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FULL PAPER

Zoospore production and motility of mangrove thraustochytrids from Hong Kong under various salinities

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Abstract We investigated the effects of salinity on the zoospore production of four mangrove thraustochytrid isolates, *Schizochytrium* sp. KF1, *Aurantiochytrium mangrovei* KF6, *Thraustochytrium striatum* KF9 and *Ulkenia* sp. KF13. The zoospore motilities, which were based on curvilinear velocity (VCL) and straight-line velocity (VSL), were monitored using the Computer-Assisted Sperm Motility Analysis (CASA) Software system. The zoospore production of four isolates was suppressed at salinity above 15‰. *Schizochytrium* sp. produced the greatest number of zoospores at 15‰, while *Aurantiochytrium mangrovei* and *Ulkenia* sp. produced abundant zoospores in diluted sea water ranging from 7.5 to 15‰. *Thraustochytrium striatum* performed relatively poorly under all salinities. Salinity and exposure time, as well as their interactions, had significant impacts on most zoospore velocity measurements. The optimal velocities of zoospore motility also varied among isolates. Zoospores of *Schizochytrium* sp. and *A. mangrovei* had similar responses to salinity, with the highest motility at 7.3‰, followed by a decrease in velocities with increasing salinity. In contrast, the zoospore of *T. striatum* had optimal motility at 12‰ and remained highly motile from 15 to 20‰. The velocities of zoospores of *Ulkenia* sp. were the lowest among the tested thraustochytrids and had optimal motility at 12‰. Zoospores of all the isolates remained active after 4 h of exposure to aqueous medium, but the optimal salinity for each mode of swimming changed. The ecological significance of these data are discussed.

Keywords *Aurantiochytrium* · Labyrinthulomycetes · *Schizochytrium* · Stramenopiles · *Thraustochytrium* · *Ulkenia*

Introduction

Thraustochytrids are obligate, marine heterotrophic fungus-like protists and belong to the eukaryotic Kingdom Stramenopiles, which includes oomycetes and diatoms (Honda et al. 1999; Tsui et al. 2009). They are ubiquitous in the ocean, and their occurrence and distribution in the water column and sediments have been well documented (Raghukumar et al. 1990; Naganuma et al. 1998; Kimura et al. 1999; Raghukumar 2002). They are important in the degradation of organic matter in the marine environment (Raghukumar 2002; Taoka et al. 2009). Their facilitation of the settlement of barnacle cyprids has also been shown (Raghukumar et al. 2000).

In the mangrove environment, thraustochytrids settle on fallen leaves, facilitated by their ectoplasmic nets, and carry out extracellular enzymatic decomposition of leafy tissues (Findlay et al. 1986; Bremer 1995; Raghukumar et al.

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1995). Thraustochytrids are thus an important component of the detrital food web, providing a source of polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA), to organisms in this food web (Findlay et al. 1986; Yongmanitchai and Ward 1989; Wong et al. 2008). Not only do thraustochytrids play a vital ecological role, but recent studies have also revealed their potential for DHA production in industry (Fan et al. 2001, 2009) and use as aquacultural feeds (Vrijmoed et al. 2003; Yamasaki et al. 2007).

Thraustochytrids produce biflagellated zoospores as their primary dispersal units in the life cycle (Moss 1986). Zoospores have a short posterior whiplash flagellum and a long anterior tinsel flagellum that is attached laterally (Kazama 1974). The flagella enable the zoospores to actively swim towards various environmental cues and settle on a substrate that provides nutrients for growth (Carlile 1993; Moss 1986). Thraustochytrids could rapidly attach to the leaves of *Kandelia obovata* Sheue, H.Y. Liu & J.W.H. Yong [formerly identified as *K. candel* (L.) Druce] around the stomatal openings within a short time of their falling to the mangrove floor (Fan 2002). Although zoospores are important to the dispersal and survival of thraustochytrids, their motility pattern and responses towards environmental changes are poorly understood, with only very few studies published. Fan et al. (2002a) reported the positive chemotactic responses of thraustochytrid zoospores towards mangrove leaf extracts, while zoospores of *Ulkenia* sp. were positively phototactic (Amon and French 2004). These tactic responses are useful for the survival of the organisms in their search for nutrients to sustain continual growth in the mangrove environment where the mangrove floor may not be inundated at all times (Fan et al. 2002a).

