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Biological identification and determination of optimum growth conditions for four species of *Navicula*

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Abstract

Four species in the genus *Navicula* were isolated using the serial dilution method. Based on scanning electron microscopy (SEM) and sequence comparisons of two segments of genes (small ribosomal subunit and large subunit of Rubisco), the species were identified as *Navicula perminuta*, *N. pseudacceptata*, *N. vara*, and *N. rhynchocephala*. Based on phylogenetic analysis and culture trials**,** there was a close relationship between *N. perminuta* and *N. vara*. Growth of these species was evaluated using measurements of optical density at 680 nm (OD_{680}) under various environmental factors. Results showed that the optimum culture conditions were 25°C, 50–100 μmol photons m^{-2} s^{-1}, pH 8.0, and salinities from 25 to 30. However, the favorable salinity for *N. perminuta* was surprisingly high at 35. Nutrient requirement analysis demonstrated that growth of *Navicula* depended on the availability of SiO_3^{2-}. Their relative growth rates (RGR) peaked at the highest tested level (0.25 mmol/L). The optimal concentrations of NO_3^- and PO_4^{3-} were 3.6 mmol/L and 0.18 mmol/L, respectively. Culture of these *Navicula* species for abalone or sea cucumber aquaculture should take these factors into consideration.

Key words: *Navicula*, SSU, *rbc*L, culture conditions

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1 Introduction

Diatoms are thought to be the most species-rich group of microalgae (Mann, 1999). They are ecologically widespread and have a global significance in the carbon cycle, accounting for at least 20% of all carbon fixed globally through photosynthesis every year (Evans et al., 2007). Some benthic diatom species have been used frequently for feeding larvae of abalone and sea cucumber in aquaculture. During the early stage, the juvenile abalone requires high quantity and quality benthic diatoms that provide sufficient nutrition for growth and survival (Wang et al., 1997; Daume and Ryan, 2004). Among these benthic diatoms, the species of *Navicula* spp. that live in all types of water from fresh to marine habitats are commonly utilized (Lebeau and Robert, 2003). It has been reported that the settlement and survival of veligers of *Haliotis discus hannai* in the benthic diatom *Navicula*-2005-A could reach 83.5% and 73.0%, respectively (Pang et al., 2006).

In establishing live cultures of benthic diatoms from the Chinese coasts, four unialgal cultures of *Navicula* spp. were isolated from four sites and identified to the species level. In China, these species were recorded only in several ecological researches (Jin et al., 1982; Cheng et al., 1993; Li, 2006) and investigations on these species were rare.

The goals of the present study were to: (a) identify the samples based on morphological observation, combined with molecular analysis of the nuclear-encoded small ribosomal subunit (18S rRNA or SSU) and the plastid-encoded large subunit of Rubisco (*rbc*L); and (b) evaluate the optimum growth conditions by different sets of culture experiments for better exploration of the species in aquaculture and long-term stock culture.

2 Materials and methods

2.1 *Sample collection and morphological observation*

Seawater samples were collected from four different locations along the Chinese coasts and vouched as mbccc 1, 2, 3, and 8 (Table 1). Pre-cultures were maintained at 18°C in 40 μmol photons m^{-2} s^{-1} under a 12 h light per day light-dark regime in temperature-controlled incubators (GXZ-260 C, Jiangnan, China) in f/2 culture medium (Guillard, 1975). The isolation procedure included one week pre-culture of seawater samples under the conditions mentioned above. Then serial dilution was performed to isolate each of the unialgal benthic diatoms (Hoshaw and Rosowski, 1973; Xu et al., 2012).

For morphological observation, a light microscope (XD-202, Jiangnan, China) and a further scanning electron microscopy (SEM) were used. For the latter, organic compounds were first cleaned off the samples, and then HNO_3 and H_2SO_4 were added into the water sample at a ratio of 1:3:1. The treated sample was then boiled for 6 min and rinsed with distilled water to remove the acid (Ki et al., 2009). The specimens were observed with SEM (KYKY2800B, Scientific Instrument Factory, CAS, China, 25 kV; Fig.1). Morphological identifications were based on Round and Mann (1990), Cheng et al. (1993), and Jin et al. (1982).

