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Research Article

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Yeasts Associated with Roots of the Endemic Plant *Mankyua chejuense*

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Abstract

BACKGROUND: Identification of endophytic yeasts inhabiting the internal roots of the *Mankyua chejuense* tree requires techniques involving biotechnology. There is a need for a culture-based method to isolate and identify yeast strains associated with *M. chejuense*.
METHODS AND RESULTS: We spread homogenized *M. chejuense* root samples onto glucose-peptone- yeast agar containing antibiotics, Triton X-100, and L-sorbose. A total of 152 yeast isolates were obtained and identified via phylogenetic analysis based on ITS gene sequencing. The results revealed that the root-associated yeast species included the genera *Cyberlindnera* (140 isolates), *Candida* (11 isolates), and *Kluyveromyces* (one isolate). Additionally, three yeast isolates showed high bioethanol production.
CONCLUSION: We identified the specific yeast community associated with *M. chejuense* roots. These yeast isolates may have industrial applications as bioethanol producers. Our findings revealed that *Cyberlindnera* isolates included *C. suaverolens* and *C. satumus*, while *Kluyveromyces* isolates showed high bioethanol production.

Key words: *Cyberlindnera*, ITS gene, *Mankyua chejuense*, Roots, Yeast

Introduction

Yeast participate in the complex processes within ecosystems such as plant tissues, insects endosymbionts, soil, as well as aquatic and extreme environments (Fonseca and Inacio, 2006; Raspor and Zupan, 2006; Botha, 2011). Industrial applications of yeast include food fermentation (Deak, 2009; Tamang and Fleet, 2009).

In this study, we explored yeast species associated with *M. chejuense*. We detected yeast colonizing *M. chejuense* roots as well as producing bioethanol. The number of previous studies reporting phylogenetic analysis of yeast isolates are limited (Rosen and Kunjappu, 2013); therefore, the results from the present study provide valuable information for future studies on yeast biotechnology.

M. chejuense, an endemic plant of the Jeju Island, was first reported (Kim 2004; Sun *et al.*, 2001) as belonging to the family Ophioglossaceae. *M. chejuense* reproduces asexually using rhizomes. Growth of the aboveground portion depends on root growth, while new leaves were produced from rhizomes. Most leaves emerge in the months of July and August, during high temperatures, and the plant enters a dormant stage by April or May the following year (Hyun *et al.*, 2014). The flat wetland habitat developed from cooled lava forms a distinctive geographical boundary to the adjacent regions and is mostly composed of trees that

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Table 1. Characteristics of *M. chejuense* habitat

Site	Altitude (m)	Vegetation & habitat characteristics	Dominant species
Seonheul-ri	90 - 200	Evergreen broad-leaved forest	*Quercus glauca, Cudrania tricspidata*
Dongbok-ri	70 - 80	Evergreen broad-leaved forest rangeland	*Quercus glauca, Ulmus parvifolia*
Gimryeong-ri	70 - 180	Evergreen broad-leaved forest rangeland	*Ulmus parvifolia, Cudrania tricuspidata*
Deokheon-ri	50 - 90	Evergreen broad-leaved forest	*Quercus glauca, Cudrania tricspidata*

thrive in a humid environment, characterized by regular water supply and abundant photosynthesis (Hyeon *et al.*, 2010; Lee *et al.*, 2012) (Table 1, Fig. 1).

There is significant research interest in ethanol production by yeast. Recently, non-conventional yeasts (NCY), excluding *Saccharomyces cerevisiae*, have been used to explore yeast distribution and function in young tree roots. In the present study, we aimed to determine 1) the presence of yeast on the tree, 2) the type and distribution of the yeast species, and 3) the function of the yeast.

Materials and Methods

Yeast isolation from roots of *M. chejuense*

Mankyua chejuense samples were aseptically collected from a Jeju Gotjawal area, using a clean trowel and forceps, and placed in clean plastic bags. The plant samples with soil were sent to the laboratory in Uljin and processed the same day. Each sample was washed three times with 10 mM potassium phosphate buffer until all traces of soil were removed and the roots were stored in an autoclaved container. Several washed root samples were placed in tubes (Falcon, Los Angeles, CA) containing a maximum of 10 mL potassium phosphate buffer (10 mM), and samples were homogenized using an autoclavable hand homogenizer (T10 basis; IKA, Germany). Homogenized and diluted samples (1 mL) were plated on sterile solid media, using a glass spreader, and incubated at 25℃ for 2–5 days. Media for yeast-screening included the following: dichloran-glycerol 18% (DG18) agar (MB Cell, Seoul), drop-out base (DOB) with complete amino acid supplement mixture (CSM) agar (MP Bio, CA, USA), glucose-peptone-yeast (GPY) agar (4% glucose, 0.5% peptone, 0.5% yeast extract, and 1.5% agar), and Sabouraud-chloramphenicol-gentamicin (SCG) agar (MB Cell, Seoul). Antibiotics (100 mg/L chloramphenicol and streptomycin) were added to each medium to inhibit bacterial growth, and 0.1% Triton X-100 and 0.4% L-sorbose were added to prevent fungal growth (Choi *et al.*, 2013).

