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RESEARCH ARTICLE

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Neodothiora pruni sp. nov., a Biosurfactant-Producing Ascomycetous Yeast Species Isolated from Flower of *Prunus mume*

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ABSTRACT

A yeast strain, designated as JAF-11^T, was isolated from flower of *Prunus mume* Sieb. *et* Zucc. in Gwangyang, Republic of Korea. Phylogenetic analysis showed that strain JAF-11^T was closely related to *Neodothiora populina* CPC 39399^T with 2.07 % sequence divergence (12 nucleotide substitutions and three gaps in 581 nucleotides) in the D1/D2 domain of the large subunit (LSU) rRNA gene, and *Rhizosphaera macrospora* CBS 208.79^T with 4.66 % sequence divergence (25 nucleotide substitutions and five gaps in 535 nucleotides) in the internal transcribed spacer (ITS) region. Further analysis based on the concatenated sequences of the D1/D2 domain of the LSU rRNA gene and the ITS region confirmed that strain JAF-11^T was well-separated from *Neodothiora populina* CPC 39399^T. In addition to the phylogenetic differences, strain JAF-11^T was distinguished from its closest species, *Neodothiora populina* CPC 39399^T and *Rhizosphaera macrospora* CBS 208.79^T belonging to the family *Dothioraceae* by its phenotypic characteristics, such as assimilation of carbon sources. Hence, the name *Neodothiora pruni* sp. nov. is proposed with type strain JAF-11^T (KACC 48808^T; MB 850034).

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1. Introduction

Ascomycota is recognized as the largest and most well-studied phylum followed by the second largest phylum *Basidomycota* in the Kingdom of Fungi. Members of *Ascomycota* are ubiquitously spread in various aquatic and terrestrial ecosystems [1,2]. At the time of writing, around 83,837 species have been described, representing more than half of all described species of fungi (Catalog of Life: http://www.catalogueoflife.org/annual-checklist/2019/; accessed June 7 2023). *Ascomycota* comprises three subphyla, including *Pezizomycotina* (filamentous fungi), *Saccharomycotina* (budding yeasts), and *Taphrinomycotina* (fission yeasts).

Among the 18 classes belonging to the phylum *Ascomycota*, *Dothideomycetes* is the most biologically and ecologically diverse with 32 orders, 113 families, 1,360 genera, and 24,729 species (Catalog of Life: http://www.catalogueoflife.org/annual-checklist/2019/ ; accessed June 7 2023) [3,4]. Members of *Dothideomycetes* are important plant pathogens, while some are used in biotechnological applications [4]. In particular, fungi belonging to the family *Dothioraceae* are characterized mainly by either medium to large, pulvinate ascostromata with one wide or several small locules [4–6]. As of this writing, the genus *Neodothiora* with a single species, *Neodothiora populina* CPC 39399^T, is the most recently proposed genus out of 25 genera in *Dothioraceae* (Catalog of Life: http://www.catalogueoflife.org/annual-checklist/2019/; accessed June 7 2023).

Over the past decades, microbe-derived biosurfactants have drawn attention for their advantages over chemically synthesized equivalents [7–9]. This is mainly because biosurfactants are composed of natural molecules giving them preferable characteristics, such as better biodegradability and lower toxicity [10]. On the other hand, molecular properties of synthetic surfactants typically complicate their removal and even increase the spread of other pollutants [11,12]. Though increasingly recognized as natural alternatives to synthetic surfactants, microbial biosurfactants remain largely undiscovered and uncharacterized. Some of studies have reported the production of biosurfactants by yeasts with the potential for industrial applications. The major biosurfactant producers include yeast species in the genera *Candida*, *Rhodotorula*, and *Saccharomyces*

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[13–15]. To the best knowledge of the authors, there are two studies investigating production of biosurfactants by yeast species belonging to the family *Dothioraceae* [16,17].

