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Comparative effects of temperature and salinity on growth of four harmful *Chattonella* spp. (Raphidophyceae) from tropical Asian waters

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In Asia, four harmful raphidophyte species, *Chattonella malayana*, *C. marina*, *C. subsalsa*, and *C. tenuiplastida*, coexist in the tropical waters but only *C. marina* was detected in temperate waters. This occurrence pattern pointed to a potentially distinct ecophysiological niche occupancy and possible species dispersion. The growth physiology of these species isolated from tropical Southeast Asia was investigated using unialgal cultures in ten temperatures (13.0–35.5°C) and five salinities (15–35) to better understand the factors driving their distribution. The highest maximum specific growth rates were observed in *C. subsalsa* (0.65 ± 0.01 d^{-1}), followed by *C. malayana* (0.47 ± 0.03 d^{-1}), *C. marina* (0.45 ± 0.02 d^{-1}), and *C. tenuiplastida* (0.39 ± 0.01 d^{-1}). Their optimal temperatures were 28.0, 30.5, 25.5, and 30.5°C, respectively, of which *C. marina* preferred colder water. *C. subsalsa* exhibited a wider growth temperature range (20.5–35.5°C), followed by *C. marina* (20.5–30.5°C), *C. tenuiplastida* (23.0–33.0°C), and *C. malayana* (25.5–33.0°C). Optimal salinities were similar between *C. subsalsa* and *C. malayana* (30), and between *C. marina* and *C. tenuiplastida* (25), but *C. subsalsa* and *C. marina* exhibited a similar growth salinity range of 15–35, while *C. malayana* and *C. tenuiplastida* was 20–35. High values of F_v/F_m were observed in *C. subsalsa* and *C. marina* (> 0.5) in all tested conditions, but F_v/F_m of *C. malayana* and *C. tenuiplastida* were significantly lower at 20.5°C. All four species achieved a maximum cell density of > 10^4 cells mL^{-1} in their optimal conditions. Optimal temperatures in *C. subsalsa* and *C. marina* were identical to previous reports. The high adaptability of *C. subsalsa* in various temperatures and salinities suggests its high competitiveness and bloom potential. The high adaptability of *C. marina* in colder waters compared to other species likely contributes to its wide distribution in the temperate Asian waters. The narrow temperature window of *C. malayana* and *C. tenuiplastida* suggests their endemicity and limited distribution in the tropical waters. This study provides evidence about the occurrences and bloom potential of *Chattonella* spp. in Asia, but the endemicity versus dispersion issue remains unresolved.

KEYWORDS

bloom potential, endemicity, harmful algae, adaptive ecology, algal growth, Fv/Fm, specific growth rate, Southeast Asia (SEA)

1 Introduction

Global warming has been perceived as a factor in the geographical expansion, intensification, and earlier timing of harmful algal blooms (HABs), especially along the coasts of tropical, subtropical, and temperate countries (Hallegraeff, 2010; Fu et al., 2012; Anderson et al., 2021; Sakamoto et al., 2021). In Southeast Asia, harmful algae are commonly found and records of HABs have been increasing (Maclean, 1984; Edvardsen and Imai, 2006; Lim et al., 2012; Azanza et al., 2017; Furuya et al., 2018; Mohammad-Noor et al., 2018; Yñiguez et al., 2021). Some of these HABs species were newly discovered in the region, and likely were introduced by anthropogenic transport or due to the adaptive strategies in the changing environment. The harmful dinoflagellate *Cochlodinium* Schütt (= *Margalefidinium* Gómez, Richlen et Anderson) is a case in point, blooms of the species have been discovered in Indonesia, Malaysia, and Philippines (Iwataki et al., 2007; Anton et al., 2008; Azanza et al., 2008; Iwataki et al., 2008; Iwataki et al., 2015); other example species are harmful raphidophytes *Chattonella* Biecheler and *Heterosigma akashiwo* (Hada) Hada ex Hara et Chihara that have been found in Indonesia, Malaysia, and Thailand (Lirdwitayaprasit et al., 1996; Gin et al., 2006; Ayu-Lana-Nafisyah et al., 2018). It is difficult to trace the origin of these HABs species due to a lack of reliable knowledge of their previous distributions, but studies have shown that they can adapt to various environments, e.g., the raphidophyte *H. akashiwo* was first detected in the USA (Hulburt, 1965), but later has been detected in Japan (Hada, 1967; Hada, 1968; Hara and Chihara, 1987), UK (Leadbeater, 1969), Norway (Throndsen, 1969), Russia and Arctic waters (Konalova, 1995; Ratkova and Wassmann, 2005; Engesmo et al., 2016). The successive adaptation of HAB species could be attributed to favorable environmental conditions such as temperature, salinity, and cyst formation ability (Marshall and Hallegraeff, 1999; Smayda, 2002; Mehnert et al., 2010; Thomas et al., 2012; Boyd et al., 2013).

The raphidophyte *Chattonella* is one of the noxious microalgae that has caused mass mortalities of coastal marine organisms, particularly farmed fish (Imai and Yamaguchi, 2012; Viana et al., 2019; Sakamoto et al., 2021; Lum et al., 2022). Recently, the wide distribution of *Chattonella* and associated fisheries damages have been clarified in the ten countries of Southeast Asia (Edvardsen and Imai, 2006; Lum et al., 2019; Lum et al., 2021; Lum et al., 2022). Moreover, recent phylogeographic studies have revealed the presence of four *Chattonella* species in Southeast Asia, including *C. subsalsa* Biecheler, *C. marina* (as the *C. marina* complex including *C. antiqua* (Hada) Ono, *C. marina*, and *C. ovata* Hara et Chihara), and two newly described species, *C. malayana* W.M. Lum, H.C. Lim, S.T. Teng, K. Takahashi, Leaw, P.T. Lim et Iwataki, and *C. tenuiplastida* W.M. Lum, H.C. Lim, K. Takahashi, S.T. Teng, Benico et Iwataki (Bowers et al., 2006; Ayu-Lana-Nafisyah et al., 2018; Lum et al., 2019; Lum et al., 2021; Lum et al., 2022). As physiological responses can vary among species, strains, and populations, the coexistence and interaction between these four *Chattonella* species arouse scientific interest in their ecophysiological traits because each may have a distinct environmental adaptability and different dispersion background (Marshall and Hallegraeff, 1999; Band-Schmidt et al., 2012; Viana et al., 2019). Furthermore, while the bloom of *C. malayana* had caused wild fish kills in Malaysia in 2016, and the distribution of *C. subsalsa* coincided with the locations where fish kills have been reported, the environmental parameters promoting their blooms have not been identified (Lum et al., 2021; Lum et al., 2022).

In situ population dynamics of HABs species are difficult to be understood without long-term observation data in specific habitats or regions, as such, data from laboratory-based growth experiments are essential to understand the effects of environmental parameters on their occurrence patterns and blooms, including *Chattonella* (Fu et al., 2012; Wells et al., 2015). Due to difficulties in the *in situ* growth rate assessment, the growth characteristics of *Chattonella* have been commonly elucidated by laboratory experiments using single or multiple unialgal culture strains under the effects of temperature, salinity, irradiance, and nutrient concentrations, i.e., *C. subsalsa* from Brazil and USA (Zhang et al., 2006; Viana et al., 2019), and *C. marina* complex (hereinafter referred to as *C. marina*) from Australia (Marshall and Hallegraeff, 1999), Japan (e.g., Nakamura and Watanabe, 1983), Korea (Lim et al., 2020), and Mexico (Band-Schmidt et al., 2012). Among these environmental parameters, temperature and salinity are two major factors in promoting/demoting the growth of *Chattonella* (Nakamura and Watanabe, 1983; Yamaguchi et al., 1991; Noh et al., 2006a; Noh et al., 2006b; Yamatogi et al., 2006; Zhang et al., 2006; Salvitti, 2010). *Chattonella marina* from different localities in Japan grew in a similar temperature range of 15–30°C (optimum 25°C), but with a slightly different salinity range of 15–35 (Yamaguchi et al., 1991;

Khan et al., 1995). On a wider scale, a similar response against temperatures was observed in *C. marina* from Australia, Japan, and Korea, but they had different optimal salinities (Yamaguchi et al., 1991; Kahn et al., 1998; Marshall and Hallegraeff, 1999; Noh et al., 2006a; Noh et al., 2006b). *Chattonella subsalsa* from the USA had a wider temperature range of 10–30°C (optimum 30°C) and salinity range of 5–30 (optimum 25) for growth while the same species from Brazil had an optimal salinity of 30 (Zhang et al., 2006; Viana et al., 2019).

