Optimal Conditions for Chromosomal DNA Isolation and Pcr Amplification of the Internal Transcribe Spacer Rdna Region of Four Riau Penicillium Isolates

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Abstract. The genus Penicillium has currently 549 described species. Many members of this genus are economically important, both in positive and negative ways. Correct molecular identification of isolated species is important for their utilization. Penicillium LBKURCC37.1, LBKURCC37.2, LBKURCC38 and LBKURCC39 are four strains isolated from Giam Siak Kecil-Bukit Batu Biosphere Reserve natural forest located in Riau. Although already identified morphology as members of Penicillium, their species identity still needs to be determined by molecular methods. The aim of this study was to determine optimal conditions for chromosomal DNA isolation and amplification of the Internal Transcribe Spacer (ITS) ribosomal DNA (rDNA) region of Penicillium LBKURCC37.1, LBKURCC37.2, LBKURCC38 and LBKURCC39, for subsequent use in molecular and phylogenetic analysis. Our results show that chromosomal DNA could be isolated from two to three days old cultures, depending on the strain. Various annealing temperatures were explored for Polymerase Chain Reaction (PCR) amplification of the ITS rDNA regions of the fungal chromosomal DNA. All strains required an annealing temperature of 42°C before producing PCR bands representing the ITS rDNA region with sizes ranging from 590 bp to 732 bp.

1. Introduction
The genus Penicillium has currently 549 described species [1]. Many members of this genus are economically important, both in positive and negative ways. Many member species produce important enzymes for the biotechnology and food industry [2-3], and produce medically important bioactive compounds such as antibiotics [4-6]. Other Penicillium species are known as plant and post-harvest pathogens, causing crop damage and economic loss [7-8]. Due to the many biotechnology potential of member species of Penicillium, and the quest to find novel antibiotics to overcome the growing number of antibiotic resistant microbes, research to isolate Penicillium species from various unique ecosystems continues. The rapid increase in the number of Penicillium species reflects the importance and interest in isolation and identification of members of this fungal genus. As of 2014 only 354 accepted Penicillium species were in the database [9]. In 2019 this number had grown 55%, becoming 549 species [1]. Along these lines, the Enzyme, Fermentation and Biomolecular Research Laboratory, at the Faculty of Mathematics and Natural Sciences, Universitas Riau, collected Penicillium isolates, from the unique ecosystem Giam Siak Kecil-Bukit Batu Biosphere Reserve peat swamp forest, located in Riau province, Indonesia. Among those collected are four Penicillium sp. strains, designated strain LBKURCC37.1, LBKURCC37.2, LBKURCC38 and LBKURCC39. These strains have only been identified to date through morphological methods [10].
Correct molecular identification of isolated species is important for their utilization. Identification of fungi by morphology and phenotyping is insufficient, since many closely related species have very similar morphology and phenotypes. Molecular identification of fungal species and phylogenetic analysis are now a recognized method for the correct identification of fungal species. The combination of molecular DNA sequences, phylogenetic analysis of certain regions of the chromosomal DNA, morphology and ecology data comprise the Consolidated Species Concept for fungal species identification [11]. One of the regions used most frequently for molecular identification and phylogenetic analysis of fungi is the DNA region known as the Internal Transcribe Spacer (ITS) of ribosomal DNA (rDNA). This is a region of the rRNA cistron that contains the genes for the 18S, 5.8S and 28S ribosome, with two internal spacers between them, known as ITS1 and ITS2. While 18S, 5.8S and 28S ribosomal gene sequences are conserved, the ITS1 and ITS2 are variable among fungal genus and species and can be used for diagnostic purposes to differentiate between species [12-13].

Molecular identification of fungal species and phylogenetic analysis using ITS rDNA sequences, requires that the fungal chromosomal DNA must firstly be isolated, the ITS rDNA region amplified by the Polymerase Chain Reaction (PCR), and sequenced. Our previous work with other fungal strains, show that the optimum age of the strain culture for chromosomal DNA isolation is variable. Also variable is the annealing temperature for PCR amplification of the ITS rDNA region [14]. Based on this, the aims of this research was to: 1) determine the optimum age of cultures of Penicillium sp. LBKURCC37.1, LBKURCC37.2, LBKURCC38 and LBKURCC39 for DNA isolation; and 2) determine the annealing temperatures to obtain good PCR products of the ITS rDNA region for cultures of Penicillium sp. LBKURCC37.1, LBKURCC37.2, LBKURCC38 and LBKURCC39.