As part of our study to investigate biosurfactant-producing yeast species, we isolated a yeast strain, designated as JAF-11^T, from the flower of *Prunus mume* Sieb. *et* Zucc [17]. According to sequence analysis of the D1/D2 domain of the large subunit (LSU) rRNA gene and the internal transcribed spaces (ITS) region, this strain represents a novel species in the genus *Neodothiora*. This strain produces a novel biosurfactant that reduces the surface tension of water with potential industrial applications, such as cosmetics, medicine, and health-functional foods. To further investigate its morphological and physiological characteristics and compare with the nearest members, type strains *Hormonema macrosporum* KACC 410064^T, *Neodothiora populina* KACC 410090^T, *Phaeocryptopus nudus* KACC 410089^T, and *Rhizosphaera macrospora* KACC 410062^T were acquired from the Korean Agricultural Culture Collection (KACC), Jeonju, Republic of Korea.

2. Materials and methods

2.1. Yeast isolation

Flower samples were collected in Gwangyang, located in the southern end of the Republic of Korea (35°3'49.2"N, 127°44'15.0"E) in March 2018. Approximately 3 g of each sample was suspended in 10 mL of sterile saline. The suspension was serially diluted by sterile saline and 0.1 mL of each dilution was spread onto Yeast Malt Agar (YMA, Difco). After incubation at 25 °C for 3–4 days, colonies with different colors and morphologies were isolated. In order to isolate and screen for potential biosurfactant-producing yeast, the isolates were examined following the procedure described in an earlier publication [17]. Briefly, they were cultivated in soybean oil medium at 25 °C for 3 days and subsequently tested using the modified drop collapse method. As a result, strain JAF-11^T, was isolated from the flower of *Prunus mume* Sieb. *et* Zucc. was selected for the present study. The pure culture of the strain was maintained in 15% (v/v) glycerol stocks at −80 °C and deposited at KACC.

2.2. DNA sequencing and phylogenic analysis

The D1/D2 domain of the LSU rRNA gene and the ITS region of strain JAF-11^T were amplified by PCR using universal primers NL1/NL4 [18] and ITS1/ITS4 primers [18], respectively. The PCR products were examined by agarose gel electrophoresis and purified and sequenced by Macrogen (Daejeon, Republic of Korea). The sequences were assembled with the SeqMan program version 7.1.0 (DNAStar Inc.). The assembled sequences of the D1/D2 domain of the LSU rRNA gene and the ITS region were 581 bp and 535 bp long, respectively. The BLAST search of pairwise sequences was conducted [19] and alignments were performed with MUSCLE [20] to match sequences from related species. Phylogenetic trees were subsequently constructed using MEGA 11 [21] based on the maximum-likelihood (ML) [22], maximum-parsimony (MP) [23], and neighbor-joining (NJ) [24] algorithms. Evolutionary distances for the ML and NJ trees were estimated according to Kimura's two-parameter model [25]. Tree topologies were evaluated by the bootstrap resampling method of Felsenstein [26] with 1,000 replicates.

2.3. Phenotypic characterization

Biochemical, morphological, and physiological characteristics of strain JAF-11^T were examined following the standard protocol described by Kurtzman et al. [27]. Cells were grown for 3 days on Glucose Peptone Yeast Agar (GPYA) at 25 °C and observed under a phase contrast microscope (AX10, Carl Zeiss). Formation of pseudohyphae and true hyphae was investigated on Potato Dextrose Agar (PDA) slide cultures at 20 °C for 3 weeks. Induction of the sexual stage and spore formation was tested by incubating cells on Corn Meal Agar (CMA) and PDA at 20 and 25 °C for up to 5 weeks with periodic microscopic examination. Color reaction with diazonium blue B (DBB, Sigma-Aldrich) was examined by dropping the DBB reagent into the colonies grown for 3 days and observing the color development after 2 min. Growth at different temperatures (4, 10, 15, 20, 25, 30, 35, 40, 45, and 50 °C) and NaCl (0-10%, w/v, in increments of 1%) was assessed on PDA for up to 3 weeks. Nitrogen assimilation was assessed on yeast carbon base agar. Carbon assimilation of strain JAF-11^T and the closely related taxa was carried out using API 20 C AUX kit (BioMérieux) according to the manufacturer's instruction. Streak culture of *Phaeocryptopus nudus* CBS 268.37^T produced fungus-like black and hard cells, and preparation of cell suspension and interpretation of the API 20 C test result were difficult. Hence, carbon assimilation profiles were not determined for *Phaeocryptopus nudus* CBS 268.37^T.