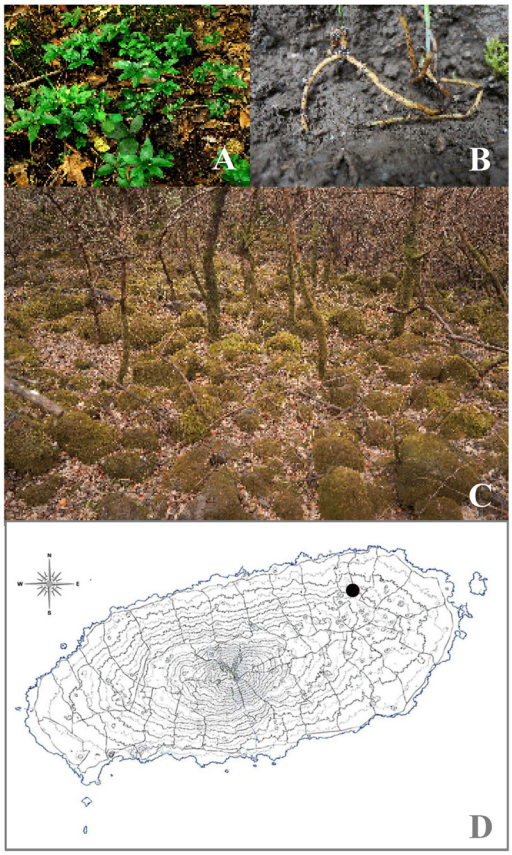

Fig. 1. A: photograph of *M. chejuense*, B: root, C: habitat of *M. chejuense*, D. a map of Jeju Island showing the sampling site.

Yeast cultivation

Yeasts were cultured on agar media in square plates (245 × 245 × 25 mm, Nunc Bio-Assay Dish; Thermo Scientific, Roskilde, Denmark). All colonies from one or two individual plates were picked up and cultured separately. A total of 152 individual isolates were transferred to fresh plates three times and then processed for sequencing of ITS (internal transcribed spacer) genes.

Sequencing and phylogenetic analysis of yeast isolates

Sequencing and phylogenetic analysis were performed as previously reported (Choi *et al.*, 2013; Kim and Kim 2015a, b; White *et al.*, 1990). Sequencing analysis was performed using a PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3730XL DNA Analyzer (Applied Biosystems) by Macrogen Inc. (Seoul, Korea). The nucleotide sequences obtained were deposited in the DNA Databank of Japan (GenBank), under the following accession numbers: LC155263-LC155414 (152 isolates).

Phylogenetic analysis of *Cyberlindnera isolates*

Nucleotide sequences of ITS genes were aligned using the Clustal Omega program on the EMBL-EBI website. The Basic Local Alignment Search Tool (BLAST) search was used to identify GenBank sequences representing the yeast strain most closely related to each isolate. Phylogenetic trees were constructed by the neighbor-joining method, using MEGA5 for Windows (Tamura *et al.*, 2011), including bootstrap analyses based on 1000 samples, and evolutionary distances were calculated by the Kimura 2-parameter method (Saitou and Nei, 1987).

Bioethanol production

Bioethanol production was analyzed from the filtrate obtained after anaerobic culture of 30 random isolates, for 48 hours. Analysis was performed by NICEM (National Instrumentation Center for Environmental Management, Seoul, Korea) by high- pressure liquid chromatography (H-column, Dionex Ultimate3000, USA), using refractive index detector (ERC, Refracto MAX520, Japan) and ultraviolet light at 210 nm.

Results and Discussion

In this study, wild-type yeast community associated with *M. chejuense* roots was isolated from homogenized root samples, analyzed, and phylogenetically identified using ITS gene sequences from genomic DNA. A total of 152 isolates were obtained, representing the genera *Cyberlindnera* (140 isolates), *Candida* (11 isolates), and *Kluyveromyces* (one isolate) (Fig. 1). *Cyberlindnera* species was the dominant colonizer. However, additional studies are required to further characterize the *M. chejuense* root. To the best of our knowledge, this is the first report identifying the yeast community associated with *M. chejuense* root, as shown in Fig. 1. Of note, three genera were found to colonize the root, warranting further studies regarding the yeast community structure. Additionally, *Cyberlindnera* and *Candida* were major colonizers, indicating possible plant-specificity of yeast.

