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Characterization of *Pythium chondricola* associated with red rot disease of *Pyropia yezoensis* (Ueda) (Bangiales, Rhodophyta) from Lianyungang, China*

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Abstract *Pyropia yezoensis* (formerly *Porphyra yezoensis*) is an economically important red alga that is cultured extensively in China. The red rot disease occurs commonly during *Pyropia* cultivation, causing serious economic losses. An incidence of red rot disease was found in a *P. yezoensis* farm from mid-November to mid-December 2015 at Lianyungang, Jiangsu Province, China. Histopathological examination revealed that the naturally infected thalli were infected apparently by a pathogen, leading to red rot symptoms. The causative agent was isolated and identified as the oomycete *Pythium chondricola* by morphological analysis and sequence analysis of the internal transcribed spacer and cytochrome oxidase subunit 1 (*cox*1). In artificial infection experiments on the *P. yezoensis* blades, the *P. chondricola* isolate was able to cause the same characteristic histopathology seen in natural infections. *P. chondricola* grew well at a wide range of temperatures in the range 8–31°C, salinities at 0–45 and pH 5–9. In an orthogonal test used to determine the effects of environmental factors (temperature, salinity, and zoospore concentration) on infection, the data revealed that temperature was the most important factor to affect red rot disease development, with the optimal conditions for disease expansion being 20°C, 35 salinity, and a zoospore concentration of 10^6 zoospores/mL. The results obtained from the present study prompted us to set up a comprehensive epidemiological study on *Pyropia*, which will provide support to maintain the healthy development of the *Pyropia* industry in China.

Keyword: *Pyropia yezoensis*; red rot; identification; *Pythium chondricola*; pathogenicity; disease expansion

1 INTRODUCTION

The red alga *Pyropia yezoensis* (formerly known as *Porphyra yezoensis*) (Bangiales, Rhodophyta), known as laver, is the most popular edible seaweed in the world, being used for both food and phycocolloid production (Gachon et al., 2010). *Pyropia* cultivation is an important industry in Asia, especially in China, Japan, and Korea. For example, laver production in China reached 115 875 tons in 2015, accounting for 16.9% of the total Asian laver production (FAO Fishstat). High profits and the development of advanced cultivation techniques have triggered investment in China, and the laver cultivation area has increased year-on-year over the past few years.

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According to the China Fisheries Statistics Yearbook, *Pyropia* culture area in China in 2016 increased by 16.7% over the previous year, reaching 65 766 hectares.

Like other marine cultural products, algae are susceptible to diseases caused by various pathogens including bacteria, oomycetes, viruses, and protists (Gachon et al., 2010). Several diseases have been described and they attack cultivated *Pyropia* at the blade stage, such as the red rot disease (Arasaki, 1947; Takahashi et al., 1977; Kerwin et al., 1992; Ma, 1996; Ding and Ma, 2005; Park et al., 2006), *Olpidiopsis* disease (Sekimoto et al., 2009; Klochkova et al., 2012), green spot disease (Fujita, 1990; Sunairi et al., 1995; Kim et al., 2016), cyanobacterial felt disease (otherwise known as filamentous bacterial disease or filamentous bacterial felt disease), and diatom felt disease (Lee et al., 2012; Kim et al., 2014). Disease at the shell-boring conchocelis stage of *Pyropia* is found seldom, with only a white spot disease being reported (Fujita, 1990; Blouin et al., 2011; Guan et al., 2013). Of these diseases, red rot disease and the *Olpidiopsis* disease are the most common, each of which can cause an average loss of 20% of the annual production in some areas (Kawamura et al., 2005; Klochkova et al., 2012). For example, during 2012–2013, an outbreak of *Olpidiopsis* spp. disease in Seocheon sea farms in South Korea resulted in losses valued at approximately US $1.6 million, accounting for ~24.5% of total potential sales.

In China, outbreaks due to various diseases regularly lead to a loss of 25%–30% of the annual production of the *Pyropia* crop (Gachon et al., 2010). Farmers have observed the red rot disease and the *Olpidiopsis* disease since 1970s; however, these diseases were not recognized and reported by scientists until 20 years later (Ma, 1992, 1996; Ding and Ma, 2005; Mo et al., 2016). The *Olpidiopsis* disease was first reported in *P. yezoensis* along the south coastal area of Jiangsu Province in 1992 (Ma, 1992), and the red rot disease caused by *Pythium porphyrae* was first reported to be a main disease in the cultured *P. yezoensis* in Jiangsu and the Zhejiang costal area (Ma, 1996). Afterwards, simultaneously infection of *Olpidiopsis* and red rot diseases was found in *P. yezoensis* (Ding and Ma, 2005). A recent study shows that an *Alternaria* species could cause the red rot like a disease in *P. yezoensis* (Mo et al., 2016).

The recent development of intensive and high-density farming practices in China has aggravated the disease outbreaks in *Pyropia*. Despite serious and longstanding economic losses in China, *Pyropia* diseases have not drawn more attention from the government and algal epidemiology scientists. The main reason may be the reluctance of farmers to report the disease problem, for fear of having their product devalued, given the relative lack of effective treatments. Currently, studies on diseases of macroalgae are uncommon in China, and only limited pathogen and epidemiological data are available. In view of the impact of red rot disease on *Pyropia* cultivation in China, the proper identification and classification of the pathogen(s) involved are warranted.

