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Research Article

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Yeasts in the Flowers of Wild Fleabane [*Erigeron annus* (L.) Pers.]

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Abstract

BACKGROUND: Yeasts associated with fleabane flowers were identified using isolation methods previously applied in yeast biotechnology. A culture-based approach was required for isolation of many yeast strains associated with fleabane.
METHODS AND RESULTS: We spread homogenized fleabane flowers onto GPY medium containing chloramphenicol, streptomycin, Triton X-100, and L-sorbose. We isolated 79 yeast strains from the flowers of wild fleabane, and identified the yeasts via phylogenetic analysis of isolates from agar plates. The yeast species included 39 isolates of *Aureobasidium pullulans*, 17 of the genus *Candida*, 14 of the genus *Rhodosporidium*, 6 of the genus *Cryptococcus*, and 3 of the genus *Rhodotorula*.
CONCLUSION: Yeast isolates associated with fleabane flowers included *A. pullulans* (39 isolates) and other yeast species (40 isolates). Such yeast isolates may have biotechnological potential.

Key words: Fleabane, *Erigeron annus* (L.) Pers., ITS gene, Wild yeast

Introduction

Erigeron annus (L.) Pers. is an annual or biennial herb of the Compositae, and is a North America native pioneer species that often colonizes disturbed areas such as pastures, vacant fields, roadsides, railways, and waste areas; the herb was introduced to Korea in 1910 (Park, 2009). Flowering occurs in June and July, cephalization is white, the ligulate flower is pistillate, and the tubular flower is yellow with a long pappus (Korea National Arboretum, 2012). The leaves are edible and are commonly used in folk medicine to treat indigestion, stomach ache, diarrhea caused by enteric pathogens, and hypoglycemia (Lee, 1996; Lee, 2003; Yoo, *et al.*, 2008). Little is known, however, about the ecological roles of plant yeasts.

Yeasts play roles in many complex processes of various ecosystems such as plant tissues (stems, flowers, and fruits); insects; soils; aquatic environments; and extreme environments (Fonseca and Inácio, 2006; Raspor and Zupan, 2006; Botha, 2011). Industrial attributes of yeasts include the primary roles they play in many food fermentations yielding beers, ciders, wines, sake, distilled spirits, bakery products, industrial enzymes, and agricultural products (Deak, 2009; Tamang and Fleet, 2009).

In this study, we characterized the yeast species associated with fleabane flowers. Previous studies have attempted to do the same, but their sampling strategies were casual or insufficient. Moreover, only minimal phylogenetic analyses of cultural yeast isolates have been performed in the past (Halloran *et al.*, 2013); therefore, our results provide a basis for

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future studies on yeast biotechnology.

Materials and Methods

Yeast isolation

Erigeron annus (L.) Pers. samples were aseptically collected from a residential area in Uljin with the aid of autoclaved scissors and forceps, and placed in clean plastic bags. Flower samples were stored in a cooler during transfer to the laboratory (in Uljin) and processed the same day. Each sample was washed three times with 10 mM potassium phosphate buffer and stored in an autoclaved container. Several washed flower samples were placed in tubes (Falcon Plastics, Los Angeles, CA, USA) filled to 10 mL with 10 mM potassium phosphate buffer and homogenized using an autoclavable hand homogenizer (T10 Basis; IKA, Staufen, Germany). Homogenized samples (1 mL) were placed on sterile solid media, plated using a glass spreader, and incubated at 25 °C for 2-5 days. Yeast colonies that grew on large plates (Nunc Bio-assay dishes, 245 × 245 × 25 mm, Thermo Scientific, Roskilde, Denmark) were selected with autoclaved toothpicks and inoculated into 96-deep-well plates (Assay Block, 2 mL/well, 96 well square v-bottom; Costar, Cambridge, MA, USA) prior to liquid culture at 25 C at 800 rpm for 48 hours (Choi *et al.*, 2013).