3. Results and discussion

3.1. Identification and delineation of a novel species

Strain JAF-11^T revealed the highest sequence similarity to *Neodothiora populina* CPC 39399^T (98.7%;

Table 1. Nuclotide substitutions in the sequences of the D1/D2 domain of the LSU rRNA gene and ITS region of strain JAF-11^T and the closely related taxa.

	D1/D2 domain		ITS region	
Yeast	Identity (%)	Nt. Substitution / Gap / Total length	Identity (%)	Nt. Substitution / Gap / Total length
Neodothiora populina CPC 39399^T	97.93	12 / 3 / 581	94.65	29 / 11 / 513
Phaeocryptopus nudus CBS 268.37^T	97.63	13 / 2 / 549	94.61	29 / 6 / 538
Rhizosphaera macrospora CBS 208.79^T	97.39	15 / 3 / 575	95.34	25 / 5 / 535
Delphinella strobiligena CBS 735.71^T	97.06	17 / 2 / 578	91.65	45 / 11 / 539
Hormonema macrosporum CBS 536.9^T	96.89	18 / 2 / 578	91.31	45 / 11 / 518
Sydowia polyspora CBS 116.29^T	96.86	13 / 1 / 578	92.21	46 / 11 / 539

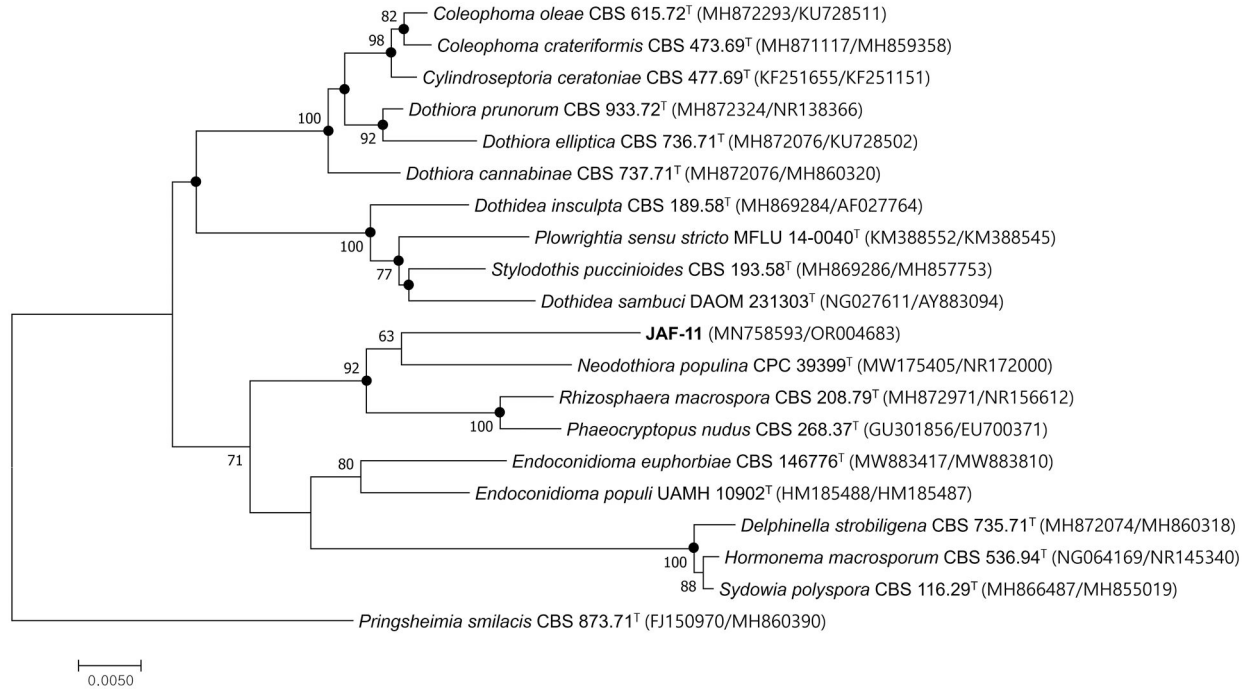

Figure 1. Neighbor-joining tree showing the phylogenetic relationships of strain JAF-11^T and closely related taxa, based on the concatenated sequences of the D1/D2 domain of the LSU rRNA gene and the ITS region. Numbers on nodes correspond to values for branches (1000 replicates); only values over 60% are indicated. Filled circles indicate the corresponding nodes that were also recovered in trees constructed using the maximum-likelihood and maximum-parsimony algorithms. Scale bar, 0.005 substitutions per nucleotide.