In Southeast Asia, there was limited studies investigating the growth characteristics of *Chattonella*, further, past studies demonstrated inconsistent conclusions (Lee, 2014; Ayu-Lana-Nafisyah et al., 2018). Lee (2014) reported *C. marina* from Sarawak, Malaysia (strain CtSb02, reported as *C. subsalsa* without molecular characterization, see Lum et al., 2022) had an optimal growth at salinity 25, which was similar to those in the temperate waters. However, Ayu-Lana-Nafisyah et al. (2018) revealed that *C. marina* from a mangrove area in Indonesia had a lower optimal salinity of 15. Whether the unique salinity preference of *C. marina* in Indonesia was a strain-specific, species-specific, or ecotypic adaptation in tropical Asian waters remains unclear. The growth characteristics of *C. marina* and other *Chattonella* species in tropical waters, including *C. subsalsa* and the two new *Chattonella* species recently described from Southeast Asia (*C. tenuiplastida* and *C. malayana*), need to be investigated and clarified to further understand the effects of the abiotic factors on their occurrence patterns and bloom dynamics. This study compared the growth responses of single unialgal cultures of the four *Chattonella* species in various temperatures and salinities to achieve the following objectives: (1) to provide baseline growth information of *Chattonella* spp. in tropical waters, (2) to recognize the ecophysiological niches (growth temperature/salinity ranges) of these *Chattonella* species, and (3) to determine their optimal temperature and/or salinity that may contribute to the formation of HABs in this region. The ecophysiological comparison in this study may reveal the bloom potentials of *Chattonella* spp. in each respective temperature and salinity and help to specify the factors promoting their blooms in the natural environment.

2 Materials and methods

2.1 Algal cultures

All *Chattonella* cultures were previously established by Lum et al. (2021; 2022). from Southeast Asia. One representative strain of *C. malayana*, *C. marina*, *C. subsalsa*, and *C. tenuiplastida* was selected (Table 1). They were grown in IMK medium (Wako, Tokyo, Japan), made up of 0.22 μm filter-sterilized, nutrient-depleted aged oceanic seawater pre-adjusted to a salinity of 30. The cultures were maintained at 23.0°C, a light intensity of 70–100 μmol photons m^{-2} s^{-1} of cool, white fluorescent light under a 12:12 h light: dark cycle regime. Note that cultures used in the growth experiments were not axenic, but culture vessels and media were sterilized by autoclaving at 120°C for 15 min, and inoculation was operated aseptically in a laminar flow cabinet.

2.2 Temperature experiment

The temperature experiment was performed in ten temperature treatments, i.e., 13.0, 15.5, 18.0, 20.5, 23.0, 25.5, 28.0, 30.5, 33.0, and 35.5°C (Yamatogi et al., 2006; Sakamoto et al., 2009). Cells of each strain were inoculated into 50 mL culture flasks with filtered caps (Sumitomo Bakelite, Tokyo, Japan) containing IMK medium prepared from aged offshore seawater pre-adjusted to a salinity of 30 by distilled water. The experiment was conducted at a light intensity of 220 μmol photons m^{-2} s^{-1} under a 12:12 h light: dark photocycle. Before the experiment, cells were pre-acclimatized to the targeted temperatures at a rate of ± 2.5°C every two days, starting from 23.0°C. After the acclimatization, an inoculum was transferred to a 50 mL fresh medium to make up for 100 ± 50 cells mL^{-1} initial cell densities for each flask. The experiment was performed in triplicate for each strain at each temperature treatment for a growth cycle of 20 days.

2.3 Salinity experiment

The salinity experiment was performed in five salinity treatments i.e., 15, 20, 25, 30, and 35. Lower salinity IMK media were prepared by pre-dilution with sterile distilled water. The experiment was conducted at 28.0°C under the same light conditions as described above. Prior to the experiment, cells were pre-acclimatized to the targeted salinities by decreasing/increasing the salinity of five every two days, starting from salinity 30. After the acclimatization, an inoculum was transferred to a 50 mL fresh medium to make up for 100 ± 50 cells mL^{-1} initial cell densities for each flask. The experiment was performed in triplicate for each strain at each salinity treatment for a growth cycle of 20 days.

2.4 Cross-factorial temperature and salinity experiment

To further understand the combined effects of temperature and salinity on the two recently described *Chattonella tenuiplastida* and *C. malayana* (Lum et al., 2022), a cross-factorial experiment was conducted with 12 treatments (in triplicate), by crossing temperatures of 25.5, 28.0, 30.5 and 33.0°C with salinities of 25, 30 and 35 in 50 mL culture flasks with non-filtered caps (Sumitomo Bakelite, Tokyo, Japan). Cells in each treatment were pre-acclimatized as described above and subsequently transferred to 50 mL fresh medium to make up for 100 ± 50 cells mL^{-1} initial cell densities for each flask. The experiment was carried out for at least 20 days and monitored until day 40.

2.5 Cell count and growth rate

Two milliliters subsamples were collected from each culture flask every two days until day 20 or day 40, and cells were fixed with HEPES-buffered glutaraldehyde before counting (Katano et al., 2009). Fixed samples were manually counted by a Sedgwick-rafter

TABLE 1 Culture strains of *Chattonella* species used in this study (Lum et al., 2021; Lum et al., 2022).

Species	Strain	Sampling location	Date of collection
C. marina	ChMi02	Miri, Sarawak, Malaysia	2013
C. subsalsa	CtSg02	St John's Island, Singapore	2014
C. malayana	CtBK02	Pantai Melawi, Bachok, Kelantan, Malaysia	20 Apr 2016
C. tenuiplastida	St1409S2	Sematan Beach, Lundu, Sarawak, Malaysia	14 Sept 2017

chamber until ≥ 100 cells. Specific growth rate (μ, d^{-1}) was calculated from the following equation, where N is the number of cells and t is time (Hall et al., 2014).

$$\mu = \frac{\ln (N_t - N_0)}{\Delta t}$$

The maximum specific growth rate ($\mu_{\max}$) was calculated from the maximum slope over a five-point window (h = 5), in other words, the highest growth rate in any eight days (Hall et al., 2014) using the package *growthrates* (Petzoldt, 2022) in R (R Core Team, 2022). Growth rates of *Chattonella* were classified into four levels; (1) negative growth where cells died after the experiment had begun and were omitted from graphs, (2) no growth indicating the survival and mortality of *Chattonella* were almost equal, and no observable μ, (3) growth where $\mu > 0.1$ d^{-1}, and (4) optimal growth with the highest μ ($\mu_{\max}$). Treatments where *Chattonella* spp. achieved high cell densities (10^4 cells mL^{-1}) were noted.

2.6 F_v/F_m measurement

To determine the effects of temperature and salinity on the *Chattonella* cell conditions, the maximum quantum yield of photosystem II (F_v/F_m) was determined by measuring chlorophyll fluorescence from selected conditions, i.e., temperatures 20.5, 25.5, and 30.5°C, and salinities 25, 30, and 35 (Yuasa et al., 2020a; Yuasa et al., 2020b). Subsamples (2 mL) were collected from each culture flask in 4-day intervals, cells were incubated in the dark for 10–15 min, and F_v/F_m values were measured subsequently by a Water-PAM fluorometer (Walz, Effeltrich, Germany). The value of F_v/F_m was calculated by the following equation:

$$F_v/F_m = \frac{F_m - F_o}{F_m}$$

where F_m and F_o are the maximum and minimum fluorescence, respectively. Measurement was conducted in triplicate.

2.7 Statistical analyses

For the single-factor experiments of the effects of temperature and salinity, the μ and F_v/F_m were analyzed by one-way variance analysis (ANOVA), followed by Tukey's *post hoc* comparison test to determine the statistical significance at $p < 0.01$. For the cross-factorial experiment of temperature and salinity, the μ was analyzed by two-way ANOVA. Tukey's *post hoc* comparison test was performed at a statistical significance of $p < 0.01$ when the result of ANOVA was significant. Statistical analyses were conducted in R ver. 4 (R Core Team, 2022).

