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Physiological Studies of Subtropical Mangrove Thraustochytrids

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Six thraustochytrids (*Schizochytrium* sp. KF-1, *Schizochytrium mangrovei* KF-2, KF-7, KF-12, *Thraustochytrium striatum* KF-9 and *Ulkenia* KF-13), isolated from fallen, senescent leaves of the mangrove tree *Kandelia candel* in Hong Kong, were identified and characterised in pine pollen seawater culture. Yeast extract peptone glucose seawater broth was used for growth studies. The pH range for growth was from 4 to 9 with optima from 4 to 7 for *Schizochytrium* species (KF-1, 2, 7, 12) and 5 to 6 for *Thraustochytrium striatum* KF-9 and *Ulkenia* sp. KF-13. Temperature and salinity optima for isolates KF-2 and KF-7 were at 25 °C and from 22.5–30 ‰ respectively while isolate KF-12 had a temperature optimum from 20–25 °C and salinity optima from 7.5–30 ‰. *Thraustochytrium striatum* KF-9 and *Ulkenia* sp. KF-13 showed strong growth at 25 °C and 15–30 ‰.

Introduction

Thraustochytrids are marine protists with monocentric thalli that possess: a multi-layered wall of scales composed predominantly of L-galactose (Darley *et al.* 1973), an organelle termed a sagenogenetsome from which the ectoplasmic net arises (Perkins 1972), and biflagellate heterokont zoospores in many of the described genera (Moss 1991). They are ubiquitously distributed and can be found on algal surfaces (Booth and Miller 1968, Haythorn *et al.* 1980, Miller and Jones 1983), in estuarine habitats (Ulken 1981), seawater (Goldstein and Belsky 1964, Bahnweg and Sparrow 1972, 1974, Gaertner 1981), and saline soils (Booth 1971a, b). Many of these ecological studies, however, were undertaken in temperate, sub-Antarctic and Antarctic regions. Studies from the tropical and subtropical regions are few, though investigations are increasing (Raghukumar 1987) especially in mangrove areas (Raghukumar 1988, Honda *et al.* 1998). In Hong Kong, there are 44 subtropical mangrove stands remaining with *Kandelia candel* (L.) Druce as the most common tree species (Tam *et al.* 1997). In Mai Po alone, *Kandelia candel* contributes an annual litter production estimated at 5.9 tonnes ha^{-1} $year^{-1}$ (Lee 1989). Thraustochytrids may play a potential role in enriching the nutritionally poor mangrove leaves and act as decomposers of mangrove litter (Bremer 1995). However, thraustochytrids must be able to withstand the continuous fluctuations in temperature and salinity in mangroves. For example, Mai Po mangrove, Hong Kong, has a salinity and pH range of 1.8–23.5 ‰ and 7.1–7.6, respectively (Sadaba *et al.* 1995), and Ting Kok mangrove has a salinity and pH range of 3–27 ‰ and 6.4–7.2, respectively (Tam and Wong 1997). This study investigated the physiological growth conditions of selected thraustochytrids isolated from Hong Kong mangroves and elucidated their adaptive survival strategy in a mangrove environment.

