Note

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Molecular identification of the algal pathogen *Pythium chondricola* (Oomycetes) from *Pyropia yezoensis* (Rhodophyta) using ITS and *cox*1 markers

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Pythium species (Pythiales, Oomycetes) are well known as the algal pathogen that causes red rot disease in *Pyropia* / *Porphyra* species (Bangiales, Rhodophyta). Accurate species identification of the pathogen is important to finding a scientific solution for the disease and to clarify the host-parasite relationship. In Korea, only *Pythium porphyrae* has been reported from *Pyropia* species, with identifications based on culture and genetic analysis of the nuclear internal transcribed spacer (ITS) region. Recent fungal DNA barcoding studies have shown the low taxonomic resolution of the ITS region and suggested the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene as an alternative molecular marker to identify *Pythium* species. In this study, we applied an analysis of both the ITS and *cox*1 regions to clarify the taxonomic relationships of Korean *Pythium* species. From the results, the two closely related *Pythium* species (*P. chondricola* and *P. porphyrae*) showed the same ITS sequence, while the *cox*1 marker successfully discriminated *P. chondricola* from *P. porphyrae*. This is the first report of the presence of *P. chondricola* from the infected blade of *Pyropia yezoensis* in Asia. This finding of the algal pathogen provides important information for identifying and determining the distribution of *Pythium* species. Further studies are also needed to confirm whether *P. chondricola* and *P. porphyrae* are coexisting as algal pathogens of *Pyropia* species in Korea.

Key Words: *cox*1; ITS region; *Pyropia yezoensis*; *Pythium chondricola*; *Pythium porphyrae*; red rot disease

INTRODUCTION

Fungal pathogens have plagued the *Pyropia* species of red algae (Rhodophyta) and seriously reduced the output of the *Pyropia* aquaculture industry in Korea and Japan (Kawamura et al. 2005, Kim et al. 2014). Red rot disease is a major algal disease that was first reported in *Porphyra tenera* (= *Pyropia tenera*) from Japan (Arasaki 1947). After that, *Pythium* species, the etiological agent of red rot disease, has been isolated and characterized from the red rot

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of infected *Pyropia* species (Takahashi et al. 1977).

Among species of the genus *Pythium*, *P. chondricola*, *P. porphyrae*, and *P. adhaerens* are closely related, based on the characteristics of a filamentous non-inflated sporangia, slow growth, and 1-4 diclinous antheridia (Levesque and De Cock 2004). However, these species show different host / substrate-specific relationships (Matsumoto et al. 1999, Levesque and De Cock 2004). *Pythium porphyrae* is recognized as the only pathogen of red rot disease in *Pyropia* species (Takahashi et al. 1977, Kim et al. 2014), while *P. adhaerens* has been isolated from soil and *P. chondricola* was discovered in *Chondrus crispus* (Levesque and De Cock 2004).

To verify the taxonomy of the genus *Pythium*, Matsumoto et al. (1999) analyzed the nuclear internal transcribed spacer (ITS) sequences of 30 *Pythium* species. More recently, Levesque and De Cock (2004) examined the phylogenetic relationship among 102 isolates using sequences of the ITS and D1, D2, and D3 regions in nuclear ribosomal DNA of *Pythium*. In addition, a DNA barcoding study for Oomycetes was conducted using ITS and cytochrome c oxidase subunit 1 (*cox*1) markers, and the taxonomic resolution of these two molecular markers was compared (Robideau et al. 2011).

Recently, Schroeder et al. (2013) suggested primers for polymerase chain reaction (PCR) based methods for the diagnosis and quantification of *Pythium* species. In a molecular phylogenetic tree from the nuclear ribosomal DNA and *cox*1 region of *Pythium*, *P. adhaerens*, *P. chondricola*, and *P. porphyrae* formed a single clade (Levesque and De Cock 2004, Robideau et al. 2011). In another approach, Park et al. (2001, 2006) developed a *P. porphyrae* specific ITS marker and applied it to forecasting red rot disease in *Pyropia yezoensis* cultivation farms.