12 nucleotide substitutions and three gaps in 581 nucleotides) in the D1/D2 domain of the LSU rRNA gene, and *Rhizosphaera macrospora* CBS 208.79^T (95.3%; 25 nucleotide substitutions and five gaps in 535 nucleotides) in the ITS region (Table 1). According to the criterion proposed by Kurtzman and Robnett [28], a yeast strain showing divergence of the D1/D2 domain of the LSU rRNA gene of more than 1% compared with its closely related species will be representative of a different species. Vu et al. proposed in 2016 that the taxonomic thresholds to discriminate yeast species are 99.51% for LSU and 98.41% for ITS [29]. Hence, strain JAF-11^T with similarities less than the thresholds should be classified as an independent yeast taxon.

The phylogenetic tree based on the concatenated sequences of the D1/D2 domain of the LSU rRNA gene and the ITS region constructed using the NJ algorithm indicated again that strain JAF-11^T was well-separated from the closely related taxa (Figure 1). Specifically, strain JAF-11^T clustered with *Neodothiora populina* CPC 39399^T, and grouped as a distinct clade with *Rhizosphaera macrospora* CBS 208.79^T and *Phaeocryptopus nudus* CBS 268.37^T with high bootstrap value (92%). The ML and MP phylogenetic trees also showed the same branching pattern concerning strain JAF-11^T (data now shown).

Based on these results, strain JAF-11^T should be considered as a representative of *Neodothiora pruni* sp. nov. within the genus *Neodothiora*. Although strain JAF-11^T showed the highest sequence similarity with *Rhizosphaera macrospora* CBS 208.79^T in the ITS region (Table 1), they were different in carbon source assimilation profiles, according to the API 20 C test. Specifically, strain JAF-11^T assimilated methyl-αD-glucopyranoside and D-lactose, while *Rhizosphaera macrospora* CBS 208.79^T did not exhibit the same traits (Table 2). On the other hand,

strain JAF-11^T did not assimilate calcium 2-ketogluconate and *N*-acetylglucosamine, but *Rhizosphaera macrospora* CBS 208.79^T had the ability to assimilate them. In addition, strain JAF-11^T was also different from its closest species, *Neodothiora populina* CPC 39399^T based on the sequence similarity in the D1/D2 domain of the LSU rRNA gene in its ability to assimilate glycerol, D-galactose, methyl-αD-glucopyranoside, and *N*-acetylglucosamine.

DBB and urease reactions were negative. Strain JAF-11^T was positive for gelatin liquefaction and able to grow in a vitamin-free medium. Production of extracellular amyloid compounds was not observed. Budding is polar, and pseudohyphae were formed on CMA after 2-week incubation at 20 °C (Figure 2). From all the results, we concluded that strain JAF-11^T represents a single novel species in the genus *Neodothiora*.

Table 2. Phenotypic comparisons between strain JAF-11^T and the closely related taxa. Strains: 1, JAF-11^T; 2, *Hormonema macrosporum* CBS 536.9^T; 3, *Neodothiora populina* CPC 39399^T; 4, *Phaeocryptopus nudus* CBS 268.37^T; 5, *Rhizosphaera macrospora* CBS 208.79^T. All strains except *Phaeocryptopus nudus* CBS 268.37^T were positive for D-glucose, L-arabinose, D-xylose, adonitol, xylitol, D-sorbitol, D-cellobiose, D-maltose, D-saccharose, D-trehalose, D-melezitose, and D-raffinose. All data are from the present study.Growth reactions: +, positive; −, negative; NA, data not available.

Characteristics	1	2	3	4	5
Growth test					
35 °C	+	−	+	−	+
10% NaCl	+	+	+	−	+
50% D-Glucose	+	+	+	−	+
60% D-Glucose	+	−	+	−	+
0.1% Cycloheximide	−	−	−	−	−
Assimilation					
Glycerol	+	+	−	NA	+
Calcium 2-ketogluconate	−	−	−	NA	+
D-Galactose	+	+	−	NA	+
Inositol	+	−	+	NA	+
Methyl-αD-glucopyranoside	+	−	−	NA	−
N-Acetylglucosamine	−	−	+	NA	+
D-Lactose	+	+	+	NA	−

3.2. Taxonomy

Neodothiora prune Kim, Lee, Heo, Kwon, Yun, & Kim, sp. nov. (pru'ni. L. gen. n. *pruni*, of *Prunus*, the tree genus from which was isolated).

