Dioszegia rishiriensis sp. nov., a novel yeast species from soil collected on Rishiri Island, Hokkaido, Japan

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Anamorphic basidiomycetous yeast strains RS090^T and RS092 were isolated from a soil sample collected on Rishiri Island in the Rishiri, Rebun, Sarobetsu National Park, Hokkaido, Japan. As the sequences of the D1/D2 domains of their large-subunit rRNA genes were identical and those of the internal transcribed spacer regions differed in only four bases, we conclude that they belong to a single species with intraspecific diversity. Phylogenetically, this species was related to *Dioszegia buhagiarii* and *Dioszegia hungarica*, in the Tremellales, Tremellomycetes, Basidiomycota, but was clearly distinct from them. Based on the results of sequence analyses and phenotypic characteristics, we conclude that they belong to a novel species in the genus *Dioszegia*, for which the name *Dioszegia rishiriensis* sp. nov. is proposed, with the type strain RS090^T (=JCM 16282^T =CBS 11844^T).

INTRODUCTION

The genus *Dioszegia* was introduced based on a single species, *Dioszegia hungarica*, by Zsolt (1957). Although he did not describe the ballistoconidium-forming ability of the strain, he observed sterigma under a microscope and drew figures as ‘short but well-marked sterigmata (neck-like connection)’. This species was moved to the genus *Cryptococcus* (Phaff & Fell, 1970) because ballistoconidia were not observed from the species and the ‘neck-like connection’ was found in various species in the genus *Cryptococcus*. With the progress of molecular techniques, the taxonomic significance of ballistoconidium-forming activity has been discredited as a result of numerous phylogenetic studies of ballistoconidiogenous and non-ballistoconidiogenous yeasts (for example, Fell *et al.*, 2000; Scorzetti *et al.* 2002; Takashima & Nakase, 1999). Based on high DNA–DNA relatedness between *Cryptococcus hungaricus* (the type species of the genus *Dioszegia*) and *Bullera armeniaca*, Takashima *et al.* (2001) stated that the two were conspecific. As a result, they reaffirmed the genus *Dioszegia* to be a distinct genus based on sequence signatures in the small-subunit (18S) rRNA gene sequence and reinstated *D. hungarica* with two new combinations for phylogenetically closely related species, *Dioszegia aurantiaca* and *Dioszegia crocea*. Since then, several species belonging to this genus

have been described, and a total of 15 species are now included in the genus.

The species described to date make a distinct cluster, the *Dioszegia* subclade *sensu* Inácio (Inácio *et al.*, 2005), in the Tremellales and are characterized by orange–yellow or orange to deep-orange colonies. Interestingly, most of the species belonging to the genus were isolated from plant leaves and only one of the heretofore described and available species, *D. hungarica*, the type species, was isolated from soil. The paper of Renker *et al.* (2004) raised the possibility of close associations between plants and *Dioszegia* species through roots, and isolation of *Dioszegia* species from soil samples has been reported by Vishniac (2006a) and Connell *et al.* (2008, 2010).

In our course of study of the biodiversity of yeasts in Japan, we isolated two strains belonging to the genus *Dioszegia* from a soil sample from Rishiri Island in the Rishiri–Rebun–Sarobetsu National Park. After sequence analyses of the D1/D2 domain of the large-subunit (LSU) rRNA gene and internal transcribed spacer (ITS) region, these yeasts were found to represent a new species, phylogenetically closely related to *Dioszegia buhagiarii* and *D. hungarica* in the Tremellales, Tremellomycetes, Basidiomycota.

METHODS

Isolation. Strains RS090^T and RS092 were isolated from a soil sample collected in July 2007 from the Minamihama marsh area of Rishiri Island. Approximately 0.5 g soil sample was suspended in 5 ml M3C broth (Vishniac, 1985) and shaken at 20 r.p.m. for 10–20 min at 15 °C; 0.1 ml soil suspension was then spread on M3C agar and DRBC agar (Difco) plates containing 50 µg chloramphenicol ml^{−1}

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the ITS regions and D1/D2 domains of the LSU rRNA genes of strains RS090^T and RS092 are AB545810 and AB494715.

A supplementary figure is available with the online version of this paper.

(Wako). The agar plates were kept at 15 °C and the colonies produced were isolated and checked.

Morphological, physiological and biochemical characteristics were generally examined according to Yarrow (1998). Assimilation of nitrogen compounds was investigated on solid media using starved inoculum. Vitamin requirements were determined by the method of Komagata & Nakase (1967).

