Nematodospora anomalae sp. nov., a novel and D-xylose-fermenting yeast species in the *Lodderomyces* clade

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Three strains of a novel species of ascomycetous yeast were isolated from the beetle species *Anomala corpulenta* (Scarabaeoidea) collected from the Baotianman and Funiu Mountains of China. These strains produced conjugated asci with a single coiled ascospore. Phylogenetic analysis of the combined sequences of the D1/D2 domains of the large subunit rRNA gene and internal transcribed spacer regions demonstrated that the three strains were closely related to *Nematodospora valgi* and an undescribed yeast strain, 13Y231. The novel strains could be differentiated from *N. valgi* CBS 12562^T by a 1.6 % sequence divergence (9 substitutions) and from the undescribed yeast strain, 13Y231, by a 1.1 % sequence divergence (6 substitutions) in the D1/D2 sequences. The ITS sequences of these strains displayed more than 4.1 % sequence divergence (12–22 substitutions and 7–8 gaps) from their two closest relatives. Interestingly, all the three strains could ferment D-xylose to ethanol effectively, a rare property among members of the *Lodderomyces* clade. Therefore, a novel yeast species, *Nematodospora anomalae* sp. nov., is proposed to accommodate these strains. The type strain of *N. anomalae* sp. nov. is NYNU 14914^T (=CICC 33059^T=CBS 13927^T). The MycoBank number is MB 816795.

The *Lodderomyces* clade, which belongs to the family *Debaryomycetaceae*, is a monophyletic group closely related to the *Spathaspora* and *Scheffersomyces* clades (Kurtzman, 2011; Urbina *et al.*, 2013). To date, the *Lodderomyces* clade comprises four ascosporic species: *Lodderomyces elongisporus* (Kurtzman, 2011), *Candida baotianmanensis*, *Candida pseudoviswanathii* (Ren *et al.,* 2015) and *Nematodospora valgi* (Gouliamova *et al.*, 2016). This clade also includes 30 additional anamorphic species *of Candida* (Lachance *et al.*, 2011; Daniel *et al.*, 2014). Among these species, *Candida albicans*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida metapsilosis*, *Candida orthopsilosis*, *Candida tropicalis* and *Candida viswanathii* are major human pathogens (Lachance *et al.*, 2011; Nitiyon *et al.*, 2011; Gouliamova *et al.*, 2016). There are also several species of yeast such as *Candida maltosa* and *Candida tropicalis* that possess the rare ability to ferment D-xylose to ethanol, a biotechnological trait of industrial interest for the production of biofuels by the fermentation of plant hydrolysates (Lohmeier-Vogel *et al.*, 1989; Lin *et al.*, 2010). Recently, Urbina *et al.* (2013) isolated several previously undescribed species, which could also ferment D-xylose, with the characteristics of some members of this clade in the gut of *Guatemalan passalids*. Their findings suggested that the D-xylose-fermenting yeasts belonging to the *Lodderomyces* clade are common in the gut of insects, particularly passalids. The discovery of new D-xylose-fermenting yeast species of the *Lodderomyces* clade may provide a source of genes, enzymes and/or sugar transporters to engineer industrial strains for the efficient production of bioethanol from renewable biomass.

In an extensive study of yeasts associated with insects in Central Chinese ecosystems, we isolated three D-xylose-fermenting yeasts. Sequence analyses of the D1/D2 domains of the large subunit (LSU) rRNA gene and internal transcribed spacer (ITS) regions showed that these isolates represented a single species closely related to *N. valgi*. In this work, we describe this novel species as *Nematodospora anomalae* sp. nov.

The host beetles were collected from two different localities in Henan Province, Central PR China. Two strains (NYNU 14905 and NYNU 14914^T) were isolated from the gut of the insect *Anomala corpulenta* (Scarabaeoidea) collected from Baotianman Mountain (33°27′N 111°48′E) in September 2014. Another strain, NYNU 15825, was found in the gut of the same insect collected from Funiu Mountain (32°45′N 113°30′E) in August 2015. The two mountains were separated from each other by 50.2 km. The methods used to

Abbreviations: ITS, Internal Transcribed Spacer; LSU, Large Sub Unit.