3 Results

3.1 Effects of temperature

None of the four *Chattonella* species grew at 13.0°C (Figure 1A). However, the effects of temperature on the growth varied among species when temperature > 13.0°C (Figures 1A, 2A and Tables 2, S1, S2). In general, growth was suppressed at 15.5 and 18.0°C, *C. subsalsa* and *C. marina* showed no growth with almost constant cell densities, while *C. tenuiplastida* and *C. malayana* exhibited negative growth (Figure 1A).

Growth temperature ranges were different among the *Chattonella* species. *Chattonella subsalsa* showed a wide temperature range of 20.5–35.5°C, while *C. malayana* demonstrated a remarkably narrow range (25.5–33.0°C). *Chattonella marina* and *C. tenuiplastida* exhibited moderate temperature ranges of 23.0–30.5°C and 23.0–33.0°C, respectively (Figures 1A, 2A). When comparing the $\mu_{\max}$ and their respective optimal temperature (Tables 2, S1), *C. subsalsa* had the highest $\mu_{\max}$ among all four *Chattonella* species (0.65 ± 0.01 d^{-1} at 28.0°C), followed by *C. marina* (0.41 ± 0.07 d^{-1} at 25.5°C), and the $\mu_{\max}$ were similar for *C. tenuiplastida* (0.38 ± 0.01 d^{-1} at 30.5°C) and *C. malayana* (0.38 ± 0.04 d^{-1} at 30.5°C).

Cells of *C. subsalsa* entered the exponential phase in day 2–8, which demonstrated the shortest lag phase among all *Chattonella* species (Figure 1A). Comparable to *C. subsalsa*, *C. marina* entered the exponential phase in day 4–10. In contrast, *C. tenuiplastida* and *C. malayana* displayed a much longer lag phase and entered the exponential phase after day 8 (Figure 1A).

The culture of *C. subsalsa* showed the highest maximum cell densities, with the maximum cell yield of 7.9×10^4 cells mL^{-1} observed at 25.5°C. High cell densities of > 6×10^4 cells mL^{-1} were recorded throughout the temperature treatments (25.5–33.0°C) (Figure 1A). The highest maximum cell density of *C. marina* was recorded at 25.5°C (> 6×10^4 cells mL^{-1}), however, cell densities were relatively high at 20.5–30.5°C (> 10^3 cells mL^{-1}). High cell densities of *C. tenuiplastida* were recorded at 28.0–30.5°C (> 10^4 cells mL^{-1}), with the maximum cell density of 2.1×10^4 cells mL^{-1} observed at 30.5°C. The culture of *C. malayana* exhibited the lowest maximum cell yield among the species examined, with the highest maximum cell density of 1.7×10^4 cells mL^{-1} observed at 30.5°C. At 28.0 and 33.0°C, cell densities remained high (10^4 cells mL^{-1}) on day

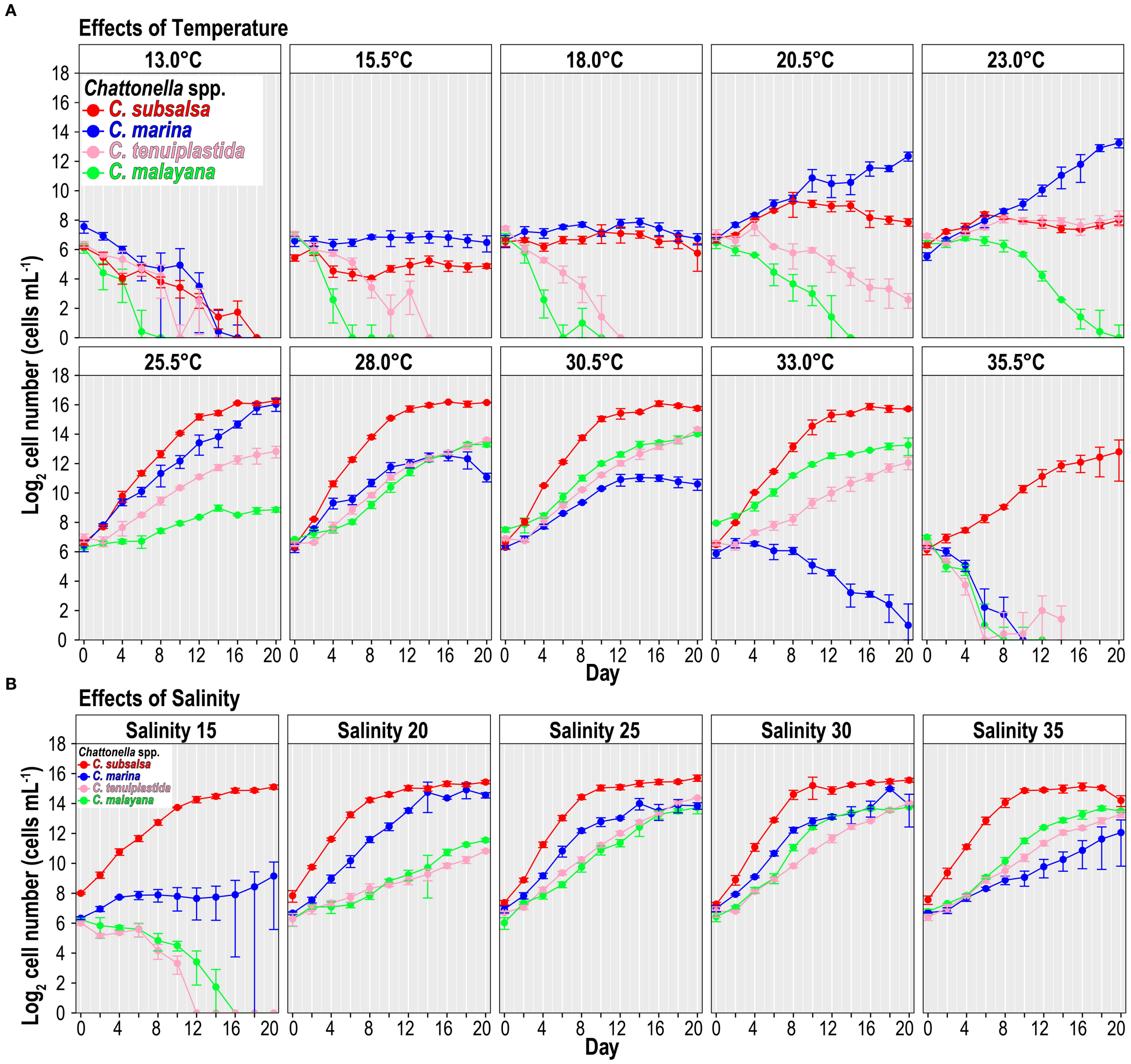

FIGURE 1
Growth of *Chattonella subsalsa*, *C. marina*, *C. tenuiplastida*, and *C. malayana* at different temperature **(A)** and salinity **(B)** treatments. Error bars represent standard deviations (*n* = 3).

20 but observation of whether the cells continued to grow was not available at the time.

The maximum quantum yield of photosystem II (F_v/F_m), an indicator of photosynthetic potential, has been commonly used to determine whether cells are under stress (Vonshak et al., 1994; Qiu et al., 2013; Katayama et al., 2017; Yuasa et al., 2020b). In this experiment, the F_v/F_m values of four *Chattonella* species were monitored in 20.5, 25.5, and 30.5°C treatments in 4-day intervals. *Chattonella subsalsa* and *C. marina* exhibited relatively high F_v/F_m (≥ 0.5) at all temperatures during the experiment period, indicating their photosynthetic potential was retained over the period (Figure 3A and Tables S5, S6). F_v/F_m of *C. subsalsa* were significantly higher at 25.5°C than that of 20.5°C throughout the experiment period ($p < 0.01$) (Figure 3A and Table S6). F_v/F_m values of *C. marina* were significantly different in all temperature treatments starting from day-8 ($p < 0.01$), the highest F_v/F_m was observed at 30.5°C (Figure 3A and Table S6). The differences of F_v/F_m among treatments were obvious in *C. tenuiplastida* and *C. malayana*. Both species showed negative F_v/F_m values at 20.5°C, implying their growth and photosynthetic potential were negatively affected by lower temperatures (Figure 3A and Table S6). At higher temperature treatments, F_v/F_m of *C. malayana* were significantly different ($p < 0.01$) between 25.5 and 30.5°C treatments after day 12, but those of *C. tenuiplastida* were not significantly different between 25.5 and 30.5°C ($p \geq 0.01$; Table S6), suggesting that *C. malayana* might be less tolerant to temperatures lower than 25.5°C.