Sequencing and phylogenetic analysis. The DNA fragment including the ITS regions and the D1/D2 domain of the LSU rRNA gene was amplified directly from yeast cells. Cells were suspended in 60 µl Prepman Ultra sample preparation reagent (Applied Biosystems) and template DNA was prepared according to the manufacturer's instructions. The ITS regions, including the 5.8S rRNA gene, were amplified by PCR according to Sugita & Nakase (1999). The D1/D2 domain of the LSU rRNA gene was amplified following Kurtzman & Robnett (1997). PCR products were sequenced directly using an ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems) and analysed with an Applied Biosystems sequencer model 3100 according to the manufacturer's instructions. Reference sequences used for phylogenetic study (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online) were obtained from the DDBJ/GenBank/EMBL database. Two subsets of the segment were made into datasets: the D1/D2 domain of the LSU rRNA gene and the ITS region plus D1/D2 domain. Multiple alignments were performed using CLUSTAL W version 1.83 (Thompson *et al.*, 1994); final alignments were adjusted manually. Ambiguous positions and alignment gaps were excluded from the analyses. Neighbour-joining trees (Saitou & Nei, 1987) were reconstructed using MEGA version 4 (Tamura *et al.*, 2007), with 1000 bootstrap replicates (Felsenstein, 1985).

RESULTS AND DISCUSSION

Taxonomic position

Strains RS090^T and RS092 were isolated from the same sample collected on Rishiri Island. The sequences of the D1/D2 domain of their LSU rRNA genes were identical; however, those of the ITS regions showed four base differences (one insertion/deletion and three transitions; 98.2 % similarity); we assume that these differences reflect intraspecific diversity. In the phylogenetic tree based on the D1/D2 domain of the LSU rRNA gene, strains RS090^T and

RS092 constituted a cluster with *D. buhagiarii* and *D. hungarica* and were included in the Tremellales, Tremellomycetes, Basidiomycota, but the bootstrap value was low (Fig. 1). The sequence similarity between RS090^T and the closest species, *D. hungarica* and *D. buhagiarii*, was 98 % in the D1/D2 domain of the LSU rRNA gene (13 substitutions with one insertion/deletion), and more than 17 base differences were observed for other species, indicating that our isolates belong to a single novel species in the genus *Dioszegia*.

Based on these results, we conclude that our isolates represent a distinct species, for which the name *Dioszegia rishiriensis* sp. nov. is proposed. The physiological and biochemical properties of *D. rishiriensis* were similar to those of *D. buhagiarii* and *D. hungarica*, but the former could be clearly differentiated from the latter two species by its assimilation patterns for DL-lactate and citrate (Table 1).

Distribution of *Dioszegia* species in soil

Although the type strain of *D. hungarica* was isolated from soil, in Tihany, Hungary (Zsolt, 1957), this species is cosmopolitan and has been isolated from other materials, for example, leaves in Hungary (M. Takashima, unpublished results), Portugal (Inácio *et al.*, 2005) and China (Wang *et al.*, 2008) and glacial melt streams of Patagonia, Argentina (de García *et al.*, 2007), and Antarctica (Vishniac, 2006b). In contrast, only these two strains of this novel species have been isolated, although we used not only soils but also plant materials as isolation sources, including samples from the Minamihama marsh area where the type strain was isolated. After checking *Dioszegia* strains isolated from plant leaves in Japan (mainly near the Tokyo area) by the ballistoconidium-falling method and kept in our laboratory or the Japan Collection of Microorganisms (JCM), we found no other strains belonging to this species. We can therefore say only that *D. rishiriensis* is a component of the population, and further study is necessary to understand the distribution of this and other *Dioszegia* species.

Fig. 1. Neighbour-joining tree of *Dioszegia rishiriensis* sp. nov. and related species based on sequences of the D1/D2 domain of the LSU rRNA gene. Evolutionary distances were calculated according to Kimura (1980). Numbers at nodes represent percentages from 1000 replicate bootstrap samplings (values less than 50 % not shown). Sequences were retrieved from the DDBJ/GenBank/EMBL databases under the accession numbers indicated. Bar, 0.01 substitutions per nucleotide position.

Table 1. Distinctive characteristics among strains of *Dioszegia rishiriensis* sp. nov. and related species

Strains: 1, *D. rishiriensis* sp. nov. strains RS090^T and RS092; 2, *D. buhagiarui* JCM 13482^T; 3, *D. hungarica* JCM 9046^T. Data were obtained in this study. +, Positive; –, negative; L, latent; w, weak; LW, latent and weak.

Assimilation of:	1	2	3
Soluble starch	+	–/w	+
L-Sorbose	LW	L	–
Inositol	–	–	+/L
DL-Lactate	L	–	–
Citrate	–	+	+/w

Latin diagnosis of *Dioszegia rishiriensis* sp. nov.