In this study, we isolated and identified a *Pythium* species associated with red rot disease of farmed *P. yezoensis* from Lianyungang, Jiangsu Province, China, and further investigated its growth, pathogenicity and the environmental factors affecting the development of the infection.

2 MATERIAL AND METHOD

2.1 Culture conditions

Unless stated to the contrary, the pathogen isolate was grown in the dark at 25°C on 50% seawater corn-meal medium (SCM) (Takahashi, 1977) or on 50% seawater glucose-glutamate medium (SGG) (Fujita and Zenitani, 1977). When required, antibiotics were added into the medium at the following concentration: 2 mg/mL streptomycin, 1 mg/mL rifampicin (dissolves in methanol). A pure line culture *P. yezoensis* RZ preserved in our laboratory was used in all infection tests. Healthy *P. yezoensis* RZ thalli were obtained by growing conchocelis in Provasoli's enriched seawater (PES) medium (Provasoli, 1968). Unless stated to the contrary, the *P. yezoensis* thallus culture conditions used were 15°C, 28–32 salinity, 62.5 μmol/(m^2·s) irradiation with a 12 h light:12 h dark illumination cycle, continuous aeration, and complete replacement of the medium once every three days.

2.2 Pathogen isolation

Diseased *P. yezoensis* thalli with evident red rot symptoms were collected and brought back to the laboratory, washed lightly with sterilized seawater to remove surface debris, and checked for infection under a light microscope. The infected sections were cut from the thallus and homogenized in sterile seawater with a grinder. The resulting homogenates were diluted with seawater in 10-fold series, and each

dilution was spread on SCM agar plates with and without antibiotics. The predominant colonies with uniform morphologies were sub-cultured on SCM plates until pure cultures were obtained. The purified pathogen isolate was transferred to an SCM slant for storage at 4°C.

2.3 Morphological and molecular identification

For morphological observation of the pathogen isolate, agar blocks (6 mm in diameter) were cut from the growing margin of the culture, and transferred onto the middle of fresh SCM plates and incubated for 7 to 30 d at 25°C. The growth characteristics were assessed, including color, size, texture, and production of zoosporangia, oospores, and antheridia. Hyphae, which developed on the medium, were examined under a light microscope. For molecular identification of the fungus, seed cultures were grown on SCM plates for 7 d at 25°C. Approximately 1-g agar blocks containing fungal mycelia were placed into a pre-chilled mortar, frozen with liquid nitrogen, and ground into a fine powder. The DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) extraction method (Zuccarello and Lokhorst, 2005). The extracted DNA was used as a DNA template in the following PCR amplification. The internal transcribed spacer (ITS) region was amplified using primers: ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al., 1990), the cytochrome C oxidase subunit 1 (*cox*1) region was amplified using primers: *cox*1-pyth-F1 (5′-ATTAGAATGGAATTAGCACAAC-3′) and *cox*1-pyth-R1 (5′-CTTAAACCWGGAGCTCTCAT-3′) (Lee et al., 2015). The cycling conditions used were 94°C for 5 min, followed by 35 cycles at 94°C for 30 s each, 60°C for 40 s for ITS or 55°C for 40 s for *cox*1, 72°C for 2 min, and a final 72°C for 10 min. The 25-μL reaction volume contained 10 ng genomic DNA, 0.5 mmol/L of each primer, and 12.5 μL Easy Taq PCR Supermix (Transgen Biotech, Beijing, China). The PCR products were subsequently sequenced by a commercial sequencing unit (Sangon Biotech, Shanghai, China), and the resulting sequence was run with Basic Local Alignment Search Tool (BLAST) in the GenBank database. For the phylogenetic analyses, the ITS and *cox*1 reference sequences of related *Pythium* species were obtained from GenBank, and all sequences were aligned using Clustal X (Thompson et al., 1997). An unrooted phylogenetic tree was constructed by the neighbor-joining algorithm in MEGA 6 (Tamura et al., 2013). The robustness of the phylogram in the maximum likelihood analysis was evaluated by 1 000 bootstrap replications.

2.4 Pathogenicity test

The representative *Pythium* isolate JS151205 was used in the infection tests. Zoospore production by JS151205 was induced as described previously, with slight modifications (Addepalli and Fujita, 2002). In brief, a seed culture of JS151205 was grown in 100 mL seawater glucose-glutamate medium liquid medium for 7 d. For the induction of zoosporangia, the JS151205 mycelia were collected and washed with a 500-mL wash medium (50% seawater, 50% distilled water, 10 mmol/L Ca^{2+}) for 5 h on an orbital shaker at 100 r/min at 15°C, with a change of wash medium every hour. After that, the 5 h-washed mycelia were incubated for 12 h at 15°C with a shaking at 100 r/min in the wash medium to induce zoospore production. For the synchronous release of zoospores, the 12 h-incubated mycelia were washed in the fresh wash medium for 1 h. The number of zoospores was calculated using a hemocytometer.

For the infection test, five healthy *P. yezoensis* RZ thalli (1–2 cm in width and 7–10 cm in length) were exposed to 200 mL of the zoospore suspension (1×10^5 zoospores/mL) in a flask containing 4 mL PES (Provasoli et al., 1968) incubated at 15°C. Thalli without the addition of the zoospores were used as the control. Each treatment was assigned to three biological replicates. After infection, the thalli from each flask were examined by eye and by light microscope (Olympus CKX41, Japan) every day for the occurrence of red rot spots. All infected thalli were collected for routine microbiological isolation and identification.