The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 domains of the large subunit rRNA gene and ITS regions of strain NYNU 14914^T are KP054269 and KP054270, respectively.

isolate the yeasts from the gut of each insect have been described previously (Nguyen *et al.*, 2007; Suh *et al.*, 2008). The insects were placed in Petri dishes for 1–3 days without food prior to dissection. Withholding food helps to eliminate some contaminating organisms that might be isolated from the gut. Surface disinfection was performed by submersion in 95 % ethanol for 1–2 min. An alcoholic wash was followed by a 0.7 % (w/v) saline rinse. The insect gut was removed aseptically under a dissecting microscope, and gut segments were streaked on acidified yeast extract–malt extract (YM) agar plates (0.3 % (v/v) yeast extract, 0.3 % (v/v) malt extract, 0.5 % (v/v) peptone, 1 % (v/v) glucose and 2 % (v/v) plain agar;adjusted to pH 3.5 with HCl) and then incubated at 25 °C for 3–4 days. The different yeast morphotypes were purified at least twice and then stored in YM agar slants at 4 °C and 15 % (v/v) glycerol at −80 °C.

The morphological observations and metabolic tests constituting standard yeast descriptions were performed in accordance with established methods (Kurtzman *et al.*, 2011). All assimilation tests were performed twice in liquid media, and the results were read after 5 and 21 days of incubation. Starved inocula were used in the nitrogen assimilation tests. Sporulation tests were performed on YM agar, 5 % (w/v) malt extract agar, corn meal agar and yeast carbon base supplemented with 0.01 % ammonium sulphate (YCBAS) agar (1.1 % yeast carbon base, 0.01 % ammonium sulphate and 1.8 % agar) in pure and mixed cultures at 25 °C for 4 weeks. The photomicrographs were made with a Leica DM5000B microscope (Leica Corporation).

Genomic DNA was extracted using an Ezup Column Yeast Genomic DNA Purification Kit, in accordance with the manufacturer's protocol (Sangon Biotech). The D1/D2 domains of the LSU rRNA gene and ITS regions were amplified by PCR with the primers NL1 and NL4 (Kurtzman & Robnett, 1998) and ITS1 and ITS4 (White *et al.*, 1990), respectively. The amplified products were purified using a QIAquick purification kit (Sangon Biotech) in accordance with the manufacturer's instructions. Direct sequencing of the purified LSU rRNA gene and ITS PCR products was performed using primers NL1/NL4 and ITS1/ITS4 with a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) by Sangon Biotech (China) in accordance with the manufacturer's protocol. Purified sequencing reaction mixtures were separated using a 3730XL automated DNA analyser (Applied Biosystems).

The BLAST program (Altschul *et al.*, 1997) from the National Center of Biotechnology Information was used to compare the sequences with available data on the D1/D2 domains of the LSU rRNA gene and ITS regions present in GenBank (http://ncbi.nlm.nih.gov) in order to determine the most closely related species. The sequences were aligned using CLUSTAL X version 1.81 (Thompson *et al.*, 1997). A phylogenetic tree, based on the combined sequences of the D1/D2 domains of the LSU rRNA gene and ITS regions, was reconstructed using MEGA software version 5.0 (Tamura *et al.*, 2011). Evolutionary distance data were calculated using Kimura's two-parameter model (Kimura, 1980) in neighbour-joining analyses. *Saccharomyces cerevisiae* NRRL Y-12632^T was used as the outgroup. Confidence limits were estimated from bootstrap analysis (1000 replicates) (Felsenstein, 1985), and only values above 50 % were recorded on the resulting trees. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Batch fermentation of D-xylose was performed as described by Cadete *et al.* (2009). D-Xylose was determined by HPLC (Waters 410), as described by Lin *et al.* (2010). Ethanol was measured with al2010cohol oxidase (Sigma) and peroxidase (Sangon Biotech). The ethanol yield coefficient ($Y_{E/xyl}$, g ethanol g^{-1} xylose) was obtained at the end of ethanol production taking into account the amount of sugar utilized. The reported values were the mean ± standard deviations obtained from independent duplicate cultures and were determined using the Student's paired *t*-test.