The standard cultures of unialgal samples in all the experiments were performed at 22°C in 60 μmol photons m^{-2} s^{-1} un-

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Table 1. List of the diatom isolates and corresponding GenBank accession number

Species	Voucher	Collection details	GenBank	
			SSU	*rbc*L
Navicula vara Hustedt	mbccc1	Xiangshan, Zhejiang Province (31.909 0°N, 121.267 71°E)	JQ045338	JX863893
Navicula rhynchocephala Kützing	mbccc2	Lvshun, Liaoning Province (38.453 8°N, 121.131 30°E)	JQ045339	JQ432374
Navicula perminuta Grunow	mbccc3	Qingdao, Shandong Province (36.022 5°N, 120.195 10°E)	JQ045340	JQ432375
Navicula pseudacceptata H. kobayasi	mbccc8	Nantong, Jiangsu Province (32.333 0°N, 121.020 74°E)	JN674064	JQ432376

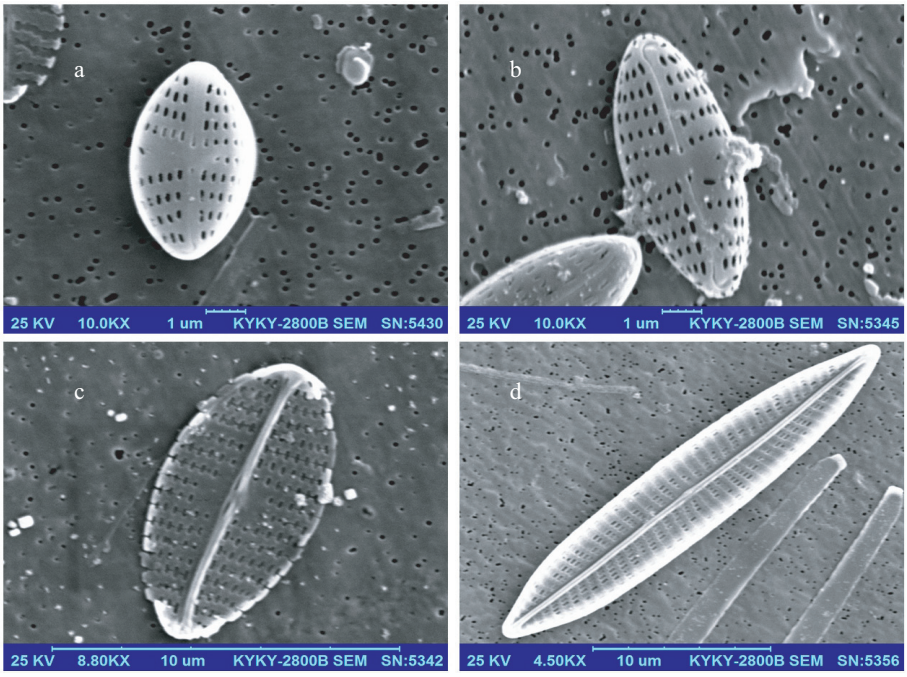

Fig.1. SEM photos of four *Navicula* spp.: *Navicula perminuta* (a), *Navicula vara* (b), *Navicula pseudacceptata* (c), and *Navicula rhynchocephala* (d).

der a 12L:12D regime in a 250-mL flask containing 100 mL sterile f/2 culture medium unless otherwise specified.

2.2 *Molecular identification and phylogenetic analysis*

Total DNA was extracted from the pure cultures of the unialgal sample. The sample was centrifuged at 8 000 *g* for 6 min in a 50-mL centrifuge tube. Then, the supernatants were discarded and the concentrated cells were collected into a 1.5-mL Eppendorf tube. DNA was extracted using the Universal Genomic DNA Extraction Kit (Takara, Japan).

For each culture, the small subunit rRNA coding gene (SSU rRNA) and the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbc*L) were amplified using the polymerase chain reaction (PCR; Saiki et al., 1988). The SSU was amplified using the forward primer 1F (5′-AACCTGGTTGATCCTGCC-3′) combined with the reverse primer 1528R (5′- TGATCCTTCTGCAGGTTCACCTACAGT-3′). The *rbc*L gene was amplified using the pair of universal primers (forward F3, 5′-GCTTACCGTGTAGATCCAGTTCC-3′, reverse R3, 5′-CCTTCTAATTTACCAACAACTG-3′) (Bruder and Medlin, 2007). PCR thermo cycling parameters (T-personal, Biometra, Germany) were also described by Bruder and Medlin (2007). SSU sequences were performed as: 94°C for 7 min, followed by 32 cycles of denaturation at 94°C for 2 min, annealing at 54°C for 4 min, and extension at 72°C for 2 min. After the cycles, final extension was completed at 72°C for 7 min. *rbc*L sequences were performed as: 94°C for 10 min, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min. After the cycles, final extension was completed at 72°C for 10 min. Each 20 μL PCR solution consisted of 10×PCR buffer, 1.5 μL $MgCl_2$ (25 mmol/L), 2 μL dNTPs (2.5 mmol/L), 0.2 μL Taq (5 U/μL), 0.5 μL each primer (10 mmol/L), and 12.3 μL ddH_2O. The PCR-products were purified by using UNIQ-10 PCR Purification Kit (Sangon, China). Sequencing reactions were completed by Sangon (Shanghai, China).