2.5 Growth under different conditions

The growth of JS151205 under different conditions was tested under different agar media, temperature, salinity and pH. Seven media were used, including 2216E marine medium, LB medium, martin medium, potato dextrose medium (PDA), 100% seawater corn-meal medium, 50% seawater corn-meal medium and 100% distilled water corn-meal medium. The temperatures tested were between 8°C and 31°C at pH 8 in 50% seawater SCM agar plates. The effects of salinity were tested at 25°C and pH 8 on SCM agar medium with a range of 0–45 salinity, adjusted with a commercial sea salt (Binghai Chemical factory, Shandong, China). The pH was tested at 25°C in 50%

Table 1 Factors and levels used in the orthogonal array design

Factors	Level 1	Level 2	Level 3
A, Temperature (°C)	10	15	20
B, Salinity	25	30	35
C, Zoospore concentration (/mL)	10^4	10^5	10^6

seawater SCM with pH ranges of 5–9, adjusted with 1 mol/L HCl or 1 mol/L NaOH. For inoculation, agar discs (8 mm in diameter), cut from the edge of a JS151205 culture, were transferred onto the middle of the respective SCM plates. Three replicates were assigned for each treatment. The growth diameter for each culture was measured with Vernier calipers after incubation for 7 d. All experiments were repeated three times, with at least two similar results. Data were presented from one of the similar results.

2.6 Environmental effects on disease development

For investigation of the combined effects of three environmental factors (temperature, salinity, and zoospore concentration) on disease development, an experimental plan was designed on the basis of the Taguchi method, as previously described (Mo et al., 2016). Each factor was assigned three levels (Table 1). An L9 (3^4) orthogonal array was designed using Statistical Product and Service Solutions (SPSS) 21.0 software (Chicago, IL, USA) (Table 2). Nine treatments were developed with different combinations of factor and level. Each treatment was performed using the same procedure as in the *Pathogenicity test*, and three replicates were assigned for each treatment. After inoculation, the incidences of infected thalli were observed by eye and by an optical microscope every day. At 14 d post-infection, the lesion areas in all thalli in the beaker flasks were measured, using a leaf area meter-1241 (Yaxin Liyi Science and Technology, Beijing, China), and leaves in three flasks were sampled as one unit. The infection rate was expressed as a mean lesion area per infected leaf. The infection results from the nine treatments and the variation contributed by each factor were evaluated with the *k* value, *R* range and variance analysis (ANOVA) via SPSS, where *k* is the mean infection level associated with each factor, and *R* range is the difference between the maximum *k* and minimum *k*. A blank column in Table 2, generated with the orthogonal design as the dummy factor with no actual factor, was included in variance analysis to eliminate variation from uncontrolled factors.

Table 2 L9 (3^4) orthogonal array design with an infection rate

Trial	*A*	*B*	*C*	Blank	Infection rate (%)
1	1	1	1	1	0.06±0.08
2	1	2	2	2	0.33±0.28
3	1	3	3	3	0.10±0.14
4	2	1	2	3	0.14±0.12
5	2	2	3	2	0.50±0.12
6	2	3	1	1	0.04±0.03
7	3	1	3	2	30.16±0.77
8	3	2	1	3	1.04±1.08
9	3	3	2	1	64.91±6.91
k_1	0.16	10.12	0.38	21.67	
k_2	0.23	0.62	21.79	10.33	
k_3	31.80	20.68	10.25	0.43	
$R_{(k_{max}-k_{min})}$	31.64	20.06	21.41	21.24	
Priority level	*A*3	*B*3	*C*2		

Infection rate is the mean lesion area per infected leaf at 14 d post-infection and expressed as mean ± SD, k_i is the value of infection level of factor level *i* in every column (*i*=1, 2, 3), *R* (range) means the difference between the maximum value and minimum value of k_i. Blank is assigned as the dummy factor in which no actual factor can be distributed.

2.7 Statistical analysis

All data from the growth and infection rate studies were expressed as the mean values ± standard deviation. Two-way ANOVA statistical analyses were conducted using SPSS 21.0 software (IBM, Armonk, NY, USA) with the level of significance set at $P<0.05$.

3 RESULT

3.1 Isolation, identification, and pathogenicity of the isolate

Red rot disease was observed in a *P. yezoensis* farm from Lianyungang (34°52′11.46″N, 119°16′37.68″E), using a half-floating culture system, during December 2015. The infected *P. yezoensis* exhibited typical red rot symptoms on the blades (Fig.1a). The infected blade tissues presented shrunken and darkened cells, occupied by colorless and translucent mycelia produced by the pathogen, which resulted in a pale red color (possibly caused by a phycoerythrobilin-like material) to be released within some cells (Fig.1b).