Due to the increasing economic significance of *Pyropia* species, concern for algal diseases in the scientific community and aquaculture industry has been raised (Gachon et al. 2010). Therefore, accurate species identification of the algal pathogen is needed to clarify the host-parasite relationship and to help find a scientific solution for the disease. Recent fungal DNA barcoding study has revealed that the ITS region cannot provide sufficient taxonomic information to discriminate between the closely related *Pythium* species (Robideau et al. 2011). This study has shown the taxonomic usefulness of the *cox*1 region at the species level and suggested it as another candidate molecular marker for DNA barcoding. In this study, we applied the ITS and *cox*1 markers to identify Korean *Pythium* species from an infected blade of *Pyropia yezoensis*, from a *Pythium* culture strain, and from environmental seawater. Using *Pythium* specific ITS primers and newly designed *cox*1 primers, we accurately identified the species of *Pythium* and revealed its taxonomic relationship among other *Pythium* species.

MATERIALS AND METHODS

We sampled a blade of *Pyropia yezoensis* infected by red rot disease in the field (Dec 2014, Biando, Gunsan, Korea). A preliminary determination of infection by an algal pathogen was conducted based on the morphological characteristics in the diseased plant. Another *Pythium* strain was also successfully isolated from an infected *Pyropia yezoensis* specimen collected from the Korean coast (Dec 2014, Aphaedo, Shinan, Korea) and cultured under indoor conditions using cornmeal agar (Park et al. 2001). This culture strain has been deposited in the Seaweed Research Center (National Fisheries Research and Development Institute, Mokpo, Korea). We also analyzed DNA templates from previous metagenomic studies of seawater (Lee et al. 2010, 2012, Yoon et al. 2012) and from environmental seawater sampled from the Nakdong River estuary near the *Pyropia* aquaculture region (Aug 5, 2011, Korea).

We applied molecular markers from the ITS and *cox*1 regions for this study. For the amplification of the ITS region, we used *Pythium porphyrae* specific ITS primers (PP-1/PP-2) (Park et al. 2001). To target the *cox*1 region of *Pythium* species, we designed *cox*1 primers having putative specificity for the genus *Pythium*. For the design of these *cox*1 primers, we downloaded the complete *cox*1 sequence of *P. ultimum* (NC_014280) and compared it with other oomycete *cox*1 sequences through the BLAST searching option in GenBank (National Center for Biotechnology Information, NCBI). From the *cox*1 sequence alignment, we found a conserved *cox*1 region among *Pythium* species. The new *cox*1 primers for the genus *Pythium* were designed basing on this conserved region.

The DNA extraction, PCR, and sequencing were conducted following methods outlined in Lee et al. (2011). Total genomic DNA was extracted from the blade of infected *Pyropia yezoensis* using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. We also extracted total genomic DNA from environmental seawater using protocols suggested by Lee et al. (2010). Amplifications were carried out in a final volume of 20 μL using *amfiXpand* (GenDEPOT, Barker, TX, USA) with PCR conditions of 2 min at 95°C, 40 cycles of 30 s at 94°C, 30 s at 48°C, and 1 min at 72°C, with a final 7 min

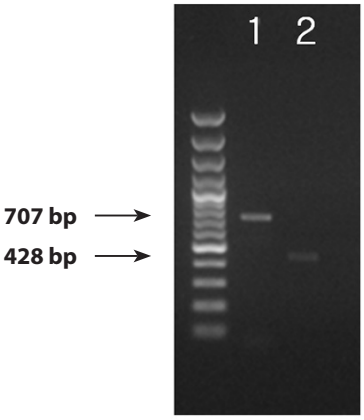

Fig. 1. Amplification of the nuclear ribosomal DNA internal transcribed spacer (ITS) region and mitochondrial DNA of the *cox*1 region using *Pythium porphyrae* specific ITS primers (1) (Park et al. 2001) and *cox*1 primers (2). Total genomic DNA was extracted from a culture strain (Shinan, Korea) and used as template DNA for polymerase chain reaction.

extension step at 72°C.