Hong Kong waters are influenced by the Pearl River estuary and the Pacific currents. Thus, the seawater salinity is highly variable near the estuary compared with locations near the open ocean (Wu 1988). The salinity of the Hong Kong mangroves varies daily and seasonally from 5 to 30‰, with an average diluted salinity ranging from 5 to 20‰ during the summer monsoon and around 30‰ in winter (Wu 1988). The thraustochytrid strains used in this study were commonly isolated from mangroves in this region. As zoospores have no cell wall, they are more likely to be vulnerable to salinity stress. Since salinity is an important factor in determining the growth of thraustochytrids (Fan et al. 2002b), fatty acid production (Fan et al. 2009) and their abundance in the ocean (Kimura and Naganuma 2001), we report here an investigation of zoospore motility of mangrove thraustochytrids under different salinity regimes using a Computer-Assisted Sperm Motility Analysis (CASA) System (CRISMAS) (Image House A/S, Denmark). The image analysis package provides a rapid, quantitative and objective assessment of sperm motility (Katz 1981; Yeung et al. 1997), such as that of fish (Lahnsteiner et al. 1997), invertebrates (Au et al. 2000) and mammals (Zinaman et al. 1996). To our knowledge, this is the first report on the application of this system in a zoospore motility investigation of labyrinthulomycetes. The effect of salinity on zoospore production for each thraustochytrid isolate was also studied by light microscopy. These findings can enhance our understanding of the survival strategies and ecological distribution of thraustochytrids in mangroves.

Materials and methods

Cultures and zoospore production

Four isolates of thraustochytrids [*Schizochytrium* sp. KF1, *Aurantiochytrium mangrovei* (Raghuk.) R. Yokoyama & D. Honda KF6, *Thraustochytrium striatum* Schneider KF9 and *Ulkenia* sp. KF13] were isolated from decaying *K. obovata* leaves from various mangrove habitats in Hong Kong (Fan et al. 2001, 2002b). They were identified based on the morphological features of vegetative cells and zoosporangia in the life cycle. The stock culture of thraustochytrid thalli was transferred to yeast extract peptone agar [YEP: 1 g yeast extract, 1 g peptone, 13 g agar in 1 l of 15‰ artificial sea water prepared from artificial sea salts (Sigma, USA)]. Two strips of agar (ca. 2 × 1 cm) from this agar plate were transferred to a fresh YEP plate for culturing of test thraustochytrids for the experiment. Zoospore suspensions were prepared from these 2-day-old culture agar plates incubated at 25°C. A cork borer (1.5 cm diameter) was used to make four wells in each plate, then flushed with 20 ml sterile distilled water (DW) or artificial seawater of different salinities (7.5, 15, 22.5 and 30‰) using different concentrations of artificial sea salt to induce zoospore release. The salinity of the water in the petri dish shifted after 2 h, and fell between 7.5 and 23‰, instead of ranging from 0 to 30‰ (Fig. 3; Table 1). The concentration of released zoospores (number/ml/petri dish) was counted using a hemocytometer after 2 h of induction. Three YEP plates were used per species per treatment (salinity levels) for observation of zoospore motility; 10 ml of the zoospore suspension from each of the three agar plates was transferred to individual autoclaved universal bottles (colony free) for the zoospore motility study. The salinity of water did not change during the investigation of the zoospore motility when the zoospore motility was investigated.

Motility study

The motility of zoospores was recorded using the image analysis system consisting of a phase contrast microscope with a lens at 20 × 10 magnification (Olympics BX50,

Table 1 The average values of percentage motility, curvilinear velocity (VCL) and straight line velocity (VSL) of four thraustochytrid isolates under various salinities (‰) and exposure times to aqueous medium, and computed *p* values (two-way ANOVA) on their effect and interactions (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, + hours exposed to aqueous medium after zoospore induction)