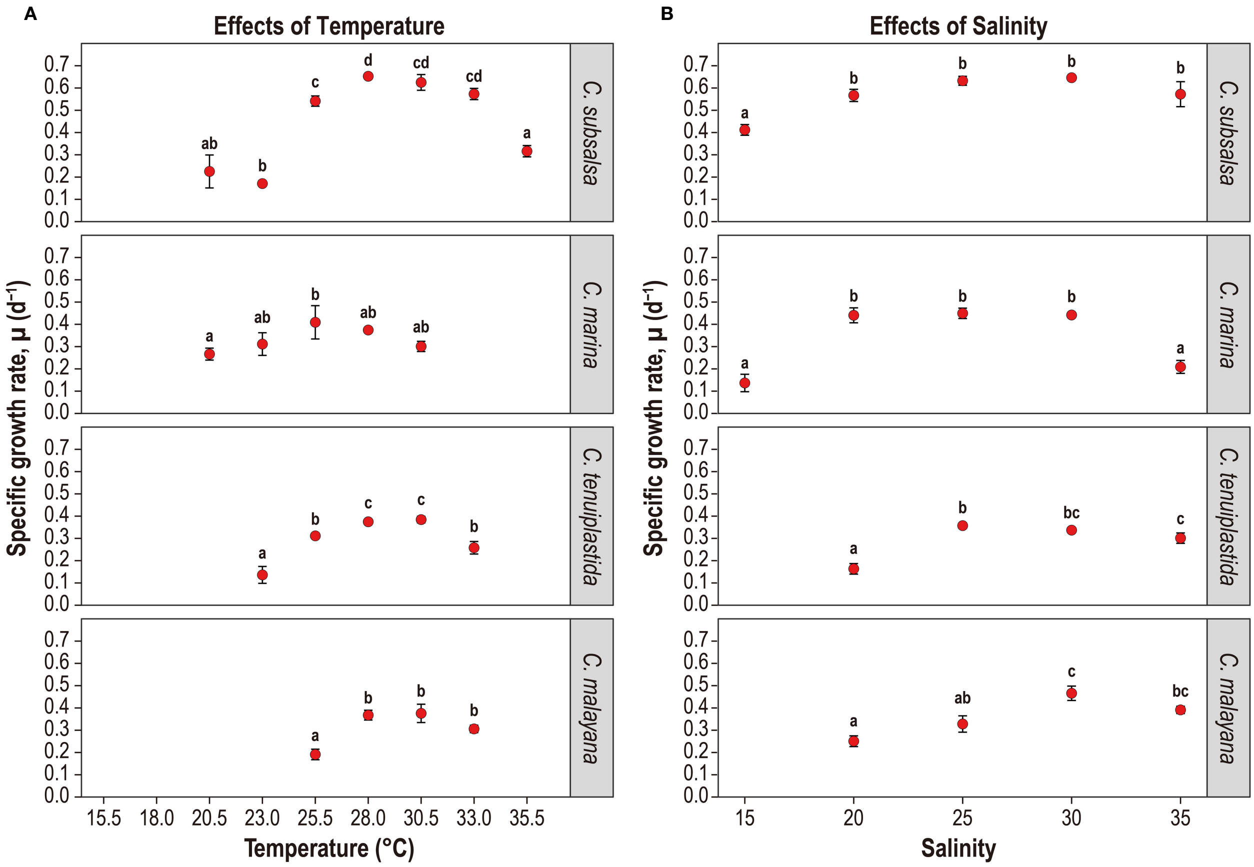

FIGURE 2

Specific growth rate, μ (d^{-1}) of *Chattonella subsalsa*, *C. marina*, *C. tenuiplastida*, and *C. malayana* at different temperature **(A)** and salinity **(B)** treatments. Note that treatments with negative or no growth were not shown, i.e., *C. subsalsa* (≤ 18.0°C), *C. marina* (≤ 18.0°C and ≥ 33.0°C), *C. tenuiplastida* (≤ 20.5°C and 35.5°C), *C. malayana* (≤ 23.0°C and 35.5°C), and *C. tenuiplastida* and *C. malayana* at salinity 15. Alphabets indicate ANOVA significant level of between-treatment comparisons (p< 0.01, Tukey's test). Error bars represent standard deviations (n = 3).

3.2 Effects of salinity

Overall, all *Chattonella* spp. showed similar growth responses to the salinity treatments (Figures 1B, 2B and Tables 2, S3, S4). Growth of *C. subsalsa* and *C. marina* was observed in the salinity range of 15–35, while *C. tenuiplastida* and *C. malayana* were in the salinity range of 20–35 (Table S3). At salinity 15, the growth of *C. tenuiplastida* and *C. malayana* were suppressed and cells died after day 12–14 (Figure 1B). Growth of *C. subsalsa* was significantly lower at salinity 15 than those at 20–35 (p< 0.01) (Figure 2B and Table S4).

Among all species, the highest μ_{max} was observed in *C. subsalsa* (0.65 ± 0.004 d^{-1} at salinity 30, Tables 2, S3), followed by *C. malayana* (0.47 ± 0.03 d^{-1} at salinity 30), *C. marina* (0.45 ± 0.02 d^{-1} at salinity 25), and *C. tenuiplastida* (0.36 ± 0.01 d^{-1} at salinity 25), respectively (Figures 1B, 2B).

For the growth phase, the exponential phase of *C. subsalsa* started on day 4 across all salinity treatments, which was the

TABLE 2 Growth ranges and maximum specific growth rates (μ_{max}) on *Chattonella* spp. in the single-factor experiments.

Species	Growth ranges		μ_{max}, d^{-1} (respective optimal growth condition)	
	Temperature (°C)	Salinity	Temperature (°C)	Salinity
C. subsalsa	20.5–35.5	15–35	0.65 ± 0.01 (28.0)	0.65 ± 0.004 (30)
C. marina	20.5–30.5	15–35	0.41 ± 0.07 (25.5)	0.45 ± 0.02 (25)
C. tenuiplastida	23.0–33.0	20–35	0.38 ± 0.01 (30.5)	0.36 ± 0.01 (25)
C. malayana	25.5–33.0	20–35	0.38 ± 0.04 (30.5)	0.47 ± 0.03 (30)

Salinity of 30 was used in the temperature treatments, whereas temperature of 28.0°C was fixed in the salinity treatments. The definition of growth range is temperature/salinity where cells could grow, and optimal growth is the temperature/salinity where cells achieved the highest μ (μ_{max}).