PCR products were subsequently sequenced in both directions by commercial sequencing (Genotech, Daejeon, Korea) and chromatograms were analyzed using Sequencher 5.3 (Gene Codes Corporation, Ann Arbor, MI, USA). The sequence similarity analyses of ITS and *cox*1 sequences were conducted BLAST in the GenBank (NCBI). 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 v.1.8 (Thompson et al. 1997). A phylogenetic tree was constructed by the neighbor-joining method (NJ) (Saitou and Nei 1987) using PAUP 4.0 (Swofford 2001). A bootstrap analysis with 2,000 replicates was conducted to assess the robustness of the NJ tree.

RESULTS

In addition to the previously developed ITS primers (Park et al. 2001), new PCR primers were designed for the selective amplification of the *cox*1 region of *Pythium* species, since the diverse fungus can be present in decaying seaweed (e.g., the infected *Pyropia* blade) and environmental seawater samples. The forward primer (*cox*1-pyth-F1; 5′-ATTAGAATGGAATTAGCACAAC-3′) is positioned at 36405-36426 of the mtDNA of *P. ultimum* (NC_014280) and the reverse primer (*cox*1-pyth-R1; 5′-CTTAAACCWGGAGCTCTCAT-3′) is bound at position 36813-36832.

We successfully obtained PCR bands from the DNA extractions from the infected blade of *Pyropia yezoensis*, the *Pythium* culture strain, and the environmental seawater samples. PCR products 707 bp and 428 bp in size were amplified by the ITS and *cox*1 primers (Fig. 1). The ITS region was successfully amplified from all three sample sources (the blade of *Pyropia*, *Pythium* culture strain, and environmental seawater). Even though the amplification yielded bands that were very weak from the ITS region of the sample from environmental seawater, the sequence was successfully determined. On the other hand, we could amplify the *cox*1 region only from the blade of *Pyropia* and the culture strain.

From the similarity analysis using BLAST, the ITS sequences showed no variation among Korean isolates (the blade of *Pyropia*, *Pythium* culture strain, and seawater). In addition, the Korean ITS sequences had 100% sequence similarity with the reference sequences of ITS in *P. porphyrae* (Korea, AB043506; Japan, AY598673, AB185111, AB043506; USA, JQ898472) and *P. chondricola* (Netherlands, AY598620, HQ643496, HQ643497, HQ643498; USA, HQ643499). The Korean *cox*1 sequences in this study showed 100% similarity with *P. chondricola* (Netherlands, HQ708542, HQ708543, HQ708544; USA, HQ708545), 99% (382/386; matched sequences / total sequences excluding the primer binding sites) with *P. porphyrae* (Japan-HQ708794) and 99% (381/386) with *P. adhaerens* (HQ708462).

In the ITS phylogenetic tree, the Korean *Pythium* ITS sequences clustered in a single clade with *P. porphyrae* (Korea, Japan, and USA) and *P. chondricola* (Netherlands), and also with a more distantly related *P. chondricola* (EF016916) from Thailand (Fig. 2A). In contrast, the *cox*1 phylogenetic tree showed that Korean *cox*1 sequences formed a single clade only with *P. chondricola* *cox*1 sequences reported from the Netherlands and USA (Fig. 2B). *Pythium porphyrae* reported from Japan formed a distant sister group of the clade including Korean *Pythium* species and *P. chondricola* (Netherlands and USA).