Colonies isolated from the SCM plates showed similar characteristics to one another, submerged with a white downy appearance (Fig.2a). Hyphae were hyaline, coenocytic, aseptate, 2.0–5.0 μm wide

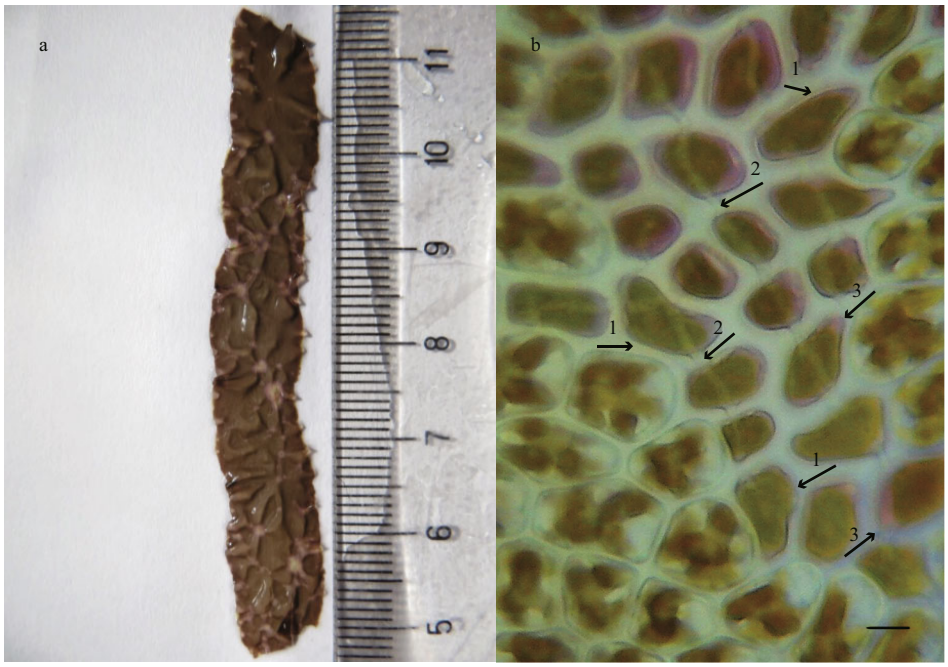

Fig.1 Clinical symptoms of red rot disease of *P. yezoensis*

a. infected *P. yezoensis* collected from Lianyungang, China; b. histopathology of the lesion area, presenting abnormal cells (arrow 1) being penetrated by fungal mycelia (arrow 2), with an accumulation of released phycoerythrobilin-like material (arrow 3). Scale bar represents 10 μm.

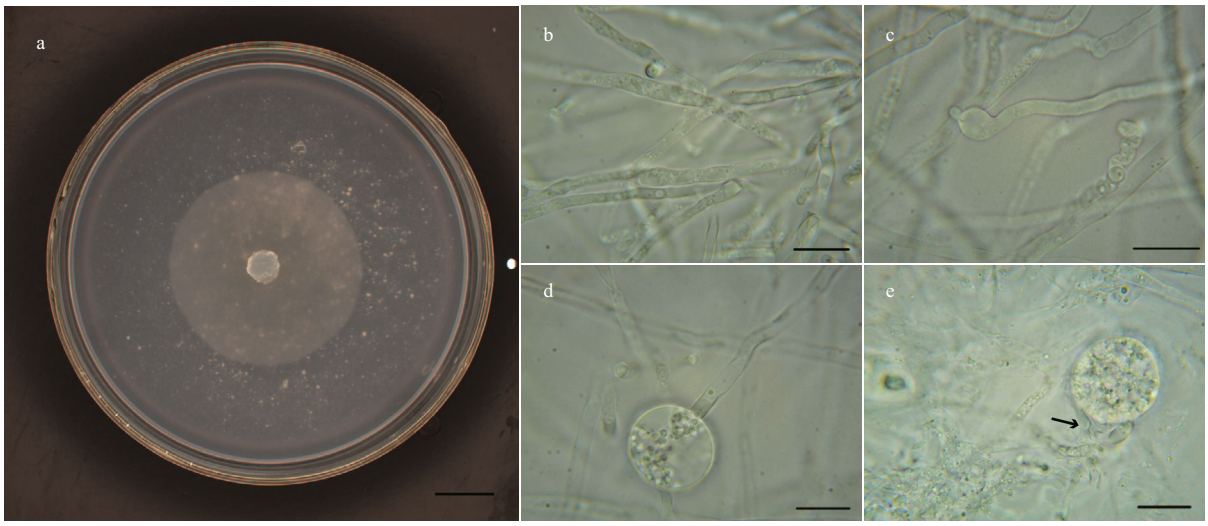

Fig.2 Morphology of pathogen isolate

Colony (a), free hyphae (b) and swollen hyphae (c) on SCM medium; zoosporangium (d) and oosporangium with a single oospore with an attached antheridium (arrowhead) (e) on 2216E medium. Scale bars represent 1 cm (a) and 10 μm (b–e).

