Fungal Biology Reviews 48 (2024) 100353

Contents lists available at ScienceDirect

Fungal Biology Reviews

journal homepage: www.elsevier.com/locate/fbr

Culturing the uncultured marine fungi in the omics age: Opportunities and challenges

Zhi-Feng Zhang [a,**], Fang Liu [b,c], Li-Rui Liu [d,e], Meng Li [b,c], Lei Cai [b,c], Shuangping Liu [a], Jian Mao [a,*]

[a] Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), Guangzhou, 511458, China
[b] State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100101, China
[c] University of Chinese Academy of Sciences, Beijing, 100049, China
[d] Archaeal Biology Center, Institute for Advanced Study, Shenzhen University, Shenzhen, 518060, China
[e] Shenzhen Key Laboratory of Marine Microbiome Engineering, Institute for Advanced Study, Shenzhen University, Shenzhen, 518060, China

ARTICLE INFO

Handling Editor: Dr Jan Dijksterhuis

Keywords:
Uncultured marine fungi
Culturability
Innovative techniques
Culturomics
Targeted isolation

ABSTRACT

Fungi are ubiquitous in all kinds of ecosystems with key ecological roles, while less than 10% of them have been described, of which, only about 1.2% are from marine habitats. Although the advance of next-generation sequencing has unquestionably improved our understanding of marine fungi, living cultures of marine fungi are important for studying the cell biology, ecological roles and evolution of microorganisms. In recent years, a number of efficient cultivation strategies, technologies, and devices have been newly developed, most of which were designed for prokaryotes and have been poorly applied to marine fungi. In this review, we give a brief discussion on the factors that may affect the isolation and cultivation of novel microorganisms, and review the omics-based innovative methods for the culturomics or targeted isolation. At last, we discuss the limitations of these approaches and their application potential on isolation and cultivation of marine fungi.

1. Introduction

Fungi are a highly diverse group and exist in all environments containing water and carbon sources on Earth, including terrestrial environments, oceans, and host-associated habitats such as animal gut systems and plant rhizospheres (Gladfelter et al., 2019; Liu et al., 2022). They play a key role in ecosystems, in which they thrive as parasites on host plants and animals, as symbionts to promote host growth, or as decomposers of organic matters driving nutrient cycling in detritus environments (Gladfelter et al., 2019; Richards et al., 2012). The number of fungal species is estimated to be 2.2–3.8 million (Hawksworth and Lücking, 2017) or even 11.7–13.2 million (Wu et al., 2019). However, the proportion of fungal species that have been described is only 3.9–6.8% (Wang et al., 2019). To a large extent, our current understanding of fungi is derived from cultured fungi, which are mostly isolated from terrestrial environments (Richards et al., 2012; Wang et al., 2019).

Oceans cover more than 70% of the Earth's surface with distinct physical and chemical characteristics (Raghukumar, 2017), harboring a great diversity of marine fungi. Marine fungi are generally recognized as "any fungus that is recovered repeatedly from marine habitats", because it is able to grow and/or sporulate (on substrata) in marine environments, forms symbiotic relationship with other marine organisms, or is shown to adapt and evolve at the genic level or be metabolically active in marine environments (Jones et al., 2019; Overy et al., 2019; Pang et al., 2016). They exist in every marine habitat, ranging from hydrothermal vents, cold seeps, deep sea sediment, arctic sea ice, to surface water, costal salt marshes and sandy beaches. Different fungal groups have adapted to free-living, parasitic or symbiotic lifestyles with other living organisms like algae, corals, sponges or even other fungi (Gladfelter et al., 2019; Overy et al., 2019; Raghukumar, 2017). The main ecological role of marine fungi is transforming and incorporating allochthonous and autochthonous organic matter into the food wed (Gladfelter et al., 2019; Grossart et al., 2019; Jones et al., 2019), which involves three major processes, namely mycoloop, mycoflux and benthic shunt. These three processes refer to parasitic or saprobic fungi rendering inedible phytoplankton edible to zooplankton grazers, fungal interaction leading to aggregation or disintegration of organic matters

* Corresponding author.
** Corresponding author.
E-mail addresses: zhangzf@gmlab.ac.cn (Z.-F. Zhang), maojian@jiangnan.edu.cn (J. Mao).

https://doi.org/10.1016/j.fbr.2023.100353
Received 23 November 2022; Received in revised form 29 November 2023; Accepted 13 December 2023

which may greatly affect aquatic carbon pump efficiency, and fungal colonization of organic litter rendering it palatable for macrozoobenthos on sediment, respectively (Grossart et al., 2019).

Given the widespread distribution and key ecological functions of marine fungi, a recurring question "how many marine fungi are there" has often been posed. However, marine fungi have been overlooked and poorly studied in comparison to their terrestrial counterparts (Gladfelter et al., 2019; Jones, 2011; Jones et al., 2019). Although recent advances in DNA sequencing technologies and metagenomics have drastically changed our understanding on the fungal diversity in marine habitats, including a number of "early diverging lineages" (Grossart et al., 2019; Li et al., 2018; Nagahama et al., 2011; Zhang et al., 2021b), most marine fungi have not yet been cultured (Jones et al., 2019). Consequently, most of our current knowledge on marine fungi is either derived from the minority of cultured lineages or from the data generated from culture-independent studies (Lewis et al., 2021; Liu et al., 2022). Although great efforts have been made to cultivate marine fungi since the mid-nineteenth century, fungal species documented from marine environments were still extremely low comparing to the overall described and estimated number of fungi (https://marinefungi.org/) (Jones et al., 2019).

With the development of sequencing-based methods, recent studies have provided numerous interesting insights into marine fungi. Isolation and cultivation are still essential to study the metabolic functions and physiological features of previously uncultured fungal lineages, and to properly understand their ecological roles in marine environments (Lewis et al., 2021; Liu et al., 2022; Marx, 2016; Overy et al., 2019). Traditional culturing methods have been continually used in fungal isolation. However, these approaches usually require substantial amounts of efforts, practices and patience to succeed. Furthermore, low abundance and specific culture preference of fungi, such as necessary substrate and growth conditions, interdependencies with other organisms, and physicochemical environmental conditions represent major challenges of marine fungi cultivation (Lewis et al., 2021). To address these limitations, some innovative technologies, such as high-throughput isolation and cultivation and targeted isolation, have been successfully applied to bring some previously uncultivated microbial "dark matter" into cultivation (Cross et al., 2019; Lewis et al., 2021; Liu et al., 2022; Zhang et al., 2021a). However, these approaches have been rarely used to culture marine fungi. In this paper, we provide an overview of progress in the cultivation of marine fungi, and innovative omics-based technologies that could facilitate the isolation, culture and characterization of poorly studied marine fungal groups.