DISCUSSION

Taxonomic resolution of ITS and *cox*1 regions

Korean *Pythium* isolates (in this study) had the same ITS sequence as *P. porphyrae* and *P. chondricola* sequences that were previously reported. Therefore, the ITS sequence did not seem to provide sufficient information to resolve the taxonomic relationship between *P. porphyrae* and *P. chondricola*. The only difference in the ITS sequences of *P. porphyrae* / *P. chondricola* was in the poly A

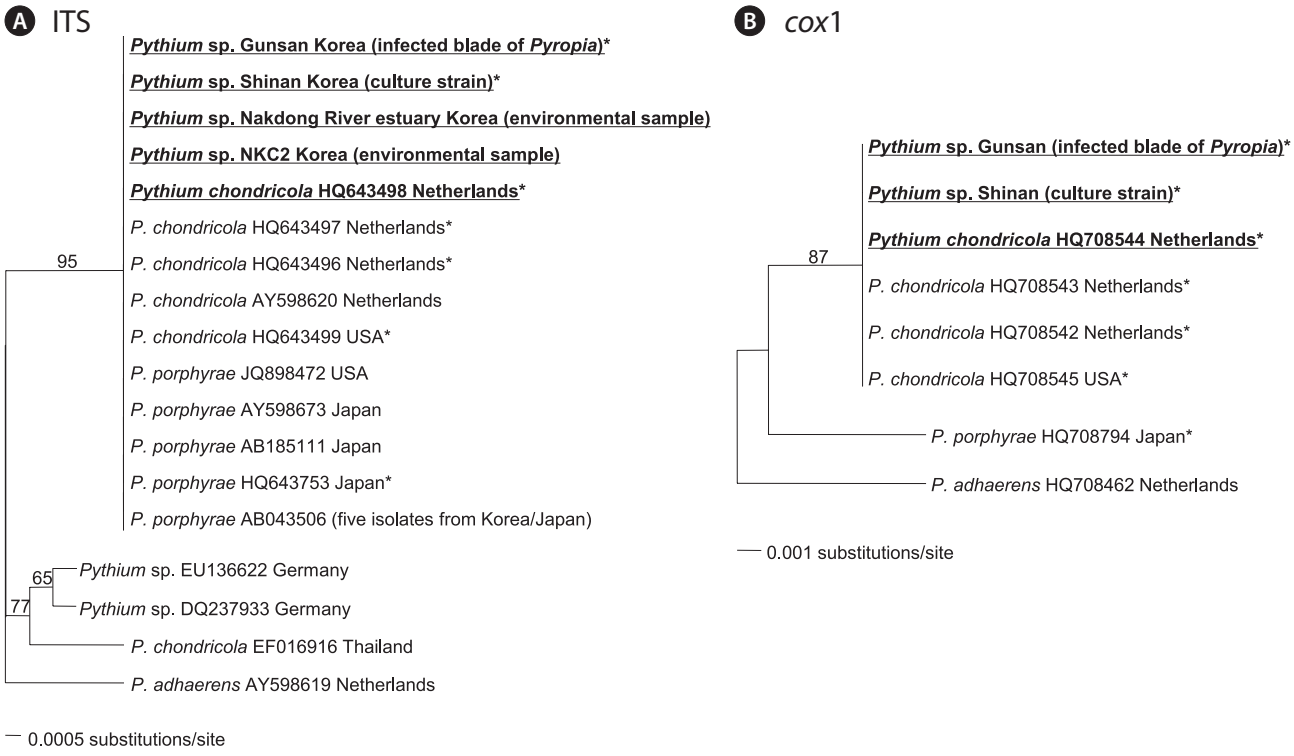

Fig. 2. Neighbor-joining phylogenetic analyses of *Pythium* species using the internal transcribed spacer (ITS) (A) and *cox*1 (B) regions. The numbers at the nodes of the tree indicate the bootstrap values (>50). Samples NKC2 (coastal seawater, Busan, Korea) and Nakdong River estuary (Busan) originated from environmental seawater. Sequences marked with asterisks represent isolates that were genetically identified by both the ITS and *cox*1 regions. ITS (HQ643498) and *cox*1 (HQ708544) sequences were isolated from a *P. chondricola* ex-type specimen (CBS 203.85) (Robideau et al. 2011).