(Fig.2b), and swollen hyphae were frequently seen (Fig.2c). Asexual structures and sexual structures were not observed on SCM agar but were observed a few times in 2216E marine agar. The zoosporangia were smooth, globose, and terminal (Fig.2d); the oosporangia were smooth, with one terminal plerotic oospore with a single antheridium (Fig.2e). These characteristics appeared to be in accordance with the description of *Pythium* spp. (Schroeder et al., 2013). All isolates had identical ITS (GenBank accession No. MF978164) and *cox*1 (GenBank accession No. MF978165) sequences. An isolate, assigned JS151205, was used in the subsequent phylogenetic analysis. JS151205 formed a cluster with *Pythium porphyrae* and *Pythium chondricola* based on ITS (Fig.3a), while formed a clade with *P. chondricola* based on *cox*1 (Fig.3b). So JS151205 was close to *P. porphyrae* and *P. chondricola* based on ITS and was highly close to *P. chondricola* based on *cox*1. Combining the morphological and phylogenetic

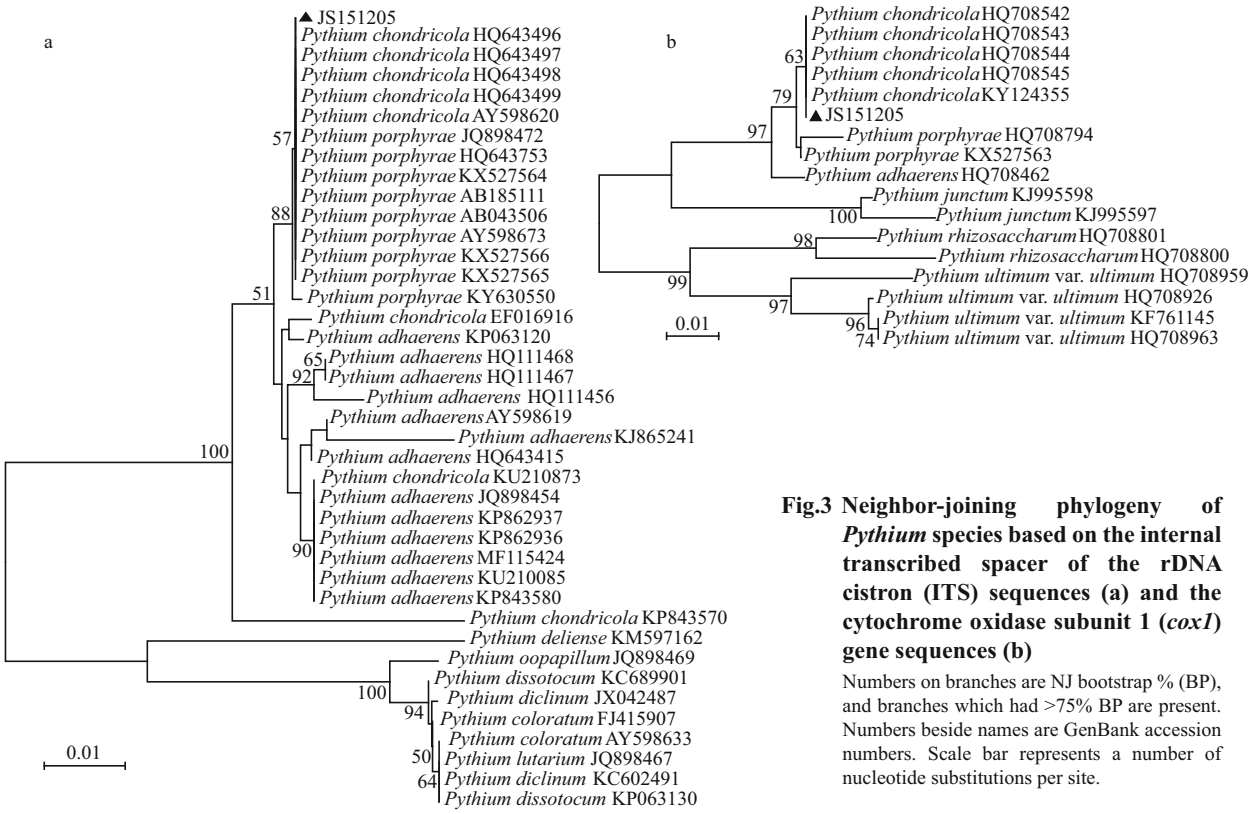

Fig.3 Neighbor-joining phylogeny of *Pythium* species based on the internal transcribed spacer of the rDNA cistron (ITS) sequences (a) and the cytochrome oxidase subunit 1 (*cox1*) gene sequences (b)

Numbers on branches are NJ bootstrap % (BP), and branches which had >75% BP are present. Numbers beside names are GenBank accession numbers. Scale bar represents a number of nucleotide substitutions per site.

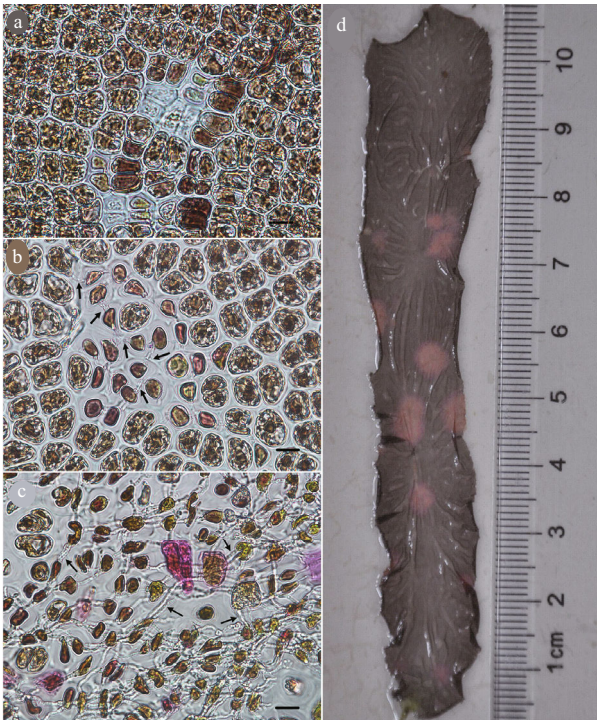

Fig.4 Infection by *P. chondricola* JS151205 of *P. yezoensis*

Infected cells of *P. yezoensis* after 1 d (a), 5 d (b), and 9 d (c), *P. chondricola* hyphae (arrowhead) between cells are visible; (d) red spots of *P. yezoensis* thallus after *P. chondricola* JS151205 challenge at 9 d. Scale bars represent 10 μm (a–c).

