Note

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Infection and *cox*2 sequence of *Pythium chondricola* (Oomycetes) causing red rot disease in *Pyropia yezoensis* (Rhodophyta) in Korea

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Red rot disease has caused a major decline in *Pyropia* (Nori) crop production in Korea, Japan, and China. To date, only *Pythium porphyrae* (Pythiales, Oomycetes) has been reported as the pathogen causing red rot disease in *Pyropia yezoensis* (Rhodophyta, Bangiales). Recently, *Pythium chondricola* was isolated from the infected blades of *Py. yezoensis* during molecular analyses using the mitochondrial *cox*1 region. In this study, we evaluated the pathogenicity of *P. chondricola* as an algal pathogen of *Py. yezoensis*. Moreover, a new *cox*2 marker was developed with high specificity for *Pythium* species. Subsequent to re-inoculation, *P. chondricola* successfully infected *Py. yezoensis* blades, with the infected regions containing symptoms of red rot disease. A novel *cox*2 marker successfully isolated the *cox*2 region of *Pythium* species from the infected blades of *Py. yezoensis* collected from *Pyropia* aquaculture farms. *cox*2 sequences showed 100% identity with that of *P. chondricola* (KJ595354) and 98% similarity with that of *P. porphyrae* (KJ595377). The results of the pathogenicity test and molecular analysis confirm that *P. chondricola* is a new algal pathogen causing red rot disease in *Pyropia* species. Moreover, it could also suggest the presence of cryptic biodiversity among Korean *Pythium* species.

Key Words: *cox*2; Koch's postulates; Pathogenesis; *Pyropia yezoensis*; *Pythium chondricola*; red rot disease

INTRODUCTION

Red rot disease was first reported on *Pyropia tenera* in Japan (Arasaki 1947), and its pathogenesis has been characterized in *Pyropia* species (Takahashi et al. 1977). Outbreaks of red rot disease on *Pyropia* species have caused major damage to commercial *Pyropia* aquaculture systems in Korea, Japan, and China (Kawamura et al. 2005, Blouin et al. 2011, Kim et al. 2014).

After the first report of red rot disease on *Pyropia tenera*, only *Pythium porphyrae* (Pythiales, Oomycetes) was identified as a pathogen of red rot disease in *Pyropia* species in Korea, Japan, and China (Park et al. 2006, Liu et al. 2012, Kim et al. 2014). Recently, we isolated *Pythium chondricola* from the infected blades of *Pyropia yezoensis* collected from commercial *Pyropia* aquaculture farms based on the mitochondrial *cox*1 region (Lee et al. 2015).

Using *cox*1 markers, *P. chondricola* was detected in *Py. yezoensis*, and was confirmed to be genetically different from the *P. porphyrae* detected in Japan. This was the first report of the presence of *P. chondricola* on *Pyropia* species, following the first taxonomic report of its occurrence on the red alga *Chondrus crispus* (De Cock 1986). However, the pathogenesis of *P. chondricola* must be proven

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to confirm that it is an algal pathogen causing red rot disease in *Pyropia* species (i.e., proven to satisfy Koch's postulates).

For precise identification of oomycete species, it is necessary to develop multiple molecular markers (Lévesque and De Cock 2004, Seifert 2009, Robideau et al. 2011). In this regard, Choi et al. (2015) recently compared the efficiency of using the pattern of sequence variation between *cox*2 and *cox*1 regions for species identification. They accordingly found that *cox*2 showed higher interspecific and lower intraspecific divergence than *cox*1, and was more successful in species identification. Therefore, the *cox*2 region has been suggested as a successful partner DNA barcode marker.

Following the detection of *P. chondricola* in an infected blade of *Py. yezoensis* using the *cox*1 region (Lee et al. 2015), a new *cox*2 gene sequence (KJ595354) of *P. chondricola* was published in GenBank (National Center for Biotechnology Information, NCBI). Therefore, we developed a new primer set to amplify the *cox*2 region that also exhibits specificity for *Pythium* species. A molecular examination was conducted based on the *Pythium*-specific *cox*2 gene marker developed in this study. *Pythium cox*2 sequences were isolated from the infected blades of *Py. yezoensis* with red rot disease, and were compared with *cox*2 sequences in GenBank. We also conducted a re-inoculation test to satisfy Koch's postulates using an isolate from the infected *Py. yezoensis*. These analyses were expected to confirm the pathogenesis of *P. chondricola* on *Py. yezoensis*.