Species delineation, classification and ecology

The three strains investigated exhibited identical sequences in both the D1/D2 domains and ITS regions, indicating their conspecificity. A phylogenetic tree was reconstructed by the neighbour-joining method, based on the combined sequences of the D1/D2 domains of the LSU rRNA gene and ITS regions (Fig. 1). The results showed that the *Lodderomyces* clade is not monophyletic, but includes several distinct clades, which may become representatives of several genera. The novel species was placed in a well supported clade with *N. valgi*, the type species of the genus *Nematodospora*, and an undescribed yeast strain, 13Y231W (Fig. 1). The D1/D2 sequences of the novel species differed from *N. valgi* CBS 12562^T by 1.6 % sequence divergence (9 substitutions) and from the undescribed strain 13Y231 by 1.1 % sequence divergence (6 substitutions). In ITS regions, the species displayed 4.1 % sequence divergence (12 substitutions and 7 gaps) from *N. valgi* CBS 12562^T, and 6.4 % sequence divergence (22 substitutions and 8 gaps) from the undescribed yeast strain, 13Y231. According to the guidelines proposed by Kurtzman & Robnett (1998) and Daniel *et al.* (2009), this extent of divergence is sufficient to justify these three strains representing a novel species of yeast.

The cells of the novel species were ovoid, proliferated by multilateral budding (Fig. 2a) and formed with pseudohyphae (Fig. 2b). As sporulation in the related *N. valgi* was observed on some common sporulation media (Gouliamova *et al.*, 2016), the novel species was also tested for ascospore formation on YM agar, 5 % malt extract agar, corn meal agar and CBAS agar (Kurtzman *et al.*, 2011). Microscopic examination revealed that all isolates of the novel species can be self-sporulating on 5 % malt extract, corn meal and YCBAS agars. Conjugation was followed by formation of a single coiled ascospore per ascus (Fig. 2c, d), a feature shared with *N. valgi* (Gouliamova *et al.*, 2016). The species is homothallic, as conjugation takes place between cells, and between

