in soil samples covered with mosses and lichens was not influenced by vegetation cover per se but, instead, was affected by location and interactions between location and vegetation cover (Yergeau et al. 2007). The authors showed that fungal communities can respond differently to changes in organic input levels and quality depending on the environmental conditions. Additionally, estimates of fungal biomass differed when using cultivation-dependent (CFU counts) versus -independent (real-time PCR) approaches, and a high correlation was found between values obtained by real-time PCR and phospholipid fatty acids (PFLA). In general, CFU counts are thought to provide a biased view of the microbial abundance in an environment.

Ji et al. (2016) studied 93 soil samples from Mitchell Peninsula that were characterized by an acidic pH (ranging from 4.82 to 6.8), moisture ranging between 1% and 10%, and low total carbon (< 0.6%). These authors found 374,996 fungal ITS gene sequences after read-quality filtering; many of the fungal OTUs could not be taxonomically assigned because of poor sequence alignment (<50% of nucleotides aligned), which represented 13% of the total filtered sequences. Additionally, 17%, 18%, and 22% of all fungal ITS sequences could not be classified at the class, order, or family level, respectively. Fungal communities were dominated by *Ascomycota* (77.1%), followed by *Basidiomycota* (9.7%) and *Chytridiomycota* (0.01%), with more than 46% of fungal sequences classified as *Lecanoromycetes*, one of the largest classes of lichenized fungi.

Wei et al. (2016) investigated the microbial functional diversity of soil and hypolith samples from Miers Valley in the McMurdo Dry Valleys by GeoChip microarray. The GeoChip microarray (with 84,000 50-mer oligonucleotide probes covering 152,000 gene variants) primarily targets bacterial genes, but some fungal genes are also represented. Among the functional gene categories analysed, fungal genes were identified associated with the carbon transformation (carbohydrate catabolism and aromatic compound catabolism), nitrogen transformation (nitrification, denitrification, ammonification, and assimilatory nitrogen reduction), and stress response (heat shock, nitrogen limitation, phosphate limitation, and oxygen stress) categories. Agaricomycetes, Leotiomycetes, Saccharomycetes, and Sordariomycetes showed the highest intensity of hybridization to genes related to stress responses. Intriguingly, *Ustilaginomycetes* and unidentified fungi showed the highest expression of genes related to nitrogen transformation. Finally, no difference was observed among the fungal groups for genes involved in carbon transformation.

2.3.2 Permafrost

Some studies suggest that the combination of deep freeze, aridity, and oligotrophy in Antarctic permafrost severely limits microbial activity and survival, resulting in very low levels of total carbon (0.01–0.05%) and total N nitrogen (undetectable to 0.09%) in samples collected from the McMurdo Dry Valleys (Goordial et al. 2016). Goordial and colleagues evaluated microbial diversity by 18S ITS pyrosequencing and RNA analyses and, even using relatively high soil amounts for RNA extraction, they could not detect RNA. They hypothesized that biomass was extremely low in their samples and suggested that the extracted DNA in these samples was mainly derived from dormant or nonviable fungi. Fungal sequences were dominated by the *Ascomycetes* fungi *Dothideomycetes* and *Eurotiomycetes*; the latter was only found on the surface of permafrost samples.

Based on cultivation-dependent and -independent methods, Kochkina et al. (2012) also reported low fungal biomass in permafrost from sites near Russian research stations. They found that the sequences of some organisms were only recovered by cultivation-independent methods, whereas some strains of *Penicillium* and *Cladosporium* were only obtained by isolation and cultivation methods, highlighting the complementarity of both approaches.

2.3.3 Lichen

Antarctic terrestrial ecosystems are dominated by lichen-symbiotic organisms that pair a mycobiont (lichenized fungi) and a photobiont (algae and/or cyanobacteria). These relationships have a certain level of specificity, as one species of mycobiont and one species of photobiont can form a symbiotic relationship in a thallus (Domaschke et al. 2012).

Taxonomic affiliations of the Antarctic lichen-associated fungi from *Cladonia borealis*, *Cladonia gracilis*, *Umbilicaria antarctica*, *Usnea aurantiaco-atra*, *Buellia granulosa*, *Amandinea coniops*, and *Ochrolechia parella* showed that they belonged to the *Ascomycota* (*Arthoniomycetes*, *Eurotiomycetes*, *Lecanoromycetes*, *Leotiomycetes*, and *Sordariomycetes*) and *Basidiomycota* (*Cystobasidiomycetes* and *Tremellomycetes*) groups (Park et al. 2015). *Lecanoromycetes* has been the most dominant fungal class identified in Antarctic lichens, including those isolated from rock substrates (Coleine et al. 2018a).