The sequences obtained were submitted to GenBank to search for homologous sequences (http://blast.ncbi.nlm.nih.gov/). Additional SSU and *rbc*L sequences were downloaded from the GenBank. These sequences were aligned for phylogenetic analysis using Clustal W (Thompson et al., 1994). Clustering trees were performed by Mega 4.0 (Tamura et al., 2007) through the Neighbor-joining (NJ) method. The evolutionary distances of NJ trees were computed using the Kimura 2-parameter method (Kimura, 1980), and the reliability of branches was evaluated with non-parametric bootstrapping (1 000 replicates). All positions that contained gaps and missing data were eliminated from dataset.

2.3 *Culture trials and measurement of relative growth rates (RGR)*

Detailed information for culture experiments is listed in Table 2. Triplicate cultures of each species were grown in a 250-mL flask containing 100 mL sterile f/2 culture medium placed in temperature- and light-controlled incubators (GXZ-260C, Jiangnan, China). In order to exclude the effects of the original culture solution, the culture was centrifuged at 7 000 *g* for 5 min before inoculation. The inoculums used were from cultures at the log-phase growth as determined by preliminary experiments. In each of the seven experiments, only the listed factor varied while others were the same. In the temperature experiment, samples were grown at temperatures ranging from 15°C to 30°C in 60 μmol photons m^{-2} s^{-1} under a 12L:12D regime. In Expt. 2, cultures were grown under light intensities ranging from 30 to 200 μmol photons m^{-2} s^{-1} at a temperature of 22°C in a 12L:12D rhythm. In Expt. 3, samples were grown under salinities ranging from 20 to 40 at standard culture conditions. The media were adjusted to designated salinities by adding distilled water or NaCl. In Expt. 4, the media were adjusted to five pH values (6.0, 7.0, 8.0, 9.0, and 10.0) by use of 0.2 mol/L HCl or NaOH. In Expts 1–4, normal f/2 medium was used. In Expt. 5, samples were grown in f/2 culture media with SiO_3^{2-} levels ranging from 0.03 to 0.25 mmol/L. In Expt. 6, samples were grown in f/2 culture media with NO_3^- levels ranging from 0.3 to 7.2 mmol/L. In Expt. 7, samples were grown in f/2 culture media with PO_4^{3-} levels ranging from 0.015 to 0.36 mmol/L.

The optical density (OD) at a wavelength of 680 nm (OD_{680}) was used as the biomass indicator for all the experiments (Wu et al., 2009). The OD value was determined by use of a T6 spectrophotometer (Pgeneral, China). All the experiments carried out in triplicates were terminated after a 12-day growth period without renewal of the culture medium in between. Then samples attached on the bottom of the flask were scraped using 1-mL pipette tips. An ultrasound was also applied for 60 s to dislodge the cells that were firmly attached to the walls (Reyes et al., 2001). RGR was calculated using the following formula:

$$K=(\lg OD - \lg OD_0) / T,$$

where OD_0 and OD represent the initial and ending OD values of the algal solution after T days culture, respectively. For the growth curve of each sample, the mean value was used.

2.4 *Statistical analyses*

All values in this study were obtained from fully independent samples. Differences were considered to be significant at a probability of 5% (p<0.05). Tests were performed using the SPSS 12.0 statistical program (SPSS Inc., Chicago, USA).

3 Results

3.1 *Identification of samples*

The partial SSU sequences of samples were sequenced (more than 1 700 bp, except *N. pseudacceptata*, 1 562 bp, Table 1). Blast analysis showed that mbccc1 had a maximal identity of 98% with *Navicula veneta* (AM501970), the same as mbccc3; mbccc2 had a maximal identity of 99% with *Navicula* sp. ArM0003 (EU090030); and mbccc8 had a maximal identity of 98% with *Navicula cryptocephala* var. *veneta* (AJ297724). In addition, species that matched our samples above 96% belonged to the *Navicula* genus.

The partial *rbc*L sequences of samples were sequenced (713 bp, Table 1). Blast analysis showed that mbccc1 had a maximal identity of 98% with *Navicula sclesviscensis* (FJ002098), the same as mbccc3. Mbccc2 had a maximal identity of 99% with *Navicula* sp. ArM0003 (EU090048), while mbccc8 had a maximal identity of 98% with *Navicula gregaria* (AM710440). The blast results had the same pattern with SSU. Based on blast analysis of the two sequences, the samples were attributed to *Navicula* spp. By analyzing the morphological characteristics (Table 3), the isolated diatom strains were also identified to be *Navicula perminuta* Grunow (mbccc3), *Navicula pseudacceptata* H. kobayasi (mbccc8), *Navicula vara* Hustedt (mbccc1), and *Navicula rhynchocephala* Kützing (mbccc2) (Fig. 1).