The media used for screening included (all % values are w/w): dichloran-glycerol 18% (DG18) agar (MB Cell, Seoul, Korea); dropout base (DOB) with complete amino acid supplement mixture (CSM) agar (MP Bio, Santa Ana, CA, USA); GPY agar (4% glucose, 0.5% peptone, 0.5% yeast extract, and 1.5% agar); and Sabouraud chloramphenicol gentamicin (SCG) agar (MB Cell). Antibiotics (100 mg/L of both chloramphenicol and streptomycin) were added to each medium to repress bacterial growth, and 0.1% (v/v) Triton X-100 and 0.4% (w/v) L-sorbose were added to repress fungal growth. Yeasts were cultured on DG18, DOB with CSM, GPY, and SCG agar media in square plates (245 × 245 × 25 mm). All colonies from plates yielding multiple colonies were picked and cultured separately. In total, 79 individual isolates were transferred to fresh plates three times and then processed for sequencing of the internal transcribed spacer (ITS) genes.

Sequencing and phylogenetic analysis

The detailed methodology of sequencing and phylogenetic analysis has been previously published (Choi *et al.*, 2013). The primer set used to amplify ITS genes from the yeast strains were the previously described ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (White *et al.*, 1990). For polymerase chain reaction (PCR), DNA was extracted from yeast colonies growing on agar plates using the Instagene Matrix method, according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA).

PCR was performed with 20 ng genomic DNA as template in a 30-μL reaction volume containing EF-*Taq* DNA polymerase (Solgent, Daejeon, Korea). The PCR program included the following steps: 95°C for 5 minutes; followed by 35 cycles of 95°C for 2 minutes, 55°C for 60 s, and 72°C for 60 seconds; and a final extension step for 10 min at 72°C. Amplification products were purified using a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing was performed using a PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Hi-Di formamide (Applied Biosystems) was added to DNA samples containing the extension products. The mixtures were incubated at 95°C for 5 minutes followed by 5 minutes on ice, and analyzed on an ABI Prism 3730XL DNA Analyzer (Applied Biosystems). DNA sequencing was performed by Macrogen Inc. (Seoul, Korea). Nucleotide sequences for the 79 isolates reported in this paper were deposited in DDBJ(DNA Data Bank of Japan)/ GenBank under the following accession numbers: LC018741-LC018819.

Phylogenetic analysis of *Aureobasidium pullulans* interspecies

The nucleotide sequences of the ITS genes were aligned using the ClustalW2 program of the EMBL-EBI website. A BLAST search was used to identify the GenBank sequences most closely related to those of the yeast isolates. Phylogenetic trees were constructed using the neighbor-joining method, using MEGA5 for Windows (Tamura *et al.*, 2011), and featured bootstrap analyses of 1,000 samples. Evolutionary distances were calculated using the Kimura two-parameter method (Saitou and Nei, 1987).

Results and Discussion

Here, yeast isolates colonizing fleabane flowers were isolated and phylogenetically analyzed. Seventy-nine strains from whole yeast isolates were identified

