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# Occurrence of fungi on deteriorated old *dluwang* manuscripts from Indonesia.

Ariyanti Oetari [a, b, *], Tamara Susetyo-Salim [c], Wellyzar Sjamsuridzal [a, b], Edvan Arifsaputra Suherman [b], Michelle Monica [a], Roni Wongso [a], Reno Fitri [a], Dafina Ghossani Nurlaili [b], Dhian Chitra Ayu [b], Teguh Prasetia Teja [a]

[a] Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia
[b] Center of Excellence for Indigenous Biological Resources-Genome Studies, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia
[c] Department of Library and Information Science, Faculty of Humanities, Universitas Indonesia, Depok 16424, Indonesia

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ABSTRACT

*Dluwang* was made from the bark of the paper mulberry (*Broussonetia papyrifera* Vent.). Old Javanese manuscripts were handwritten on *dluwang*. Fungi samples from deteriorated *dluwang* manuscripts from the libraries of royal palaces in Indonesia and from the faculty library of Universitas Indonesia were collected by using cotton swabs and adhesive tape. Fungal isolates were obtained by the culture-dependent method. Identification of 38 fungal isolates was carried out by the molecular method using the internal transcribed spacer (ITS) regions of ribosomal DNA. Phenotypic description of the isolates was obtained by classic culturing and direct microscopic observation. The results showed that fungi from the genera of *Aspergillus* and *Penicillium* were commonly found on old *dluwang* manuscripts. According to the molecular identification, species obtained were *Aspergillus awamori*, *Aspergillus clavatus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus jensenii*, *Aspergillus niger*, *Aspergillus pulvericola*, *Aspergillus ruber* (*Eurotium rubrum*), *Aspergillus versicolor*, *Penicillium citrinum*, *Penicillium oxalicum*, *Penicillium rubens*, *Pseudocercospora chiangmaiensis*, and *Trichoderma longibrachiatum*. The isolated fungi were xerophilic and cellulolytic in nature. All isolates were able to grow on *dluwang* paper, 29 isolates (76%) were able to utilize carboxymethyl cellulose (CMC), and 18 isolates (47%) were able to utilize CMC and microcrystalline cellulose (MCC). The present study showed that the deterioration of the written heritage was potentially caused by diverse fungi.



## 1. Introduction

*Dluwang* was made from beaten tree bark, and it was mainly used as a writing material from Java and the Madura Islands in Indonesia. The plant material was identified as *Broussonetia papyrifera* Vent., popularly known as the paper mulberry (Teygeler, 1995). The oldest known manuscript on *dluwang* is a Javanese Islamic text from the end of the 16th century (Teygeler, 2002). The historical manuscripts were written in archaic scripts (i.e., *Arab-Jawa*, *Jawa Pegon*, *Aksara Jawa Baru*) and in old Javanese language (Widiarti, 2011). Old *dluwang* manuscripts are important documents consisting of religious scripts, prayers, Islamic stories, historical scripts, horoscopes, drug formulas, magical words, calendars, town legends, traditions, puppets and puppet literature, laws, myths, tales, royal genealogies, and administration management (Teygeler, 2002). Centuries-old *dluwang* manuscripts are held in the Museum Sonobudoyo of Yogyakarta Palace in Central Java, the library of the Kraton Kasepuhan Palace Cirebon in West Java, and the former Library of Faculty of Humanities at the Universitas

* Corresponding author. Current address: Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia; Center of Excellence for Indigenous Biological Resources-Genome Studies, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia.

*E-mail addresses:* aoetari@gmail.com (A. Oetari), tamara_susetyo@yahoo.com (T. Susetyo-Salim), sjwelly@hotmail.com (W. Sjamsuridzal), edvan.arifsaputra@yahoo.com (E.A. Suherman), sinurat.michelle@gmail.com (M. Monica), roniwongso@gmail.com (R. Wongso), reno.fitri@yahoo.com (R. Fitri), dafina.ghossani@gmail.com (D.G. Nurlaili), dhianchitra@gmail.com (D.C. Ayu), tprasetiia@gmail.com (T.P. Teja).

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Indonesia, West Java. These manuscript collections are invaluable because they contain the ancient history of the Indonesian people. Today, very few scholars are able to read them.

In museums and collections, as well as in libraries, fungi play a major role in biodeterioration (Pinheiro et al., 2011; Montanari et al., 2012; Sterflinger and Piñar, 2013). The fungal hyphal networks penetrate the materials deeply, resulting in a substantial loss due to acid corrosion, enzymatic degradation, and mechanical attack (Sterflinger and Pinzari, 2012). Fungal biodeterioration in archives and library collections depends mainly on environmental conditions (Pinzari et al., 2004; Pinheiro et al., 2011). Paper presents an abundant carbon source for fungi (Sequeira et al., 2012). Fungi that colonize documents made of paper are caused by slow-growing species and xerophilic fungi (fungi that thrive on materials with a low water activity, i.e., $a_w = 0.70–0.85$) (Sterflinger and Piñar, 2013). Xerophilic fungi can cause brown spots or *foxing* on cultural heritage books and paper. Foxing is formed when fungi produce organic acids, saccharides, and amino acids which react with paper (Arai, 2000). Fungi growing on paper art objects often secrete a variety of pigments. These pigments may be encrusted in spores or mycelium, or they may be secreted onto the paper which caused staining. Degradation of cellulose fibers on paper depends on the cellulase enzyme complex and results in tears (Szczepanowska and Lovett, 1992).