Materials and Methods

Isolation

Fallen leaves (black and brown) of *Kandelia candel* were collected from the floors of three mangroves – Mai Po, Three Fathoms Cove and Ting Kok in Hong Kong. The leaf samples were returned to the laboratory for isolation of thraustochytrids within the day of collection. Collected leaf samples were cut into leaf discs (1.5 cm diameter) and washed three times with 15 ‰ sterile natural seawater (NSW) supplemented with 1 mg mL^{-1} of each of penicillin G and streptomycin sulphate to suppress bacterial growth. Washed leaves were plated onto yeast extract-peptone (YEP) agar composed of 1 g yeast extract (Oxoid), 1g mycological peptone (Difco), 13 g of agar (Difico) and 1 L of 15 ‰ NSW, then a few drops of 15 ‰ sterile NSW were added before incubation at 25 °C for 1–2 days. During the incubation period, the plates were periodically observed for thraustochytrid colonies. The latter were aseptically transferred to fresh YEP agar plates containing antibiotics and repeatedly subcultured until axenic cultures were obtained. To confirm the purity of the axenic cultures, the thraustochytrids were induced to sporulate with 15 mL of 15 ‰ sterile NSW. After 2 hours, zoospores were collected (0.1 mL contained 500 zoospores) and introduced to 15 mL of 15 ‰ sterile natural seawater containing *Pinus* pollen grains (heat sterilised at 100 °C for 1 day) as bait. The life cycle of the zoospores of each strain was followed and identified. Stock cultures are maintained on YEP agar slopes with 1 mL of 15 ‰ sterile NSW at 25 °C and subcultured every month.

Physiological experiments

Six thraustochytrid isolates (KF-1, KF-2, KF-7, KF-9, KF-12 and KF-13) were used for the physiological study (Table I). Thraustochytrid life cycles were observed in natural seawater baited with heat sterile pine pollen (*Pinus* sp.) and zoospores were fixed in 2.5% glutaraldehyde in phosphate buffer at pH 6 before examination. Photomicrographs were taken with an Olympus C-35AD-4 camera attached to an Olympus BH-2 light microscope (Japan).

For preparation of inoculum, YEP agar cultures were used. Zoospore suspensions were obtained by using a cork borer (1.5 cm diameter) to make four wells in a 2-day old YEP culture plate (25 °C) which was flushed with 15‰ sterile NSW. Zoospores accumulated in the wells after 2–3 hours. One mL of the zoospore suspension was transferred into 50 mL aliquot of broth (1 g yeast extract, 1 g mycological peptone, 10 g glucose and 1 L of 15‰ artificial seawater [ASW]) in a 250 mL flask, plugged with cotton wool and wrapped with aluminium foil. The culture was shaken at 200 rpm at 25 °C for 40hour. A 5% (v/v) inoculum of the 40-hour old culture broth was used as initial inoculum for all subsequent growth, pH, temperature and salinity experiments. Such a volume (5% v/v) was equal to a 2.5 mL of the initial inoculum inoculated into a flask containing 50 mL of yeast extract-peptone-glucose seawater (YPGS) broth.

Growth experiment

Yeast extract-peptone-glucose seawater broth composed of 10 g glucose, 10 g yeast extract and 1g peptone was prepared at a salinity of 15‰ using ASW prepared from diluted sea salts (Sigma). The pH was adjusted to 6.0 using 2 N HCl. The culture flasks were shaken at 200 rpm at 25 °C for a 6-day period. Triplicate flasks were harvested at 24-hour intervals for biomass determination.

pH experiment

In the pH experiment, the initial pH of the YPGS broth was adjusted to pH 7, 8, and 9 using 2 N KOH and to pH 4, 5, and 6 using 2 N HCl and measured using a calibrated pH meter (Hanna instrument HI 8521, Portugal). After pH adjustment, the broth was filter-sterilised using a 0.45 µm-membrane filter. At the end of the incubation period, the pH of the broth was also measured with the pH meter after the cells were harvested. Triplicate flasks were incubated and shaken at 200 rpm at 25 °C for 4 days.

Temperature and salinity

Yeast extract-peptone-glucose seawater broth prepared in a litre of distilled water (DW) or ASW with different salinities (7.5, 15, 22.5, 30‰) at pH 6 were used in this experiment. Triplicate flasks were incubated and shaken at 200 rpm at different temperatures (15, 20, 25 and 30 °C) for 4 days.