In liquido YM, post dies 3 ad 17 °C cellulae globosae, ovoideae, ellipsoideae aut apiculatae, (3.8–6.3) × (4.5–10) µm, singulae vel binae; post unum mensem ad 17 °C annulus fragilis, insulae et sedimentum formantur. In agar YM, post unum mensem ad 17 °C cultura aurantiaca, glabra, subnitida, mollis aut butyracea, et margo glabra. Mycelium et pseudomycelium non formantur. Post unum mensem ad 17 °C in CMA, ballisporae nullae. Fermentatio nulla. Glucosum, galactosum, L-sorboseum (lente et exiguum), sucrosum, maltosum, cellobiosum, trehalosum, raffinoseum, melezitoseum, amyllum solubile, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum (lente), L-rhamnosum, D-glucosaminum, N-acetyl-D-glucosaminum, ethanolum (lente et exiguum), ribitolum (lente et exiguum), galactitolum, D-mannitolum, D-glucitolum (lente et exiguum), salicinum (lente), glucono-δ-lactonum (lente et exiguum), acidum D-gluconicum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum DL-lacticum (lente), acidum succinicum (lente), acidum saccharicum (lente), xylitolum (lente et exiguum), L-arabinitolum (lente et exiguum), acidum D-glucuronicum et acidum D-galacturonicum assimilantur, at non lactosum, melibiosum, inulinum, methanolum, glycerolum, erythritolum, methyl-α-D-glucosidum, acidum citricum, inositolum, hexadecanum, propane-1,2-diolum nec butane-2,3-diolum. Natrium nitrosum et L-lysinum assimilantur, at non kalium nitricum, ethylaminum nec cadaverinum. Cultura crescens ad 25 °C at non ad 30 °C. Ad crescentiam thiaminum necessarium est. Ureum hydrolysat. Commutatio coloris per diazonium caeruleum B positiva. Teleomorphosis ignota.

Holotypus: isolatio RS090 ex luto, uligo Minamihama, Rishiri, Hokkaido, Japonia, M. Takashima et B. H. Van, Julius 2001, in collectionibus culturarum ut JCM 16282 quas Japan Collection of Microorganisms (JCM), RIKEN, Wako, Saitama, Japonia conservata, item in collectionibus culturarum ut CBS 11844 quas Centraalbureau voor Schimmelcultures (CBS), Utrecht, Hollandia conservata.

Description of *Dioszegia rishiriensis* sp. nov.

Dioszegia rishiriensis (ri.shi.ri.en'sis. N.L. fem. adj. *rishiriensis* of or belonging to Rishiri Island, because the type

strain of the species was isolated from soil collected on Rishiri Island).

In YM broth, after 3 days at 17 °C, cells are globose, oval, ellipsoidal, apiculate, (3.8–6.3) × (4.5–10) µm, single or in pairs (Fig. 2). A sediment is formed. After 1 month, a complete and fragile ring, islets and a sediment are produced. On YM agar, after 1 month at 17 °C, the streak culture is orange, smooth, semi-shining, soft to butyrous and has an entire margin. In Dalmau plate culture on cornmeal agar, mycelium and pseudomycelium are not formed. After 4 weeks of incubation on cornmeal agar at 17 °C, ballistoconidia are not produced. Teleomorph is unknown. Does not ferment glucose. Assimilates glucose, galactose, L-sorbose (latent and weak), sucrose, maltose, cellobiose, trehalose, raffinose, melezitose, soluble starch, D-xylose, L- and D-arabinose, D-ribose (latent), L-rhamnose, D-glucosamine, N-acetyl-D-glucosamine, ethanol (latent and weak), ribitol (latent and weak), galactitol, D-mannitol, D-glucitol (latent and weak), salicin (latent), glucono-δ-lactone (latent and weak), D-gluconate, 2-ketogluconic acid, 5-ketogluconic acid, DL-lactic acid (latent), succinic acid (latent), saccharic acid (latent), xylitol (latent and weak), L-arabinitol (latent and weak), D-glucuronic acid and D-galacturonic acid. Does not assimilate lactose, melibiose, inulin, methanol, glycerol, erythritol, methyl α-D-glucoside, citric acid, inositol, n-hexadecane, propane-1,2-diol or butane-2,3-diol. Assimilates sodium nitrite and L-lysine hydrochloride. Does not assimilate potassium nitrate, ethylamine hydrochloride or cadaverine dihydrochloride. Thiamine is required for growth. Grows at 25 °C but not 30 °C. Starch-like substances are produced. Urease reaction is positive. The diazonium blue B reaction is positive.

The type strain, RS090^T (=JCM 16282^T =CBS 11844^T), was isolated from mud collected in July 2007 from the Minamihama marsh area on Rishiri Island in the Rishiri-Rebun-Sarobetsu National Park, Hokkaido, Japan, by M. Takashima and B. H. Van. The culture from the holotype strain of this species has been deposited in the Japan

Fig. 2. Vegetative cells of strain RS090^T grown in YM broth for 3 days at 25 °C. Bar, 10 µm.