Fungal species' identities and composition are essential to understanding the colonization and biodegradation of paper stored in a closed environment (Michaelsen et al., 2010; Montanari et al., 2012). For the identification and inventory of fungal isolates that contaminate documents and art objects, the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) has frequently been used (Michaelsen et al., 2006; Mesquita et al., 2009).

This study focused on the isolation, identification, and characterization of fungi from deteriorated historical *dluwang* manuscripts from Indonesia. These findings will contribute to the understanding of the damage problems that occur with old manuscripts and the conservation of library collections, and they can aid in the prevention or restriction of fungal growth on old manuscripts, especially materials made from tree bark paper.

## 2. Materials and methods

### 2.1. Sampling

The samplings were conducted in June 2009 at the Museum Sonobudoyo of Yogyakarta Palace, in December 2011 at the library of Kraton Kasepuhan Palace, Cirebon, and in March 2011 at the former Library of Faculty of Humanities, Universitas Indonesia. Fungal growth on the surface of manuscripts was detected by the presence of caespituli and brown spots. The adhesive tape method and sterile cotton swabs were used to obtain samples suitable for further fungal culturing and identification (Michaelsen et al., 2010; Pinzari et al., 2010). The swab culture was performed on plate count agar (PCA) (Britania, Laboratorios Britania, Buenos Aires, Argentina) and potato dextrose agar (PDA) (Difco, Becton Dickinson Company, New Jersey, USA) containing 0.05% (w/v) tetracycline. The plates were incubated at 25 °C. Fungi were purified at least twice, and representative colonies of each morphological type were selected on PDA containing 0.05% (w/v) tetracycline. All fungal isolates were preserved in 10% (v/v) glycerol and 5% (w/v) trehalose in screw cap micro tubes, stored at −80 °C, and preserved by L-drying in lyophilized ampoules. The cultures obtained in this study were deposited in the Universitas Indonesia Culture Collection (UICC), Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Indonesia.

### 2.2. Xerophilic test

Fungal isolates were inoculated on dichloran 18% glycerol (DG18) agar (Oxoid, Basingstoke, Hampshire, England) and incubated at 25 °C for 7 days (Hocking and Pitt, 1980). The experiment was carried out in triplicate.

### 2.3. Dluwang paper as a substrate

Newly made dluwang paper was obtained from a local artisan in Bandung. The *dluwang* paper strip method was carried out according to Pinzari et al. (2006). Fungal isolates were inoculated on PDA and incubated at 25 °C for 3 days. *Dluwang* paper was cut into strips of 1.5 × 1.5 cm and sterilized in an autoclave at 121 °C for 15 min. The three-day old culture was suspended in 5 ml of sterile water, and fungal spores and hyphae were obtained by scraping the surface of the culture with a round inoculating loop. Spore/hyphal suspension was obtained at a concentration of approximately $10^7$ cells/ml. The *dluwang* paper strips were placed inside Petri dishes containing modified Czapek Dox agar (medium composition in Samson et al., 2004) without carbon source. Each strip was inoculated with 20 μl of spore/hyphal suspension. Positive, negative, and blank controls were *dluwang* strips inoculated with *Aspergillus niger* UICC 371, *Rhizopus oryzae* UICC 24B, and sterile water, respectively. *Aspergillus niger* UICC 371 was a cellulose producer, and *R. oryzae* UICC 24B was a non-producer. The plates were incubated at 25 °C for 5 days. The experiment was carried out in triplicate.

### 2.4. Cellulolytic activity test

The isolates were grown in a single point inoculation on modified Czapek Dox agar plates (Samson et al., 2004) without carbon source, and 1% carboxymethyl cellulose (CMC) or 1% microcrystalline cellulose (MCC) was added as a sole carbon source. The plates were incubated at 25 °C for 7 days, then they were flooded with 1% (w/v) aqueous congo red and destained with 1 M NaCl. Confirmation of cellulose utilization and cellulolytic activity was made by observing the clear zone formed around each fungal colony (Pointing, 1999). The experiment was carried out in triplicate.

### 2.5. Morphological and molecular characterizations of fungal isolates

Genera of isolated fungi were examined under a light microscope and were identified on the basis of the monographs (Raper and Fennell, 1973; Domsch et al., 1980; Klich, 2002; Samson et al., 2004).

For DNA extraction, fungal isolates were incubated in potato dextrose broth (PDB) (Difco) at 25 °C for 2 days. Formed fungal mycelia in the PDB were suspended in sterile 200 μl milliQ water, boiled at 95 °C for 10 min and then vortexed. Fungal DNA was extracted using a commercial kit (Genomic DNA Mini Kit, Blood/Cultured Cell; Geneaid Biotech Ltd., New Taipei City, Taiwan).

Polymerase chain reactions (PCRs) were executed to amplify the ITS regions of rDNA samples using a commercial kit (MyTaq Red Mix; Bioline, London, UK). Fragments of approximately 450–600 bp in size were amplified with the primer pairs using ITS5 forward and ITS4 reverse (White et al., 1990). The PCR condition was as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 30 s. The PCR products were analyzed by means of electrophoresis in a 1% (w/v) agarose gel, stained with 1% (v/v) ethidium bromide for 20 min, and