2. Methodology

2.1. Chromosomal DNA extraction
Fungal cultures were grown as lawns on PDA in petri dishes. Daily, 0.3 g mycelia were collected from the PDA plates using a sterile spatula and moved to a sterile microtube. To the tube was added 293 µL 0.5M EDTA pH 8 and 7.5 µL Arthrobacter luteus lyticase (SIGMA-Aldrich, St. Louis, USA, Cat. No. G5711). After homogenizing the mixture, it was incubated at 37°C for 60 minutes. The tubes were then centrifuged for 2 minutes at 13,000 rpm. The supernatant was discarded. 300 µL of nuclei lysis solution from the Wizard® Genomic Purification Kit (Promega, USA, Cat no. A1120) was added to the precipitate. All subsequent steps followed the protocol described in the kit standard protocol instruction manual for fungal DNA isolation. Fungal DNA extracted were stored at -20°C until further use.

2.2. PCR amplification of ITS rDNA
PCR was done using the primer pairs ITS5 (forward) and ITS4 (reverse) with primer sequences as described by Raja et al. [15]. PCR amplification was done in a Techne TC-312 Thermal Cycler. Total PCR reaction volumes were 50 µL, comprising 2 µL fungal chromosomal DNA as template, 25 µL MyTaq™ Red Mix solution (Bioline, London, UK, Cat. No. BIO-25043), 1 µL of 10 pmol forward primer, 1 µL 10 pmol reverse primer, and 21 µL H2O. The PCR reaction was initiated using a five minute hot start at 95°C, followed by denaturation at 94°C for 30 seconds, annealing at temperatures starting from 45°C for 30 seconds, extension for 1 minute at 72°C, with a final extension of 5 minutes at 72°C. If no PCR product was obtained, the annealing temperature was lowered 1°C at a time.

2.3. DNA fragment analysis by agarose gel electrophoresis
Chromosomal DNA were electrophoresed on 30 mL 0.8% agarose gels supplemented with 3 µL GelRed® Nucleic acid stain (Biotium, San Francisco, CA, USA). After PCR, the PCR products were electrophoresed on 30 mL 1% agarose gels also supplemented with 3 µL GelRed® Nucleic acid stain. After electrophoresis the gels where visualized using a UV transilluminator, and photographed in a hood by digital camera using an orange gel filter. All samples were electrophoresed using 1 kb DNA ladder (Bioline, London, UK, Cat. No. BIO-33053; or Promega, Madison, WI, USA, Cat. No. G5711) as standard markers. Distance to the original starting well of each band of the 1kb ladder visualized.
was measured to create a standard logarithmic plot of MW as a function of distance travelled. Using this plot size of sample chromosomal DNA or PCR products were calculated.

3. Results and Discussion

Isolation of fungal chromosomal DNA was affected by the age of the cultured fungal lawns on PDA plates. The optimum age was different for the fungal strains, as shown on Table 1. For slow growing and slow sporulating fungi, the optimum age of the cultured lawn was 3 days, as shown for *Penicillium sp.* LBKURCC37.2. When *Penicillium sp.* LBKURCC37.2. mycelia were collected at a younger age, i.e. lower than 3 days colony growth, chromosomal DNA could not be extracted sufficiently. If the amounts of DNA extracted is insufficient, no DNA bands representing chromosomal DNA will be detected in the electrophoresis assay following extraction.

For the other three strains, that is LBKURCC37.1, LBKURCC38 and LBKURCC39, the optimum age for chromosomal DNA extraction was 2 days, reflecting these strains as faster sporulating fungi than LBKURCC37.1. For fast sporulating fungi, if the mycelium is collected too late, that is if too much spores have developed, lysis of the fungal cell walls become difficult, and so does the DNA extraction. No DNA could be sufficiently extracted when the LBKURCC37.1, LBKURCC38 and LBKURCC39 cultures were older than 2 days. On the other hand, no chromosomal DNA was sufficiently extracted when the LBKURCC37.1, LBKURCC38 and LBKURCC39 cultures were younger than 2 days.

Similarly, in a former study of other fungal species and strains, we found the same phenomena [14]. In that study, the optimum culture age for DNA extraction from 8 different fungal strains studied, varied from 1 to 3 days. It is therefore recommended, that before DNA extraction from fungal mycelia, a preliminary study should be done to ensure optimum culture age for DNA extraction of a given strain.

| Penicillium strain | Culture Age (days) |
|-------------------|-------------------|
| LBKURCC37.1       | 2                 |
| LBKURCC37.2       | 3                 |
| LBKURCC38         | 2                 |
| LBKURCC39         | 2                 |

Chromosomal DNA extracted from all fungal strains varied in size, ranging from 10,093 bp to 15,929 bp (Figure 1 and Table 2). LBKURCC38 had the largest chromosomal DNA extracted, and LBKURCC37.2 the smallest. These were chromosomal DNA’s extracted under optimal conditions, that is optimal culture age. As seen in Figure 1, the bands representing the chromosomal DNA’s of each strain were strong bands, showing successful DNA extraction in amounts enabling further use as template DNA for PCR amplification of genomic segments.
Figure 1. Agarose gel electrophoresis results for extracted fungal chromosomal DNA. Strains in panel A are LBKURCC37.1 and LBKURCC37.2; panel B LBKURCC38; and Panel C LBKURCC39. Bands in lanes 1 are DNA from 1 kb standard marker. Bands in lanes 2 are chromosomal DNA samples.