3.2 *SSU and rbcL sequence analyses*

In the SSU tree (Fig. 2), *N. vara* was sister to *N. perminuta*, judged by divergences of the SSU gene. *N. lanceolata*, *N. ramo-*

Table 2. Details of the culture trials

Expt. 1 Temperature /°C	Expt. 2 Irradiance /μmol photons·m^{-2}·s^{-1}	Expt. 3 Salinity	Expt. 4 pH	Expt. 5 SiO_3^{2-} levels/ mmol·L^{-1}	Expt. 6 NO_3^- levels / mmol·L^{-1}	Expt. 7 PO_4^{3-} levels / mmol·L^{-1}
15	30	20	6.0	0.03	0.3	0.015
20	50	25	7.0	0.05	0.6	0.03
25	100	30	8.0	0.10	0.9	0.06
30	150	35	9.0	0.15	1.8	0.09
	200	40	10.0	0.20	3.6	0.18
				0.25	7.2	0.36

Table 3. Morphological features of the four diatoms

Species	Cell length/μm	Cell width/μm	Striae (in 10 μm)	Cell shape
Navicula vara (mbccc1)	7–8.5	3.5–4.5	18–20	ellipse
Navicula rhynchocephala (mbccc2)	30–55.5	5–12	12–13	lanceolate
Navicula perminuta (mbccc3)	6–13	3–3.5	16–18	boat-like
Navicula pseudacceptata (mbccc8)	6–10	3–4.5	16–22	ellipse