Species	Time+	Salinity (‰)					ANOVA	*P*
		7.5	12	15	20	23		
Motility (%)								
Schizochytrium sp.KF1	2	100	100	99.63	90.33	100	Salinity	<0.0001***
	4	75.5	81.34	78.89	64.70	75.82	time	<0.0001***
							Interaction	0.8126
Aurantiochytrium mangrovei KF6	2	92.38	68.07	89.21	94.44	87.59	Salinity	0.0021**
	4	58.28	63.83	70.58	83.06	72.66	Time	<0.0001***
							Interaction	0.1023
Thraustochytrium striatum KF9	2	100	99	100	100	94.44	Salinity	0.2112
	4	92.51	77.54	84.97	79.42	89.4	Time	<0.0001***
							Interaction	0.0508
Ulkenia sp. KF13	2	94.6	97.12	93.97	89.39	97.5	Salinity	0.1368
	4	65.01	72.35	81.69	73.33	82.06	Time	<0.0001***
							Interaction	0.3
VCL (μm/s)								
Schizochytrium sp.KF1	2	127.87	83.26	85.35	57.04	76.07	Salinity	<0.0001***
	4	90.73	90.87	87.02	90.10	103.64	Time	0.0243*
							Interaction	<0.0001***
Aurantiochytrium mangrovei KF6	2	151.28	93.37	121.89	80.66	106.50	Salinity	0.0025**
	4	74.32	63.25	106.61	94.28	101.01	Time	0.0011***
							Interaction	0.0008***
Thraustochytrium striatum KF9	2	73.04	128.31	102.92	102.42	65.58	Salinity	<0.0001***
	4	107.27	101.57	118.08	109.83	122.42	Time	<0.0001***
							Interaction	<0.0001***
Ulkenia sp. KF13	2	60.16	82.69	71.70	66.64	76.07	Salinity	<0.0001***
	4	67.03	75.59	72.99	62.86	73.97	Time	0.3840
							Interaction	0.0300*
VSL (μm/s)								
Schizochytrium sp.KF1	2	93.33	63.67	55.04	35.25	56.74	Salinity	<0.0001***
	4	55.98	67.39	58.84	57.59	58.15	Time	0.6782
							Interaction	<0.0001***
Aurantiochytrium mangrovei KF6	2	125.25	47.58	95.20	53.56	86.08	Salinity	0.0059**
	4	42.72	35.20	74.66	68.77	78.14	Time	0.0148*
							Interaction	0.0098**
Thraustochytrium striatum KF9	2	49.45	113.19	76.75	81.32	44.89	Salinity	<0.0001***
	4	58.77	63.64	78.38	67.93	78.40	Time	0.4215
							Interaction	<0.0001**
Ulkenia sp. KF13	2	35.01	40.68	41.43	33.24	45.37	Salinity	0.0175*
	4	28.74	34.81	35.70	31.02	31.42	Time	0.1391
							Interaction	0.6929

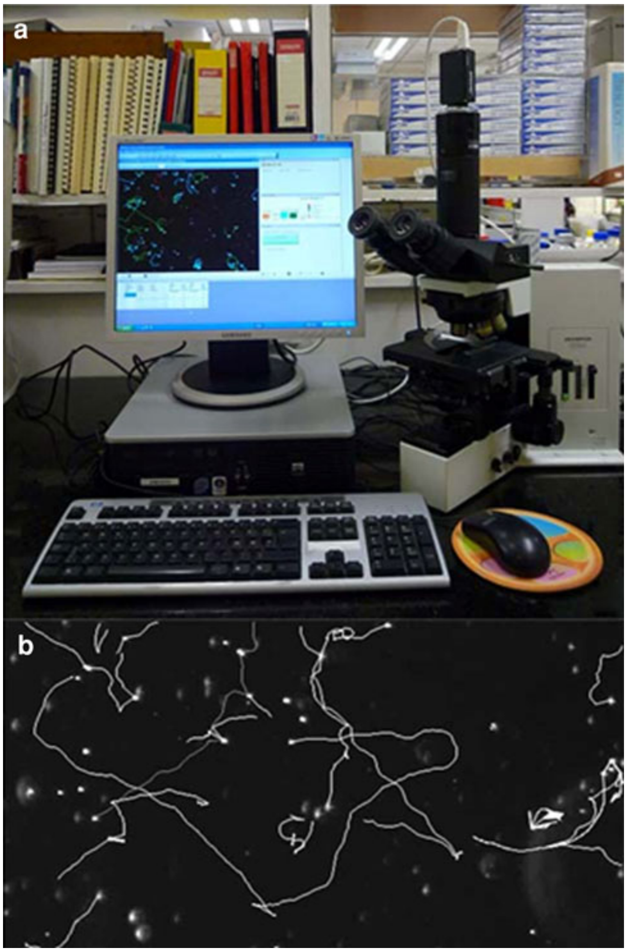


Fig. 1 **a** A *photo* showing the image analysis system consisting of a phase contrast microscope equipped with a progressive scan CCD camera, which was connected to a PC installed with the Copenhagen Rigshospitalet Imagehouse Sperm Motility Analysis System (CRISMAS) software. **b** Swimming pattern of *A. mangrovei* zoospores tracked by CASA; *lines* representing paths of various zoospores