MATERIALS AND METHODS

Sample preparation

We used *Pythium*-infected blades of *Py. yezoensis* collected from aquaculture farms in Korea (Biando [Gunsan] and Aphaedo [Shinan] in December 2014). *Pyropia* samples with signs of red rot disease were also collected from Gaeyado (Dec 2014, Gunsan, Korea). The presence of *P. chondricola* was confirmed in these *Pyropia* samples (Lee et al. 2015). Strains of *P. chondricola* were isolated from *Py. yezoensis* collected from Biando and Aphaedo (Dec 2014, Korea) and were cultured in the Seaweed Research Center (National Institute of Fisheries Science, Mokpo, Korea). In addition, a culture strain of *P. chondricola* (Biando, Korea; NIFS-PC-001) has been deposited in the Marine BioResources Bank (MBRB) in the Seoul National University, Korea (SFC20170403-M01). Species identification of the cultured strain was conducted using the *cox*1 marker (Lee et al. 2015).

Re-inoculation experiment

We inoculated the *P. chondricola* strain onto cornmeal agar and Arasaki B medium (Arasaki et al. 1968), and maintained the culture (Biando) at 15°C. A re-inoculation test was performed in accordance with Koch's postulates. Blades of *Py. yezoensis* used in the re-inoculation test were cultured at 15°C under white fluorescent irradiation of approximately 20 μmol photons m^{-2} s^{-1} and a 12 : 12-h light : dark cycle. Morphological observations during the infection of *P. chondricola* on the healthy blade of *Py. yezoensis* were conducted using a microscope (Nikon Eclipse Ni-u, Tokyo, Japan) (Fig. 1).

Development of a *Pythium*-specific *cox*2 marker and molecular analyses

We developed a new primer set to amplify the *cox*2 region of *P. chondricola*. For primer design, we compared two *cox*2 gene sequences of *P. chondricola* (KJ595354) and *P. porphyrae* (KJ595377) from GenBank. We initially searched the conserved regions of the two species. We excluded the *cox*2 region conserved among other oomycetes, except for *Pythium* species.

Because these two species have a close taxonomic relationship (Lévesque and De Cock 2004), a new *cox*2 marker was designed for putative specificity in *P. chondricola*, *P. porphyrae,* and *P. adhaerens*. To evaluate the specificity of the newly developed *cox*2 primers for *Pythium* species, we compared their specificity against that of previously reported *cox*2 primer pairs (COX2F/COX2R, FM66/FM58) (Hudspeth et al. 2000, Villa et al. 2006, Senda et al. 2009, Choi et al. 2015).

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing processes followed the methods described by Lee et al. (2015). Total DNAs were extracted from a culture strain of *P. chondricola* and the infected field samples of *Py. yezoensis*. The putative infection of *P. chondricola* in *Pyropia* samples was investigated using the *cox*1 marker (Lee et al. 2015). Similarity analysis was conducted using the BLAST searching tool in GenBank.