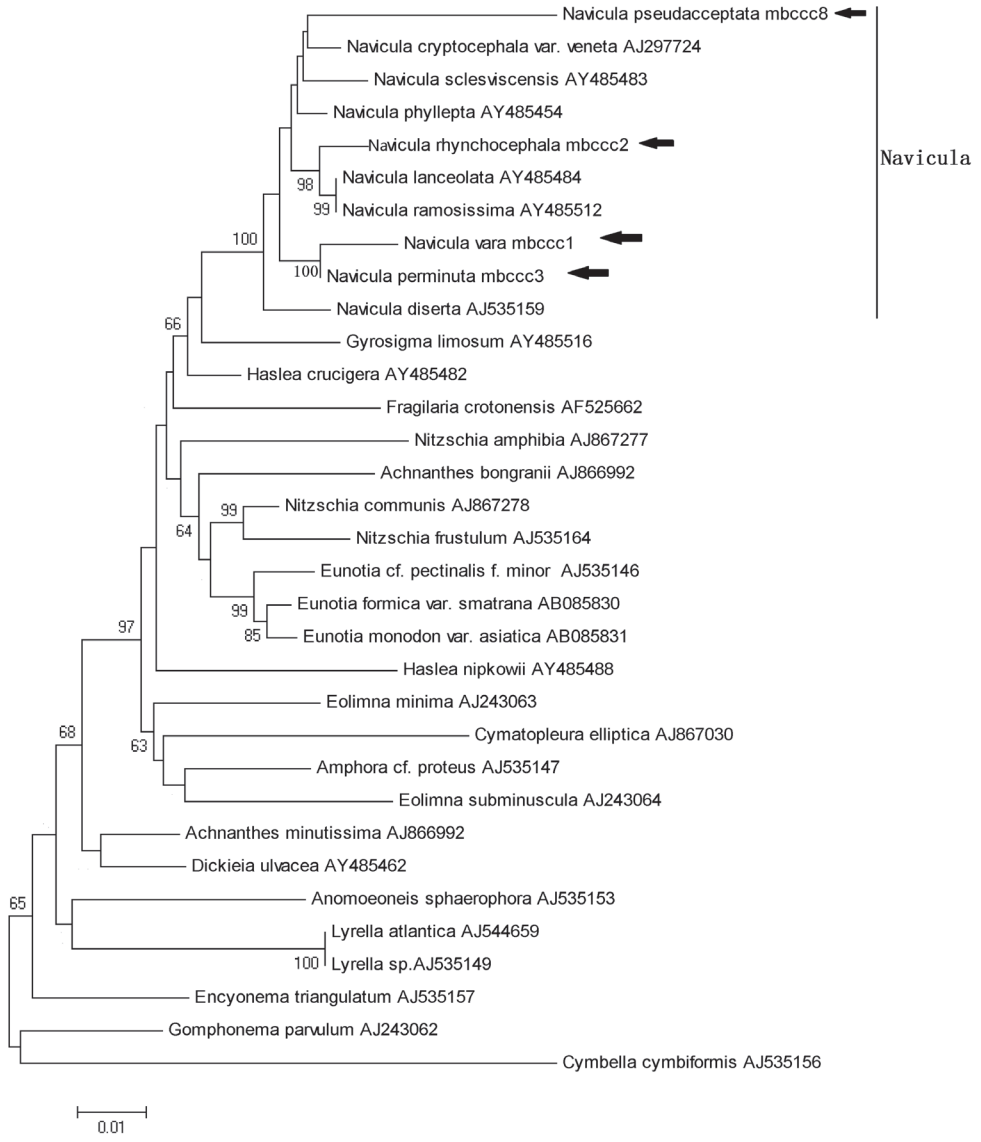

Fig.2. Phylogeny inferred with NJ analysis using SSU rRNA sequences from GenBank. Bootstrap values were obtained from 1 000 replications. The direction arrow represents the species studied in this experiment. Numbers near each clade refer to the NJ bootstrap value, and are displayed when the bootstrap value is greater than 60.

sissima, and *N. rhynchocephala* were grouped together. The *Navicula* spp. clade was sister to *Gyrosigma limosum*.

In the NJ tree based on *rbc*L sequences (Fig. 3), all species of *Navicula* formed a clade. *N. vara* was sister to *N. perminuta*; and *N. rhynchocephala*, *N.* cf. *duerrenbergiana*, and *N. pseudacceptata* were grouped into the *Navicula* spp. clade. In the combined analysis of SSU and *rbc*L, *N. vara* might have a close relationship with *N. perminuta*, since the divergences were 0.8% and 1%, respectively.

3.3 *Relative growth rates in different culture conditions*

3.3.1 *Effects of temperature and light intensities*

Four species of *Navicula* could grow under all the test conditions with varied RGR. The highest RGR was recorded at 25°C for the samples (Fig. 4a, $p<0.05$). Except for *N. pseudacceptata*, no significant difference was found between 20°C and 25°C. There were negative relationships between RGR and temperature above 25°C for the four samples. *N. perminuta* and *N. vara* had similar RGR values under the different temperatures. In the irradiance test (Fig. 4b) for *N. rhynchocephala*, the maximum RGR was obtained at 100 μmol photons m^{-2} s^{-1}, followed by 150, 50, 200, and 30 μmol photons m^{-2} s^{-1} ($p<0.05$). *N. pseudacceptata* had the maximum RGR, occurring at 50 μmol photons m^{-2} s^{-1} ($p<0.05$). For the other two species, *N. perminuta* and *N. vara*, the highest RGRs were achieved at 50 μmol photons m^{-2} s^{-1}, but no significant difference was found in comparison with rates under 100 mol photons m^{-2} s^{-1}.

3.3.2 *Effects of salinity and pH*

The highest RGRs were recorded at the salinity of 25 and 35 for *N. vara* and *N. perminuta*, respectively ($p<0.05$). For the oth-

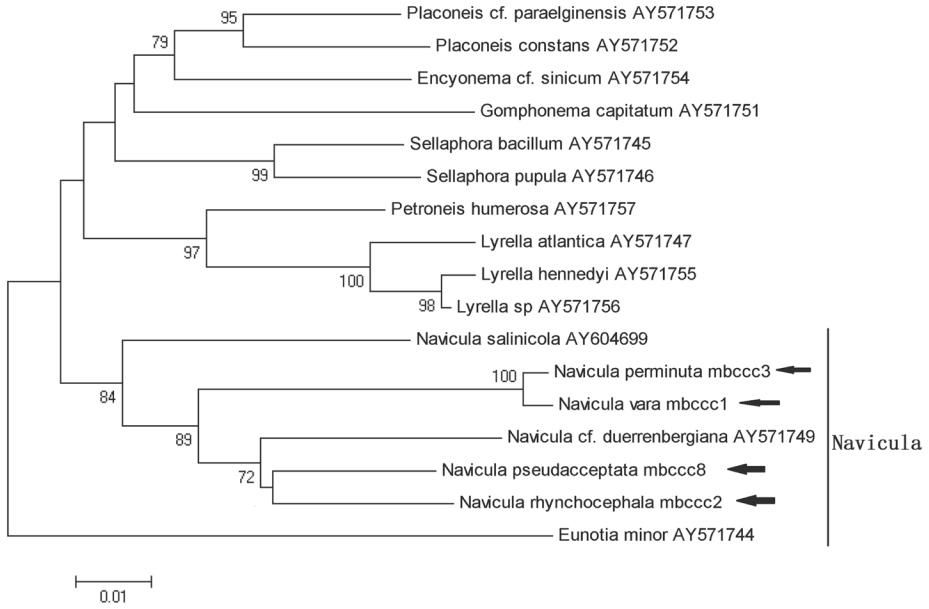

Fig.3. Phylogeny inferred with NJ analysis using *rbc*L gene sequences from GenBank. Bootstrap values were obtained from 1 000 replications. The direction arrow represents the species studied in this experiment. Numbers near each clade refer to the NJ bootstrap value, and are displayed when the bootstrap value is greater than 60.

