Salinity and temperature affect growth rate of Alphamyces chaetifer and Gorgonomyces haynaldii (Chytridiomycota) isolated from coastal habitats of Taiwan

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Salinity and temperature affect growth rate of *Alphamyces chaetifer* and *Gorgonomyces haynaldii* (Chytridiomycota) isolated from coastal habitats of Taiwan

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Abstract: Salinity and temperature are two of the key environmental variables influencing the distribution of fungi. Results from the limited research available on the effects of salinity and temperature on growth and reproduction of chytrids were inconclusive. This study investigated the combined effects of salinity (0, 1, 2, 4, 8, 16, 32) and temperature (17, 24, 30 °C) on the growth rate of *Alphamyces chaetifer* (isolates IMB230, IMB231, IMB232) and *Gorgonomyces haynaldii* (IMB233, IMB237, IMB239) cultured from low-salinity water samples collected from coastal wetlands/ponds of eastern Taiwan. All isolates grew well at salinity 4 or below, irrespective of the incubation temperatures, although some grew significantly faster at 30 °C. No growth was observed at salinity 16 or 32. At or below 4, *A. chaetifer* IMB230 and IMB231, isolated from the same collection site where salinity was 2, produced the fastest growth rate at these salinities while *G. haynaldii* IMB237 and IMB239, isolated from water samples of zero salinity, had the slowest growth rate. These results agree with previous research that chytrids are sensitive to salinity, and may explain why only 27 culturable Chytridiomycota species and allied taxa have been documented from the marine environment.

Keywords: coastal fungi; ecophysiology; rDNA; Rhizophydiales; zoosporic fungi

1 Introduction

Significant progress has been made in the classification of fungi, from four phyla (Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota) to nineteen phyla (Aphelidiomycota, Ascomycota, Basidiobolomycota, Basidiomycota, Blastocladiomycota, Calcarisporiellomycota, Caulochytriomycota, Chytridiomycota, Entomophthoromycota, Glomeromycota, Kickxellomycota, Monoblepharomycota, Mortierellomycota, Mucoromycota, Neocallimastigomycota, Olpidiomycota, Rozellomycota (or Cryptomycota: Jones et al. 2021), Sanchytriomycota, Zoopagomycota) (Voigt et al. 2021). This substantial change was brought about by recent phylogenetic studies (Tedersoo et al. 2018; Wijayawardene et al. 2018) and environmental DNA methodology (Picard 2017; Zhang et al. 2021) which revealed the highly diverse lineages of basal fungi and zoosporic fungi (Chytridiomycota and allied taxa). In particular, a greater diversity of marine Chytridiomycota and allied taxa has been revealed in the epipelagic to deep-sea zones using environmental DNA (Hassett et al. 2020; Le Calvez et al. 2009). Although this high diversity of marine zoosporic fungi is recognised, their ecological role and their interaction with other organisms and the environment are largely unknown (Cunliffe 2022).

Salinity and temperature are two of the major environmental factors influencing the distribution of fungi, including zoosporic fungi (Booth 1971; Jones 2000). Booth (1971) studied the combined effects of salinity (0, 5, 15) and temperature (10, 20, 30 °C) on the growth (dried weight) of 57 isolates of zoosporic fungi from soils of Arctic and western north America across various habitats, and discovered species-specific and isolate-specific responses to salinity and temperature, which correlated with habitat types. Generally, temperature exerted a stronger effect on growth of the fungi.
than salinity, while better growth was observed at low salinities (0, 5; Booth 1971).

Sodium ions appear to be a key element affecting the growth of marine Chytridiomycota; for example, a Phlyctochytrium species produced the best growth at 237 mM sodium, with poor growth at 0 mM or concentrations higher than 560 mM (Amon 1976). Terrestrial zoosporic fungi were also sensitive to sodium chloride concentration, and only grew up to 10 g L$^{-1}$ sodium chloride in a solid medium; only one isolate each of Chytridiomycetes hyalinus and Rhizophydiurn sp. could grow at 4.375 g L$^{-1}$ sodium chloride in a liquid medium (Gleason et al. 2006).