1.1. The uncultured majority of marine fungi

Approximately 150,000 fungal species have been described so far (https://nmdc.cn/fungalnames/) (Wang et al., 2021, 2022; Wijayawardene et al., 2022), while the uncultured proportion accounts for 98.7%–98.9% of the total estimated global fungal species (Hawksworth and Lücking, 2017; Wu et al., 2019). Of the described fungal species, more than 97% belong to two higher fungal phyla, *Ascomycota* and *Basidiomycota*, while the remaining proportion belong to other 18 fungal phyla (Galindo et al., 2021; James et al., 2020). However, a high-throughput sequencing-based study on environmental DNAs (eDNA) suggested that currently cultured representatives cannot represent the natural fungal diversity (Amend et al., 2019). Specifically, marine fungi are a remarkably underrepresented group, as there are only 1857 cultured fungal species belonging to 769 genera retrieved from marine environments, accounting for only ~1.2% of the cultured fungi worldwide (ca. 150,000 species). This is rather unexpected as the ocean covers 70% of the earth surface and fungi are ubiquitous in every habitat from intertidal zone and surface water to depth of kilometers (Raghukumar, 2017; Richards et al., 2012).

Molecular analyses of marine eDNA have revealed an unexpectedly large diversity of undescribed fungi, including the "dark matter fungi (DMF)" which have never been observed in culture (Grossart et al., 2016; Jones et al., 2019; Lok, 2015). For instance, in costal sea water and sediment in North Carolina, USA, a high proportion of early-diverging fungi (ca. 39.5% of total OTUs and 33.6% of total reads) were detected (Picard, 2017). Through sequencing the entire internal transcribed spacer (ITS) region using Pacific Biosciences (PacBio) single molecule real-time (SMRT) sequencing, we have found a large proportion of basal fungal lineages, of which, *Rozellomycota* and *Chytridiomycota* comprised ca. 35.5% of the total fungal OTUs, indicating a high diversity of DMF in mangrove sediments (Zhang et al., 2021b). Currently, several conventional methods, such as direct detection followed by single spore isolation, tissue isolation, baiting followed by culturing, and dilution plating method, have been adopted to isolate and cultivate marine fungi from substrates such as plant, animal, seawater, and sediment in coast, open ocean, seafloor, and even the hadal trench (Table 1) (Jones, 2011; Kumar et al., 2021; Overy et al., 2019; Raghukumar, 2017; Rédou et al., 2016). However, similar to terrestrial fungi, the majority of recorded marine fungi are fast growing osmotolerant/halotolerant generalist genera, and belong to Ascomycota and Basidiomycota, while diverse and abundant chytrids and aligned lineages have been rarely cultivated (Grossart et al., 2019; Hassett et al., 2021; Ilicic and Grossart, 2022; Overy et al., 2019; Raghukumar, 2017).

1.2. Factors influencing the cultivation of marine fungi

It has been well known for over a century that only a small fraction of microbial population is culturable on synthetic media *in vitro* (Nichols et al., 2010; Rappe and Giovannoni, 2003; Rinke et al., 2013), and the percentage of culturable microorganisms in comparison with total cell counts is ca. 1% (Amann et al., 1995; Kaeberlein et al., 2002). The discrepancy between microorganisms presented in samples and those could be cultured in laboratory was referred to as the "great plate count anomaly" (Lewis et al., 2021; Staley and Konopka, 1985). We summarized several possible explanations for the difficulty to isolate and culture marine fungi.

Necessary substrate and growth conditions. In laboratory, the media most commonly used for marine fungi are similar to these for terrestrial fungi, including PDA, MEA, SDA, CDA, and CMA (Table 1), which cannot adequately simulate all essential components of the natural environment (Mu et al., 2020). Specific environmental conditions and growth factors include vitamins, amino acids, nucleotides, inorganic compounds and other undetermined components, such as metals, sulfur and nitrogen compounds, which are difficult to be identified and simulated (Davis et al., 2005; Kappler and Bryce, 2017; Lewis et al., 2021; Overmann, 2013). For example, by adding additional vitamins such as vitamin C (Ascorbic acid), vitamin B1 (Thiamine), vitamin B2 (Riboflavin), vitamin B6 (Pyridoxine), vitamin H (Biotin), and vitamin B12 (Cobalamin), Zhou et al. obtained a significantly higher diversity of fungal isolates from an artificial lake compared to using traditional culture media (Zhou et al., 2020). In some cases, sterilized seawater is supplemented to media, providing necessary salts and other components for marine fungi (Kjer et al., 2010; Mitchison-Field and Gladfelter, 2021). Another approach involves membrane-covered plates incubated in a seawater pool with constant seawater flow, which only allow small molecules to pass through the membrane and thus provide a simulated environment for fungi in the plates (Hagestad et al., 2019). For culturing some symbiotic fungi, media made up of homogenized host algae or plant and seawater is a feasible solution (Table 1) (Garzoli et al., 2018; Gnavi et al., 2017).

Additionally, new evidences suggested that the high nutrient concentration in artificial media has a significant effect on the microbial cultivation, since numerically abundant microbes in nonhost systems are oligotrophs (Cho and Giovannoni, 2004; Gao and Wu, 2018). In general, oligotrophs are hard to culture because of their slow growth rate and low adaptability (Carini, 2019). The nutrient-rich media may generate a hypertonic condition and kill the oligotrophic microbes

Table 1
A summary of traditional cultivation research on marine fungi.