Collection of Microorganisms (JCM), Wako, Saitama, Japan, and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Strain RS092 (=JCM 16283) is a second strain of the species.

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REFERENCES

- Connell, L. B., Redman, R., Craig, S., Scorzetti, G., Iszard, M. & Rodriguez, R. (2008). Diversity of soil yeasts isolated from South Victoria Land, Antarctica. *Microb Ecol* **56**, 448–459.
- Connell, L. B., Redman, R., Rodriguez, R., Barrett, A., Iszard, M. & Fonseca, Á. (2010). *Dioszegia antarctica* sp. nov. and *Dioszegia cryoxerica* sp. nov., psychrophilic basidiomycetous yeasts from polar desert soils in Antarctica. *Int J Syst Evol Microbiol* **60**, 1466–1472.
- de Garcia, V., Brizzio, S., Libkind, D., Buzzini, P. & van Broock, M. (2007). Biodiversity of cold-adapted yeasts from glacial meltwater rivers in Patagonia, Argentina. *FEMS Microbiol Ecol* **59**, 331–341.
- Fell, J. W., Boekhout, T., Fonseca, A., Scorzetti, G. & Statzell-Tallman, A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**, 1351–1371.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Inácio, J., Portugal, L., Spencer-Martins, I. & Fonseca, Á. (2005). Phylloplane yeasts from Portugal: seven novel anamorphic species in the Tremellales lineage of the Hymenomycetes (Basidiomycota) producing orange-coloured colonies. *FEMS Yeast Res* **5**, 1167–1183.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Komagata, K. & Nakase, T. (1967). Microbiological studies on frozen foods. V. General properties of yeasts isolated from frozen foods. *J Food Hyg Soc Jpn* **8**, 53–57.
- Kurtzman, C. P. & Robnett, C. J. (1997). Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. *J Clin Microbiol* **35**, 1216–1223.
- Phaff, H. J. & Fell, J. W. (1970). *Cryptococcus* Kützing emend. Phaff et Spencer. In *The Yeasts, A Taxonomic Study*, 2nd edn, pp. 1088–1145. Edited by J. Lodder. Amsterdam: North-Holland.
- Renker, C., Blanke, V., Börstler, B., Heinrichs, J. & Buscot, F. (2004). Diversity of *Cryptococcus* and *Dioszegia* yeasts (Basidiomycota) inhabiting arbuscular mycorrhizal roots or spores. *FEMS Yeast Res* **4**, 597–603.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Scorzetti, G., Fell, J. W., Fonseca, A. & Statzell-Tallman, A. (2002). Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Res* **2**, 495–517.
- Sugita, T. & Nakase, T. (1999). Non-universal usage of the leucine CUG codon and the molecular phylogeny of the genus *Candida*. *Syst Appl Microbiol* **22**, 79–86.
- Takashima, M. & Nakase, T. (1999). Molecular phylogeny of the genus *Cryptococcus* and related species based on the sequences of 18S rDNA and internal transcribed spacer regions. *Microbiol Cult Collect* **15**, 35–47.
- Takashima, M., Deak, T. & Nakase, T. (2001). Emendation of *Dioszegia* with redescription of *Dioszegia hungarica* and two new combinations, *Dioszegia aurantiaca* and *Dioszegia crocea*. *J Gen Appl Microbiol* **47**, 75–84.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Vishniac, H. S. (1985). Yeast biomass in Ross Desert soils: evaluation of quantitation methods and sample transport effects. *Antarct J US* **19**, 174–176.
- Vishniac, H. S. (2006a). A multivariate analysis of soil yeasts isolated from a latitudinal gradient. *Microb Ecol* **52**, 90–103.
- Vishniac, H. S. (2006b). Yeast biodiversity in the Antarctic. In *Biodiversity and Ecophysiology of Yeasts*, pp. 419–440. Edited by G. Péter & C. Rosa. Heidelberg: Springer.
- Wang, Q. M., Jia, J. H. & Bai, F. Y. (2008). Diversity of basidiomycetous phylloplane yeasts belonging to the genus *Dioszegia* (Tremellales) and description of *Dioszegia athyri* sp. nov., *Dioszegia butyracea* sp. nov. and *Dioszegia xingshanensis* sp. nov. *Antonie van Leeuwenhoek* **93**, 391–399.
- Yarrow, D. (1998). Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts, A Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Zsolt, J. (1957). Egy új élesztő: *Dioszegia hungarica* nov. gen. et sp. *Bot Kozl* **47**, 63–66 (in Hungarian).