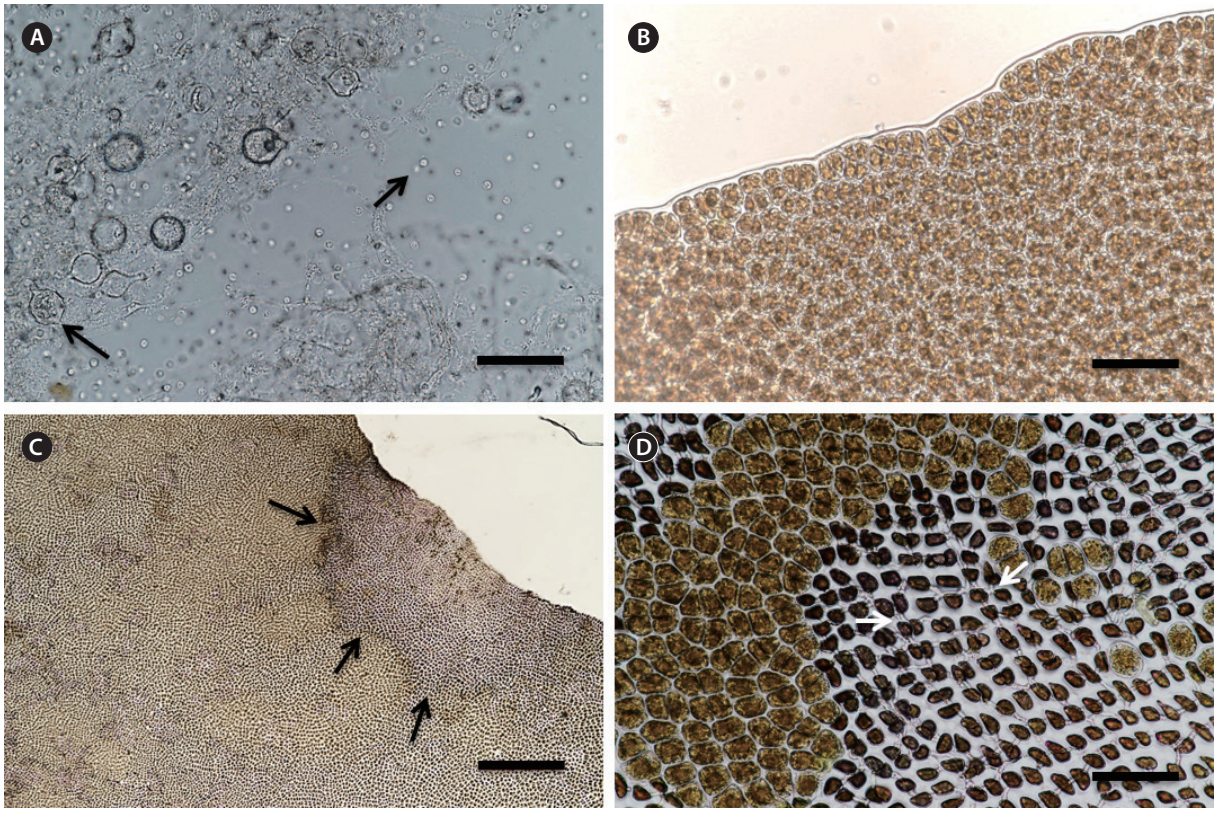

Fig. 1. (A) Zoosporangium formation and zoospore release (arrows) of the *Pythium chondricola* culture strain isolated from an infected blade of *Pyropia yezoensis*. (B) A healthy blade of *Py. yezoensis*. (C) *Py. yezoensis* 2 days after artificial infection with the released zoospores of *P. chondricola*. *Pyropia yezoensis* cells showed symptoms of red rot disease. The lesioned area lost its original color and developed a purple and greenish color (arrows). (D) Mycelia of *P. chondricola* formed over the lesioned area (arrows). Scale bars represent: A, B & D, 50 μm; C, 500 μm.

RESULTS

Infection of *Pyropia yezoensis* by *Pythium chondricola*

The morphological features of *Py. yezoensis* blades were observed during infection by *P. chondricola*. The formation of a zoosporangium and the release of zoospores were accordingly observed (Fig. 1A). Two days after re-inoculation, *Pyropia* cells in the blade showed symptoms of red rot disease. The area of the lesion in the infected blade lost its original color and developed a purple and greenish color (Fig. 1C). *P. chondricola* mycelia were also observed in the lesioned area (Fig. 1D).

Pythium-specific *cox*2 marker development for the detection of *Pythium chondricola*

In this study, we developed the following primer pair: forward primer, cox2-PACP-F1 (5′-GATGTTATTTAAAACAATAGTTC-3′); and reverse primer, cox2-PACP-R1 (5′-TAAAGAAGGAATAGCCCAA-3′). PCR was successfully conducted on the infected blades of *Py. yezoensis* using this new primer pair. We also acquired PCR products from the culture strain. The COX2F/COX2R and FM66/FM58 primer pairs used in previous studies showed amplicons of different sizes, approximately 600 bp.