analyses, we preferred to identify isolate JS151205 as the oomycete *P. chondricola*.

The pathogenicity of JS151205 was determined by challenging healthy *P. yezoensis* RZ thalli with a dose of 2.3×10^5 zoospores/mL of JS151205. The infection by JS151205 on blades of *P. yezoensis* led to the dark coloration of infected cells, and the tissues were infected quickly by the mycelia (Fig.4a–d). The symptoms shown by the experimentally infected blades were similar to those shown by the naturally infected laver. From the diseased blades, the pathogen was re-isolated and identified to be JS151205, according to the morphology, ITS, and *cox*1 sequences.

3.2 JS151205 growth under different conditions

JS151205 was able to grow on all seven agar media, including PDA, LB, 2216E, Martin, a corn-meal medium prepared with 100% seawater (CM1), 50% seawater (CM2), and distilled water (CM3). Best growth occurred on 50% seawater corn-meal medium (Fig.5a). JS151205 was able to grow within a temperature range of 8–31°C, with maximal growth being achieved between 22°C and 25°C (Fig.5b). Additionally, JS151205 was able to grow within a

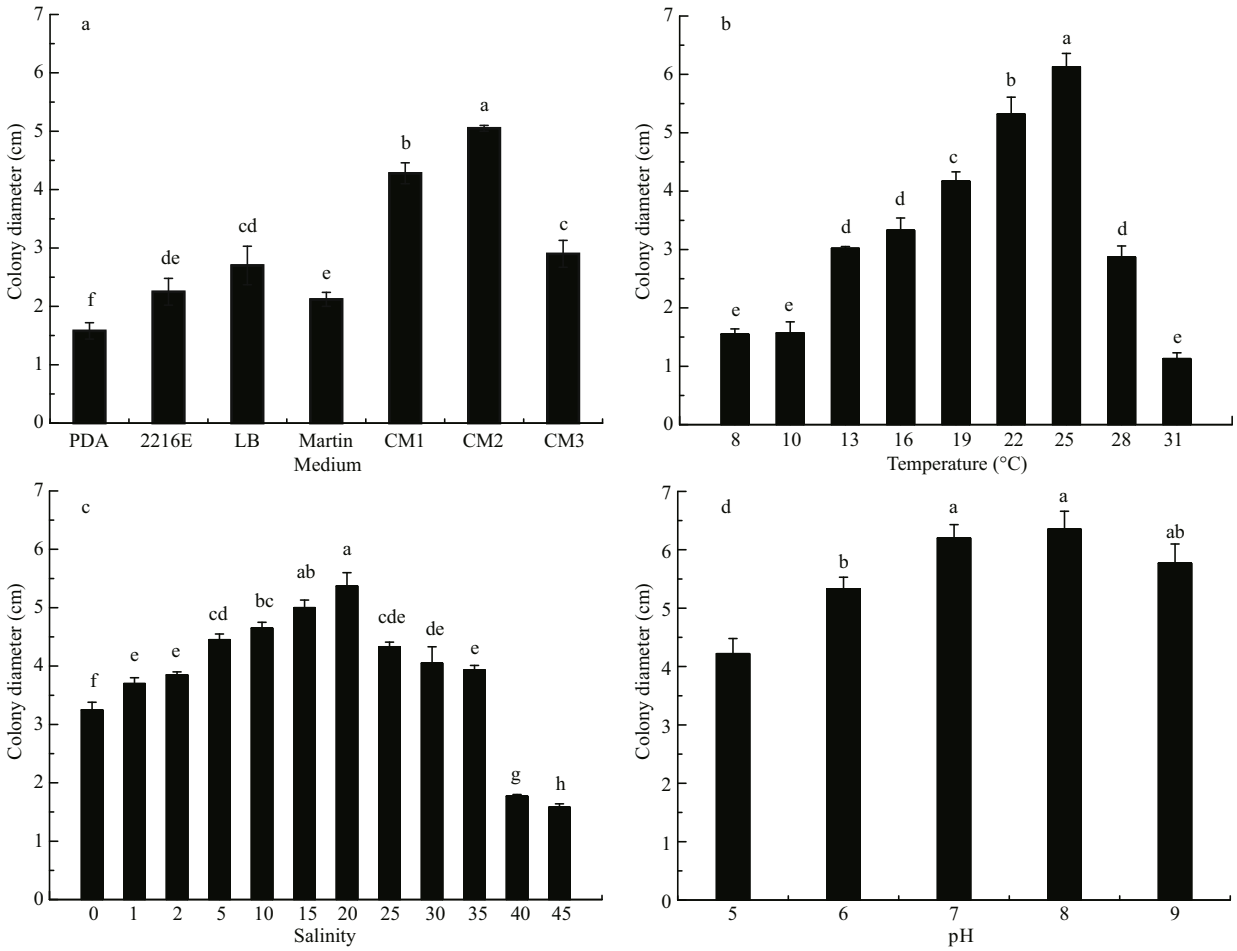

Fig.5 Growth of JS151205 under different conditions

The growth of JS151205 on seven agar media (a), a temperature range of 8–31°C (b), salinity range of 0–45 (c), and pH range of 5–9 (d). The colony diameter was measured at 7 d and data are expressed as mean ± SD. Under "Medium", CM1, CM2, and CM3 are corn-meal agar plates prepared with 100% sea water, 50% sea water and distilled water, respectively.

salinity range of 0–45, salinity being optimal at 20 (Fig.5c). Moreover, JS151205 was able to grow within a pH range of pH 5–9, with the optimal value for growth being pH 7–8 (Fig.5d).

3.3 Effects of environmental factors on disease level

In the orthogonal tests, *P. yezoensis* RZ blades were exposed to nine treatments to assess the disease levels at different combinations of temperature, salinity, and inoculum concentration. Based on the mean value *k* and variance analysis indicated in Table 2 and Table 3, the temperature was the most important factor affecting disease development, followed by zoospore concentration and salinity. The infection rate increased significantly ($P<0.01$) with increasing temperature, zoospore concentrations or salinity, the highest infection rate being 31.8% at 20°C, 21.8% at 10^5 conidia/mL and 20.7% at 35 salinity. Based on *R* range data in Table 2, the factor order affecting the infection rate was temperature > zoospore concentration > salinity, with *R* values of 31.6, 21.4, and 20.1, respectively, which was in agreement with the *k* variance analysis. By combining the above results, the optimal conditions for red rot disease expansion were 20°C, 10^5 zoospores/mL and 35 salinity.

Table 3 Analysis of variance (ANOVA) of infection rate

Factor	Sum of squares	Degree of freedom	Mean squares	*F* value	*P* value
A	6 083.042	2	3 041.521	552.432	0.000
B	2 067.232	2	1 033.616	187.736	0.000
C	2 002.505	2	1 001.253	181.858	0.000
Error	99.103	18	5.506		

Since the orthogonal design did not include the inoculum concentration of 10^6 zoospores/mL into the combination of 20°C and 35 salinity, we carried out

additional experiments to compare the infection rates between 10^6 zoospores/mL and 10^5 zoospores/mL under the conditions of 20°C and 35 salinity. The results showed that the infection rate was 71.7% for the combination of 20°C, 35 salinity and 10^6 zoospores/mL, and 44.8% for the combination of 20°C, 35 salinity and 10^5 zoospores/mL. This result further supported the finding that high inoculum concentration of JS151205 caused high infection rates in *P. yezoensis*. Thus, the optimal conditions for red rot disease expansion were updated to 20°C, 10^6 zoospores/mL and 35 salinity.