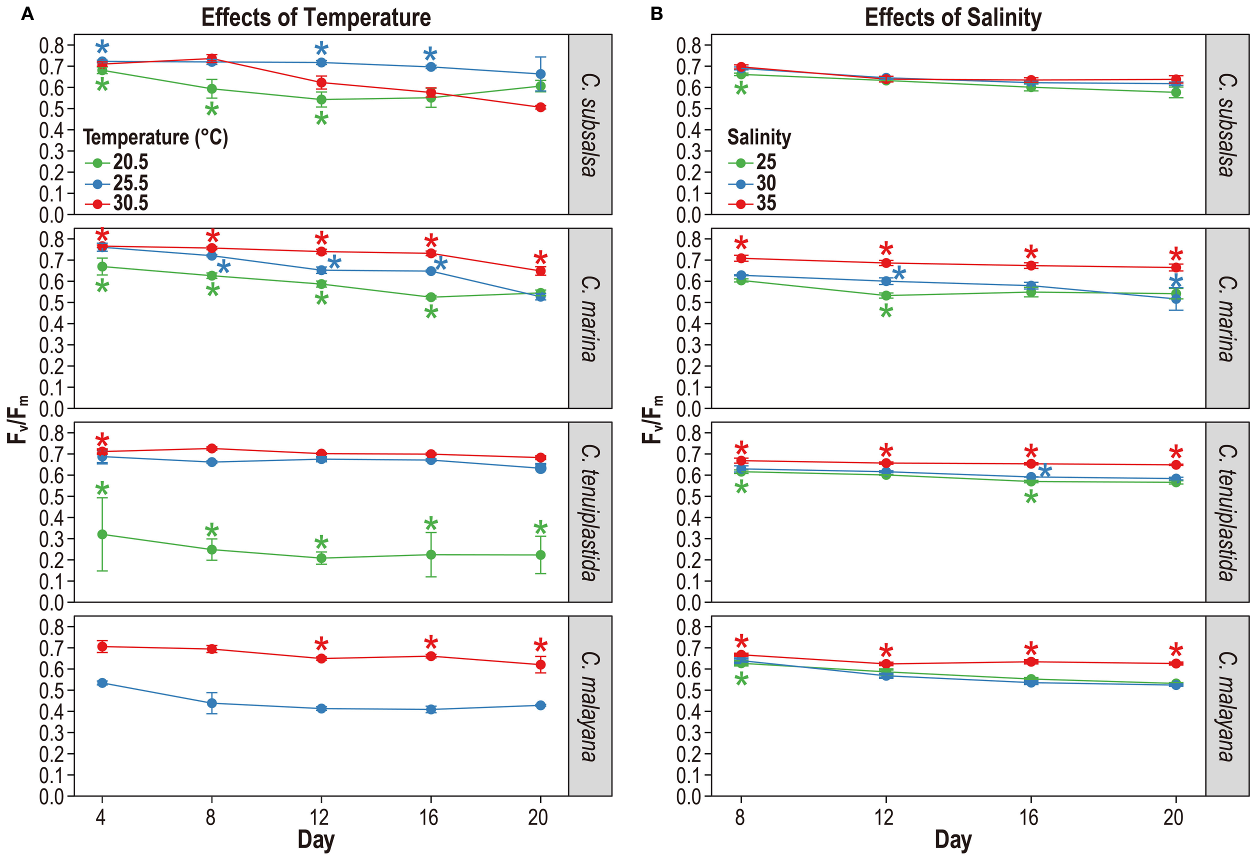

FIGURE 3
The maximum quantum yield of photosystem II (F_v/F_m) of *Chattonella subsalsa*, *C. marina*, *C. tenuiplastida*, and *C. malayana* at temperature treatments of 20.5, 25.5, and 30.5°C **(A)**, and salinity treatments of 25, 30, and 35 **(B)**. Asterisks indicate ANOVA significance differences ($p< 0.01$) between different temperature or salinity treatments on the sampling days. Note that F_v/F_m of *C. malayana* at 20.5°C (< 0.1) was excluded, and no data for day 4 of the salinity experiment due to equipment failure. Error bars represent standard deviations (n = 3).

shortest among all species (Figure 1B). *Chattonella marina* entered the exponential phase on day 6 at salinities 20–30, but with a longer lag phase (10 days) at salinities 15 and 35 (Figure 1B). *Chattonella malayana* also started to grow exponentially on day 6, similar to *C. marina*, at salinity 30 (Figure 1B). The culture of *C. tenuiplastida* exhibited the longest lag phase, as its exponential phase only started after day 8 (Figure 1B).

The maximum cell densities of *C. subsalsa* exceeded 3.5 × 10^4 cells mL^{-1} at all salinity treatments, which was much higher than other species (Figure 1B). The maximum cell densities of *C. marina* exceeding 10^4 cells mL^{-1} were recorded at salinities 20–30 but were lower (< 5 × 10^3 cells mL^{-1}) at salinities 15 and 35. Maximum cell densities of both *C. tenuiplastida* and *C. malayana* were comparable to *C. subsalsa* and *C. marina* (> 10^4 cells mL^{-1}) at salinities 25–35.

The maximum quantum yield of photosystem II (F_v/F_m) was observed for salinities 25, 30, and 35 in 4-day intervals, and the results showed that the effects of salinity on the photosynthetic potential of the four *Chattonella* species were minor (Figure 3B and Tables S7, S8). High F_v/F_m (> 0.6) was observed in *C. subsalsa* in all salinity treatments, and they were not significantly different ($p \geq 0.01$; Figure 3B and Table S8). At salinity 35, *C. marina*, *C. tenuiplastida*, and *C. malayana* had lower μ but higher F_v/F_m as compared to those at salinities 30 and 25, suggesting that the three species could tolerate high salinity of 35 (Figures 2B, 3B and Table S8).

3.3 Cross-factorial effects of temperature and salinity

The combined effects of temperature and salinity produced similar results from the single-factor experiments, and no significant differences were observed among treatments, including the interaction effects of temperature and salinity (Figure 4 and Tables 3, 4). The only statistically significant result was detected in temperature (p = 0.0064, two-way ANOVA) in *C. tenuiplastida*, but almost all results showed no statistical differences ($p \geq 0.01$), except for 25.5-30.5°C (p = 0.0055, Tukey's test) (Tables 4, S9). Cells of *C. tenuiplastida* did not grow at 33.0°C across the salinities 25, 30, and 35, except for one replicate, which grew at salinity 35 (μ = 0.12 d^{-1}). *Chattonella malayana* grew in all tested conditions but μ was extremely low (0.03 ± 0.02 d^{-1}) at 33.0°C and salinity 25. μ_{max} of *C. malayana* (0.40 ± 0.10 d^{-1}) was higher than that of *C. tenuiplastida* (0.39 ± 0.05 d^{-1}), comparable to previous experiments (Table 3).

The optimal growth of *C. malayana* was observed at 28.0°C and salinity 25, with a remarkable μ of 0.51 d^{-1} observed in a replicate

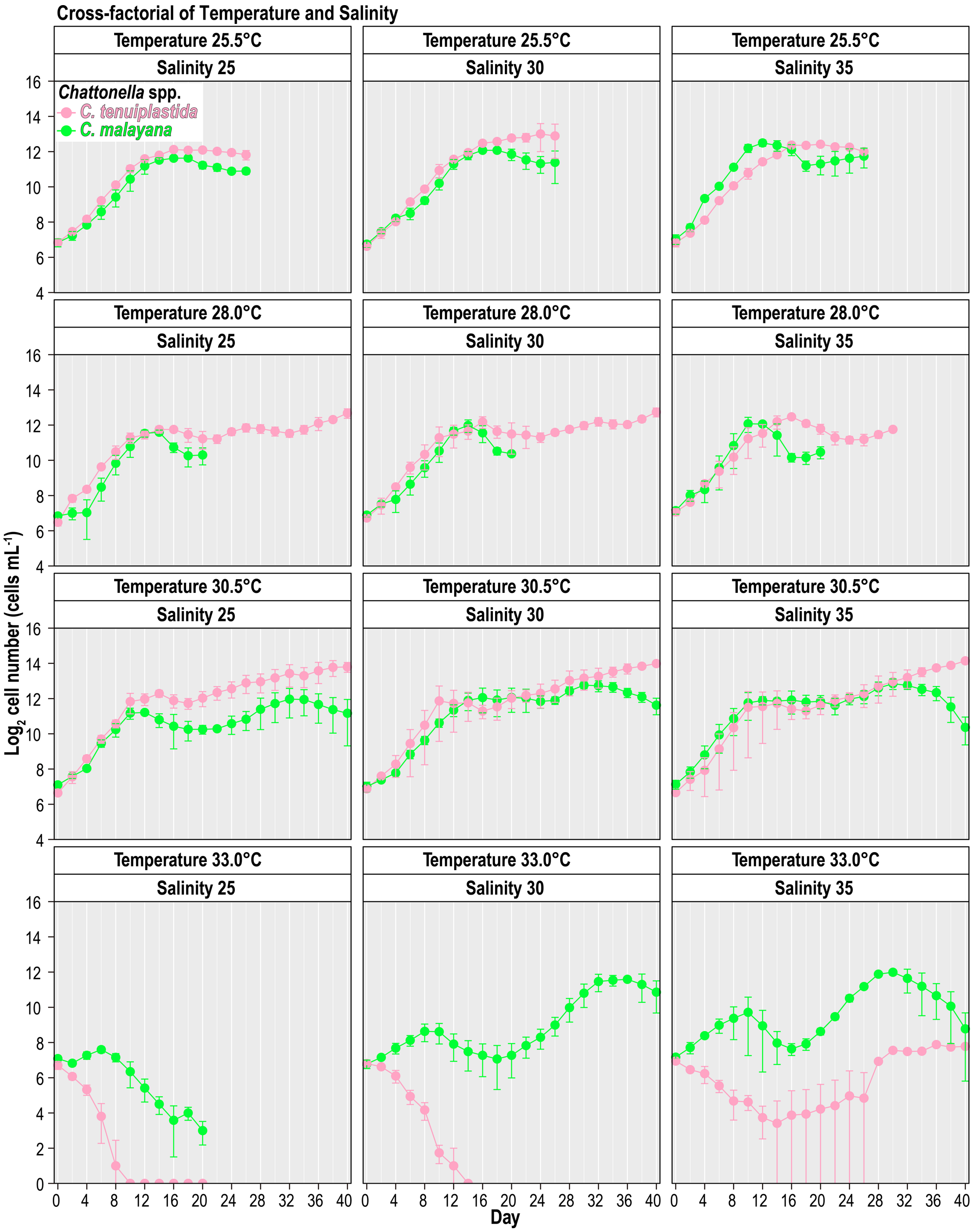

FIGURE 4
Growth of *Chattonella tenuiplastida* and *C. malayana* in the cross-factorial experiment of various temperatures and salinities. Error bars represent standard deviations (n = 3).