Novel yeast species belonging to phylum *Ascomycota*, subphylum *Pezizomycotina*, class *Dothideomycetes*, order *Dothideales*, family *Dothioraceae*.

Colonies grown on GPYA for 3 days at 25 °C are undulate, cream-colored, mucoid, and raised (Figure 2). Cells are ovoid to ellipsoid (2.2–6.69 um x 1.48–3.93 um) after 3 days on GPYA at 25 °C. Budding is polar budding. Pseudohyphae are observed on PDA after 3-week incubation at 20 °C. No true hyphae are produced.

Assimilate nitrogen sources including creatine, ethylamine, glucosamine, L-lysine, nitrate, but do not assimilate nitrite.

Assimilate carbon sources including D-glucose, glycerol, L-arabinose, D-xylose, adonitol, xylitol, D-galactose, inositol, D-sorbitol, methyl-αD-glucopyranoside, D-cellobiose, D-lactose, D-maltose, D-saccharose, D-trehalose, D-melezitose, and D-raffinose, but do not assimilate calcium 2-ketogluconate and *N*-acetylglucosamine.

Cells grow at 4–35 °C (optimum, 25 °C) and can tolerate up to 10% NaCl in Potato Dextrose Broth (PDB). Growth occurs on GPYA, YMA, Yeast Extract Peptone Dextrose (YPD), and Yeast Malt Extract Agar (YMEA) medium. Growth is observed in media containing 50 and 60% (w/v) glucose, but not in 0.01% of cycloheximide. Production of extracellular amyloid compounds and DBB and urease reactions are negative. Gelatin liquefaction and growth in a vitamin-free medium are positive.

The holotype, JAF-11^T, isolated from the flower samples in Gwangyang, Republic of Korea, is preserved in a metabolically inactive state at KACC, Republic of Korea as KACC 48808^T. The GenBank/EMBL/DDBJ accession numbers for the D1/D2 domain of the LSU rRNA gene and ITS region for JAF-11^T are MN758593 and OR004683, respectively. The mycobank accession number is MB 850034.

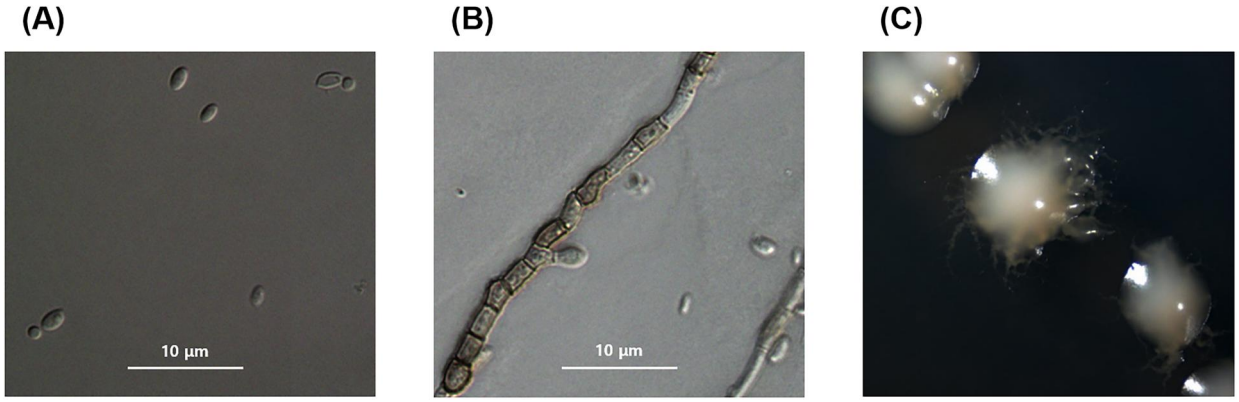

Figure 2. *Neodothiora pruni* sp. nov. (A) Polar budding cells on PDA after three days at 20 °C; (B) Pseudohyphae with chlamydospores on CMA after 2-week incubation at 20 °C; (C) Colonies on GPYA after three days at 25 °C.

Disclosure statement

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