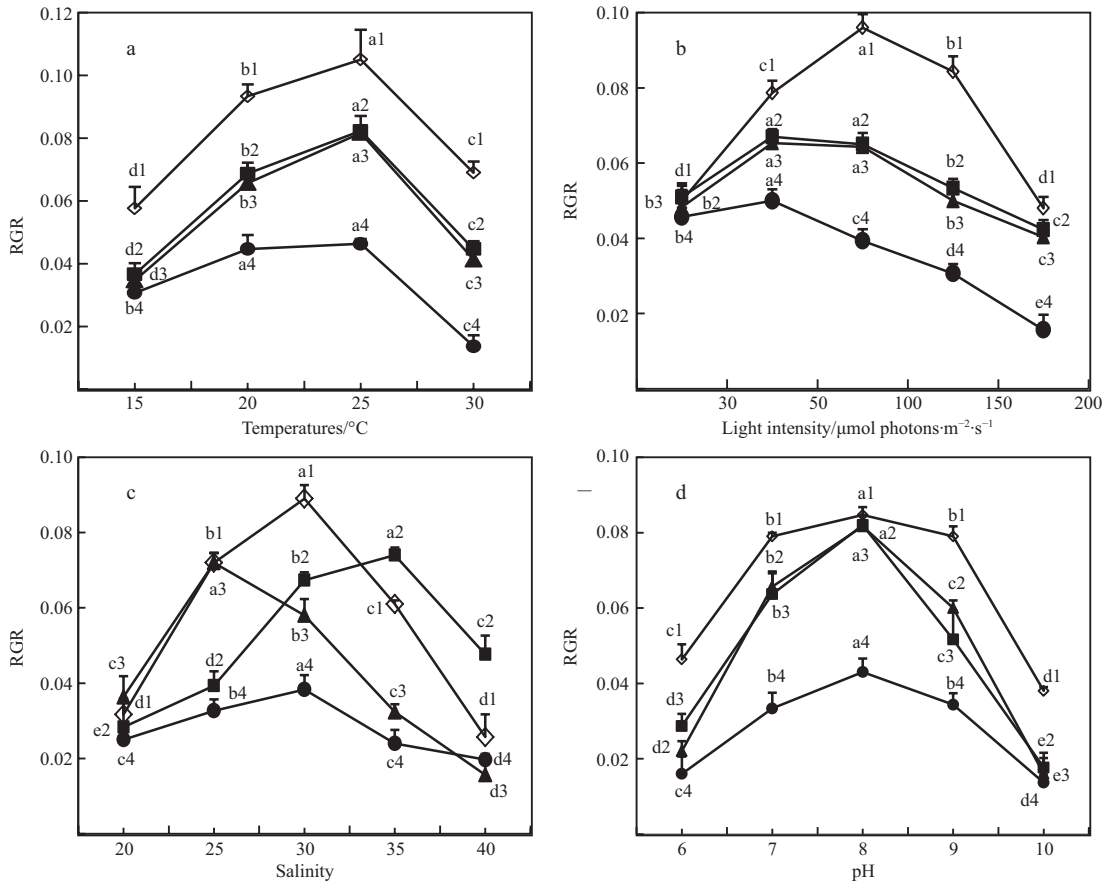

Fig.4. Relative growth rate (RGR) of *Navicula* spp. under different temperatures (a), light intensities (b), salinities(c), and pH (d). In the figure, ▲, ■, ● and ◇ refer to *N. vara, N. perminuta*, *N. pseudacceptata*, and *N. rhynchocephala*, respectively. The significant differences (p<0.05) of RGR in the same species under different conditions are indicated by different letters. The same numerals represent the same species. Vertical bars represent standard deviation (n=3).

er two species, the optimal salinities were at 30 (Fig. 4c, p<0.05). In the pH tests (Fig. 4d), all the samples shared identical RGR curves under different pH, and the highest RGR value was observed at pH 8.0 (p<0.05). Higher pH was shown to inhibit the growth of all species. pH 10.0 was nearly lethal for *N. pseudacceptata, N. vara*, and *N. perminuta*.