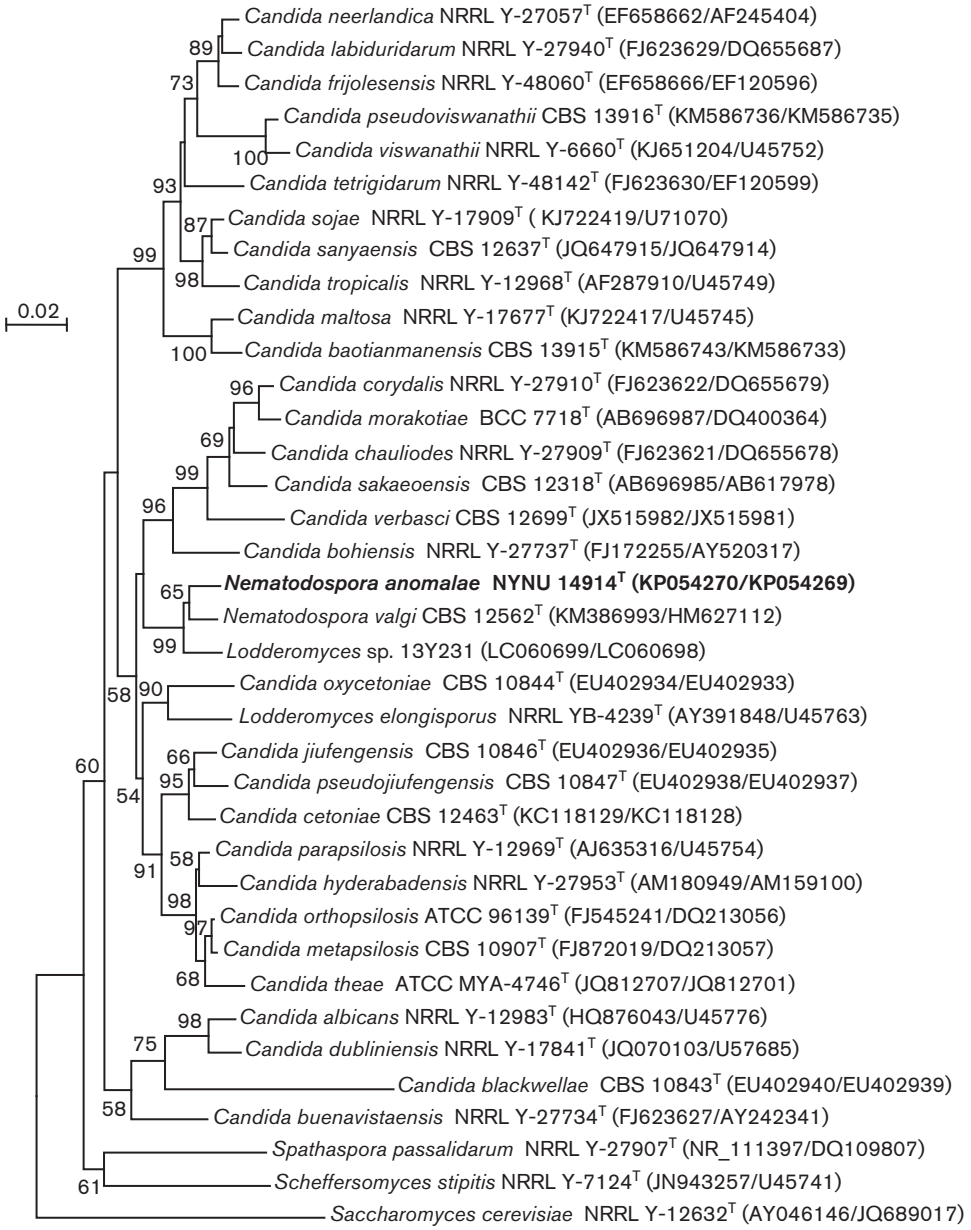

Fig. 1. Phylogenetic tree based on combined sequences of the D1/D2 domains of the LSU rRNA gene and ITS regions, showing relationships between *Nematodospora anomalae* sp. nov. and members of the *Lodderomyces* clade. *Saccharomyces cerevisiae* NRRL Y-12632^T was used as the outgroup. Bootstrap values above 50 % are given at nodes based on 1000 replications. Bar, 2 % sequence difference.

cells and the buds of a single strain. Fermentation tests in Durham tubes indicated that this novel species can ferment D-xylose. A detailed analysis of batch fermentation by these yeasts showed that all can produce the same amount of ethanol in rich YP (1 % yeast extract, 2 % peptone) medium supplemented with 2 % xylose ($Y_{E/xyl}$ = 0.34 ± 0.04). This finding confirmed that the novel species of yeast can ferment D-xylose to ethanol effectively, as can other species of yeast (Cadete *et al.*, 2009).

The three strains of the novel species were well separated from the most closely related and previously known species, *N. valgi* (Gouliamova *et al.*, 2016) on the basis of carbon assimilation and other physiological characteristics. The novel species could be distinguished from *N. valgi* by its ability to assimilate D-glucosamine and grow at 35 °C, as well as its inability to assimilate sucrose, methyl α-D-glucoside, melezitose, ribitol and citrate. The novel species also differed from *N. valgi* in terms of the positive