4 DISCUSSION

Although the red rot disease is widely distributed in *Pyropia* farms in China, the true causative agent of this disease had not been confirmed up to this point, due to the lack of epidemiological data. We carried out the present study to identify and characterize the causative agent of red rot disease in a *P. yezoensis* farm located in the main *Pyropia*-producing area. Our results showed that the pathogen was isolated and identified to be *P. chondricola*, and confirmed that this *P. chondricola* isolate was pathogenic on *P. yezoensis*, causing symptoms similar to those from field observations. The oomycete *P. chondricola* was capable of growing over a wide range of environmental conditions, including temperatures between 8 and 31°C salinity between 0 and 45, and pH between 5 and 9. The temperature was the most important factor affecting the development of red rot disease, and the optimal conditions for disease development were 20°C, 35 salinity and an inoculum concentration of 10^6 zoospores/mL. These results indicated that *P. chondricola* was the causative agent resulting in *P. yezoensis* red rot disease in the farm at Lianyungang, China.

Based on our results, the pathogen was easily isolated from the infected blades of *P. yezoensis* that exhibited red rot symptoms. The isolates exhibited similar morphology such as aseptate hyphae, hyphal-terminal zoosporangium, and oosporangium with one antheridium. These characteristics accorded with descriptions of *Pythium* (LéVesque and De Cock, 2004). Molecular analysis showed that the isolates had identical ITS sequences with *P. porphyrae* and *P. chondricola* sequence and that these sequences were phylogenetically close to those from *P. porphyra*e and *P. chondricola*. *P. porphyrae* and *P. chondricola* are close related species based on morphological and genetic characteristics. Morphologically, no stable difference was available for discrimination between *P. chondricola* and *P. porphyrae*. Genetically, DNA molecular including ITS and *cox*1 have been suggested to use as identified oomycete DNA barcodes (Choi et al., 2015). Our analysis showed that ITS sequences could not provide information for discrimination between *P. chondricola* and *P. porphyrae* (Fig.3a), while *cox*1 could discriminate between these two species (Fig.3b). This result was in consistent with previous studies that *cox*1 was a potential DNA marker for identification of *P. chondricola* and *P. porphyrae* (Robideau et al., 2011; Lee et al., 2015). A recent study on *cox*2 sequences analysis also supported the genetic difference between *P. chondricola* and *P. porphyrae* (Lee et al., 2017). In our study, JS151205 formed a single clade with *P. chondricola* species based on *cox*1, thus we identify JS151205 as *P. chondricola*. However, some researchers suggested that *P. chondricola* was a taxonomic synonym for *P. porphyrae* due to low variation in *cox*1 sequences in two species (Diehl et al., 2017; Klochkova et al., 2017). Actually, the close genetic similarity was common among *Pythium* (Robideau et al., 2011). In this regard, more DNA sequences and sequences variation ranges are required to include in phylogenetic analysis for corrective and effective identification of *Pythium* species.