From sequencing, we successfully isolated the *cox*2 region using our newly developed primer pair (cox2-PACP-F1/cox2-PACP-R1). The *cox*2 sequences isolated from culture strains of *P. chondricola* and the infected host blades of *Py. yezoensis* (Biando and Aphaedo) have an identical sequence (GenBank accession number KU754146 [373 bp]). With the exception of the primer binding sites, the *cox*2 regions showed 100% similarity (373/373) with that *P. chondricola* (KJ595354; CBS:203.85, The Netherlands), 98% (367/373) with that of *P. porphyrae* (KJ595377; CBS:369.79, Japan), and 97% (362/373) with that of *P. adhaerens* (KJ595386; CBS:520.74, The Netherlands). The *cox*1 sequences of these three *Pythium* strains

(CBS:203.85, CBS:369.79, CBS:520.74) were also found in GenBank, and these sequences were analyzed by Lee et al. (2015). We have deposited the *cox*1 sequences of *P. chondricola* reported by Lee et al. (2015). All Korean samples have an identical *cox*1 sequence (GenBank accession number KY124355, 386 bp). Whereas the interspecific sequence variations identified using the *cox*1 region were consistent, the *cox*2 marker showed more variable sequences.

The primers used in previous *cox*2 studies could not amplify the *cox*2 region of *Pythium* species. The primer pairs COX2F/COX2R (631 bp) and FM66/FM58 (613 bp) amplified the *cox*2 region of the host, *Py. yezoensis*. The sequence of the amplicons had 100% similarity with the *cox*2 sequence of *Py. yezoensis* (JQ736809; each 581 bp and 573 bp).

DISCUSSION

The re-inoculation test successfully confirmed that the isolated *P. chondricola* causes pathogenesis in *Py. yezoensis*. The infection by *P. chondricola* caused cell death in healthy blades of *Py. yezoensis* maintained under indoor culture. The infected blades of *Py. yezoensis* showed characteristics typical of red rot disease (Takahashi et al. 1977).

We previously isolated *cox*1 and internal transcribed spacer (ITS) sequences from culture strains of *P. chondricola* and infected samples of *Py. yezoensis* collected from aquaculture farms (Lee et al. 2015). However, the ITS sequences failed to discriminate *P. chondricola* from *P. porphyrae*. In contrast, the *cox*1 region successfully provided useful genetic information for species identification. It has been suggested that a recently identified oomycete DNA barcode (Choi et al. 2015) could be used with the *cox*2 region, serving as a partner DNA barcode, because of its efficiency for PCR and sufficient variation for species identification. The *cox*2 region from the culture strain of *P. chondricola* is identical to that from the infected blade of *Py. yezoensis*.

The *cox*2 region has been used for molecular taxonomic studies of oomycetes (Hudspeth et al. 2000, Villa et al. 2006, Senda et al. 2009), and effective primer combinations were also developed. In these studies, most of the DNA samples were extracted from *Pythium* culture strains, and not from environmental samples such as infected *Pyropia* blades from aquaculture farms. Therefore, in the case of DNA analysis for host species of pathogens, the primer pair used to amplify the *cox*2 region should be verified to specify the target pathogen species.

In this study, we assessed the efficacy of universal primers of the *cox*2 region for oomycetes to isolate the *cox*2 region from the infected blades of *Py. yezoensis*. However, we were unable to acquire the *Pythium cox*2 sequence. Furthermore, the universal primers did not show specificity for *Pythium* species, since this primer pair also amplified the *cox*2 region of the host *Py. yezoensis*. Therefore, *cox*2 primers that are appropriate for broad taxonomic groups of oomycetes might fail to detect the presence of *Pythium* species in the infected blades of *Py. yezoensis*.

We developed a new *cox*2 primer set that exhibited specificity for *Pythium* species, thereby enabling us to isolate the *Pythium cox*2 sequence from host samples showing red rot disease. The primer pair (cox2-PACP-F1/cox2-PACP-R1) developed in this study could successfully amplify the *Pythium cox*2 sequence with high efficiency and specificity.