Sample type	Location	Method	Media[a]	Culture condition	Mode of Nutrition	Fungal lineage	References
Sediment	Abyssal/hadal Trench	Dilution plating method	WSA, PDA, R2A, MEA, CDA, YPD, SDA, CMA, YM, Martin, RBA	4 °C, 28 °C, anaerobic, aerobic	Saprophytic	Ascomycota, Basidiomycota	(Chen et al., 2021; Gao et al., 2020; Xu et al., 2018b)
		Pressurized vessels	WSA, PDA, R2A, CDA, YPD, SDA	28 °C, anaerobic, 100 MPa	Saprophytic	Ascomycota	Chen et al. (2021)
	Hydrothermal vent	Dilution plating method	MEA, CDA, CMA, SDA, PDA, YM, GYP,	4 °C, 10 °C, 25 °C, 35 °C	Saprophytic	Ascomycota, Basidiomycota, Mucoromycota	(Pang et al., 2019; Xu et al., 2017, 2018b)
	Coast/seafloor	Dilution plating method	YPD, MEA, PDA, CMA, RBA, CZA, SDA, TSM, SNA	10 °C, 18, 24 °C, 30–40 °C	Saprophytic	Ascomycota, Basidiomycota, Mucoromycota, Mortierelomycota	(Bovio et al., 2017; Cecchi et al., 2021; Ettinger and Eisen, 2020; Florio Furno et al., 2022; Luo et al., 2020; Mitchison-Field and Gladfelter, 2021; Ogaki et al., 2020; Redou et al., 2015; Singh et al., 2012)
			SWA, NA, MV, MC, KMV+, YP	20–30 °C	Saprophytic, Parasitic	Thraustochytrid	Lyu et al. (2021)
		Baiting method	Pine, bone shrimp, and sweet gum pollen; then GPY	20 °C, 25 °C	Saprophytic, Parasitic	Thraustochytrid	(Lee Chang et al., 2012; Lyu et al., 2021; Unagul et al., 2017)
		Dillution plating and in-situ culture	CMA, SWA	*In-situ*	–	Ascomycota, Basidiomycota, Mucoromycota, Oomycota	Hagestad et al. (2019)
		Pressurized enrichment	PDB, DSB, MEB, Marine broth	5 °C, 25–45 °C, 4–40 Mpa	Saprophytic	Ascomycota, Basidiomycota	(Redou et al., 2015; Singh et al., 2012)
Sand	Coast	Dilution plating method	SDA	28 °C, 37 °C	Saprophytic, Pathogenic	Ascomycota, Basidiomycota	(Frenkel et al., 2022; Sabino et al., 2014; Velez et al., 2022)
Seawater	Coast/Ocean	Dilution plating method	YPD, MEA, PDA, CMA, GPYA	18–20 °C, 25 °C	Saprophytic	Ascomycota, Basidiomycota, Mucoromycota	(Bovio et al., 2017; Ettinger and Eisen, 2020; Fotedar et al., 2022; Mitchison-Field and Gladfelter, 2021)
		Membrane filter method	SDA, GYP	25 °C, 28 °C	Planktonic, Saprophytic	Ascomycota, Basidiomycota, Mucoromycota	(Fotedar et al., 2022; Pham et al., 2021)
		Dilution to extinction method	YM-IO agar	22 °C	Saprophytic	Ascomycota, Mucoromycota	Overy et al. (2014)
		Baiting method	Pine, bone shrimp, and sweet gum pollen	20 °C, 25 °C	Saprophytic, Parasitic	Thraustochytrid	(Gupta et al., 2013; Lyu et al., 2021)
			SWA, NA, MV, MC, KMV+, YP	20–30 °C	Saprophytic, Parasitic	Thraustochytrid	Lyu et al. (2021)
	Hydrothermal vent	Dilution plating method	CDA, GYP,	25 °C	Saprophytic	Ascomycota, Basidiomycota	Pang et al. (2019)
Organism: Algae	Coast/Ocean	Algal mass culture/dual culture	Host algal culture	19 °C, Light: dark photoperiod	Parasitic	Chytridiomycota and allied Phyla	(Lepelletier et al., 2014; Letcher et al., 2017; Seto and Degawa, 2018)
		Homogenize and dilution plating method	CMA, STD, PDA, Host medium	15 °C, 18 °C, 25 °C	Saprophytic, Endophytic	Ascomycota, Basidiomycota, Mucoromycota	(Francis et al., 2016; Gnavi et al., 2017)
		Sterile swab method	STD, PDA	18 °C	Saprophytic, Endophytic	Ascomycota, Basidiomycota	Francis et al. (2016)
		Tissue isolation	PDA, YM	18 °C	Endophytic	Ascomycota, Basidiomycota	Loque et al. (2009)
Organism: Plant/ Debris	Tidal zone, Mangrove, Salt marsh	Tissue isolation	MEA, PDA, CMA, PCA, TA, POM, LM, Host medium	20 °C, 25–28 °C	Endophytic	Ascomycota	(Cha et al., 2021; Chi et al., 2019; Collado et al., 2007; Ettinger and Eisen, 2020; Goncalves et al., 2021; Khalmuratova et al., 2015; Kjer et al., 2010; Pasqualetti et al., 2020; Supaphon et al., 2017; You et al., 2012)

(*continued on next page*)

Table 1 (*continued*)

Sample type	Location	Method	Media[a]	Culture condition	Mode of Nutrition	Fungal lineage	References
		Grind/ homogenize and dilution plating method	YPD, MEA, PDA, CMA, GPYA, RBA, Host medium	15 °C, 18–20 °C, 24 °C	Saprophytic, Endophytic	Ascomycota, Basidiomycota, Mucorromycota	(Garzoli et al., 2018; Mitchison-Field and Gladfelter, 2021; Nasr et al., 2017; Panno et al., 2013)
		Single spore isolation	MEA, PDA, CMA, CDA	25 °C	Saprophytic, Eathogenic, endophytic	Ascomycota, Basidiomycota	(Calado et al., 2019; Dayarathne, 2020; Dayarathne et al., 2020; De Padua and Dela Cruz, 2021; Devadatha, 2018; Devadatha et al., 2019; Fryar et al., 2020; Preedanon et al., 2017; Suetrong et al., 2017; Tibell et al., 2020)
Organism: Animal	Coast/ocean	Grind/ homogenize/ scrap and dilution plating method	YPD, MEA, PDA, DYA, RBA, LB, CMA, GA, GYP, CDA	15 °C, 18–20 °C, 25–27 °C	Symbiotic	Ascomycota, Basidiomycota, Mucorromycota	(Abd El-Rahman et al., 2020; Bovio et al., 2018; Mitchison-Field and Gladfelter, 2021; Mouton et al., 2012; Pang et al., 2019; Shaumi et al., 2021; Valderrama et al., 2021; Wang et al., 2011)
		Tissue isolation	DYA, RBA, SWA, CMA	15 °C, 25–27 °C	Symbiotic	Ascomycota	(Abd El-Rahman et al., 2020; Bovio et al., 2018)

[a] Medium abbreviation: CDA, Czapek dox agar; CMA, Corn meal agar; DYA, Dextrose yeast agar; GA, Gelatin agar; GPY, Glucose peptone yeast agar; Host medium, host homogenates with seawater and agar; LB, Luria bertani agar; LM, Lecithin medium; Martin, Martin broth; MC, Modified mar chiquita medium; MEA, Malt extract agar; MEB, Malt extract broth; MV, Modified vishniac's medium; NA, Nutrient agar; PCA, Potato carrot agar; PDA, Photo dextrose agar; PDB, Photo dextrose broth; POM, Palm oil media; RBA, Rose-Bengal agar; SDA, Sabouraud dextrose agar; SDA, Sabouraud dextrose broth; SNA, Synthetic nutrient-poor agar; STD, Standard marine agar; SWA, Sea water agar; TA, Tryptone agar; TSM, Trichoderma selective medium; WSA, Glucose, soybean extract, yeast extract, malt extract, NaCl, agar; YEP, Yeast extract-peptone agar; YM, Yeast maltose agar; YPD, Yeast extract peptone dextrose medium.

(Connon and Giovannoni, 2002). Moreover, filamentous fungi with faster growth rate in nutrient-rich media would occupy culturing space and deplete essential nutrients, inhibiting the growth of slow-growing fungi. To reduce the growth rate of fast-growing fungi, oligotrophic media and low culturing temperature have been widely applied to increase the efficiency of isolation and cultivation in different environments (Chen et al., 2021; Connon and Giovannoni, 2002; Ferrari et al., 2011; Hagestad et al., 2019; Jiang et al., 2017; Nagahama et al., 2011; Sun et al., 2020).

Interdependencies with other organisms. In natural environments, the survival of some microorganisms depends on the nutrient exchange (including essential molecules or electrons) with other organisms. This interspecies dependency is known as symbiosis (Govindarajulu et al., 2005; Limpens and Bisseling, 2003; McInerney et al., 2008; Spribille et al., 2016), which is quite common for marine fungi (Gladfelter et al., 2019; Grossart et al., 2019). Symbiotic relationships include mutualism, commensalism, and parasitism. It is proposed that obligate symbiotic microorganisms lose some necessary functions and depend on partners for their survival (Mu et al., 2020). In this case, methods that can simulate biotic exchange between partners or co-isolate both microbial partners could be advantageous for culturing these symbiotic microorganisms (Lewis et al., 2021; Mu et al., 2020; Overy et al., 2019).