We previously showed that *Meyerozyma* and *Cryptococcus* constituted 83.5% (56 isolates) and 13.4% (nine isolates), respectively, of isolates obtained from *Aloe vera*, whereas *Rhodosporidium* was predominant at 97.6% (41 isolates) of isolates from *Aloe saponaria* (Choi *et al.*, 2013). Yeast isolates from the tiger lily flower have been reported to comprise of *Aureobasidium pullulans* (97.9%, 95 isolates) (Kim and Kim, 2015a), whereas those associated with fleabane flowers include *A. pullulans* (49.3%, 39 isolates), *Candida* (21.5%, 17 isolates), *Rhodosporidium* (17.7%, 14 isolates), *Cryptococcus* (7.5%, six isolates), and *Rhodotorula* (3.7%, three isolates) (Kim and Kim 2015b). In addition, *A. pullulans* from flowers have also been found to produce several biosurfactants, depending on their phylogenetic class (Kim *et al.*, 2015; Kim *et al.*, in press).

The *M. chejuense* habitat falls within the subtropical region and vegetation of the area includes mainly evergreen, broad-leaved forest trees. Evergreen, broad-leaved forest trees such as *Quercus glauca* dominate the vegetation of Seonheul-ri and Duckcheon-ri forest areas. Although these forests are at close proximity, grazing and scrubland formation has given rise to differences in vegetation. The *M. chejuense* habitat is located in the east area of Gotjawal, Jeju Island. Main habitats include Jocheon-eup Seonheul-ri, Gujwa-eup

Table 2. Bioethanol productivity of representative root yeasts associated with *M. chejuense*

Yeast	Lactic acid	Acetic acid	Ethanol
		(ppm)	
Kluyveromyces sp. EY12111-1-2	352.86	199.99	18074.85
Cyberlindnera sp. EY12111-1-30	306.73	424.34	14925.95
Cyberlindnera sp. EY12111-1-33	303.78	417.73	15038.29