Tokyo, Japan) equipped with a progressive scan charged-coupled device (CCD) camera (Basler Scout, SCA640-70FM, Ahrensburg, German). The CCD camera was adapted to a Firewire board mounted in a PC installed with the Copenhagen Rigshospitalet Imagehouse Sperm Motility Analysis System (CRISMAS) software (Research Version 5.0) (Gade-Nielsen 1998) (Fig. 1a). Images of thallic cells or encysted zoospores were filtered from video analysis using the GIPS object feature analysis provided by the CRISMAS system (Fig. 1a, b).

For measuring spore motility, a zoospore suspension (50 µl each) from each universal bottle was prepared for each treatment (salinity levels) after 2 and 4 h of zoospore induction and mounted on clean, concave glass slides, each covered with a cover slip. In each treatment, zoospore motility was recorded for 10 s in five random microscopic fields providing a total of 15 samples (3 replicates × 5 field) for each treatment at each time interval. Zoospore movement was captured via the CCD camera at 25 frames per second and stored at AVI sequence. Abnormal zoospore swimming patterns were also followed, traced and recorded. Zoospore swimming attributes assessed included: (1) percentage of motile spores, (2) curvilinear velocity (µm/s) (VCL), the time-average velocity of the zoospore head along its actual trajectory and (3) straight line velocity (µm/s) (VSL), the time-average velocity of the zoospore head along the straight line between its first detected position and its last position (Figs. 1b, 2).

The effects of salinities and exposure time to the aqueous medium on the motile percentage (data normalized), as well as motility patterns of zoospores (VCL and VSL) were analyzed using two-way analysis of variance (ANOVA) according to Zar (1996). A significant difference was determined at $P \leq 0.05$.

Results

An apparent drop in zoospore production was observed for all thraustochytrid isolates at salinity above 15‰ (Fig. 3). *Schizochytrium* sp. KF1 produced abundant zoospores at 15‰, but considerably less at other salinity regimes. In contrast, water of salinity 7.5‰ had the greatest zoospore induction (55,400/ml/per dish) in *A. mangrovei* KF6, and zoospore production gradually dropped with increasing saline concentration. *Ulkenia* sp. KF13 produced many zoospores at various salinities below 15‰ (65,900/ml/per dish at 7.5‰), and the number decreased with increased salinity. This isolate produced the highest yield of the zoospores among the tested isolates, with optimum zoospore production at 7.5–15‰. For *T. striatum*, zoospore production was relatively poor (under 10,000/ml/per dish) over all the tested salinity regimes.

Zoospores of all four isolates were shown to swim in the typical pattern of straminipilous organisms, with straight swimming paths and occasional turns (Carlile 1993) (Fig. 1b). The predominant straight swim pattern may be useful for long-range movement and selecting suitable habitats. Zoospores were also observed to swim occasionally in an abnormal pattern such as circular and irregular helical patterns at higher salinities (data not shown). The zoospores of the four thraustochytrid isolates remained motile (>90%) in most sampling measurements 2 h after zoospore induction at various salinity levels, except *Aurantiochytrium mangrovei* KF6. The motile percentage was significantly affected by salinity in *Schizochytrium* sp. KF1 and *A. mangrovei* KF6 (ANOVA, $P < 0.0001$ and $P = 0.0021$, respectively) (Table 1), whereas those of *T. striatum* KF9 and *Ulkenia* sp. KF13 were not affected at all. However, in all four isolates, the percentage of motile zoospores dropped significantly after 4 h of aqueous