In terms of reproduction, Höhnk and Aleem (1953) inoculated Ophidiurn maritimum-infected Ruppia pollen in water of different salinities (0, 4, 13, 17, 28) with fresh pollen, and found that most sporangia were produced from 0 to 17 with an optimum of 13, while minimal production of sporangia was observed at 28. Scholz (1958) tested reproduction of a number of zoosporic fungi and Thraustochytridium pachydermum isolated from saline soils; the majority of the species could reproduce in freshwater, seawater and sodium chloride solution, suggesting their plasticity towards salinity.

The effects of temperature on chytrids were found to be taxon-specific and determined by environmental conditions; for example, the Blastocladiales and the Spizellomycetales could withstand higher temperatures for growth than the Chytridiales and Rhizophydiales (Gleason and McGee 2008). Temperature can affect growth, reproduction and infection dynamics of chytrids (Scholz et al. 2017). For some species, higher temperatures can accelerate their reproductive cycles, leading to faster growth rate; in contrast, lower temperatures may slow down or inhibit reproductive processes (Maier and Peterson 2017).

To further understand the effects of salinity and temperature on the physiology of chytrids, we tested the combined effects of salinity (0, 1, 2, 4, 8, 16, 32) and temperature (17, 24, 30 °C) on the growth (rate) of isolates of Alphamyces chaetifer (Sparrow) Letcher and Gorgonomyces haynaldii (F. Schaarschm.) Letcher, cultured from low-salinity water samples collected in coastal ponds and wetlands in the eastern counties of Taiwan, using a spectrophotometric method (Langvad 1999; Su et al. 2021).

2 Materials and methods

2.1 Sampling, isolation and culture

Surface water samples were collected in 50 mL centrifuge tubes from Zhiben Wetland, Guanshan Waterfront Park, Pipa Lake of Beinan Estuary Wetland in Taitung, and Waterbird and Wetland Conservation Park in Hualien, Taiwan. Collection details of the samples are provided in Table 1. Samples were kept in an ice box and transported to the laboratory at National Taiwan Ocean University within 24–36 h. Water samples (20 mL) were dispensed into Petri dishes supplemented with pine pollen, snakeskin and antibiotics (Penicillium G and streptomycin sulfate, 0.2–0.5 g L$^{-1}$), and incubated at 24 °C. Another water sample (100 μL) was spread onto 1/4 YpsS agar (5 g L$^{-1}$ soluble starch, 0.25 g L$^{-1}$ yeast extract, 0.25 g L$^{-1}$ KH2PO4, 0.125 g L$^{-1}$ MgSO4·7H2O, 15 g L$^{-1}$ agar technical, 0.2–0.5 g L$^{-1}$ each of Penicillin G and streptomycin sulfate). The inoculated samples were checked daily. When zoospores were observed in the water sample, 50 μL of the water were taken and spread onto fresh 1/4 YpsS agar medium. Infected pollen was picked up with a fine needle and dragged on fresh 1/4 YpsS agar medium. Colonies developed on the spread plates were checked for sporangia and zoosporangia under a light microscope and subcultured onto fresh 1/4 YpsS agar medium. Pure cultures were kept at 24 °C and subcultured every 2 weeks.

2.2 Molecular identification

Colonies were scraped from the agar plate and ground into fine powder in liquid nitrogen with pestle and mortar. Genomic DNA extraction was performed with DNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer’s instructions. Internal transcribed spacer (ITS) regions of the rDNA was amplified with the primers ITS4 and ITS5 (White et al. 1990) to obtain the complete ITS1-5.8S-ITS2 region. PCR reactions were conducted with the following ingredients: ca. 20 ng DNA, 0.2 μM of each primer, 12.5 μL Gran Turismo PreMix (Ten Giga BioTech, Taipei, Taiwan) and PCR water (topped up to 25 μL). The amplification cycle consisted of an initial denaturation of 95 °C for 5 min, followed by 35 cycles of (1) denaturation (95 °C for 30 s), (2) annealing (52 °C for 30 s), (3) extension (72 °C for 30 s), and a final extension at 72 °C for 7 min. The PCR products were confirmed with agarose electrophoresis and sent to Genomic (New Taipei City, Taiwan) for sequencing with the same primers. Sequences were checked and assembled in MEGA 11 (Tamura et al. 2021) and submitted to NCBI for a nucleotide BLAST search.