Dry weight determination

For dry weight biomass determination, the entire content (50 mL) of the flask was transferred to a pre-weighed centrifuge tube and harvested by centrifugation at 3500 g for 10 min and the supernatant discarded. Harvested cells were subsequently washed with 50 mL sterile distilled water, followed by manual continuous agitation for 10 min. The cells were then collected by centrifugation at 3500 g for 10 min and the supernatant discarded. Cell samples were examined under the microscope to check for cell integrity and no morphological disruption was observed. This rinsing-centrifugation process was repeated three times, then the washed cells were freeze-dried for 24 hours before weighing. Biomass was expressed as mg freeze-dried weight 50 mL^{-1} of growth broth.

Table I. Thraustochytrid strains isolated from various mangroves in Hong Kong.

Strain	Site of isolation	Substrate[1]	Salinity (‰)[2]
Schizochytrium sp. KF-1	Three Fathom Cove	Brown	5
Schizochytrium mangrovei Raghukumar KF-2	Three Fathom Cove	Brown	5
Schizochytrium mangrovei Raghukumar KF-7	Mai Po	Black	11
Thraustochytrium striatum Schneider KF-9	Mai Po	Black	11
Schizochytrium mangrovei Raghukumar KF-12	Ting Kok	Brown	34
Ulkenia sp. KF-13	Ting Kok	Brown	34

[1] All the thraustochytrids were isolated from submerged *Kandelia candel* leaves in various stages of decay (brown or black) in the mangroves.
[2] Salinity of water in which the leaves were collected.

Statistical analysis

Significant differences in vegetative growth were compared using single ANOVA and differences among means were compared using the Student Newman-Keul's test at $\alpha = 0.05$ according to Zar (1996).

Results

Table II summarises the morphological characters of the six thraustochytrid isolates used in this study. All isolates produced biflagellate zoospores and mature cells of isolates KF-1, KF-2, KF-7 and KF-12 divided by successive binary divisions to form diads, tetrads and clusters. Isolates KF-1, KF-2, KF-7 and KF-12 can be assigned to *Schizochytrium* because they divided by repeated binary divisions and produced biflagellate zoospores (Raghukumar 1988).

Schizochytrium isolate KF-1 differed from KF-2, KF-7 and KF-12 in that each cell developed into a zoosporangium producing several zoospores. Even though the number of zoospores released from each zoosporangium is one of the most important characters in species delimitation within the genus *Schizochytrium*, zoospore numbers could not be accurately determined in the zoosporangia of isolate KF-1. Therefore, isolate KF-1 is hereafter referred to as *Schizochytrium* sp. KF-1.

Schizochytrium isolates KF-2, KF-7 and KF-12 developed a single zoospore in the zoosporangium. The zoospore inside the zoosporangium became obvious prior to its release and discharge of the zoospore commenced. This developmental pattern fits the description of *S. mangrovei* Raghukumar (Raghukumar 1988).

Isolate KF-9 was identified as a *Thraustochytrium* species with a globose sporangium and cytoplasm that cleaved into zoospores, commencing at the periphery by centripetal invagination of the plasma membrane. Later the zoosporangium appeared to be radially cleaved and zoospores were formed. Only occasionally was there a central, persistent, proliferation body observed within the zoosporangium. This isolate was identified as *T. striatum* Schneider (Schneider 1967) and hereafter is referred to as *T. striatum* KF-9.

Isolate KF-13 had globose to subglobose sporangia, developed amoeboid cells before the protoplast cleaved into zoospores and no proliferation bodies were observed. This isolate is referred to the genus *Ulkenia* (Gaertner 1977).

Growth

All test isolates grew rapidly in YPGS broth (Fig. 1). Maximum yields were obtained one to two days after incubation for all isolates with the exception of *Schizochytrium mangrovei* KF-7 and *Ulkenia* sp. KF-13, which required three days incubation. For all these isolates, day 4 corresponded to the late stationary

Table II. Morphological characteristics of the six thraustochytrids[a] used in this study.