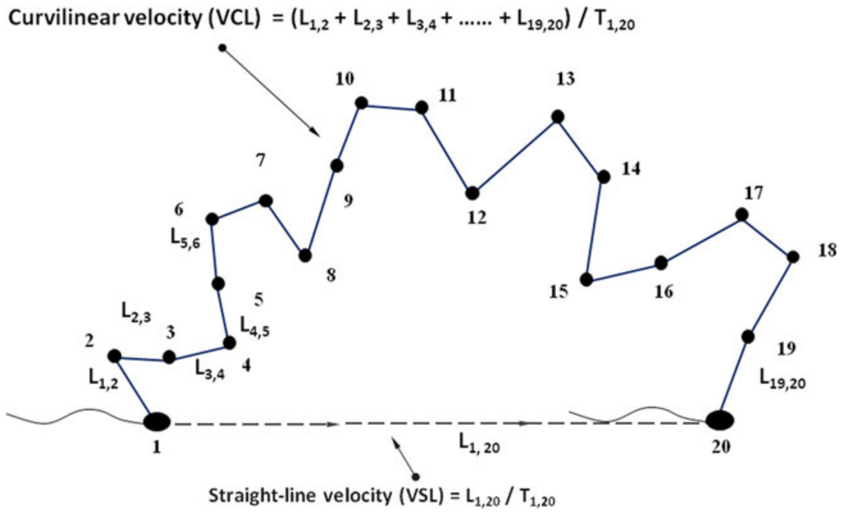


Fig. 2 A diagram showing the two velocity parameters measured and the calculation of velocity parameters

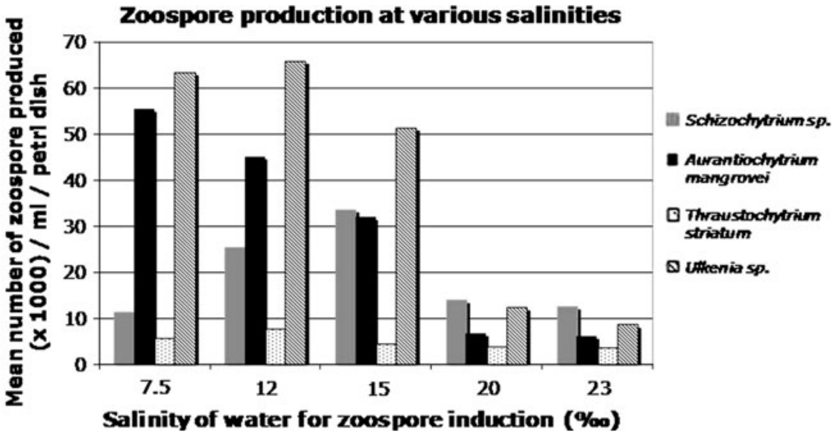


Fig. 3 The zoospore production of all test thraustochytrids under various salinities. *Each bar* shows the average number of zoospores from three replicate petri dishes

medium exposure (ANOVAs, $P < 0.0001$, in all cases) (Table 1).