2.3 Growth study

Three isolates each of the species A. chaetifer (IMB230, IMB231, IMB232) and G. haynaldii (IMB233, IMB237, IMB239) were selected for a growth study and inoculated onto 1/4 YpsS agar medium for 5–7 days. Sterile water (3 mL) was added on top of the colonies and left for a few hours until zoospores were released. Zoospores were then collected in suspension, counted with a hemocytometer and adjusted to 3 × 10$^6$ spore mL$^{-1}$. The modified protocol used for growth measurement in Langvad (1999) and Su et al. (2021) was followed. The zoospore suspension (20 μL) was inoculated into YpsS liquid medium (180 μL) adjusted to salinities of 0, 1, 2, 4, 8, 16 and 32 in wells of microtiter plates (Costar 3595, Corning, Maine, USA), and incubated at 17, 24 and 30 °C. Six replicates were performed for each salinity-temperature combination. The control wells consisted only of the liquid medium. Absorbance at 600 nm (Ishibashi et al. 2019) was measured daily for 2 weeks by the multi-detection microplate readers (Synergy HT, BioTek). Fungal growth (zoosporangia and rhizoids) was estimated by subtracting the absorbance of the inoculated wells from that of the control wells.
Growth curves of the fungi were constructed using the sigmoidal function, Logistic1. Modeling was conducted using OriginPro 2021 (OriginLab Corporation, USA). It is a generalized logistic model with the following formula,

\[ y = \frac{a}{1 + e^{-k(x-x_c)}} \]

where \( y \) represents the optical density at time \( x \); \( a \) is the maximum value of the curve; \( x_c \) is the \( x \)-value of the sigmoid’s midpoint; \( k \), the growth rate constant; \( e \), the base of natural logarithms. The maximum growth rate was described with the formula, \( ka/4 \).

A non-parametric one-way ANOVA test was performed in SPSS 25 (IBM Corp., Armonk, New York, USA), using Tukey test post hoc comparison based on average maximum growth rate, to determine the effects of different salinities and temperatures on growth. A heatmap with dendrograms was generated using RStudio (v2022.12 + 353; Posit team 2022) with package pheatmap (v1.0.12; Kolde 2019) to show growth rate under different salinities and temperatures.

### 3 Results

All isolates used in this study were cultured from water samples collected at wetlands/ponds in eastern Taiwan (5 from Taitung County, 1 from Hualien County) (Table 1). Three isolates were identified by morphology and sequence analysis as *A. chaetifer* and the other three as *G. haynaldii* (Figure 1). *A. chaetifer* was distinguished by an abundance