sequence at the 5′ end of the ITS region (6 in HQ643499 vs. 7 in other isolates). On the other hand, the *cox*1 sequence showed sufficient variation to discriminate between *P. porphyrae* (HQ708794) and its close relatives, *P. chondricola* (HQ708545) and *P. adhaerens* (HQ708462) (Fig. 2).

A recent DNA barcoding study also reported the greater discriminative power in the *cox*1 region than in the ITS region, allowing a clarification of the taxonomic relationships among the closely related *Pythium* species (Robideau et al. 2011). From our results, the molecular analysis based on the ITS region could not provide specific information for the identification of *P. porphyrae*. Therefore, the ITS sequence was not a suitable barcoding marker to discriminate *P. chondricola* from *P. porphyrae*.

Presence of *Pythium chondricola* / *P. porphyrae* in the aquatic ecosystem

From the PCR analysis of the environmental seawater samples using the *Pythium* specific ITS primer set, we detected *P. chondricola* / *P. porphyrae* ITS sequences from the coastal seawater of Korea. We did not obtain a *cox*1 amplicon from these samples. Because nuclear ribosomal DNA has a high number of tandem repeat sequences (Rogers and Bendich 1987), PCR amplification with ITS primers could be more efficient than *cox*1 region in detecting *Pythium*.

The ITS sequences from environmental samples could have originated from fungal zoospores of *P. chondricola* / *P. porphyrae* in the seawater. Kawamura et al. (2005) also reported the distribution of *P. porphyrae* in the seafloor sediment (Ariake Sea, Japan) basing on an ITS sequence analysis. Therefore, *P. chondricola* / *P. porphyrae* appears to be present in the Korean aquatic ecosystem and could infect *Pyropia* species. This study provides important information to monitor the distribution of red rot disease. In addition, more sensitive, high efficiency methods should be developed to detect *Pythium* species from environmental seawater samples because relatively little biomass of the zoospore exists in the aquatic ecosystem.

Taxonomic entity of Korean *Pythium* species

Pythium chondricola was first isolated from decaying *Chondrus crispus* collected in and near the salt lake Grevelingen in the Netherlands (De Cock 1986). Since it was as a new species, *P. chondricola* has only been reported from the Netherlands and USA using molecular markers (Robideau et al. 2011). De Cock (1986) examined different fungal isolates from the same origin and locality as earlier isolates of *P. chondricola*, and discovered diverse sources of *P. chondricola*, including aquatic plants such as *Zostera marina* (flowering plants), *Ulva lactuca* (green algae), and an unidentified red alga. This implies that *P. chondricola* can infect a variety of host plants and that *Pyropia* species is one of them.

Robideau et al. (2011) reported the presence of *P. porphyrae* (HQ708794) from Japan as a separate taxonomic entity from *P. chondricola*. *Pythium chondricola* has not been reported from *Pyropia* species or aquatic environments of Korea or Japan (Kawamura et al. 2005, Park et al. 2006, Uzuhashi et al. 2015). To our knowledge, the finding of *P. chondricola* in this study is the first report of this species in the Asian region (Global Catalogue of Microorganisms [GCM]; The Barcode of Life Data System [BOLD]) (Ratnasingham and Hebert 2007, Wu et al. 2013).

We could hardly find *P. porphyrae* from *Pyropia* blades during this study. Therefore, further studies targeting the *cox*1 region are strongly requested and should include the wide survey for red rot disease in *Pyropia* cultivation sites and in the natural aquatic ecosystem to confirm whether *P. chondricola* and *P. porphyrae* are coexisting as algal pathogens of *Pyropia* species in Korea.

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