3.3.3 *Effects of nutrients*

Higher concentrations of SiO_3^{2-} up to 0.25 mmol/L facilitated the growth of all four species, with *N. rhynchocephala* being the fastest one in RGR (p<0.05). A close to linear relationship between the SiO_3^{2-} concentrations and RGRs was observed in *N. pseudacceptata* (Fig. 5a). Maximal RGRs in the NO_3^- and PO_4^{3-} tests were observed to occur at 3.6 and 0.18 mmol/L, respectively, in all four species (p<0.05) (Figs 5b and c).

4 Discussion

An often encountered problem in investigating and utilizing *Navicula* spp. in aquaculture or benthic diatom research is the accurate biological identification. In aquaculture, the feeding and settlement-inducing effects of larvae of abalone or sea cucumber are often species-specific. Thus, misidentification or misapplication of the species will lead to a slow-down of larvae growth or a decrease in settlement rate. Morphological identification alone often is not as accurate as expected (Evans et al., 2007). The development of molecular markers has provided an effective solution (Mann et al., 2001). In this investigation, results of sequencing and comparisons of SSU and *rbc*L genes with the data of GenBank confirmed the results based on morphological features depicted by the images of SEM.

All molecular and SEM results confirmed that the samples in this investigation belonged to *Navicula* spp. In the two sequences, SSU was proposed for helping identification of diatom samples, even though it was not as variable as expected (Jahn et al., 2007; Moniz and Kaczmarska, 2010). SSU could be used initially in most cases to discriminate samples to genus or family level. Ki et al. (2009) reported the dominant diatoms in summer Arctic reservoirs of Svalbard (Norway). Based on 18S rDNA sequences, these diatoms were identified as *Diatoma*, *Navicula*, and *Fragilaria*. In Taiwan, ten strains of diatoms were isolated by Tsai (2008); these diatoms were discriminated to three genera using SSU as *Amphora*, *Navicula*, and *Cyclotella*. Xu (2012) also employed SSU to identify microalgae stored in her laboratory: in all 19 strains of samples, 16 were identified to genus level. The