(Figure 5A and Table 3). The maximum cell density of 8.3 × 10^3 cells mL^{-1} was observed at 30.5°C and salinity 30, which was lower than those observed in the single-factor experiments (Figure 4). In contrast, the optimal growth of *C. tenuiplastida* was at 30.5°C and salinity 30, with its highest μ_{max} of 0.39 ± 0.05 d^{-1} (Figure 5B and Table 3). In the cross-factorial experiment with temperatures set at 28.0 and 30.5°C, a fluctuating pattern of growth was observed where cell densities declined slightly around day 20 and gradually increased to higher cell densities, resulting in two peaks in the growth curves (Figure 4). This type of growth curve was not observed at 25.5°C, as cells entered the exponential phase from day 4 and then stationary/death phases before day 20. High cell densities (> 10^4 cells mL^{-1}) were recorded at their optimal temperature of 30.5°C across all salinities 25–35.

TABLE 3 Specific growth rate (μ) in the cross-factorial experiments on *Chattonella malayana* and *C. tenuiplastida*, n = 3.

Species	Temperature (°C)	Salinity	Mean μ ± s.d. (d^{-1})	Range of μ (d^{-1})
C. malayana	25.5	25	0.30 ± 0.03	0.27–0.34
	25.5	30	0.30 ± 0.004	0.296–0.305
	25.5	35	0.37 ± 0.004	0.366–0.373
	28.0*	25*	0.40 ± 0.10	0.32–0.51
	28.0	30	0.33 ± 0.04	0.29–0.37
	28.0	35	0.36 ± 0.04	0.32–0.40
	30.5	25	0.32 ± 0.03	0.29–0.35
	30.5	30	0.30 ± 0.02	0.28–0.32
	30.5	35	0.35 ± 0.03	0.32–0.38
	33.0	25	0.03 ± 0.02	0.005–0.05
	33.0	30	0.29 ± 0.06	0.25–0.35
	33.0	35	0.28 ± 0.03	0.26–0.31
C. tenuiplastida	25.5	25	0.30 ± 0.01	0.29–0.31
	25.5	30	0.31 ± 0.05	0.28–0.36
	25.5	35	0.31 ± 0.01	0.30–0.33
	28.0	25	0.34 ± 0.03	0.30–0.36
	28.0	30	0.33 ± 0.09	0.23–0.40
	28.0	35	0.32 ± 0.04	0.29–0.37
	30.5	25	0.37 ± 0.01	0.36–0.38
	30.5*	30*	0.39 ± 0.05	0.33–0.44
	30.5	35	0.38 ± 0.01	0.37–0.40
	33.0	35	0.12	–

Asterisks mark the optimal conditions for each species when the highest μ ($\mu_{\max}$) was achieved.

4 Discussion

4.1 *Chattonella* in the tropical and temperate waters

Studies of temperature effects on the growth of *C. subsalsa* and *C. marina* focus mainly on temperate waters (Australia, Japan, Korea, and the USA), only a few reports had investigated the growth characteristics of *Chattonella* in tropical waters (Lee, 2014; Ayu-Lana-Nafisyah et al., 2018; Kok et al., 2019) (Table 5). In this study, we demonstrated that all four species, *C. subsalsa*, *C. malayana*, *C. marina*, and *C. tenuiplastida* from tropical Asian waters exhibited comparable growth ($\mu_{\max}$) and maximum quantum yield of photosystem II (F_v/F_m) to those of temperate *C. subsalsa* and *C. marina* (Salvitti, 2010; Qiu et al., 2013; Yuasa et al., 2020b) (Table 5). This study revealed that *C. subsalsa* achieved its $\mu_{\max}$ at 28.0°C and salinity 30 (single-factor), *C. malayana* at 28.0°C and salinity 30 (single-factor), *C. marina* at 28.0°C and salinity 25 (single-factor), and *C. tenuiplastida* at 30.5°C and salinity 30 (cross-factor).

When comparing the tolerance of two distinct geographical populations of *C. subsalsa*, the tropical Singapore strain from this study showed a narrower temperature range (20.5–35.5°C), in contrast to the temperate USA strain which showed the range of 10–30°C (Zhang et al., 2006; this study; Table 5). As for *C. marina*, past studies have revealed a lower temperature tolerance limit (10–30°C) of the temperate strains, while the tropical *C. marina* strain demonstrated an upper temperature tolerance limit in the range of 20.5–34°C (Yamaguchi et al., 1991; Khan et al., 1995; Ayu-Lana-Nafisyah et al., 2018; this study). In both cases, tropical *C. subsalsa* and *C. marina* have higher growth temperature ranges than the temperate ones, suggesting that they have well adapted to the local environment and intraspecific variability by latitudinal differences is prominent.

Previous studies have reported higher optimal temperatures in *C. subsalsa* (28–30°C) as compared to *C. marina*, which was 25°C (Yamaguchi et al., 1991; Khan et al., 1995; Kahn et al., 1998; Marshall and Hallegraeff, 1999; Noh et al., 2006a; Noh et al., 2006b; Zhang et al., 2006; Wang et al., 2011; this study). This is in agreement with the present study. A study by Band-Schmidt et al.

TABLE 4 Two-way ANOVA for cross-factorial experiments on *Chattonella malayana* and *C. tenuiplastida*, *n* = 3.

	df	Sum of Squares	Mean Square	F	Sig
C. malayana					
Temperature	3	0.0236	0.00785	4.14	0.0181
Salinity	2	0.00818	0.00409	2.16	0.140
Temperature : Salinity	5	0.0121	0.00241	1.27	0.311
Residuals	22	0.0418	0.00190		
C. tenuiplastida					
Temperature	2	0.0243	0.0122	6.78	0.0064*
Salinity	2	0.000160	0.00008	0.045	0.956
Temperature : Salinity	4	0.000550	0.000139	0.077	0.988
Residuals	18	0.0323	0.00179		

df, degree of freedom; F, F-statistic; Sig, significance level.
Only the temperature factor of *C. tenuiplastida* was statistically different ($p < 0.01$), indicated by an asterisk. Note there were no significant differences in the combined effects of temperature and salinity.

(2012) also showed that the Mexican *C. marina* strain grew better in colder water than *C. subsalsa* strain. The adaptability to a broader temperature range of *C. marina* explained its wide latitudinal distribution across the tropical Southeast Asia and temperate East Asia.