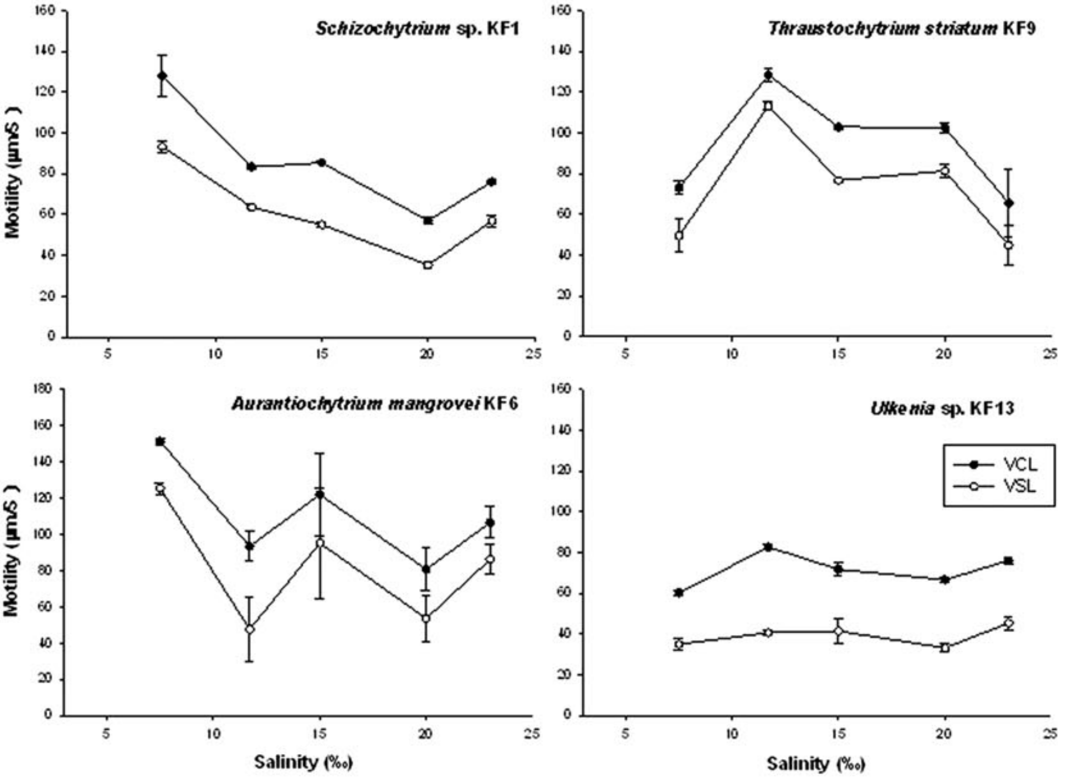
Both salinity and exposure time (duration of exposure to aqueous medium) exhibited significant impacts on zoospore motilities of all test thraustochytrids except the response of *Ulkenia* sp. KF13 to time (Table 1). There were also significant interactions ($P < 0.0001$) between salinity and exposure time.

The optimal salinity level for motility differed with isolates. The VCL and VSL of zoospores of *Schizochytrium* sp. KF1 and *A. mangrovei* KF6 were similar in their responses to the tested salinity regimes, in general a drop in VCL and VSL with increasing salinity of the medium (Fig. 4a). The VCL and VSL of zoospores of *Schizochytrium* sp. KF1 were 127.87 and 93.33 μm/s, respectively, at 7.5‰, but reduced to 76.07 and 56.74 μm/s, respectively, at the highest tested salinity level of 23‰. Zoospores of *A. mangrovei* KF6 also had the highest VCL and VSL at 151.28 and 125.25 μm/s, respectively, at the salinity level of 7.5‰, and lower velocities were recorded below this level, although the reduction in velocity was not gradual with decreased salinity levels (Fig. 4a).

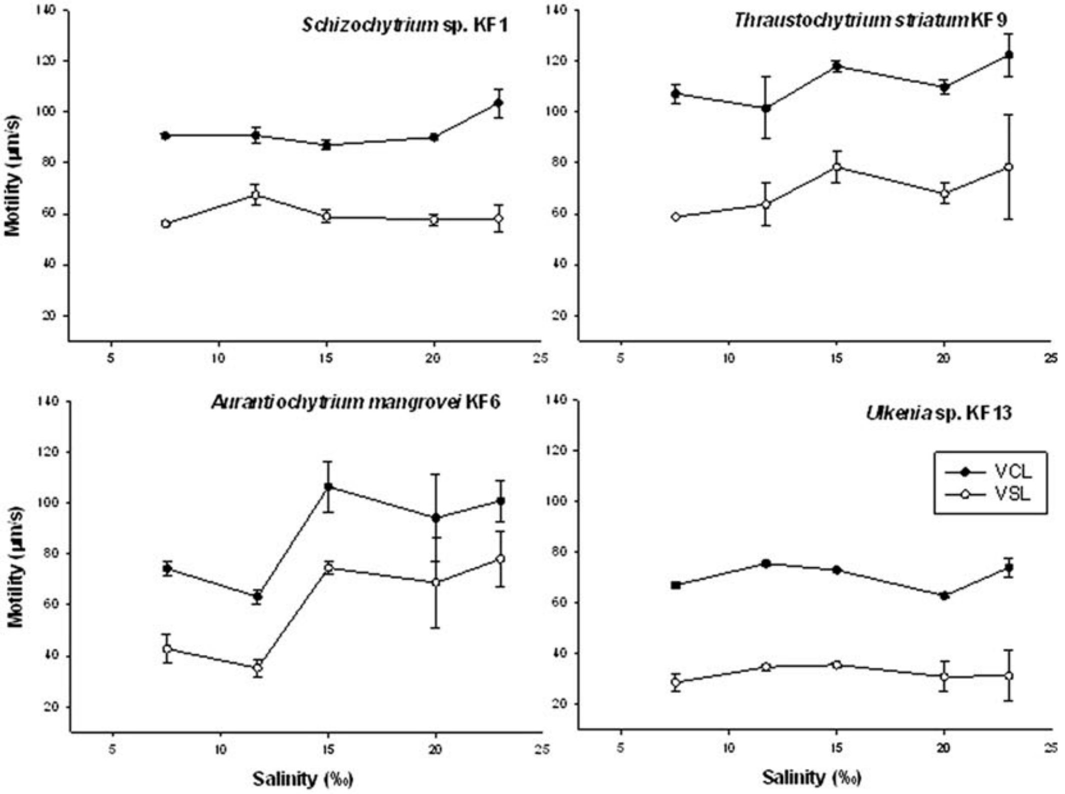
Zoospores of *T. striatum* KF9 were relatively slow at 7.5‰ (VCL and VSL at 73.04 and 49.45 μm/s, respectively), but both velocities increased sharply at 12‰, followed by a subsequent decline with increasing salinity levels, and maintained a fairly constant speed ranging from 102.42 to 128.31 μm/s in VCL and from 76.75 to 113.19 μm/s in VSL, respectively, between 12 and 20‰, reaching a low velocity again at the highest tested salinity level at 23‰ (Fig. 4a). The responses of zoospores to salinity in *Ulkenia* sp. KF13 were relatively small in intensities compared to the other three isolates (Fig. 4a). The motility was low at 7.5‰ and remained stable for the tested range.