![Figure 1](image_url): Zoospores and sporangia of two species of Chytridiomycota from coastal habitats of Taiwan. *Alphamyces chaetifer* IMB232: (A) zoospore with a single flagellum; (B–C) developing sporangia; (D) emptied sporangium after zoospore release. *Gorgonomyces haynaldii* IMB239: (E) zoospore with a single flagellum; (F–G) immature sporangia with rhizoids; (H) mature sporangium with papilla (arrows). Scale bars: (E–F) = 30 \( \mu \)m, (A–D, G–H) = 10 \( \mu \)m.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Taxon</th>
<th>Sampling site</th>
<th>Coordinates</th>
<th>Sampling date</th>
<th>Salinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMB230</td>
<td><em>Alphamyces chaetifer</em></td>
<td>Zhiben Wetland, Taitung</td>
<td>22°41′35.1″N 121°04′00″E</td>
<td>6/5/2021</td>
<td>2</td>
</tr>
<tr>
<td>IMB231</td>
<td><em>Alphamyces chaetifer</em></td>
<td>Zhiben Wetland, Taitung</td>
<td>22°41′35.1″N 121°04′00″E</td>
<td>6/5/2021</td>
<td>2</td>
</tr>
<tr>
<td>IMB232</td>
<td><em>Alphamyces chaetifer</em></td>
<td>Pond in Guanshan Waterfront Park, Taitung</td>
<td>23°02′36.9″N 121°10′00″E</td>
<td>6/5/2021</td>
<td>2</td>
</tr>
<tr>
<td>IMB233</td>
<td><em>Gorgonomyces haynaldii</em></td>
<td>Pond in Guanshan Waterfront Park, Taitung</td>
<td>23°02′36.9″N 121°10′00″E</td>
<td>6/5/2021</td>
<td>2</td>
</tr>
<tr>
<td>IMB237</td>
<td><em>Gorgonomyces haynaldii</em></td>
<td>Pipa Lake in Taitung Forest Park of Beinan Estuary Wetland, Taitung</td>
<td>22°45′26.2″N 121°09′00″E</td>
<td>16/5/2022</td>
<td>0</td>
</tr>
<tr>
<td>IMB239</td>
<td><em>Gorgonomyces haynaldii</em></td>
<td>Pond in Waterbird and Wetland Conservation Park, Hualien</td>
<td>23°56′15.9″N 121°36′00″E</td>
<td>17/5/2022</td>
<td>0</td>
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</table>
of long, slender, branched or unbranched hairs on the sporangium (Chen and Chien 1995; Letcher et al. 2008) when grown on pollen. The developing or mature zoosporangia were spherical, with diameters of 9.8–12.3 μm (n = 28). The delicate and extensive rhizoidal system was 2.4–6.5 μm in length and 0.6–1.2 μm in width. Zoospores were released through an apical pore, globose, 2.2–4.0 μm (n = 14) in size, and with flagella 9.5–15.1 μm (n = 13) in length (Figure 1A–D).

Sporangia of G. haynaldii were subglobose to oval when young, while turning globose to subglobose when mature, with one to several papillate or tube-like structures, 11.2–22.0 × 13.0–22.0 μm in size. Rhizoids were cylindrical at the base of sporangia and slightly branched. Zoospores were spherical, 2.1–3.4 μm (n = 15) in size, and the flagella were 13.2–16.2 μm (n = 9) in length (Figure 1E–H). The nucleotide BLAST search of the ITS regions of rDNA also confirmed the

Figure 2: Average maximum growth rate (optical density per day) of Alphamyces chaetifer (IMB230, IMB231, IMB232) and Gorgonomyces haynaldii (IMB233, IMB237, IMB239) cultured at 7 salinities and 3 temperatures for 14 days. Data plotted are means ± standard deviation (n = 6). At any salinity, temperatures with different Roman numerals (i or ii) are significantly different at p = 0.05. At any temperature, salinities with different letters are significantly different at p = 0.05.
species identity of the isolates (Supplementary Table S1). Both species belong to the Rhizophydiales, Chytridiomycota, and were among the first chytrids reported from Taiwan (Chen and Chien 1995).

The combined effects of salinity (0, 1, 2, 4, 8, 16, 32) and temperature (17, 24, 30 °C) on growth rate of the six fungal isolates are shown in Figure 2. No growth was observed at salinities of 16 and 32 under any incubation temperatures. All isolates grew well at salinities at or below 4, but they generally grew faster at 0 and 1. G. haynaldii IMB233 and IMB237 also showed minimal growth at a salinity of 8. At or below 4, the isolates grew significantly faster at 30 °C than at 17 °C and 24 °C.

The colour scheme in the heat map shown in Figure 3 indicates different growth rates of the fungi. The cluster analysis-based column dendrogram formed by average linkage of a Bray–Curtis similarity matrix shows the similarity in growth rate of the isolates cultured under the different culture conditions. Two main clusters were observed; one cluster consisted of salinities at or below 2, while the higher salinities formed the other cluster.