Strain	Zoosporangium[b]	Biflagellate zoospore[b]	Successive binary division	Diad[b]	Tetrad[b]	Release of amoeboid cells prior to formation of zoospores	Formation of zoospores inside zoosporangium	Number of zoospores per zoosporangium
Schizochytrium sp. KF-1	n.m.[c]	3.2–4.4 × 2.0–3.2 (3.5 × 2.7)	yes	8.0–12.4 × 7.0–10.0 (10.0 × 8.6)	9.0–11.0 × 9.4–12.0 (9.4 × 10.0)	no	yes	not observed
S. mangrovei KF-2	none	6.0–7.2 × 3.2–4.4 (6.5 × 3.8)	yes	7.0–14.4 × 6.2–10.0 (12.0 × 8.0)	8.0–16.4 × 9.4–16.0 (14.0 × 12.0)	no	yes	1
S. mangrovei KF-7	none	4.8–7.2 × 2.8–4.4 (5.8 × 3.5)	yes	8.0–14.0 × 6.0–12.0 (12.4 × 9.0)	8.0–16.0 × 10.0–16.0 (12.0 × 12.0)	no	yes	1
Thraustochytrium striatum KF-9	17.0–28.0 × 18.0–29.0 (23.0 × 23.0)	3.8–6.8 × 3.0–5.0 (4.0 × 3.4)	no	nil	nil	no	yes	numerous
S. mangrovei KF-12	none	5.0–7.0 × 3.0–4.2 (5.6 × 3.9)	yes	8.0–14.0 × 5.4–12.0 (13.4 × 8.4)	7.0–16.0 × 10.0–16.0 (10.0 × 12.0)	no	yes	1
Ulkenia sp. KF-13	8.0–16.0 × 8.0–16.0 (11.0 × 11.0)	3.8–5.0 × 3.8–5.0 (4.3 × 4.0)	no	nil	nil	yes	no	not observed

[a] Sample size for each isolate = 30
[b] All the measurements are in µm. Mean values are in brackets.
[c] n. m. = no measurement