At 4 h after zoospore release induction, with the exception of *Ulkenia* sp., noticeable changes in both VCL and VSL in the three test thraustochytrid isolates were observed; a decrease as well as increase in both modes of swimming was detected (Table 1; Fig. 4a, b). Moreover, the optimal salinity for each mode of swimming was also different compared with the measurement at 2 h after zoospore release induction. For example, for *Thraustochytrium* sp. KF9, the VCL at 7.5‰ increased from 73.04

a 2 hrs after zoospore induction



b 4 hrs after zoospore induction



◀ **Fig. 4** Changes in curvilinear velocity (VCL) and straight line velocity (VSL) of the zoospores of four thraustochytrid isolates on exposure to different salinity levels measured after 2 h (**a**) and 4 h (**b**) of zoospore induction. Each *dot* shows the average plus standard error (*error bar*) of 15 zoospores in three replicate petri dishes

to 107.27 µm/s, and the optimal salinity changed from 20 to 23‰ in 2 h. Similar increases in either VCL or VSL could be detected, though to a lesser extent, but the change in salinity optima can be observed in the three thraustochytrids.

Discussion

The mangroves are a dynamic ecosystem comprising trees and shrubs colonizing the estuarine area (Lee 1995), with their fallen leaves being significant resources of nutrients and energy. These fallen leaves provide the necessary substrata for the thraustochytrids to colonize, grow and propagate. The exposure to a change of salinity was a precondition to asexual reproduction of *Halophytophthora* spp. (Nakagiri et al. 1989). Their abilities to produce abundant, motile zoospores, although in varying quantities, in different salinities are adaptations and survival strategies in the fluctuating saline environment. Their zoospores were released from sporangia to attach and colonize the decaying leaves within 24–48 h (Newell et al. 1987; Newell and Fell 1992).

The zoospore production was triggered strongly at low salinity (0–15‰) in at least two isolates, *A. mangrovei* KF6 and *Ulkenia* sp. KF13, whereas *Schizochytrium* sp. KF1 produced the highest number of zoospores at 7.5 and 15‰. Thus, these isolates are well adapted to the mangrove environment for zoospore production where salinity usually varies from 0 to 15‰ in summer. Zoospore formation and discharge of marine *Halophytophthora* spp. and many *Phytophthora* species were also favored by the exposure of the thallic cells in distilled water or diluted salt solutions (Carlile 1986; Nakagiri et al. 1989).