For the *Py. yezoensis* blades collected from Biando and Gaeyado (Gunsan), a universal primer pair showed the non-specific band of *Py. yezoensis*. Nevertheless, the *cox*2 primer pair developed in the present study successfully amplified the *cox*2 region of *Pythium* from the Biando sample. The sequencing results show that the *cox*2 amplicons obtained using the universal primer pair exhibited the *Py. yezoensis cox*2 sequence instead of a *Pythium* sequence. These results strongly suggest the specificity of this new *cox*2 primer pair.

To confirm *P. chondricola* as an algal pathogen, a re-inoculation experiment performed in accordance with Koch's postulates is required. In addition, the DNA barcode method is needed to detect *Pythium* species from samples in various states (e.g., decaying host samples or environmental samples, such as seawater and sediment). Here, we examined the pathogenesis of *P. chondricola* through the re-inoculation test and a novel molecular marker with high efficiency and specificity for *Pythium*.

Several *cox*1 sequences have been reported under the names of *P. chondricola* and *P. porphyrae* from Japan, Korea, The Netherlands, New Zealand, and the USA (Robideau et al. 2011, Lee et al. 2015, Diehl et al. 2017, Klochkova et al. 2017). The *cox*1 sequences (HQ708542, HQ708543, KY124355) were identical to that of the type culture of *P. chondricola* (HQ708544, CBS 203.85). Moreover, three *cox*1 sequences showed high similarity: HQ708545 (1 bp difference, 679/680 [99%]), KX527563 (2 bp, 576/578 [99%]), and KY650705 (2 bp, 676/678 [99%]). In contrast, the *cox*1 sequence of the type culture of *P. porphyrae* (HQ708794; CBS369.79) showed a 7 bp difference (673/680, 99%) from that of the type culture of *P. chondricola* (HQ708544).

The reported *Pythium* species show a diverse range of host infection (*P. chondricola* from *Chondrus crispus* [red algae], *Ulva lactuca* [green algae], *Zostera marina* [flowering plants] in De Cock (1986)); as *P. porphyrae* (KX527563) on the embryonic roots of various higher plants (e.g., carrot, cucumber, lettuce, and rice) in the artificial infection test of Klochkova et al. (2017); as *P. porphyrae* (KY650705) on *Porphyra* and *Pyropia* species in Diehl et al. (2017); as *P. porphyrae* on *Py. tenera* / *Py. yezoensis* in Kim et al. (2014); and *P. chondricola* (KY124355) on *Py. yezoensis* in Lee et al. (2015, this study). Interestingly, this broad range of host infection was also reported from the original description of *P. chondricola* (De Cock 1986).

Diehl et al. (2017) recently treated *P. chondricola* as a heterotypic synonym of *P. porphyrae* based on the identical ITS region and the high sequence similarity of the *cox*1 region in the two species. However, the close genetic similarity among *Pythium* species has previously been reported from molecular taxonomic and DNA barcoding studies on the genus *Pythium* (Lévesque and De Cock 2004, Robideau et al. 2011). On the basis of the phylogenetic analyses of intra/interspecific variations, Robideau et al. (2011) treated *P. chondricola* as an independent species separate from *P. porphyrae*. Moreover, they proposed that the *cox*1 region could be used as a molecular marker to discriminate between *P. chondricola* and *P. porphyrae*.

Of course, taxonomic re-examination will need to be conducted to clarify the taxonomic relationship among three allied *Pythium* species (*P. adhaerens*, *P. chondricola*, and *P. porphyrae*). However, for that purpose, more samples, including type cultures and more DNA sequences, should be examined. Therefore, for further studies, the distinctive genetic features of Korean *P. chondricola* should be conserved as an independent taxonomic entity apart from *P. porphyrae*.

The recent findings regarding a novel algal pathogen of *Pyropia* species causing red rot disease could suggest the cryptic biodiversity of oomycetes pathogens (Mo et al. 2016). Moreover, Park et al. (2003) investigated the genetic variation among Korean and Japanese isolates of *P. porphyrae* using random amplified polymorphic DNA and detected interesting genetic heterogeneity among Korean isolates. These results also support the possibility of intra/interspecific genetic diversity among Korean *Pythium* species and the existence of *P. chondricola* as an independent species. Therefore, this research is expected to provide an accurate and reliable method for monitoring the distribution and infection pattern of *P. chondricola* / *P. porphyrae* in *Pyropia* aquaculture farms.

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