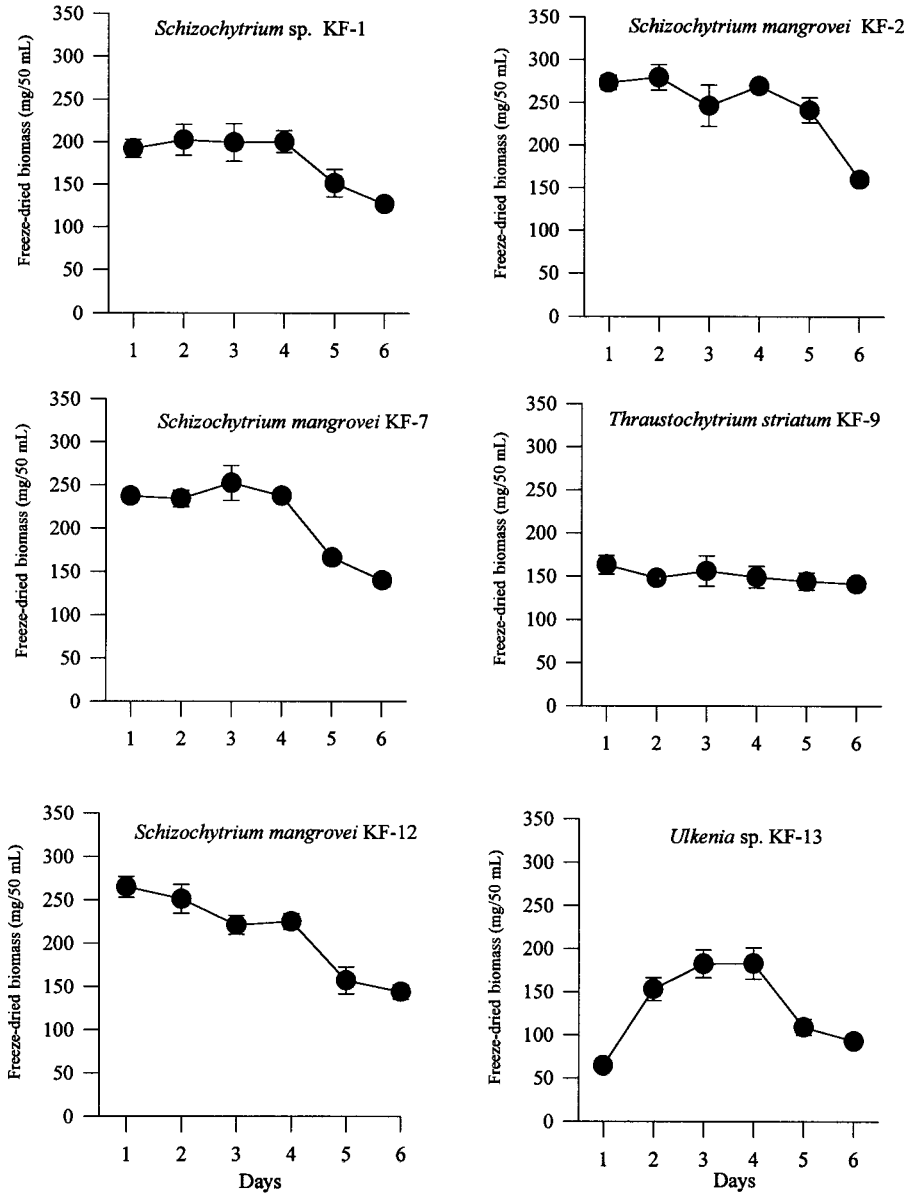

Fig. 1. Growth (± S. E. M.) of six thraustochytrid strains in YPGS broth for 6 days at 25 °C.

Table III. Growth of thraustochytrids in YPGS broth at different pH levels.

Initial pH	Total biomass (mg freeze-dried weight/50 mL of YPGS broth)											
	KF-1[1]		KF-2		KF-7		KF-9		KF-12		KF-13	
4*	180[2]a	(7.78)[3]	230a	(7.63)	220a	(7.46)	180a	(7.59)	190a	(7.84)	nvg[4]	(4.24)
5	170a	(7.76)	250a	(7.83)	230a	(7.99)	140bc	(7.77)	150a	(8.04)	180b	(7.95)
6	190a	(8.00)	250a	(7.98)	230a	(8.24)	170ac	(7.97)	210a	(8.20)	180b	(7.79)
7	180a	(7.82)	220a	(7.88)	230a	(8.10)	140bc	(7.93)	150a	(8.23)	100a	(7.80)
8	90b	(7.99)	80b	(8.11)	200ab	(8.37)	90d	(8.00)	100b	(8.24)	60a	(7.15)
9	120c	(8.16)	70b	(8.32)	140b	(8.25)	60d	(8.13)	100b	(8.40)	50a	(8.17)

Thraustochytrids were grown at 25 °C for four days on a reciprocal shaker at 200 rpm.
a, b, c – indicates statistical significance. Mean values with the same letter within each column are not significantly different ($P < 0.01$).
* Value recorded after filter-sterilized through 0.45 μm membrane filter. [1] Refer to Table I for details of the test strains. [2] The data are expressed as means of triplicate flasks. [3] Final pH. [4] nvg = no vegetative growth

phase of growth in batch culture, as the biomass of all isolates declined after this day. Therefore, the growth period for all subsequent experiments was 4 days.

pH

Based on the initial pH values, all isolates grew at between pH 4–9 (Table III), with the exception of *Ulkenia* sp. KF-13 which did not grow at pH 4. High biomass was recorded in broth cultures with initial low pH values, although it is interesting to observe that the final pH value of most culture broths was alkaline.