Another reason that may affect the abundance of zoospore production is likely due to the pattern of zoospore formation. For example, representatives of *Aurantiochytrium* and *Schizochytrium* produce zoospores by successive binary division, leading to rapid formation and release (Raghukumar 1988, 2002). However, *Thraustochytrium striatum*, a non-proliferous thraustochytrid (the entire sporangium cleaves to form zoospores), required at least 10 min from sporangial cleavage to zoospore discharge. This is in sharp contrast with proliferous thraustochytrids, which can release zoospores rapidly (20–30 s) (Harrison and Jones 1974).

The percentage of motile zoospores (Table 1) as well as motilities of thraustochytrid isolates dropped in response to various salinity treatments after 4 h of zoospore induction (Fig. 4). Motility, osmoregulation and the maintenance of correct internal ion concentrations have metabolic costs (Carlile 1986). Prolonged swimming may use up their energy reserves, which are generally in the form of neutral lipids (Reed et al. 1999). The lipid level also dropped quickly during the motile phase in the life cycle of a thraustochytrid (Jain et al. 2007). The energy reserve in zoospores of *Phytophthora palmivora* Butler also dropped after 6 h of swimming (Bimpong 1975).

The abilities to maintain high zoospore velocity among species may be related to the amount of total lipids in the cell (Fan et al. 2001). *A. mangrovei* cells had a relatively high lipid content (percentage freeze-dried biomass) of 38.1–53.2%, while the total lipid contents (percentage freeze-dried biomass) of *T. striatum* KF9 and *Ulkenia* sp. KF13 were 4.5 and 11.9%, respectively (Fan 2002). Thus, *A. mangrovei* could be expected to have a higher lipid reserve for motility in terms of higher VCL and VSL compared with *T. striatum* KF13 and *Ulkenia* sp. KF13, as demonstrated by our results.

In the current study, *A. mangrovei* had higher zoospore production and motility in terms of VCL and VSL compared with *T. striatum* and *Ulkenia* sp., especially at low saline conditions (7.5‰) (Table 1; Figs. 3, 4). In Hong Kong, where there is a daily occurrence of two low tides between a high tide (Wu 1988), it is likely that *A. mangrovei* are exposed to daily fluctuating saline conditions that are compatible with their physiology of spore production and discharge. The same observation has been reported by Newell and Fell (1996) for marine halophytophthoras. Their zoospores also had a strong chemotactic response to various leaf extracts and chemicals, suggesting their high efficiency in attachment on natural substrata (Fan et al. 2002a), leading eventually to proliferation of vegetative cells. These results are in agreement with the fact that *A. mangrovei* was the most common thraustochytrid in the Hong Kong mangrove (Fan 2002). *Aurantiochytrium mangrovei* has also been commonly found along the coastal habitats in southeast Asia (Leano 2001); for example, *A. mangrovei* was considered the most predominant species in Japan in summer during a recent survey (Ueda et al. 2009). This species can be the most adaptable and competitive species among the various mangrove thraustochytrids (Fan et al. 2002a).

Statistical analysis of the motility data has demonstrated there is a significant impact of time and varying salinity on the VCL and VSL of the zoospores of the tested thraustochytrids with the exception of *Ulkenia* sp. KF13. Although a decrease in VCL and VSL was observed in the remaining three species, which can be explained by the gradual depletion of energy within the cells, the increase of some of these values in the same isolates after 4 h of zoospore release induction is difficult to explain. Moreover, the phenomena of different salinity optima for VCL and VSL of these species are equally puzzling as there is insufficient information from the literature on their cellular physiology to salinity changes. Nevertheless, these experiments have further proven that the zoospores of thraustochytrids are well equipped to survive in the mangrove environment with abundant zoospore production and maintenance of motility at varying salinities, even after 4 h of zoospore release. Further study of their physiological response at the cellular level in relation to their ecological adaptation to the mangrove environment is warranted.

Conclusion

Our data indicated that not all isolates are equally adapted to the mangrove environment, at least in terms of zoospore motility and production. All tested isolates had wide saline tolerance, but *Schizochytrium* sp. KF1 was highly motile in seawater of 15‰, whereas *A. mangrovei* and *Ulkenia* sp. produced the greatest number of zoospores in diluted sea water. The adaptation of zoospore production and motility could be important in determining their abundance in the mangrove environment. The Computer-Assisted Sperm Motility Analysis System (CASA) was effective in assessing zoospore motility parameters of the thraustochytrids.

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