The row dendrogram (complete Linkage-Euclidean distances) represents the similarity of growth rates of isolates based on hierarchical clustering of culture conditions. Three groupings were noticeable. A. chaetifer IMB230 and IMB231 had the fastest (best) growth, while G. haynaldii IMB237 and IMB239 had the slowest (poorest) growth. IMB232 did not grow as fast as IMB230 and IMB231, although they were all identified as A. chaetifer. Among the G. haynaldii isolates, IMB233 had the fastest (best) growth.

4 Discussion

This study is the first to use a spectrophotometric method to study growth of the Chytridiomycota under the influence of salinity and temperature. This is a technically simple method which can be used to study the combined effects of multiple environmental factors (e.g., pH, temperature, salinity) on growth of chytrids. Enumeration of zoosporangia and zoospore production in other studies can be labour-intensive, as is the use of dried weight to represent growth, which requires inoculation of a large number of flasks and a large space for their incubation, making study of multiple variables impossible (Booth 1971; Höhnk and Aleem 1953; Scholz 1958).

The A. chaetifer and G. haynaldii isolates cultured from low-salinity water samples collected at coastal ponds or wetlands in this study grew at all three incubation temperatures (17, 24 and 30 °C), but showed growth only at or below salinity 8 (little growth at 8 for two isolates). Gleason et al. (2006) tested the growth of five isolates of zoosporic fungi under a range of sodium chloride (NaCl) concentrations (0, 4.375, 8.75, 13.125, 17.5, 26.25 g L⁻¹) in a synthetic liquid

![Figure 3: Heat map showing the combined effects of temperature (17, 24 and 30 °C) and salinity (0, 1, 2, 4, 8, 16, 32) on growth of isolates of Alphamyces chaetifer and Gorgonomyces haynaldii. The colour spectrum represents the average maximum growth rate (increase in optical density per day); red and blue indicate high and low growth rate, respectively.](image-url)
medium composed of glucose and metal salts, and only two isolates were able to grow up to 4.375 g L⁻¹. In a study of fungal communities along a salinity gradient in the Baltic Sea, a higher abundance of Chytridiomycota was found at salinities below 8 (Rojas-Jimenez et al. 2019). These results collectively suggest that species of the Chytridiomycota are sensitive to environmental salinity.

Booth (1971) found that temperature was the main factor influencing growth of zoosporic fungi. In the present study, maximum growth rate at 30 °C for A. chaetifer IMB230, IMB231, IMB232 and G. haynaldii IMB233 was observed at 0–2 salinity (Figure 2), suggesting that their growth rate under this temperature was salinity dependent.

Different growth responses were observed for isolates of the same species. At salinity 4 or below, A. chaetifer IMB230 and IMB231 had the fastest growth rate, while G. haynaldii IMB237 and IMB239 had the slowest growth rate. IMB237 and IMB239 were cultured from water samples of zero salinity, while IMB230, IMB231, IMB232 and IMB233 were cultured from water samples with a salinity of 2, so their growth responses towards salinity may correspond to their ecotypes (Table 1).

The results of this study confirm that salinity affects the growth responses of chytrids. Can these observations explain why only 27 species of culturable Chytridiomycota and allied taxa have been recorded from the marine environment (Jones et al. 2019)? It would be ingenious to suggest that a single factor controlled the ability of a fungus to tolerate life in the marine environment. Jones et al. (2022) highlighted the many interacting physiological features that play a role in enabling fungi to grow in the marine milieu, salinity of the water being only one factor. Motile zoospores are unwalled but have a carbohydrate coat that protects against desiccation (Longcore and Simmonds 2020), as opposed to the thick chitinous cell walls of mycelial fungi. Zoospores, therefore, are more permeable to the uptake of cations/anions and may require different mechanisms for their exclusion. However, it does not mean that zoospores cannot survive in saline waters. Fungi, and especially filamentous fungi, are known to synthesize compatible solutes (salt-out strategy) to balance the osmotic pressure of their surrounding environment (Gostinčar et al. 2011; Jones et al. 2022). Whether fungi of the Chytridiomycota produce compatible solutes to counteract salinity stress, and the types of compatible solutes produced by the Chytridiomycota, are topics requiring further study.

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References


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Bionotes

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