On the other hand, the salinity ranges for growth were inconclusive, as intraspecific variation occurred in *Chattonella* species from similar climates (Table 5). For example, the *C. subsalsa* strain from the USA had a broad salinity range of 5–30 but the *C. subsalsa* strain from Brazil was unable to grow at a salinity below 20 (Zhang et al., 2006; Viana et al., 2019). As for *C. marina*, the temperate Korean strains grew in a salinity of 7.5 (Noh et al., 2006a; Noh et al., 2006b), which was the lowest salinity ever reported for *C. marina*, whereas the Japanese and Australian strains grew in higher salinity ranges (Yamaguchi et al., 1991; Khan et al., 1995; Kahn et al., 1998; Marshall and Hallegraeff, 1999) (Table 5). The tropical *C. marina* strains also showed slightly different salinity ranges, i.e., *C. marina* from Malaysia grew at salinity ranges of 10–

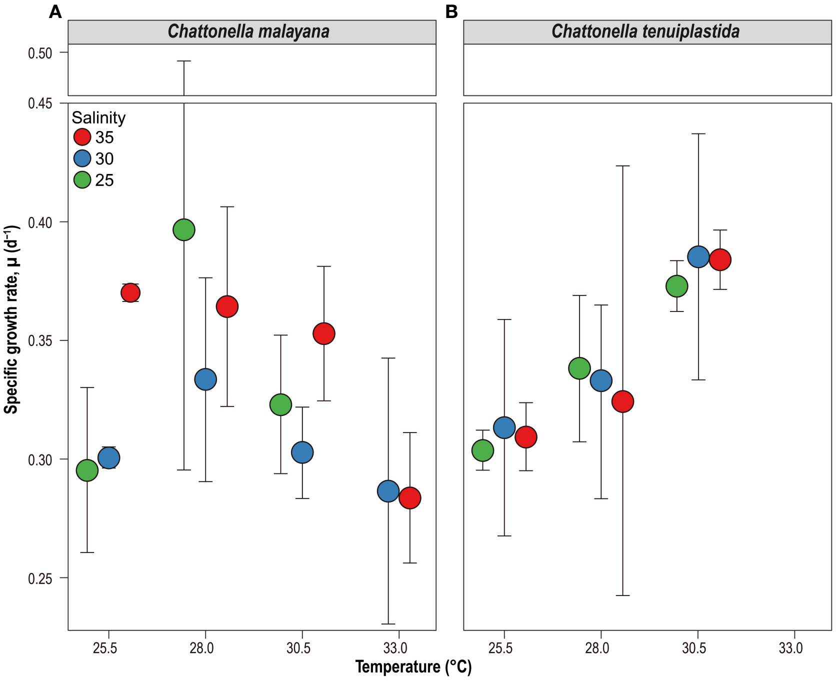

FIGURE 5
Specific growth rate, μ (d^{-1}) of *Chattonella malayana* **(A)** and *C. tenuiplastida* **(B)** and in the cross-factorial experiments of temperatures (x-axis) and salinity (colored circles). Note that *C. tenuiplastida* showed no growth at 33.0°C at salinities of 25 and 30, and only one replicate grew at salinity 35 ($\mu = 0.12\ d^{-1}$), which is not included in the plot. Error bars represent standard deviations (*n* = 3).

TABLE 5 Growth ranges, optimum growth conditions, and maximum specific growth rates (μ_{max}) of *Chattonella* spp.

Species	Growth ranges		Optimal growth		μ_{max} (d^{-1})	Origin
	Temperature (°C)	Salinity	Temperature (°C)	Salinity		
C. subsalsa	20.5–35.5	15–35	28.0[a]	30[a]	0.65	Singapore[†]
	10–30	5–30	30[a]	25[a]	0.60	Delaware, USA[7]
	–	20–30	–	30	0.54	Guanabara Bay, Brazil[10]
C. marina	20.5–30.5	15–35	25.5[a]	25[a]	0.45	Sarawak, Malaysia[†]
C. marina var. *antiqua*	15–30	10–35	25	25	0.67[b]	Seto Inland Sea, Japan[1]
	15–30	15–40	25[a]	35[a]	0.45[b]	Seto Inland Sea, Japan[2]
	15–30	10–40	25[a]	35[a]	0.49[b]	Kagoshima Bay, Japan[2]
C. marina var. *marina*	15–30	10–35	25	20	0.42[b], 0.56[b]	Seto Inland Sea[1] and Yatsushiro Sea, Japan[3]
	10–30	15–45	25[a]	30[a]	0.33[b]	Boston Bay, Australia[4]
	15–30	10–35	25[a]	25[a]	0.64	Gamak Bay, Korea[6]
	–	10–30	–	25[a]	0.28	Sarawak, Malaysia[8]
	25–34	15–33	–	15[a]	0.83	East Java, Indonesia[9]
C. marina var. *ovata*	15–30	7.5–40	25[a]	30[a]	0.47	Jangheung, Korea[5]
C. tenuiplastida	23.0–33.0	20–35	30.5[a]	25[a]	0.39	Sarawak, Malaysia[†]
C. malayana	25.5–33.0	20–35	30.5[a]	30[a]	0.47	Kelantan, Malaysia[†]

[1]Yamaguchi et al. (1991); [2]Khan et al. (1995); [3]Kahn et al. (1998); [4]Marshall and Hallegraeff (1999); [5]Noh et al. (2006a); [6]Noh et al. (2006b); [7]Zhang et al. (2006); [8]Lee (2014); [9]Ayu-Lana-Nafisyah et al. (2018); [10]Viana et al., 2019; [†]Present study.

[a]Optimal temperature/salinity from single-factor experiments.

[b]Data recalculated from the divisions per day reported in the original paper.

30 or 15–35 (both were optimal at 25), but those from Indonesia had a salinity range of 15–33, with an optimum salinity of 15 (Lee, 2014; Ayu-Lana-Nafisyah et al., 2018; this study). The specific growth rates of the tropical and temperate *C. marina* varied at salinity 35, i.e., the tropical strains had lower growth rates (0.2–0.4 d^{-1}) as compared to the Japanese strains (0.4–0.7 d^{-1}) (Nakamura and Watanabe, 1983; Khan et al., 1995; Lee, 2014; Ayu-Lana-Nafisyah et al., 2018; this study). In short, the salinity ranges are not species-specific and could not be explained by the latitudinal differences.

As for *C. tenuiplastida* and *C. malayana*, where their ecophysiology was firstly examined in the present study, relatively narrower growth ranges of temperatures and salinities were observed. The strains exhibited longer lag phases, slightly lower growth rates, and affected F_v/F_m at low temperature (20.5°C) when compared to the tropical *C. subsalsa* and *C. marina* in this study. This suggested that the two species have lower adaptability to colder water and thus restricted their distributions in warm waters. However, their μ_{max} were comparable to those of *C. subsalsa* from Brazil, Malaysia, and Mexico, and *C. marina* from Australia, Japan, and Korea, suggesting competitive growth potentials in the two species (Table 5).

Combined effects of temperature and salinity on the optimal growth have been observed in *Chattonella* (Smayda, 1969; Tomas, 1978; Yamaguchi et al., 1991; Yamatogi et al., 2006). In our single-factor experiments, results showed that *C. tenuiplastida* and *C. malayana* had relatively slower growth (longer lag phase) and relatively lower μ than *C. subsalsa* and *C. marina*, but since *Chattonella* usually could grow very dense (Marshall and Hallegraeff, 1999), the interaction effect of temperature and salinity on these two species had been determined to better understand their growth characteristics. Although our cross-factorial experiments did not show significant differences among all tested conditions (Table 4), the optimal temperature and salinity differed from that observed in the single-factor experiments, i.e., the optimum growth in *C. malayana* had changed from 30.5°C (when salinity was 30) and salinity 30 (when the temperature was 28.0°C) to the combined factors of 28.0°C and salinity 25 (Table 3). The optimal temperature of *C. tenuiplastida* remained the same at 30.5°C but its optimal salinity changed from 25 to 30 in the cross-factorial experiments (Table 3). The changes in the optimal temperature and salinity suggested that other factors not considered in this study might have affected the physiological responses, e.g., the use of non-filtered caps that limited the gaseous exchange. The accumulation of high carbon dioxide (CO_2) concentration resulting in acidic conditions has produced different growth responses in *C. marina* var. *marina* and *C. marina* var. *ovata* (Lim et al., 2020). Increased growth of *H. akashiwo* has also been observed in higher concentrations of CO_2 (Fu et al., 2008).

The μ_{max} of these two species, however, was similar to those of the single-factor experiments, further supported their optimal growths and adaptability. In the cross-factorial experiments, there was no significant difference in the growth response of *C. tenuiplastida* and *C. malayana* in the treatments of salinities 25, 30, and 35, unlike those demonstrated in the single-factor salinity experiments (Figures 2B, 4 and Tables 4, S4). Unfortunately, no constructive conclusion can be drawn from the cross-factorial experiments, except the evidence that the two species exhibited narrow windows of temperature and salinity tolerances. The results from the cross-factorial experiment somewhat agree with those in the single-factor experiments, which suggested the limited distribution of *C. tenuiplastida* and *C. malayana* in the tropical Asian waters.