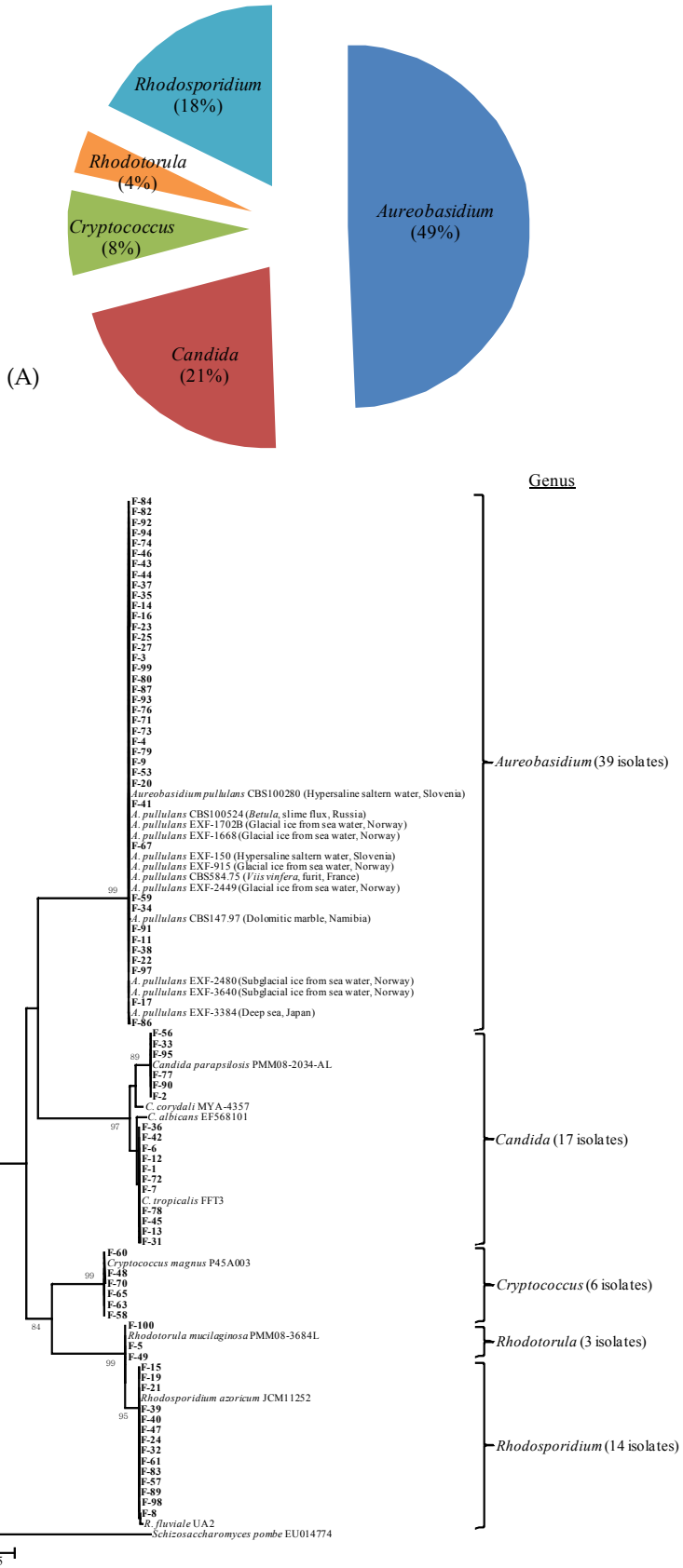

Fig. 1. (A) Pi chart, and (B) a phylogenetic tree of yeast isolates from flowers of the wild fleabane *Erigeron annus* (L.) Pers., based on internal transcribed spacer sequences. Strains described in the present study are in bold font. The numerals are the confidence levels derived from 1,000 replicate bootstrap samplings.

from a single plate of homogenized flower samples. The strains included, *A. pullulans* (39 isolates), *Candida* (17 isolates), *Rhodosporidium* (14 isolates), *Cryptococcus* (6 isolates), and *Rhodotorula* (3 isolates) (Fig. 1). Shown in Fig. 1 is a summary phylogram of the yeast isolates. *A. pullulans*, *Candida* spp., and *Rhodosporidium* spp. dominated the yeast composition of the plant flower, representing 49%, 21%, and 18% of the isolates, respectively. The least abundant yeasts were *Cryptococcus* spp. (8%) and *Rhodotorula* spp. (4%). Among the isolates were several species, including *C. tropicalis* (11 isolates), *C. parapsilosis* (6 isolates), *Rhodosporidium azoricum* (13 isolates), and *R. fluvial* (1 isolate) for which there was little prior knowledge regarding their habitat and function (Käppeli and Fiechter, 1977). Karatay and Dönmez (2010) showed that *C. tropicalis* and *R. mucilaginosa* produce biodiesel in media containing molasses, while *Rhodosporidium* isolates were also shown to produce biodiesel in a snow crab study (unpublished data). As shown in Fig. 1, this is the first yeast composition to be defined in fleabane flowers. Specially, five genera colonized the flowers, and future studies should explore community structure. In addition, *A. pullulans*, *Candida*, and *Rhodosporidium* were major colonizers, suggesting that yeast strains may be plant-specific.