Temperature and salinity

The effects of temperature and salinity on the growth of the test isolates are presented in Figure 2 and summarised in Table IV. Analysis of interactions showed that salinity and temperature affected growth significantly ($P < 0.001$). The data indicate most species are able to tolerate a wide range of temperatures (Table IV). Temperature and salinity optima for isolates KF-2 and KF-7 were observed at 25 °C at 22.5–30 ‰ salinity while isolate KF-12 had a temperature optimum between 20 and 25 °C and salinity optima between 7.5 and 30 ‰. *Thraustochytrium striatum* KF-9 and *Ulkenia* sp. KF-13 showed strong growth at 25 °C and in a salinity range of 15–30 ‰.

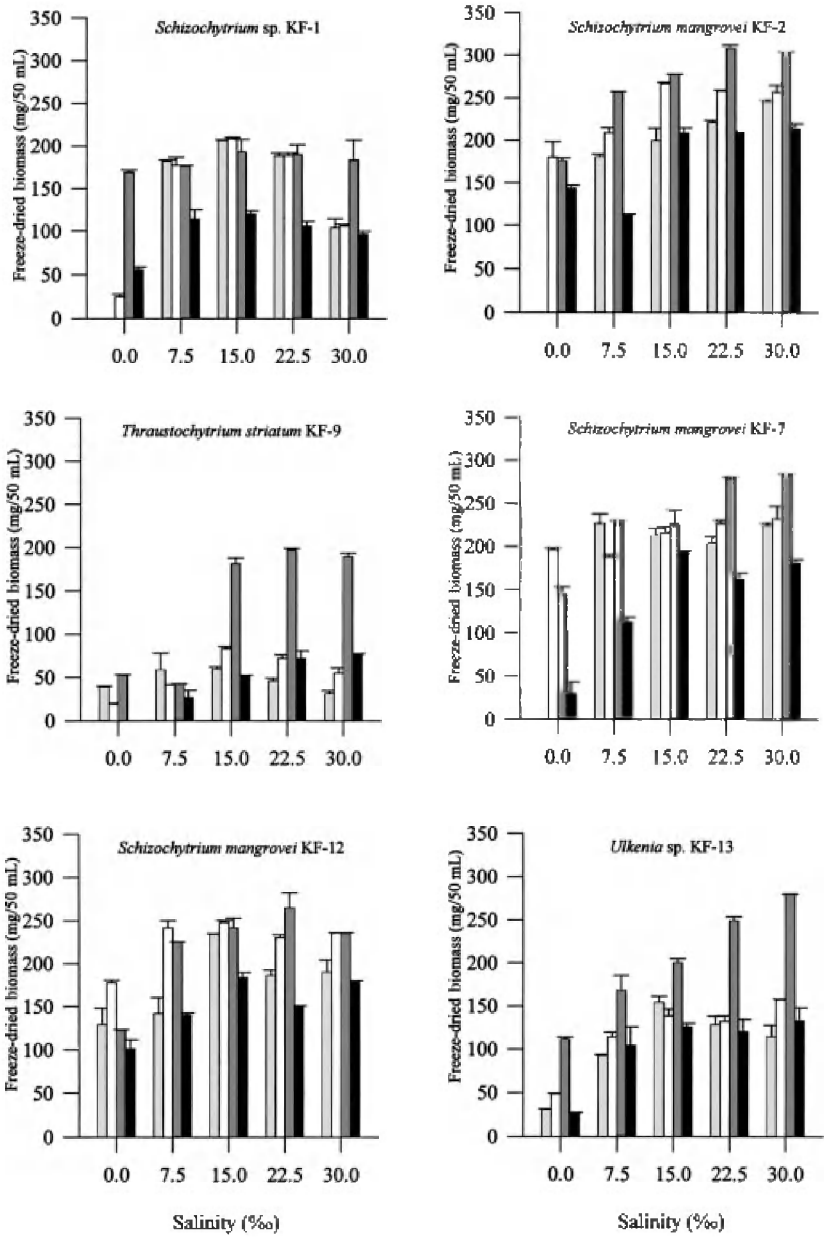

Fig. 2. Growth (± S. E. M.) of six thraustochytrid strains in YPGS broth at different salinity and temperature levels after 4 days of incubation. ▭ = 15 °C, ▭ = 20 °C, ▭ = 25 °C, ■ = 30 °C.