4.2 Indigenous or alien *Chattonella* spp.?

It is difficult to ascertain the origin and the geographical introduction of these *Chattonella*, but the ability to adapt to a new environment has been suggested as an important driver for the widespread distribution of some phytoplankton especially the bloom-forming raphidophytes and dinoflagellates (Richlen et al., 2010; Zerebecki and Sorte, 2011; Sala-Pérez et al., 2021). They can adapt to warmer temperatures, less saline water, lower nutrient availability, and/or high CO_2 concentration (Cubillos et al., 2007; Hallegraeff, 2010; Thomas et al., 2012; Sala-Pérez et al., 2021). As a case in point, the harmful raphidophyte *H. akashiwo* is widely distributed globally, likely owing to its adaptability to a wide growth temperature and salinity range, where it has high growth in warmer temperatures (Zhang et al., 2006; Fu et al., 2008). Another example is *Margalefidinium polykrikoides* (Margalef) Gómez, Richlen et Anderson, which is a euryhaline and eurythermal bloom-forming dinophyte that could grow in a wide range of temperatures and salinities (Richlen et al., 2010; Kudela and Gobler, 2012). Its widespread global distribution has been well-known, and many newly detected locations have been reported since its first discovery (Richlen et al., 2010; Kudela and Gobler, 2012; Thoha et al., 2019). Similarly, the adaptation of *Gymnodinium aureolum* (Hulburt) Hansen in the Black Sea since its first report in the USA has also been pointed to its euryhaline traits (Sala-Pérez et al., 2021).

The first reports of *Chattonella* in Asia and Southeast Asia were in 1969 and 1983, respectively, which were 30 years later than the first discovery of *C. subsalsa* in France (1933) and 10 years after the discovery of *C. marina* in India (1949), but the understanding on the origin and dispersion of these *Chattonella* was limited (Biecheler, 1936; Subrahmanyan, 1954; Khoo, 1985; Okaichi, 2003). Among the four *Chattonella* species tested in our experiments, we anticipated that *C. subsalsa* and *C. marina* could have been transported in or out of tropical Asian waters and adapted to the new environments as they exhibited high adaptability to broad temperature ranges. Cyst formation in *C. subsalsa* and *C. marina* has also been observed, and these cysts could survive for months in the dark and be transported by ballast water, further suggesting the chance of them being transported (Imai and Itoh, 1988; Imai, 1989; Portune et al., 2009; Jeong et al., 2013; Satta et al., 2017).

In contrast to *C. subsalsa* and *C. marina*, *C. tenuiplastida* and *C. malayana* exhibit narrower temperature and salinity ranges for growth (Lum et al., 2022; this study). In addition, their growths were unparalleled to those observed in *C. subsalsa*, suggesting that these two species may have a weaker ability to spread to other regions. The ability of cyst formation in *C. tenuiplastida* and *C. malayana* has yet to be clarified.

4.3 Potential harmful algal bloom formation

Cell densities of *Chattonella* species ranging from 35 × 10^3 to 28 × 10^7 cells L^{-1} have been reported to be associated with fish kill events (Okaichi, 2003; Barraza-Guardado et al., 2004; Cortés-Altamirano et al., 2006; Martínez-López et al., 2006; Jugnu and Kripa, 2009; Satta et al., 2017). In this study, the cell density of all four *Chattonella* species exceeded 10^7 cells L^{-1} in their respective temperatures and salinities, suggesting that they can form high cell biomass when conditions are favorable. The growth rates of the *Chattonella* spp. in this study are comparable to those of *C. marina* estimated from an *in situ* mesocosm experiment (0.1–0.36 d^{-1}) and during a HAB incident in Yatsushiro Sea, Japan (~1 division d^{-1}, Watanabe et al., 1995; Nakashima et al., 2019).

One of the major factors affecting *Chattonella* blooms in their natural habitat is the water temperature (Imai and Yamaguchi, 2012; Satta et al., 2017). Blooms of *C. subsalsa* occurred in tropical Guanabara Bay, Brazil at 21–31°C, and in Santa Giusta Lagoon, Italy at 20–30°C (Satta et al., 2017; Branco et al., 2019; Viana et al., 2019). *Chattonella subsalsa* in our study demonstrated high growth rates in a wide range of temperatures (25.5–33.0°C), which overlaps with the known bloom temperature of this species. *Chattonella tenuiplastida* and *C. malayana* also showed better growth in warmer temperatures, which were ≥ 25.5°C and ≥ 28.0°C, respectively. These three species could possibly achieve high growth and form HABs in Southeast Asia as the coastal water ranges from 27.2–32.5°C fit their preferred temperature (Lau et al., 2017; Hii et al., 2021). Blooms of *C. marina* have been observed in slightly lower temperatures, such as in southwest India (25–27°C, Sanilkumar et al., 2012), and Mexico (17–22.7°C, Cortés-Altamirano et al., 2006; García-Mendoza et al., 2018). Our tropical *C. marina* maintained these growth characteristics, where it preferred colder water and may have a lower chance to form HABs in Southeast Asia. However, its ichthyotoxicity should not be underestimated as it had caused fish kills in low cell densities (Nishikawa et al., 2014).

Another factor, salinity, has seldom been related to the blooms of *Chattonella*, except when there is a nutrient influx from the river discharge (Onitsuka et al., 2011; Aoki et al., 2012). A wide range of salinities has been reported during their blooms, i.e., blooms of *C. subsalsa* at 38.5 (Mexico, Martínez-López et al., 2006), and 50 (Salton Sea, Tiffany et al., 2001), and blooms of *C. marina* at 35.3–36.9 (Australia, Marshall, 2002), 30–32.8 (Japan, Nakamura et al., 1988), 11.6–33.2 (Korea, Jeong et al., 2013), 33–38.9 (Mediterranean Sea, Ismael and Halim, 2001), and 34.5–34.7 (Mexico, Cortés-Altamirano et al., 2006). In Southeast Asia, heavy rainfalls and low salinity conditions in the coastal areas are common (Leong et al., 2015; Lau et al., 2017; Razali et al., 2022). In such cases, salinity adaptability is important for the survival of these HABs species and the chance to bloom (Nakamura, 1985;

Onitsuka et al., 2011; Aoki et al., 2012; Kok et al., 2019; Hii et al., 2021). In the present study, *C. subsalsa* and *C. marina* could grow in lower salinity and the growth ranges were larger than *C. tenuiplastida* and *C. malayana*, suggesting the former two had higher potential to proliferate and form blooms in the tropical Southeast Asian region.

Based on the historical records of *Chattonella* blooms and fisheries damages in Southeast Asia, *C. subsalsa* has been suggested as the responsible species in the region because of its occurrence in the fish-kill locations (Lum et al., 2021). Its higher growth and maximum cell densities in a wider range of temperatures and salinities as in this study, compared to the other three species, have further supported this claim. Aside from *C. subsalsa*, *C. malayana* had been reported to cause a harmful algal bloom resulting in massive wild fish mortality in Malaysia (Lum et al., 2022). It is interesting to note that, however, the cell density of *C. malayana* examined in this study was relatively low as compared to the other three species. This implied that there are other bloom-promoting factors that may affect the species but have yet to be unveiled. García-Mendoza et al. (2018) suggested that changes in the phytoplankton community must also be considered in understanding the bloom dynamics of *Chattonella*. In future studies, other factors such as irradiance, nutrient availability, CO_2 concentration, strain variability, and their interaction effects should be clarified (Yamaguchi et al., 1991; Marshall and Hallegraeff, 1999; Yamaguchi et al., 2008; Salvitti, 2010; Band-Schmidt et al., 2012; Wells et al., 2015; Kok et al., 2019; Viana et al., 2019; Lim et al., 2020; Yuasa et al., 2020a). To truly explain the ichthyotoxicity of these *Chattonella* species, the linkage between bloom potential and the production of reactive oxygen species (ROS) or other allelopathic chemicals should also be explored (Liu et al., 2007; Imai and Yamaguchi, 2012; Qiu et al., 2013; Yuasa et al., 2020a; Yuasa et al., 2020b; Ahumada-Fierro et al., 2021; Shikata et al., 2021; Cho et al., 2022).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

WL conducted the experiments, and wrote and finalized the manuscript. SS and KY assisted in data collection. TKo, TKa, and KyT assisted in data analyses. KK, CL and PL provided and maintained cultures. KtT and MI conceptualized, reviewed and finalized the manuscript, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor ZH declared a past collaboration with the author MI.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1127871/full#supplementary-material

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