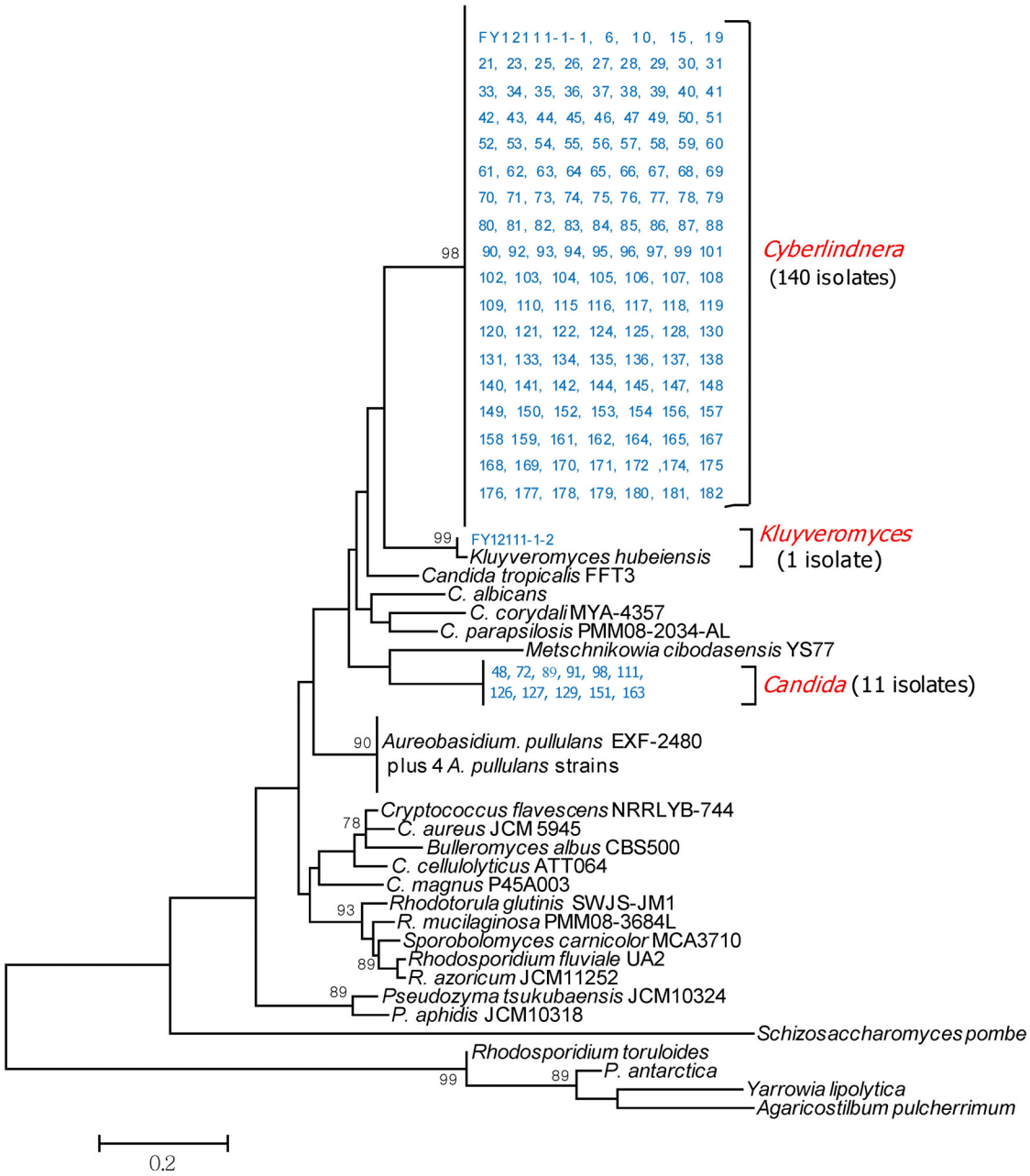

Fig. 2. A neighbor-joining tree of yeast isolates based on ITS gene, using sequences obtained from cultivated representative isolates from *M. chejuense* roots. The numerals represent confidence levels from 1000 replicate bootstrap samplings.

Dongbuk-ri, Gimryung-ri, and Deokcheon-ri, Jeju, where these areas are located at 80 to 200 m above sea level (Table 1). *Cyberlindnera* isolates included the species *C. suaverolens* and *C. satumus* (Fig. 2). Poomtien *et al.*, (2013) reported that *Cyberlindnera samutprakarnensis* JP52 produces a sophorolipid, a type of glycolipid biosurfactant. *Cyberlindnera* isolates obtained in this study need to be further characterized to understand whether the isolates produce biosurfactants.

BE tests were conducted on 30 random isolates of *Cyberlindnera*, *Candida*, and *Kluyveromyces* after anaerobic incubation for 48 hours. As listed in Table 2, *Kluyveromyces* showed highest bioethanol production, followed by *Cyberlindnera* and *Candida* with similarly high production. The results indicate that the isolates converted 37% to 45% glucose to bioethanol.

In conclusion, we isolated yeast from roots of an endemic tree, *M. chejuense*, found exclusively at

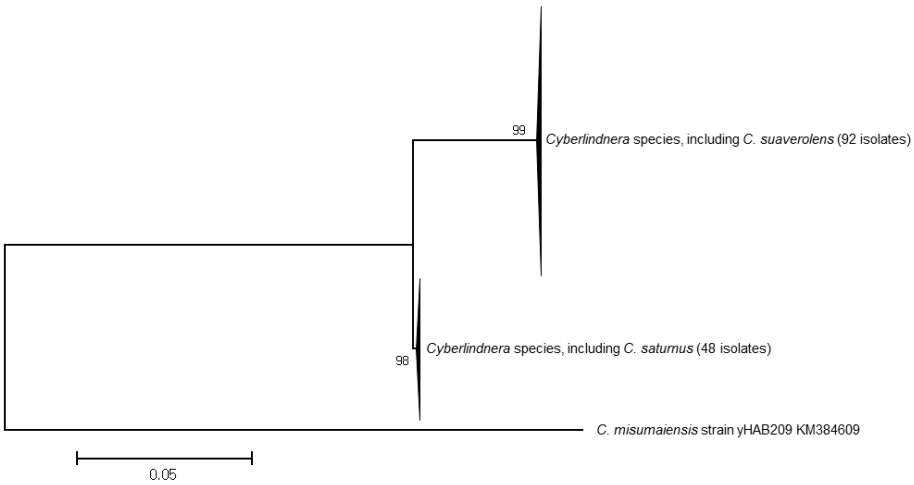

Fig. 3. Molecular phylogenetic analysis of 140 isolates of *Cyberlindnera* genera by maximum likelihood method. Evolutionary history was traced by the Maximum Likelihood method based on the Tamura-Nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search was obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, followed by selection of the topology using the superior log likelihood value. The tree is drawn to scale, with branch lengths indicating the number of substitutions per site. The analysis involved 143 nucleotide sequences including three type strains. All positions containing gaps and missing data were eliminated. There were a total of 456 positions in the final dataset. Evolutionary analyses were conducted using MEGA6 software.

Gotjawal, in the east region of Jeju Island. *Cyberlindnera* and *Candida* comprised the dominant genera, of which some species showed potential for bioethanol production.

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