Table IV. Temperature and salinity requirements of selected thraustochytrids from Antarctic, temperate and tropical/subtropical regions compared with strains from the present study.

Species	Vegetative growth					
	Temperature (°C)		Salinity (‰)		Water salinity (‰) or substrate/habitat of isolation	Ref.
Schizochytrium sp. KF-1	15–30	(15–25)*	0–30	(7.5–22.5)*	5	1
Schizochytrium mangrovei						
-KF-2	15–30	(25)	0–30	(22.5–30)	5	1
-KF-7	15–30	(25)	0–30	(22.5–30)	11	1
-KF-12	15–30	(20–25)	0–30	(7.5–30)	11	1
Thraustochytrium striatum KF-9	15–30	(25)	0–30	(15–30)	34	1
Ulkenia sp. KF-13	15–30	(25)	0–30	(15–30)	34	1
Antarctic species						
Thraustochytrium antarcticum Bahnweg *et* Sparrow	0–17	(4–9)	5–35	(15–30)	seawater	2
Thraustochytrium kerguelensis Bahnweg *et* Sparrow	0–17	(4–9)	15–35	(20–30)	seawater	2
Thraustochytrium rossii Bahnweg *et* Sparrow	0–17	(4–9)	15–35	(15–20)	seawater	2
Temperate species						
Althornia crouchii E. B. G. Jones *et* Alderman	20–30	(30)	15–35	(30–35)	seawater	3
Labyrinthuloides haliotidis Bower	5–24	(5–15)	15–45	(30)	abalone tissue	4
Thraustochytrium aureum S. Goldstein	4–30	(20–25)	5–35	(20–35)	littoral water	5
Thraustochytrium motivum S. Goldstein	4–37	(12–25)	5–35	(25–35)	littoral water	6
Thraustochytrium multirudimentale S. Goldstein	4–37	(15–25)	10–35	(25–35)	littoral water	6
Thraustochytrium roseum S. Goldstein	4–30	(25–30)	5–35	(25–35)	littoral water	7
Tropical/Subtropical species						
Schizochytrium limacinum Honda *et* Yokochi	10–35	(25)	0–30	(15–30)	mangrove water	8

* Values in brackets are optimum levels.

1 This study
2 Bahnweg (1979a)
3 Alderman and Jones (1971)
4 Bower (1987)
5 Goldstein (1963b)
6 Goldstein (1963a)
7 Goldstein (1963c)
8 Yokochi *et al.* (1998)

Discussion

Salinity and temperature

Temperature and salinity requirements of selected thraustochytrids from various geographical regions (Antarctic, temperate and subtropical) are summarised in Table IV. The isolates from Hong Kong (KF-1, 2, 7, 9, 12, 13) demonstrate for the first time that thraustochytrids can be isolated from low salinity waters while the remaining strains listed (Table IV) were isolated from environments with salinity levels close to full strength seawater. However, all strains examined here could grow over a wide range of salinities from 5–35‰ with strong growth at the higher salinity levels (15–35‰). This indicates that the salinity of the site of isolation has no effect on their salinity tolerance for vegetative growth under laboratory conditions. Bahnweg (1979a) reported *Thraustochytrium aureum* and *Schizochytrium aggregatum* Goldstein *et* Belsky 3401 could grow at a salinity as low as 1‰, but no growth was observed in a medium prepared from distilled water. All the Hong Kong isolates used in this present study also showed stronger growth at higher salinity levels, but they differed from the temperate and Antarctic species in being able to grow, though at a much reduced rate, in a medium prepared with distilled water that contained only a minimal amount of salts originating from the 5% v/v inoculum. This is a feature also shared by the *Schizochytrium limacinum* isolate from Japan (Table IV). Absence of growth or reduced growth in medium with a minute quantity of sea salts (e.g. as represented by sea salts contained in the inoculum) may be largely due to ion deficiencies. Seawater contains many major ions (Na, Ca, K, Mg) which are essential for the growth of marine fungi (Jennings 1983). The absence of potassium ions caused reduced growth in thraustochy-