Based on BLAST searches and phylogenetic analyses,

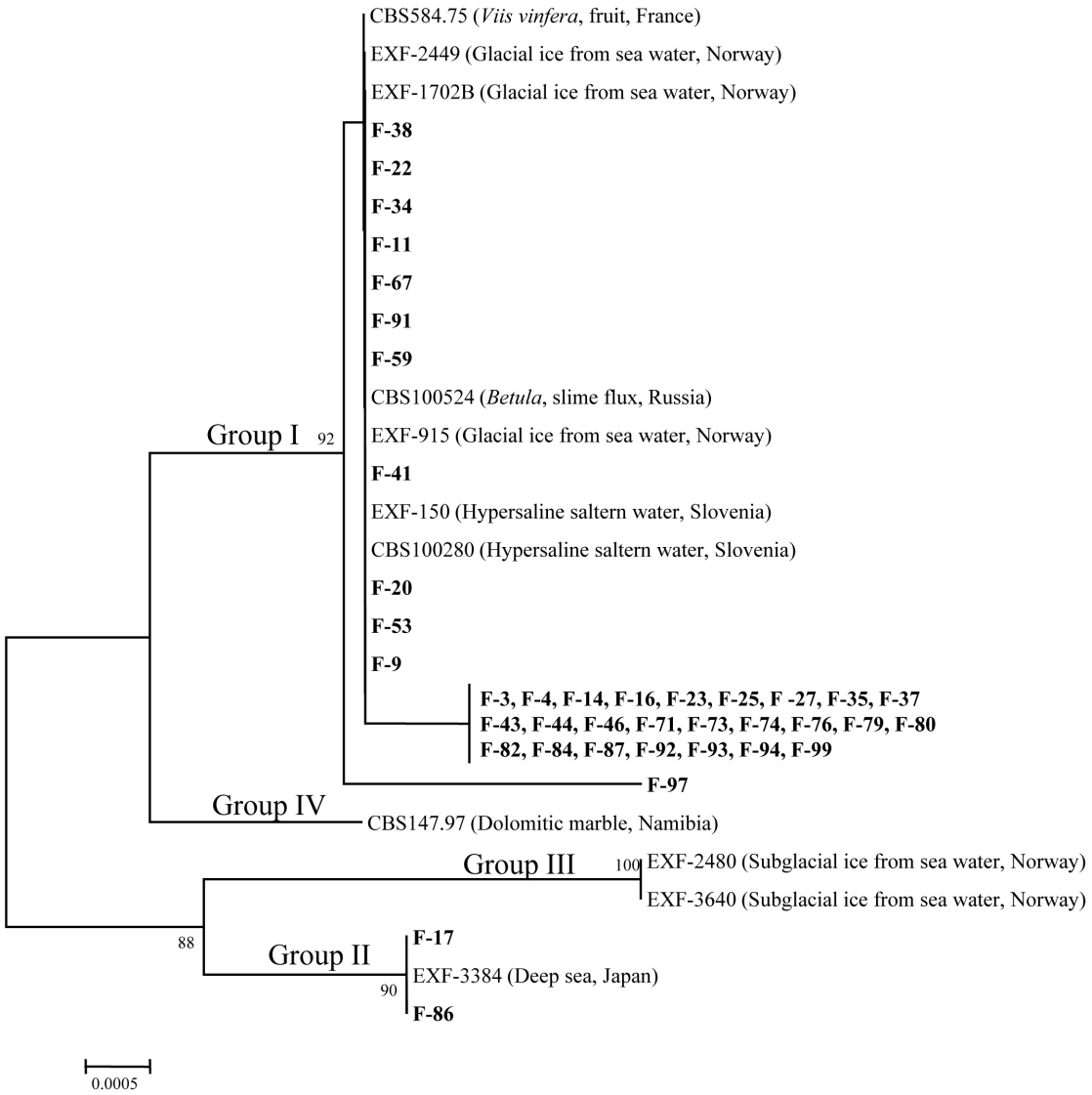

Fig. 2. *Aureobasidium pullulans* interspecies. A neighbor-joining tree of *A. pullulans* isolates from flowers of the wild fleabane Erigeron annus (L.) Pers., based on internal transcribed spacer sequences. Strains described in the present study are in bold font. The numerals are the confidence levels derived from 1,000 replicate bootstrap samplings.