PCR product amplification of the ITS rDNA region of all four *Penicillium sp.* strains in this study was successful only after the annealing temperatures had been lowered from 45°C to 42°C. This is markedly different from a previous study of other *Penicillium sp.* strains, also collected from Giam Siak Kecil-Bukit Batu Biosphere Reserve, but from different longitude and latitude locations in the 70,000 hectares forest. In the previous study, 5 *Penicillium sp.* strains had varying optimal annealing temperatures for the PCR amplification of the fungal complete ITS rDNA regions, between 44°C to 47°C, giving ITS rDNA region product sizes varying between 604 bp to 702 bp [14]. In this study, the ITS rDNA PCR product size for the four *Penicillium sp.* strains also was varied, ranging between 590 bp for strain LBKURCC39 to 732 bp for strain LBKURCC37.2 (Figure 2 and Table 2). This variation in size may reflect different species among the four strains tested. Damirel *et al.* [16] reported ITS rDNA PCR product sizes of 570 bp for 9 strains of *Penicillium sp.* isolated from Turkish soil. In another study by the same authors, they reported product sizes of 700 bp for 23 *Penicillium sp.* strains also isolated from Turkish soil [17]. Another group also reported variation of ITS rDNA size in the macrofungi, with combination of ITS1 and ITS2 having average lengths of 536±93 bp [18]. If added with the size of the conserved 5.8S rDNA that lies between ITS1 and ITS2, then the average length of ITS rDNA for macrofungi would be 693±93 bp. Our findings in this study is inline with these reports. Further confirmation of correct amplification of the ITS rDNA region awaits DNA sequencing results of the PCR products.

Lower bands that separated from the main PCR products of ITS rDNA region were seen faintly for LBKURCC38 and LBKURCC39. For sequencing purposes of the ITS rDNA region, this can be overcome with ease, by carefully cutting out the bands to sequence from an agarose electrophoresed gel, followed by extraction and cleaning of the DNA from the agarose gel band. This is a common practice now days prior to sequencing PCR products, so it is unnecessary to search an annealing temperature with only a single PCR product.
Figure 2. Agarose gel electrophoresis results for PCR products of ITS rDNA region using primer pairs ITS5 (forward) and ITS4 (reverse). Strains in panel A are LBKURCC37.1 and LBKURCC37.2; panel B LBKURCC38; and Panel C LBKURCC39. Bands in lanes 1 are DNA from 1 kb standard marker. Bands in lanes 2 and 3 are PCR products.

Table 2. Summary of chromosomal DNA and ITS rDNA PCR products.

| Fungal identity       | Chromosomal DNA size (bp) | ITS rDNA PCR product size (bp) |
|-----------------------|---------------------------|-------------------------------|
| *Penicillium sp.* LBKURCC37.1 | 10,730                     | 673                           |
| *Penicillium sp.* LBKURCC37.2 | 10,093                     | 732                           |
| *Penicillium sp.* LBKURCC38   | 15,929                     | 613                           |
| *Penicillium sp.* LBKURCC39   | 11,091                     | 590                           |

In this study we amplified by PCR the ITS rDNA region of the fungal strains for future sequencing, followed by phylogenetic analysis of fungi for their molecular identification. ITS rDNA region was chosen because it is the universal diagnostic region for fungi with the highest number of entries in the GenBank data base. Currently there are more than 30,000 ITS rDNA sequences for fungi in GenBank, 16,864 belonging to *Ascomycetes* of which the genus *Penicillium* is a member. There are 4,751 ITS rDNA reference sequences from verified and described TYPE strains, of which 1,164 sequences belong to the *Ascomycetes*, in a specialized data base of GenBank for ITS rDNA sequences. This ITS reference sequence (RefSeq) data base can be used for BLAST analysis by accessing https://www.ncbi.nlm.nih.gov/bioproject/PRJNA177353 [15]. Due to the existence of this large data base set, for preliminary molecular identification and phylogenetic analysis, ITS rDNA sequences are recommended. When ambiguity arises, or for further verification, other fungal gene sequences can be used for multiple gene and phylogenetic analysis verification. Among gene sites recommended for
multiple gene molecular identification of *Penicillium* species are the *tef-1* [15], *cmdA* and *benA* gene sequences [19].

4. Conclusion

Successful chromosomal DNA isolation of fungal strains depend on the culture age of mycelia used for DNA extraction. In this study it can be concluded that the optimum mycelia culture age for DNA extraction of *Penicillium* LBKURCC37.1, LBKURCC38, and LBKURCC39 is 2 days, while for *Penicillium* sp. LBKURCC37.2 is 3 days. The optimal annealing temperature for PCR amplification of the ITS rDNA region for all *Penicillium* strains in this study is 42°C. The PCR product size obtained in this study were in line with average ITS rDNA PCR product sizes for *Penicillium* sp.

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