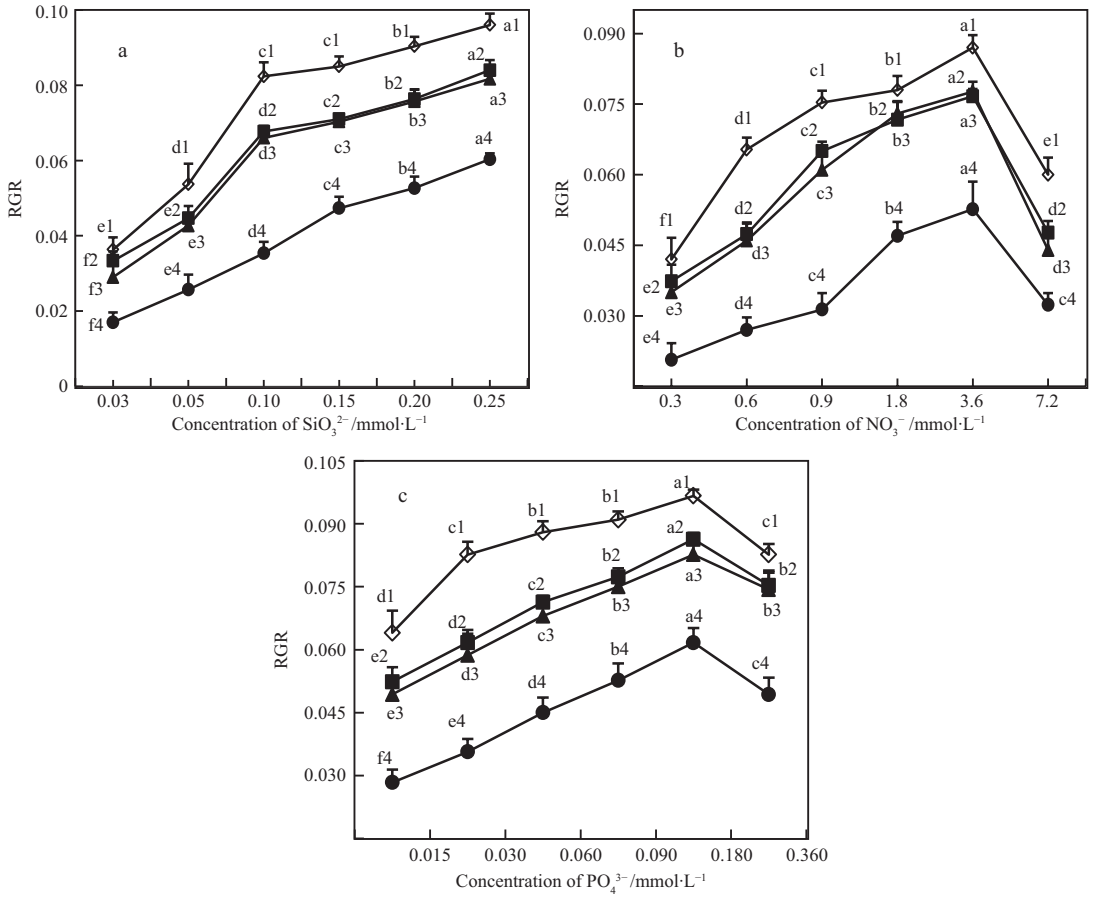

Fig.5. Relative growth rate (RGR) of *Navicula* spp. under different concentrations of SiO_3^{2-} (a), NO_3^- (b), and PO_4^{3-} (c). In the figure, ▲, ■, ● and ◇ refer to *N. vara, N. perminuta, N. pseudacceptata*, and *N. rhynchocephala*, respectively. The significant differences (p<0.05) of RGR in the same species under different conditions are indicated by different letters. The same numerals represent the same species. Vertical bars represent standard deviation (n=3).

failed cases were mainly due to the lack of reference sequences (Jahn et al., 2007). It is noteworthy to point out that sequencing the SSU gene has helped us to correct a few misidentified benthic diatoms in our comprehensive effort to establish a national stock culture of benthic diatoms. Other advantages of SSU include the ease to get PCR products with universal primers and the abundance of sequences available in NCBI for SSU for comparison (Sorhannus, 2007). The *rbc*L gene can also discriminate diatoms to genus or family level (Bruder and Medlin, 2007; Evans et al., 2007). In addition, insertions or deletions in the *rbc*L are extremely rare (Bruder and Medlin, 2007). It also evolves faster than SSU rRNA (Soltis and Soltis, 1998). However, fewer sequences are available in NCBI for *rbc*L than SSU.

Diatoms serve as an essential food source in aquaculture. If the diatoms are overgrazed, the growth of spats will be limited. Thus, for the selection of diatom species with a potential to be used in aquaculture, relatively higher growth rates are very much expected. Screening of fast-growing strains of *Navicula* spp. has been the target of most abalone or sea cucumber farms. The growth rate of diatoms has been shown to be influenced principally by temperature, salinity, irradiance level, and the supply of nutrients (Thessen et al., 2005). Qian et al. (2009) found that 20–25°C was the best temperature condition for the growth of *Navicula corymbosa*, which was similar with *N. pseudacceptata*, but different from three other samples in this investigation.

The optimal light intensities for species in *Navicula* could be different. In a previous report, 30 μmol photons m^{-2} s^{-1} was optimal for growth of *Navicula mollis* (Wang et al., 1997). However, our research got a different result, which had the maximal RGR occurring at 50–100 μmol photons m^{-2} s^{-1}. The optimal irradiance for vegetative growth of *Navicula* is thus species-specific, also depending on the corresponding niches in which they are living. In the present investigation, the optimal pH and salinities for growth were found to be at 8.0 and 25–30, respectively. Except *N. perminuta*, the favorable salinity was found at 35. The result of the salinity trial explains why these four species often occur in sea water (Cheng et al., 1993). *N. perminuta* was isolated in Qingdao, a place where the mean value of salinity ranged from 31 to 33. It has adapted to the high salinity environment. Results of this investigation were slightly different from that of a previous study by Kang et al. (2011), who observed the optimum pH and salinity conditions for the growth of *Navicula incerta* were obtained at pH 8.3 and salinity 33.

Concentration of silicon in culture is believed by many researchers to be the important limiting factor for the growth of diatoms (Guillard et al., 1973; Thomas and Dodson, 1975; Egge and Aksnes, 1992). Our results that concentrations of SiO_3^{2-} up to 0.25 mmol/L were shown to greatly facilitate the growth of all species well confirmed this point. In a study, Li and Wang (1998) also found that the RGR of samples reached the highest level at the highest concentration of silicon, while decreased silicon was omitted. The availability of silicon may also strongly affect diatom population composition in nature (Brzezinski et al., 1990).

At the highest concentration of nitrogen and phosphorus, the RGR decreased. It may be due to the self-shading effect, in which too much nitrogen and phosphorus restrain the photosynthesis of cells (Brown et al., 1996; Guo et al., 2002). This evidence indicates that the concentration of any specific nutrition factor should be considered for a specific species culture.

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