Moreover, lichenized fungal species are important components of Antarctic soil biodiversity and may have a widespread distribution across Antarctic sites, as reported by Czechowski et al. (2016) for a study of samples from Prince Charles Mountain analysed using high-throughput sequencing. Notably, global warming mainly affects the maritime Antarctic and, in this sense, can also affect the fungal diversity of lichens in Antarctic soils.

2.3.4 Lake and Ice-Covered Lake Water

Lake Vostok is the largest of the nearly 400 subglacial Antarctic lakes and has been continuously buried by glacial ice for 15 million years. Metagenomic and metatranscriptomic analyses of Lake Vostok accretion ice revealed sequences that belong to the three domains of life (94% Bacteria, 6% Eukarya, and only two sequences of Archaea). The predominant eukaryotic sequences were similar to those from *Ascomycota*, followed by uncultivated fungi, *Basidiomycota* and *Mucorales* fungi (Rogers et al. 2013).

Among such pristine environments, analyses of ice-covered lake and water from five lakes showed that fungal taxa represented between 0.93% and 60.32% of the eukaryotic sequences (by 18S rRNA), with *Cryptomycota* and *Chytridiomycota* as the most abundant phyla of the fungal communities in all lakes, followed by members of *Ascomycota*, *Basidiomycota*, *Zygomycota*, and *Blastocladiomycota* (Rojas-Jimenez et al. 2017).

2.3.5 Snow

Antarctic snow pack harbours diverse, active, and viable microbial communities that represent almost all of the major phylogenetic groups, as described by Antony et al. (2016). Antony et al. found that sequences related to Ascomycota (Aspergillaceae), including *Aspergillus* and *Penicillium* strains, accounted for 82% of fungal communities in snow collected in Princess Elizabeth Land (East Antarctica). The remaining 18% of sequences were affiliated with Cryptomycota, which is a distinct fungal group that lacks a chitin-rich cell wall. Additionally, the cultivation-dependent approach used allowed the isolation of 17 fungi belonging to the genus *Cryptococcus*.

2.3.6 Wood and Historic Structures

Many structures were introduced into Antarctica by explorers, including woods and other organic structures, such as roof planks, fragmented wooden boxes, and wooden boats (Fig. 2.1). This material, which naturally favours the growth of fungi, has been widely used in studies of fungal diversity and was a likely source of exogenous microorganism introduction into Antarctica (Ludley and Robinson 2008; Arenz et al. 2006, 2011; Blanchette et al. 2010; Arenz and Blanchette 2011). In this sense, fungal strains have been long recovered from sites of human activity, as Antarctic soils have been contaminated with petroleum and discarded wood at sites near Antarctic stations, as described by Kerry (1990).

Wood samples from Lake Fryxell Basin (Dry Valley) and remote sites at Mt. Fleming and Allan Hills were subjected to DGGE using the ITS regions of ribosomal DNA for further identification. A total of 48 samples analysed by DGGE revealed 100 ITS sequences belonging to 71 fungal profiles and 28 taxa that were not detected by cultivation. Conversely, 25 fungal taxa were only detected using cultivation-dependent methods. Among filamentous fungi, most sequences showed high similarity with Ascomycota, followed by *Zygomycota*. For yeasts, most sequences were related to *Basidiomycota*, followed by Ascomycota. The most abundant taxa were *Cadophora*, *Cladosporium*, *Cryptococcus, Epicoccum*, and *Hormonema* (Arenz et al. 2006). Representatives of the genera *Cladosporium*, *Hormonema*, *Penicillium*, and *Lecythophora* were isolated from wood samples at the historic expedition huts on Ross Island (Held et al. 2005).

2.4 Conclusions and Perspectives

Although Antarctic fungal diversity remains incompletely characterized, recent studies have been illuminating. Although the preponderance of studies has focussed on understanding Antarctic bacterial diversity rather than fungal diversity, this might simply reflect the markedly greater abundance of bacteria at most environmental sites.

Knowledge of Antarctic fungal communities has been mainly gained through cultivation-dependent techniques and, more recently, cultivation-independent approaches that employ either direct microscopic analysis or NGS strategies. Notably, studies of Antarctic fungal diversity are essential to understand the impact of global warming and how the introduction of structures by explorers could affect biodiversity in such a remote and pristine place. Additionally, they may help to inform how the transport and dissemination of species can occur in Antarctica, as there is a continuum of environments that is often ignored. For example, fungal species can share similar lichens and be found in various types of soil samples.

Future strategies to cultivate Antarctic fungi that are currently considered to be noncultivable are promising. If successful, they may add up to our knowledge of endemism, dispersal, and fungal colonization in Antarctica and drive discovery of new taxa and metabolic routes. Additionally, many of these fungi possess unique properties and an array of putative new molecules to be used in biotechnological processes with the potential to improve the quality of life in modern society.

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