For instance, *Chytridiomycota*, informally known as chytrids, represents an early diverging fungal group. Some of them are phytoplankton parasites, showing great diversity, abundance and wide distribution in marine environments (Comeau et al., 2016; Gutierrez et al., 2016; Ilicic and Grossart, 2022; Scholz et al., 2014). To discover members of chytrids and aligned lineages, targeted isolation approaches have been employed (Grossart et al., 2019). For instance, algal mass cultures (AMC) method uses cultured host algae to provide habitats for parasitic fungi including chytrids and aphelids (Carney and Lane, 2014; Grossart et al., 2019). By using this method, many new parasitic chytrids such as *Collimyces mutans*, *Rozella rhizoclosmatii*, and *Rhizophydium jobii* have been cultivated from marine algae hosts (Lepelletier et al., 2014; Letcher et al., 2017; Seto and Degawa, 2018). Furthermore, substrates on a highly nutritious agar and pine pollen baiting in seawater with antibiotics have been commonly used to cultivate thraustochytrids (Hassett et al., 2021; Lyu et al., 2021; Lyu et al. 2021, 2021) (Table 1).

Physicochemical environmental conditions. In addition to biotic factors, abiotic factors, mainly environmental physicochemical variables such as temperature, pH, salinity and redox conditions, are essential for the growth of microorganisms and are important selective forces for the structure and dynamics of microbial community (Mu et al., 2020; Tedersoo et al., 2014; Thompson et al., 2017). For example, salinity is a character that distinguishes marine habitats from freshwater and terrestrial environments. As a consequence, fungi in these habitats vary greatly in response to salinity. Usually, marine fungi are tolerant to a wider range of salinities than terrestrial and freshwater fungi. Zoosporic fungal-like organisms are more sensitive to salinity than Ascomycota and Basidiomycota (Jones et al., 2022). To culture marine fungi, sea salt or (filter-) sterilized seawater is commonly used to make media of suitable salinity (Dayarathne, 2020; Dayarathne et al., 2020; Kjer et al., 2010; Mitchison-Field and Gladfelter, 2021).

Furthermore, marine habitats like abyssal sea sediment, hydrothermal area, and cold seep, are characterized by high or low temperature, low pH, high pressure, and anaerobic conditions. These environmental physicochemical factors vary sharply in natural environments across microscale distances. Therefore, they are important determinants for microbial isolation and cultivation (Lewis et al., 2021). In particular, high pressure and anaerobic conditions in abyssal sea represent major challenges to obtain microbes in these habitats (Chen et al., 2021; Lewis et al., 2021; Mu et al., 2020). In this context, pressurized vessels are developed to simulate the high pressure and anaerobic conditions. These devices have been mostly applied for prokaryotes (Duperron et al., 2016; Timmers et al., 2015; Zhang et al., 2015b), while only a few studies adopted them to culture marine fungi (Table 1) (Chen et al., 2021; Rédou et al., 2016). By using a device with a hydrostatic pressure of 100 Mpa (equal to the water pressure in 11,000 m below ocean surface, about 1000 times standard atmospheric pressure), 19 pressure-tolerant and anaerobic fungal strains were cultivated from the hadal Challenger Deep, which belonged to terrestrial genera *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Exophiala*, *Gymnoascus*, *Microascus*, *Penicillium*, *Purpureocillium*, and mostly were found to be newly specialized in this specific habitat (Chen et al., 2021).

Resuscitation of dormancy. There are three physiological statuses for microbial cells, *i.e.* active, dormant, and dead (Kell and Young, 2000). Although microbes in dormant status remain metabolically active, they are commonly regarded as viable but nonculturable (VBNC),

and most of them have not been cultured (Barer and Harwood, 1999; Oliver, 2005). Dormancy is pervasive and may represent the default state of microbial life in extreme habitats, such as deep sub-surface sediments (Gonnella et al., 2016; Hoehler and Jorgensen, 2013; Lewis et al., 2021). Therefore, resuscitation of dormancy represents an essential step in microbial cultivation (Lewis et al., 2021; Mu et al., 2018). Generally, the ability to undergo cell division can be used to evaluate whether a microbe can be cultured. However, we know little about transition of microorganisms from dormant to active states, although a number of studies on microbial dormancy have been reported (Lewis et al., 2021; Mu et al., 2020). Meanwhile, it has been suggested that the transition from dormancy to active status may be a stochastic process (Epstein, 2013), affected by certain signal molecules, such as short peptides and cell wall muropeptides (Dworkin and Shah, 2010; Nichols et al., 2008). Hence, future research on the mechanism of dormancy recovery will facilitate the cultivation of marine fungi.

Low relative abundance. The low abundance is another obstacle to culturing microorganisms (Lewis et al., 2021; Mu et al., 2020). It is reported that microbial communities in natural environments are comprised of a small proportion of dominant taxa and a large proportion of rare taxa, which represent high and low relative abundance of microbial taxa, respectively (Chen et al., 2021; Zhang et al., 2021b). Although rare fungal taxa may be of great ecological functions, few have been isolated by the traditional dilution plating method (Lynch and Neufeld, 2015; Mu et al., 2020; Pedrós-Alió, 2012), possibly due to their low abundance. Another one is that, microorganisms with low abundance might be habitat-specific and result from abiotic and biotic factors as illustrated above (Jousset et al., 2017; Lynch and Neufeld, 2015). Microorganisms in a community have different metabolic ability and different niches. It means that a microorganism with low-abundance in a specific habitat may grow fast and be abundant under other conditions (Gudelj et al., 2010; Zhou and Ning, 2017). While, there may be some rare species that always grow slowly in any environments (Mu et al., 2020). To overcome this barrier, strategies including high-throughput isolation, identifying suitable growth conditions, inhibiting fast growing fungi, and separating isolation space provide solutions have been adopted. Specifically, dilution-to-extinction and isolation chip (iChip) have been applied to cultivate endophytic, soil (Collado et al., 2007; Ferreira et al., 2021; Unterseher et al., 2013), freshwater fungi (Zhou et al., 2020), and marine fungi (Overy et al., 2014), which have great potential in the isolation of rare marine fungi.

1.3. *Omics-based innovative techniques*

With the advance of metagenomics, two omics-based strategies, culturomics and targeted isolation, have been developed for the cultivation of uncultured microorganisms (Figs. 1 and 2).