According to our results from growth studies, the growth of JS151205 was influenced by temperature, salinity, and pH. Maximal growth was found at 20 salinity, pH 7–8 and temperatures of 22–25°C. These characteristics were similar to those of *P. porphyrae* isolates from Japan and Korea (Fujita and Zenitani, 1977; Park et al., 2000; Klochkova et al., 2017) except salinity. Following the distribution law of ocean salinity, the seawater salinity decreases respectively from a subtropical sea area to the high and the low latitudes, indicating that the seawater salinity in Jiangsu Province at a lower latitude is higher than that of Korea and Japan at higher latitudes. The Korean strain of *P. porphyrae* grew best in half seawater salinity condition (15) (Klochkova et al., 2017) while the optimal salinity of JS151205 was 20, suggesting the *Pythium* strains from *Pyropia* farms along the East Pacific are well adapted to the marine environment, while JS151205 grew well in 0 salinity, indicating that this oomycete isolate has the potential to grow in a terrestrial environment. Whether *P. chondricola* can survive on land is not known; a recent study has shown that *P. porphyrae* could infect 11 land plant seedlings, causing several of them to die

(Klochkova et al., 2017). These findings raised the possibility that land-adapted *P. porphyrae* could be the inoculum which initiates red rot disease in *Pyropia* farms, as a result of terrestrial runoff. Much work needs to be done to address this possibility, including investigating the presence of *Pythium* on the sea floor and in the sediment around the sea farm, the genotype of *Pythium* strains from different origins, the species-specific detection of *Pythium* strains, etc.

Our results from the orthogonal test showed that temperature was the most important factor to affect red rot disease level in *Pyropia* thalli after infection with JS151205; the higher the temperature, the higher the infection rate. This result was in agreement with a previous study that *Pyropia* red rot disease was induced by temperature (Kazama and Fuller, 1973). Indeed, increased temperature stress has a great impact on *P. yezoensis* physiological and developmental processes. A recent study, on the comparative transcriptome of *P. yezoensis* in response to temperature stresses, has shown that *P. yezoensis* has much more differentially expressed unigenes in response to high temperature than at low temperatures (Sun et al., 2015). Amongst those unigenes active at high temperatures, most were involved in replication and repair of DNA, and in protein processing in the endoplasmic reticulum, while several unigenes encoding metacaspases were also up-regulated. Metacaspase is an important regulator of programmed cell death (PCD); abnormal regulation of PCD in animals has been shown to be associated with immunological and developmental disorders (Fuchs and Steller, 2011). Although the real role of PCD in algal physiology is not known, we hypothesize that elevated temperatures could induce excessive PCD activity, resulting in defensive and developmental disorders of *P. yezoensis* and make them more susceptible to various opportunistic pathogens that commonly exist in the environment. Further studies along these lines should be encouraged.

The orthogonal test showed that the optimal conditions for JS151205 infection and development were 20°C, an inoculum concentration of 10^5 zoospores/mL, and 35 salinity. An additional experiment showed that that a higher zoospore concentration (10^6 zoospores/mL) increased *P. chondricola* infection under conditions of 20°C and 35 salinity. This result indicated that higher numbers of zoospores caused a higher incidence of *Pyropia* infection, supporting an epidemiological study conducted in Wando, Korea (34°19′0″N, 126°45′0″E) (Park et al., 2006). In Park's research, a trend was observed of an increased rate of infection of *Pyropia* as the zoospore concentration of *P. porphyrae* increased, and when the zoospore concentration in seawater reached above 4 000 zoospores/L, *P. porphyrae* thallus appeared to be so heavily infected that it disintegrated. This scenario could also happen in the *Pyropia* farm at Lianyungang, which is located at the same latitude as Wando. In fact, the optimal salinity and temperature determined for red rot disease development in our growth room studies were similar to those prevailing at the Lianyungang *Pyropia* farm during mid-November to mid-December, when the red rot outbreak occurred, with the temperatures being 18°C–20°C and salinity 28–32, which are favorable for the growth of JS151205. Therefore, the field observations that *P. yezoensis* red rot disease commonly occurs at this period might be due to increased susceptibility of heat-stressed algae to *Pyropia* infection.

In conclusion, the present study demonstrated that *P. chondricola* JS151205 was the causative agent of red rot disease on *P. yezoensis* thalli and that this disease occurred most often during temperature stress conditions. These results should encourage additional studies on the epidemiology and pathogenesis of *P. chondricola* so that its impact on *P. yezoensis* culture can be reduced.

5 CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

6 DATA AVAILABILITY STATEMENT

All the sequence using in this study have been deposited in GenBank, with accession No. MF978164 and MF978165. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

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