we found several interspecies of *A. pullulans* (Fig. 2). *A. pullulans* is known as black yeast because it produces melanin. Previous data showed that this yeast is the dominant species on the flower, leaf and stem of tiger lily, covering 97%, 35%, and 42% of each of these surfaces, respectively (Kim and Kim, 2015). To identify species and/or interspecies, deep-clade phylogenetic analyses of the *A. pullulans* isolates was performed, revealing that the yeast strains were most closely related to Group I (37 isolates) and Group II (2 isolates), while none belonged to Groups III or IV. In Group I, isolate F-97 was separated from the rest of the group. Isolates F-3 through F-99 and the remaining 25 isolates were not related to Group I. Generally, *A. pullulans* produces polysaccharides, including pullulan and β-glucan, which find industrial and medical applications (Yurlova and de Hoog, 1997; Cheng *et al.*, 2011; Muramatsu *et al.*, 2012). Recently, *A. pullulans* has been shown to produce (poly)malic acid (Nagata *et al.*, 1993), lipase (Leathers *et al.*, 2013), laccase (Rich *et al.*, 2011), mannitol oils (Price *et al.*, 2013), biocontrol agents (Mari *et al.*, 2012), biosurfactants (Kim *et al.*, 2015), valuable lipids (Turk *et al.*, 2004), and siderophores (Ma *et al.*, 2012). *A. pullulans* from plant flowers have also been found to produce several biosurfactants, depending on their phylogenetic class (unpublished data).

The present study identified the main yeast isolates from fleabane, including a phylogenetic assessment of the *A. pullulans* isolates. However, the roles played by yeasts (including *A. pullulans*) in host plants still require further exploration. Future studies should address the distribution of yeast communities using an extensive sampling strategy for more robust group assessments.

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References

Botha, A. (2011). The importance and ecology of yeasts in soil. Soil Biology and Biochemistry, 43(1), 1-8.

Cheng, K. C., Demirei, A., & Catchmark, J. M. (2011). Pullulan: biosynthesis, production, and applications. Applied Microbiology and Biotechnology, 92(1), 29-44.

Choi, S. C., Kim, M. U., & Kim, J. S. (2013). Selective isolation and phylogeny of the yeast species associated with *Aloe vera* and *Aloe saponaria*. Korean Journal of Environmental Agriculture, 32(3), 240-243.

Deak, T. (2009). Ecology and biodiversity of yeasts with potential value in biotechnology. Yeast biotechnology: diversity and applications (ed. Satyanarayana, G., Kunze, G.), pp. 151-168. Springer Science + Buisiness Media B.V., Dordrecht, Netherlands.

Fonseca, A., & Inácio, J. (2006). Phylloplane yeasts. Biodiversity and ecophysiology of yeast (ed. Rosa, C. A., Peter, G.), pp. 263-301. Springer, Berlin, Germany.

Halloran, S. T., Mauck, K. E., Fleischer, S. F., & Tumlinson, J. H. (2013). Volatiles from intact and *Lygus*-damaged *Erigeron annuus* (L.) Pers. are highly attractive to ovipositing *Lygus* and its parasitoid *Peristenus relictus* Ruthe. Journal of Chemical Ecology, 39(8), 1115-1128.

Käppeli, O., & Fiechter, A. (1977). Component from the cell surface of the hydrocarbon-utilizing yeast *Candida tropicalis* with possible relation to hydrocarbon transport. Journal of Bacteriology, 131(3), 917-921.

Karatay, S. E., & Dönmez, G. (2010). Improving the lipid accumulation properties of the yeast cells for biodiesel production using molasses. Bioresource Technology, 101(20), 7988-7990.

Kim, J. S., & Kim, D. S. (2015). Phylogeny of the yeast species isolated from wild tiger lily (*Lilium lancifolium* Thunb.). Korean Journal of Environmental Agriculture, 34(2), 149-154.

Kim, J. S., Lee, I. K., & Yun, B. S. (2015). A novel biosurfactant production by *Aureobasidium pullulans* L3-GPY from a tiger lily wild flower, *Lilium lancifolium* Thunb. PLoS One, 10(4), e0122917.

Korea National Arboretum, (2012). *Field Guide Naturalized Plants of Korea*, p. 239, GeoBook, Seoul, Korea.