Culturomics approaches. Culturomics is a high-throughput culturing approach that adopts multiple culture conditions for isolation, which can be combined with MALDI-TOF mass spectrometry and

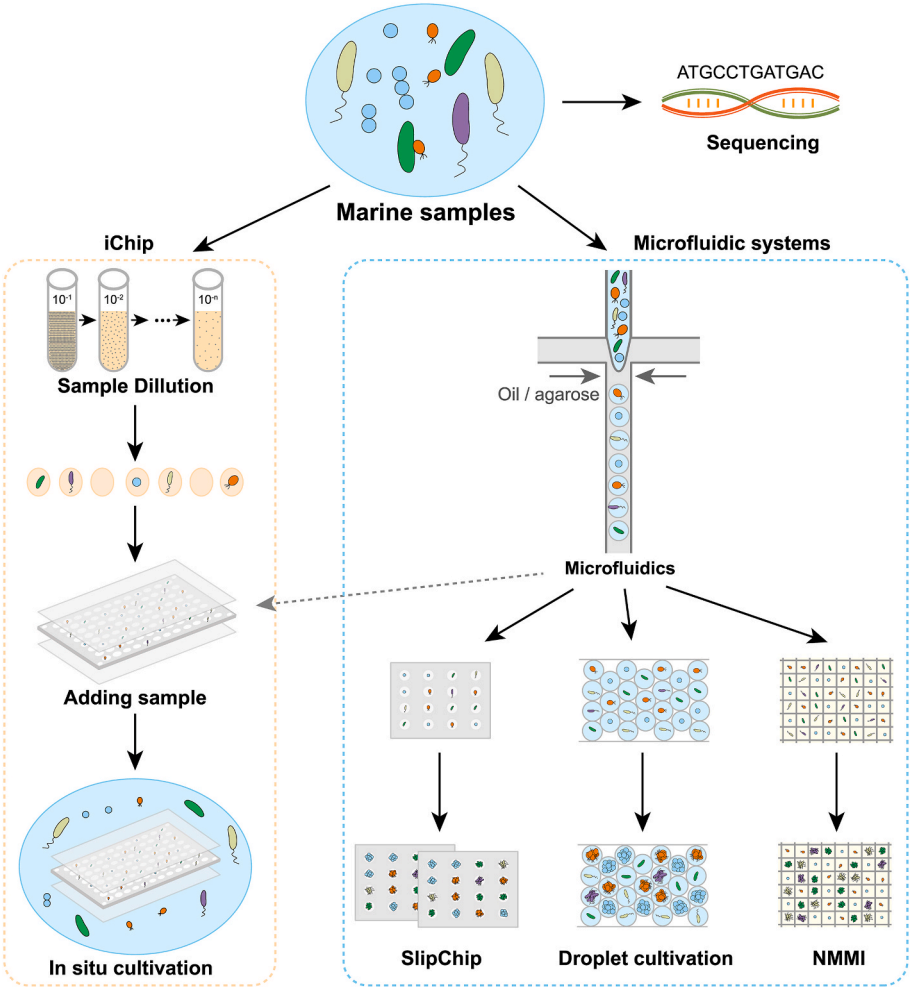

Fig. 1. Illustration of the culturomic approaches for the isolation and cultivation of marine microorganisms. Sequencing on the right of marine samples means that microbial diversity and community can be analyzed to find microorganisms of interest before isolation. iChip: isolation chip; NMMI: nanoporous microscale microbial incubators.

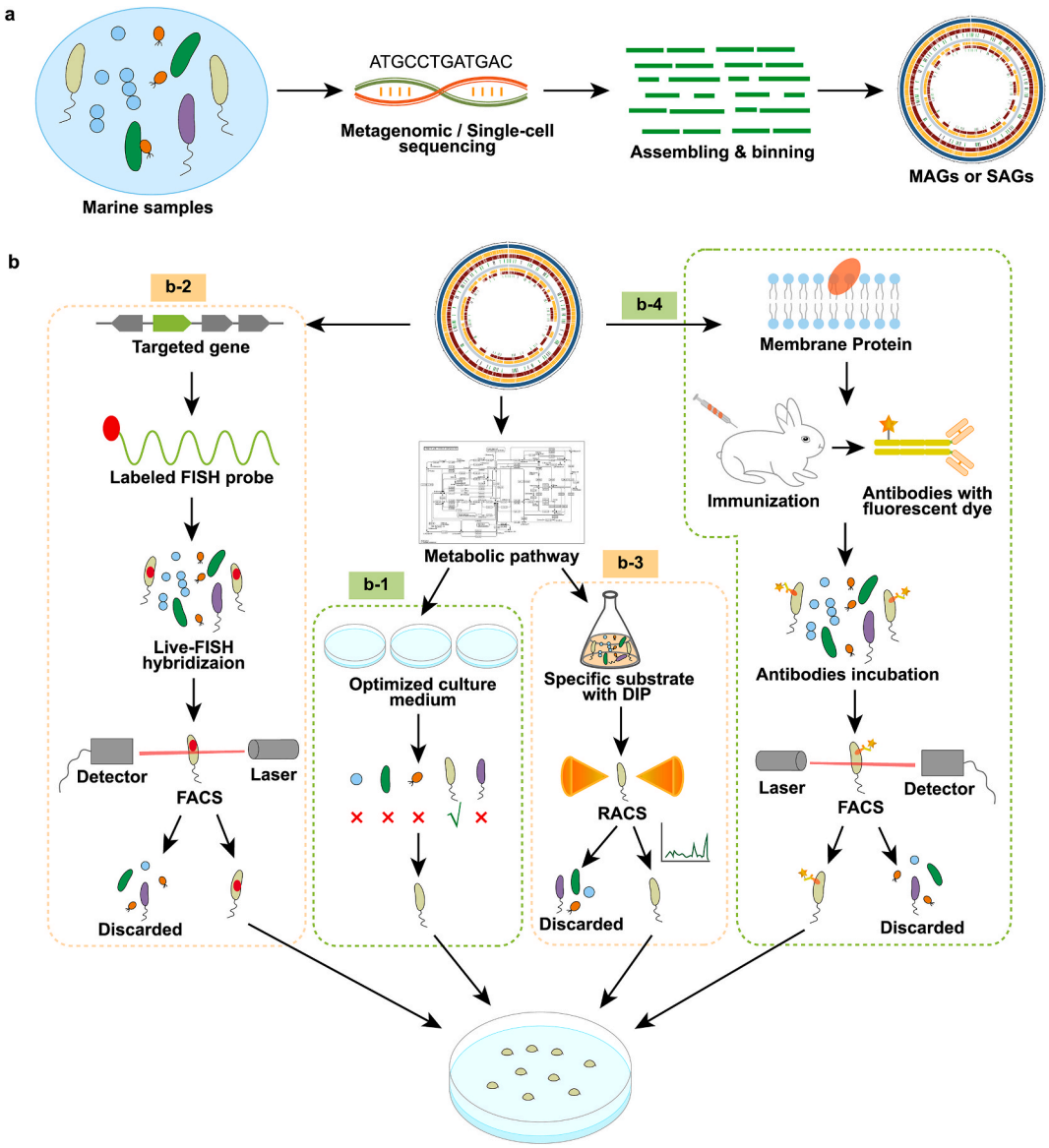

Fig. 2. Illustration of the innovative omics-based targeted methods for the isolation and cultivation of novel marine microorganisms. a. A brief pipeline for metagenomic analysis from environmental samples to metagenomic assembled genomes (MAGs) or single-cell amplified genomes (SAGs). b. A brief schema demonstrating the omics-based targeted approaches, including: b-1, culture medium optimization; b-2, gene probing guided fluorescence-activated cell sorting (FACS); b-3, Stable-isotope probing guided Raman-activated microbial cell sorting (RACS); b-4, reverse genomics guided isolation. Picture for metabolic pathway is derived from KEGG database as an example (https://www.kegg.jp/pathway/map00500).