trids (Bahnweg 1979a) and Siegenthaler *et al.* (1967a, b) suggested that sodium ions facilitated phosphate uptake and were required in macro-quantities for strong growth of thraustochytrids. Garrill *et al.* (1992) confirmed that sodium could not be replaced by potassium for the growth of thraustochytrids.

Thraustochytrid strains used in this study showed growth responses to varying salinity and temperature levels. *Schizochytrium* species, especially *S. mangrovei* (KF-2 and KF-7), displayed stronger overall growth compared with *Thraustochytrium striatum* KF-9 and *Ulkenia* sp. KF-13 (Figs 1, 2). Temperature affected the ability of the test strains to grow under different salinity levels, and was more marked for *Thraustochytrium striatum* KF-9 and *Ulkenia* sp. KF-13 when strong growth at most salinity levels was only achieved at the optimum growth temperature of 25 °C. In comparison, *Schizochytrium* spp. appeared to be more tolerant of varying temperature and salinity levels for strong vegetative growth, showing little difference in biomass production at all salinities with the exception of growth at 30 °C. No *Phoma* pattern (higher salinity growth optimum with higher temperatures, Ritchie 1957) was detected in any of the species. The optimum growth temperature recorded for most of the test species was 25 °C; this was also observed in many of the thraustochytrids from temperate regions (Jones and Harrison 1976). Species isolated from Antarctic and sub-Antarctic regions, however, exhibited a lower temperature optimum with *Thraustochytrium rossi* and *T. antarcticum* growing from 0–9 °C but never above 17 °C (Table IV).

pH

All the test strains tolerated a wide pH range for growth. It is intriguing to observe that although stronger growth was recorded at the lower initial pH level for most strains, the final pH recorded for all culture filtrates was alkaline, with the exception of *Ulkenia* sp. KF-13 when no growth was recorded. The pH of water of the Hong Kong sites when the test strains were isolated ranged from 6.3–7.6 (Sadaba *et al.* 1995, Tam and Wong 1997), which is in good agreement with the experimental findings.

Ecological implications

The findings indicate the Hong Kong thraustochytrid isolates can tolerate the fluctuating physical conditions in the mangrove environment, though some strains, especially the *Schizochytrium* species appear to be more adapted than *Thraustochytrium* and *Ulkenia* species. This is complemented by a preliminary survey of the abundance of thraustochytrids on decayed mangrove leaves on the mangrove floor in one of the test sites – Mai Po Nature Reserve – revealing *Schizochytrium* spp. are indeed the most abundant species (Fan *et al.* 2000). *Schizochytrium* spp. were detected from 47 out of 50 YEP agar plates when each contained four 1-cm square pieces of senescent *Kandelia candel* leaves while *Thraustochytrium* spp. and *Ulkenia* spp. were detected from only 9 out of 50 YEP agar plates. The role of thraustochytrids in nutrient recycling in mangroves has also been suggested: thraustochytrids can secrete degrading enzymes such as cellulases and amylases (Raghukumar *et al.* 1994, Bremer and Talbot 1995) and utilise a wide range of carbon and nitrogen sources (Bahnweg 1979b). The presence of abundant n-3 fatty acids in thraustochytrids isolated from mangrove leaves (Findlay *et al.* 1986, Fan *et al.* 2000) raises a question as to the importance of these protists as a food source for marine organisms, such as crabs, shrimps and fish that live in the mangroves.

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