Leathers, T. D., Rich, J. O., Anderson, A. M., & Manitchotpisit, P. (2013). Lipase production by diverse phylogenetic clades of *Aureobasidium pullulans*. Biotechnology Letters, 35(10), 1701-1706.

Lee, T. B. (2014). *Coloured Flora of Korea*, Vol. II, p. 327, Hayangmunsa, Seoul, Korea.

Lee, W. T. (1996). *Coloured Standard Illustrations of Korean Plants*, p. 360, Academy Publishing Co., Seoul, Korea.

Ma, Z. C., Chi, Z., Geng, Q., Zhang, F., & Chi, Z. M. (2012). Disruption of the pullulan synthetase gene in siderophore-producing *Aureobasidium pullulans* enhances siderophore production and simplifies

siderophore extraction. Process Biochemistry, 47(12), 1807-1812.

Mari, M., Martini, C., Spadoni, A., Rouissi, W., & Bertolini, P. (2012). Biocontrol of apple postharvest decay by *Aureobasidium pullulans*. Postharvest Biology and Technology, 73, 56-62.

Muramatsu, D., Iwai, A., Aoki, S., Uchiyama, H., Kawata, K., Nakayama, Y., Nikawa, Y., Kusanbo, K., Okabe, M., & Miyazaki, T. (2012). β-Glucan derived from *Aureobasidium pullulans* is effective for the prevention of influenza in mice. PLoS One, 7(7), e41399.

Nagata, N., Nakahara, T., & Tabuchi, T. (1993). Fermentation production of poly(β-L-malic acid), a polyelectrolytic biopolyester, by *Aureobasidium* sp. Bioscience, Biotechnology, and Biochemistry, 57(4), 638-642.

Park, S. H. (2009). *New illustration and Photographs of Naturalized Plants of Korea*, p. 124, Ilchokak, Seoul, Korea.

Price, N. P. J., Manitchotpisit, P., Vermillion, K. E., Bowman, M. J., & Leathers, T. D. (2013). Structural characterization of novel extracellular liamocins (mannitol oils) produced by *Aureobasidium pullulans* strain NRRL 50380. Carbohydrate Research, 370(5), 24-32.

Raspor, P., & Zupan, J. (2006). Yeast in extreme environments. Biodiversity and ecophysiology of yeasts. (eds. Rosa, C. A., Peter, G.), pp. 370-417. Springer, Berlin, Germany.

Rich, J. O., Manitchotpisit, P., Peterson, S. W., & Leathers, T. D. (2011). Laccase production by diverse phylogenetic clades of *Aureobasidium pullulans*. Rangsit Journal of Arts and Sciences, 1(1), 41-47.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4(4), 406-425.

Tamang, J. P., & Fleet, G. H. (2009). Yeasts diversity in fermented foods and beverages. Yeast biotechnology: diversity and applications (ed. Satyanarayana, G., Kunze, G.), pp. 169-198. Springer Science + Buisiness Media B.V., Dordrecht, Netherlands.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28(10), 2731-2739.

Turk, M., Méjanelle, L., Šentjurc, M., Grimalt, J. O., Gunde-Cimerman, N., & Plemenitaš, A. (2004). Salt-induced changes in lipid composition and membrane fluidity of halophilic yeast-like melanized fungi. Extremophiles, 8(1), 53-61.

White,T. J., Bruns, T., Lee, S., & Taylor, J. W. (1990). Amplicationand direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications. (eds. Innis, M. A., Gelfand, D. H., Sninsky, J. J., White, T. J.), pp. 315-322. Academic Press, San Diego, USA.

Yoo, N. H., Jang, D. S., Yoo, J. L., Lee, Y. M., Kim, Y. S., Cho, J. H., & Kim, J. S. (2008). Erigeroflavanone, a flavanone derivative from the flowers of *Erigeron annuus* with protein glycation and aldose reductase inhibitory activity. Journal of Natural Products, 71(4), 713-715.

Yurlova, N. A., & de Hoog, G. S. (1997). A new variety of *Aureobasidium pullulans* characterized by exopolysaccharide structure, nutritional physiology and molecular features. Antonie Van Leeuwenhoek, 72(2), 141-147.