16S rRNA gene sequencing for microbial identification (Lagier et al., 2012, 2016, 2018). Using 212 different culture conditions, Lagier et al. studied the culturable microbial composition of stools from three persons, and obtained 32,500 colonies belonging to 340 bacterial species (Lagier et al., 2012). Although culturomics was introduced for the human microbiota at first, here we can extend its definition for the cultivation of marine fungi. The new definition is a culturing approach that use high-throughput cultivation and high-throughput identification of microorganisms. Thus, membrane diffusion-based and microfluidics-based high-throughput cultivation methods fall into the culturomics category (Lewis et al., 2021).

Membrane diffusion-based cultivation. The inability to provide the growth condition and necessary growth factors in natural habitats remains a key barrier for microbial cultivation. Accordingly, *in situ* culturing methods by physically separating cells and natural habitats have been developed (Bollmann et al., 2010; Lewis et al., 2021; Mu et al., 2020; Nichols et al., 2010). To achieve the separation, a filter or membrane was designed, with a pore size small enough for growth factors to diffuse in and inhibiting metabolites to diffuse out, while cells are not allowed to go through (Zhou et al., 2020, 2021). In this manner, the separated chambers mimic natural environments for microbial cells retained inside (Berdy et al., 2017; Lewis et al., 2021).

These devices include the isolation chip (iChip) (Kaeberlein et al., 2002; Nichols et al., 2010), a hollow-fibre membrane chamber device (Aoi et al., 2009), and other related devices (Chaudhary et al., 2019; Ferrari et al., 2005; Pudasaini et al., 2017). IChip is a representative of membrane diffusion-based technology with capacity to perform high-throughput cultivation (Fig. 1). It consists of a plate with an array of small holes that function as micro-chambers to accommodate and cultivate one cell per chamber, and two sealing membranes with 20–30 nm pore size (Berdy et al., 2017; Lewis et al., 2021). Once diluted microbial samples were added, the chamber is placed in natural environments. This approach could result in a 300-fold increase in microbial recovery compared to conventional plating method (Berdy et al., 2017;

Kaeberlein et al., 2002). Although iChip is commonly used for the isolation of marine bacteria (Kaeberlein et al., 2002; Nichols et al., 2010), it can be applied to isolate marine fungi (Overy et al., 2019). For example, in partnering with Professor Lei Cai' group in Institute of microbiology, Chinese Academy of Sciences, an iChip-based investigation on fungal diversity in mangrove sediments was performed. In total, more than 700 fungal stains were obtained. About 70% of them were isolated only by iChip, and 45 strains were potential novel fungal species (Li et al., 2023). Despite of the high discovery rate of novel microbial isolates, there are some limitations. Since several plates with hundreds of micro-chambers are required for each sample at a time, it can be time- and effort-consuming to find a suitable dilution ratio when processing multiple samples. To increase work efficiency, the following microfluidic systems may be combined.

Microfluidic systems. Microfluidic systems manipulate small volumes of liquids and gases in integrated channels with dimension of tens of micrometres, which have been increasingly applied in microbial cultivation and other fields (Fig. 1) (Convery and Gadegaard, 2019; Shang et al., 2017; Whitesides, 2006). The devices are characterized by high scalability, miniaturized overall experimental set-ups, as well as the capability to manipulate large numbers of single cells from samples in parallelization under a range of substrates or under different physicochemical conditions, including both aerobic and anaerobic conditions, and therefore high throughput cultivation (Lewis et al., 2021; Ma et al., 2014a, 2014b). The encapsulation methods of microfluidic systems provide the cells with separated growth space from each other, which decrease the competition between species. Hence, these systems are reported to recover more phylogenetically diverse microorganisms than traditional cultivation methods in most cases (Watterson et al., 2020).

Commonly used microfluidic systems include droplet cultivation (Kaminski et al., 2016; Shang et al., 2017), nanoporous microscale microbial incubators (NMMI) (Ge et al., 2016), and SlipChip (Du et al., 2009; Ma et al., 2014a, 2014b) (Fig. 1). Droplet cultivation manipulates single microbial cells or small populations of cells in micro liquid or gel droplets, and the cell-containing droplets are incubated in a microfluidic device (Lewis et al., 2021). The NMMI device creates micro-well arrays, namely diffusion chamber, on transparent glass slides using nanoporous hydrogels, which allow chemical diffusion between diffusion chambers, and each microbial cell occupies a single well and grow within the well (Ge et al., 2016). Although SlipChip was originally designed for chemical and biochemical analyses, it was later redesigned for high-throughput cultivation of microorganisms (Du et al., 2009; Lewis et al., 2021; Ma et al., 2014a). The redesigned SlipChip device consists of two plates etched with ducts acting as fluid conduits, and thousands of wells acting as individual microcompartments that contain various media and substrates, where microorganisms are confined and cultivated at single-cell level. Once a single microbial cell is inoculated in the microcompartment, the chip is incubated to multiply the cell. After micro-sized cultures formed, the plates are then slipped apart and divided into two identical replicate microcultures. Subsequently, one replicate plate can be individually screened or destructed for growth, taxonomic identification or genomic analysis, and the corresponding plates with living cells can be preserved for continued cultivation (Lewis et al., 2021; Ma et al., 2014b). These microfluidic devices hold enormous opportunities for marine microbiological studies, including high-throughput cultivation of microbes and discovery of novel natural products and enzymes (Cario et al., 2022; Hu et al., 2021; Leung et al., 2012; Rusconi et al., 2014; Xu et al., 2018a).

Omics-based targeted isolation. Metagenomics has significantly enhanced our understanding of environmental microorganisms, not only expanding our knowledge on the microbial tree of life, but also providing a relatively unbiased view of the community structure and functional (metabolic) potential (Cross et al., 2019; Hugenholtz and Tyson, 2008). Besides metagenome-assembled genomes (MAGs), single-cell amplified genomes (SAGs) have led to a deluge of proposed new microbial lineages (Cross et al., 2019; Gawad et al., 2016; Hug et al., 2016). Based on the information generated from MAGs and SAGs, a number of specific targeted strategies, including culture medium optimization, gene targeted isolation, stable-isotope probing guided Raman-activated microbial cell sorting (RACS), and reverse genomics, have been proposed to bring uncultured microorganisms into cultivation (Fig. 2) (Lewis et al., 2021; Mu et al., 2020). These novel strategies can be complementary to the traditional culture methods and culturomics methods described above.

The first step for metagenomics-guided targeted isolation is metagenomic assembling (Fig. 2a). The assembled contigs are subsequently binned to reconstruct MAGs (Quince et al., 2017; Thomas et al., 2012). After bin refinement and quality evaluation, high quality MAGs could be used for the following functional annotation and metabolic reconstruction. The former step involves gene predication and gene annotation via sequence similarity or hidden Markov models (HMMs) of functional domains referring to a range of databases. Metabolic pathways can be predicated and reconstructed by methods such as BlastKOALA, GhostKOALA (Kanehisa et al., 2016), and RAST (Aziz et al., 2008). The analyses of MAGs and SAGs provide valuable information on microbial traits such as primary metabolism, substrate utilization, oxygen requirements, resistance to antibiotics, cell structures, and even interspecies interactions, which are pivotal for targeted isolations of uncultured microorganisms (Cross et al., 2019; Fan et al., 2012; Liu et al., 2022).

Culture medium optimization. Understanding the nutritional requirements and metabolic characteristics is important to isolate and stably cultivate uncultured microorganisms (Liu et al., 2022). MAGs and SAGs provide opportunities to confirm specific nutritional requirements, antibiotic resistance and growth conditions for fastidious microorganisms, which can be used to optimize the culture media and culture condition (Figs. 2b–1) (Liu et al., 2022). This culture medium optimization strategy has been widely adopted in the cultivation of specific microorganisms in various habitats (Karnachuk et al., 2021; Lugli et al., 2019; Pope et al., 2011). For example, Pope et al. (2011) successfully enriched a dominant *Succinovibrionaceae* group, WG-1, from the wallaby digesta samples, by supplying starch as the sole carbon source, urea as the nitrogen source, and antibiotic bacitracin in medium, all of which were predicated from reconstructed MAGs. Likewise, when analyzing the samples from deep subsurface aquifers, Karnachuk et al. obtained a novel MAG with genes encoding starch utilization enzymes and membrane transporter protein. Accordingly, a strain of *Brevinematales* was isolated by using modified spirochete medium with maltose and starch (Karnachuk et al., 2021). For growth condition optimization, Sauer & Wang developed a useful tool to predict the optimal growth temperature of prokaryotes based on genomic information (Sauer and Wang, 2019). Knowledges of these physicochemical factors such as temperature, pH, salinity, or oxygen concentration derived from genomic data are crucial for isolation of extremophiles from extreme environments (Liu et al., 2022).

Gene probing guided FACS. Fluorescence-activated cell sorting (FACS) is a common method to sort cells based on fluorescence signals (Liu et al., 2022). Despite of the intrinsic fluorescence properties of some microorganisms, cellular targets such as DNA and phospholipid membranes can be stained by some fluorescent dyes. The cells with distinguished fluorescence can be separated using FACS (Liu et al., 2022). Fluorescence *in situ* hybridization (FISH) is the most widely used fluorescent labelling method to identify and quantify cells of specific microbial groups (Lewis et al., 2021; Wagner and Haider, 2012). However, in traditional FISH protocols, procedures of cell fixation and permeabilization would kill living cells, thus is not appropriate for cultivation experiments. To overcome this obstacle, a method called "live-FISH" was demonstrated, which could introduce labelled DNA probes into living bacterial cells by means of chemical transformation instead of traditional cell fixation and permeabilization, and thus maintain the viability of labelled cells for subsequent cultivation

(Fig. 2b–) (Batani et al., 2019). For microorganisms of interest in a complex community, the FISH probes can be designed using the specific DNA or gene sequences derived from metagenomic data. However, since the survival rates of cells in live-FISH experiments remain relatively low, the method may be unsuitable for isolating microorganisms of low abundance (Lewis et al., 2021).

Stable-isotope probing guided RACS. Stable-isotope probing guided Raman-activated microbial cell sorting (RACS) is another highly efficient isolation approach (Lee et al., 2019, 2021). Heavy water (D_2O), which is proportionally incorporated into the synthesized lipids of active cells, is an economic and effective deuterium isotope probe (DIP) for Raman detection (Berry et al., 2015; Lee et al., 2019). In regular isolation process using RACS, microbial cells are shortly incubated using designed medium with specific substrate and D_2O. Physiologically active microbial cells will absorb D_2O and generate C-D fingerprint peaks in the single-cell Roman spectra (2040–2300 cm^{-1}) in a microfluidic device, then the corresponding cells will be captured and immediately sorted to a sterile end of the capillary with optical tweezers (Figs. 2b–3) (Berry et al., 2015; Lee et al., 2019; Lewis et al., 2021). This approach can detect metabolically activate cells in a sample without destructive fixation, which are then committed to downstream applications, including whole-genome sequencing, targeted cultivation or complementary microscopic analyses (Hatzenpichler et al., 2020; Lee et al., 2019; Zhang et al., 2015a). Therefore, the possible microbial traits derived from metagenomic data such as necessary growth factors, substrate utilization, oxygen requirements, and antibiotic resistance, provide possibility to label, sort, and isolate targeted microorganisms using RACS (Lee et al., 2019; Liu et al., 2022). This strategy has been applied using samples of mammalian guts (Berry et al., 2015; Wang et al., 2020), wastewater treatment plants, and marine environments (Lee et al., 2019, 2021).

Reverse genomics guided isolation. A recent technique described by Cross et al. (2019), namely reverse genomics, was capable to capture previously uncultured microbes of interest from complex communities following procedures in Figs. 2b–4. First, the target microorganisms belonging to novel or important lineages are identified, and their genomes are reconstructed from metagenomic data; Second, based on these retrieved genomes, highly expressed membrane proteins with extracellular domains can be predicated and identified; Accordingly, a target-protein domain antigen is synthesized and inoculated into a suitable animal for antibody production; The raised antibodies are subsequently purified, coupled to a fluorescent dye, and added to corresponding environmental samples; After labelling antibodies, target cells or symbionts are then sorted onto liquid or solid growth media via FACS based on the antibody-conferred signal (Cross et al., 2019; Lewis et al., 2021). In contrast to traditional destructive FISH, reverse genomics can label cells while maintaining their viability (Lewis et al., 2021; Liu et al., 2022). Using this method, Cross et al. cultivated four previously uncultured human oral bacterial lineages (Cross et al., 2019). Besides the antibodies labelled FISH, some antibiotics that directly bind to specific bacterial surface structures can also be used as probes for bacteria sorting (Lin et al., 2021; Miao et al., 2020).

1.4. Limitations of omics-based cultivation on marine fungi

Although culturomics and omics-based targeted isolation strategies have great potentials and some of these technologies have been successfully applied on marine bacteria (Kaeberlein et al., 2002; Lee et al., 2019, 2021; Nichols et al., 2010), they have been rarely applied to marine fungi. There are challenges and limitations that need to be overcame in general and especially for marine fungi.

General limitations and challenges. Theoretically, these innovative methods target diverse taxa, but, the practical application is complicated in some cases (Lewis et al., 2021). For example, microorganisms in biofilms could be difficult to separate for cell sorting (Müller and Nebe-von-Caron, 2010). In addition, samples from nutrient-rich or non-biological particle-rich sedimentary habitats may influence the performance of molecular and flow-based methods. In this case, samples should be pre-processed to separate cells from particles (Lewis et al., 2021; Morono et al., 2013).

Another challenge for some of these methods is regarding to the isolation and cultivation of microorganisms in anoxic and high-pressure conditions (Chen et al., 2021). Inoculation devices with these functions are usually difficult to fit currently available cell sorters due to the size of the device. In this case, developing smaller fluorescence-based cell sorters that can be used under anaerobic condition would be a possible solution (Lewis et al., 2021; Liu et al., 2022). Meanwhile, the supplement of gaseous substrates, such as H_2, CO_2, and CH_4, is difficult to incorporate into culturomic methods, because of the unavailability of the device. Furthermore, an intrinsic challenge regarding the cultivation of thermophiles is liquid evaporation during cultivation, which dries out the culture and prevents the real-time monitoring of microbial growth because of condensation (Lewis et al., 2021).

In our cultivation experiment with mangrove sediment using iChip, some of cultivated fungal strains could not maintain growth continuously when inoculated to new medium in laboratory. It represents a common phenomenon in microbial study, and suggests that cultivation using suitable medium and physicochemical conditions is pivotal to obtain isolated microorganisms (Lewis et al., 2021). As introduced above, inferring phenotypic features from metagenomic, proteomic and transcriptomic data provides an opportunity to define the optimal medium and culture conditions for uncultured target microorganisms (Gutleben et al., 2018; Lavy et al., 2014; Sauer and Wang, 2019). Nevertheless, genome data alone may not be sufficient to accurately predict all necessary requirements for microbial growth (Lewis et al., 2021). In this case, sophisticated 'next-generation' physiology approaches can be an alternative or supplementary to obtain these information (Hatzenpichler et al., 2020; Lewis et al., 2021).

Reverse genomics and similar methods also have a few shortcomings. For instance, the prediction and identification of suitable surface exposed epitopes may be not possible. In addition, the post-translational modification of the target epitope such as glycosylation may sterically hinder the recognition and combination of antibodies. One possible solution for these challenges is to select several epitopes or use entire protein domains as antigens (Liu et al., 2022).

Specific limitation and challenges for marine fungi. To isolate and cultivate marine fungi using these innovative technologies, the characteristics of fungi that differ from bacteria and archaea may cause additional problems and should be taken into consideration.

Because fungal genomes are difficult to be reconstructed from metagenomic data, it is a challenge to apply omics-based targeted isolation method to marine fungi. On one hand, most fungi contain multiple sets of chromosomes, and their complex genomes are usually much larger than prokaryotes and contains long repeats (West et al., 2018). On the other hand, the abundance of marine fungi is much lower than that of marine prokaryotes. As revealed by one of our metagenomic studies, the relative abundance of fungi is less than 10% of overall microbial community in mangrove sediment (Zhang et al., 2023). New analytic strategy for eukaryotic metagenomics, new sample processing procedures, and single-cell genomics may be combined to address these limitations. By developing a new prediction algorithm for eukaryotic contigs, namely EukRep, researchers in Professor Banfield's group retrieved 21 fungal MAGs from several different environments (Olm et al., 2019; West et al., 2018). Using Tara Oceans metagenomic datasets and single-cell eukaryotic genomic data, Delmont et al. reconstructed 713 eukaryotic MAGs and SAGs. 23 of them have completeness over 90%, 97 have completeness over 80%, and 200 have completeness over 50%. However, only two of these genomes were fungi (Delmont et al., 2022). In addition, the long reads generated by third generation sequencing, represented by PacBio SMRT and Oxford Nanopore technologies, can partly resolve ambiguous repetitive regions and improve genome contiguity in fungal metagenomics.

Another challenge is associated with the filamentous growth of many fungi for microfluidic systems, in particular droplet cultivation devices. Currently, the most common materials employed to encapsulate and immobilize single-cell droplet in microfluidic devices are oil, agarose, and polymer hydrogels (Lagus and Edd, 2013; Lin et al., 2011). Unlike bacteria, hyphae of filamentous fungi are usually strongly destructive and deeply invade into agar medium, which may destroy the wall of droplets or wells and invade into neighboring cultivation unites. Therefore, new materials that can encapsulate filamentous fungi is needed for droplet cultivation devices. As discussed above, a modified device that combines microfluidics and iChip *in situ* cultivation may greatly benefit the processing efficiency and recover more phylogenetically diverse fungal isolates.

Several previous studies have found that different fungal lineages in liquid and solid media have different nutrient utilization ability, metabolic activity, and growth rate (Novotna et al., 2023; Pradeep et al., 2013; Viniegra-González et al., 2003). Therefore, which media to be selected, liquid or solid, should also be considered for the cultivation of marine fungi. In traditional culture method, solid media are the most commonly selected, possibly because of two reasons: 1) filamentous fungi develop mycelium on the sides of flasks or form clumps of mycelium, and their morphology is difficult to observe (Droce et al., 2013); 2) Fungi can form single colonies in solid plates, which are convenient for morphological observation and colony selection. As a consequence, liquid media are usually used to enrich specific fungal groups instead of isolation (Singh et al., 2012), except for dilution-to-extinction method, which is similar to the high-throughput cultivation method for root bacteria community (Zhang et al., 2021a). Besides, liquid media seems to be practicable for some culturomics methods, such as iChip, SlipChip, Droplet cultivation and NMMI, which provide separating spaces for single fungal colony.

Overall, the culturomics and omics-based targeted isolation approaches have bright prospects in the cultivation of uncultured marine fungi.

2. Conclusions

Metabarcoding and metagenomic sequencing have become a standard assay to provide a complete census of microbial diversity, but often the census data have gaping holes, and these methods generate mostly fragmented genomes (Marx, 2016). Meanwhile, living microbial cultures are essential to understand their metabolic functions, physiological features, and ecological roles. These shortages and requirements lead to a wide-scale need for microbial isolation and cultivation, and as a consequence, numerous innovative methods have been developed. The omics-based innovative methods summarized herein adopt either a high-throughput or a targeted strategy to isolated microbial cells by trying to resolve or at least partly resolve the influencing factors of culturability. Although these innovative strategies are theoretically feasible, few of them have been successfully applied in the cultivation of marine fungi. There are possible explanations for this case (Lewis et al., 2021). The first explanation is that these methods may fail to overcome currently limiting biological processes, and results in a small number of captured fungi. The second one is that these methods are rarely applied to marine environments, especially for marine fungi, which is commonly neglected. Considering the current situation of marine fungi, the second explanation may be more important. Therefore, the optimization of these innovative technologies is required to convert uncultured marine fungi into cultivable ones. In addition, the further development and maturation of advanced cultivation technologies may lead to new breakthroughs in capturing the uncultured marine fungi.

Author contributions

Conceptualization, Z.-F.Z.; writing—original draft preparation, Z.-F. Z.; writing—review and editing, F.L., L.-R.L., M.L., L.C., S.-P.L., and J.M. All authors have read and agreed to the published version of the manuscript.

Funding

This work was financially supported by the Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou) (GML2021GD0806).

Declaration of competing interest

All authors declared no conflict of interest and no relevant relationships.

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