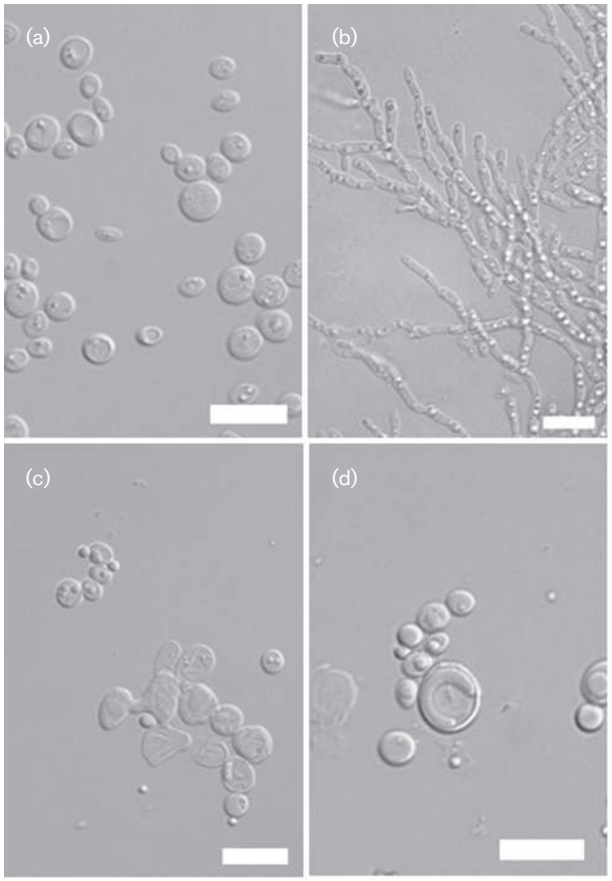

Fig. 2. Photomicrographs of *Nematodospora anomalae* sp. nov. NYNU 14914^T. (a) Budding cells, 3 days, YM broth, 25 °C. (b) Pseudohyphae, 2 weeks, corn meal agar, 25 °C. (c) Conjugating cells, 2 days, corn meal agar, 25 °C. (d) Asci with coiled ascospore, 2 weeks, corn meal agar, 25 °C. Bar, 10 μm.

fermentation of galactose and maltose. The rDNA sequence and phenotypic comparisons made above demonstrate that these strains represent a novel species of the genus *Nematodospora* in the *Lodderomyces* clade; the name *Nematodospora anomalae* sp. nov. is proposed.

Members of the *Lodderomyces* clade have been isolated from various sources, such as marine habitats, food, plants, soil, soft drinks, humans and animals (Kurtzman, 2011; Lachance *et al.*, 2011; Nitiyon *et al.*, 2011; Gouliamova *et al.*, 2016). Previous studies conducted in different geographic regions showed that phytophagous insects frequently host yeasts belonging to the *Lodderomyces* clade, including human pathogens (Nguyen *et al.*, 2007; Suh *et al.*, 2008; Ji *et al.*, 2009; Urbina, 2013; Ren *et al.*, 2015). In the present study, three strains of *N. anomalae* sp. nov. were repeatedly isolated from the beetle species, *A. corpulenta* (*Scarabaeoidea*), collected from two different regions of PR China (Baotianman and Funiu Mountains). Phylogenetic analysis placed *N. anomalae* sp. nov. as a sister taxon of *N. valgi*, which was also isolated from the beetle *Valgus hemipterus* (*Scarabaeoidea*) collected from Psogovska Planina Mountain and Nature Park, Zlatni Pyasatsi, Bulgaria (Gouliamova *et al.*, 2016). On the basis of our results, we suggest that the yeast species *N. anomalae* sp. nov., and *N. valgi*, as well as their phytophagous *Scarabaeoidea* beetle hosts may form an intimate ecological relationship. The living culture derived from the type strain is preserved by lyophilization as strain CBS 13927^T in the Yeast Division of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands, and as strain CICC 33059^T in the China Centre of Industrial Culture Collection, Beijing, PR China.

Description of *Nematodospora anomalae* sp. nov.

Nematodospora anomalae (a.no.ma′lae. N.L. fem. gen. n. *anomalae* referring to the genus of the host beetles, *Anomala corpulenta*, from which it was isolated).

In YM broth after 3 days at 25 °C, cells are spherical or ovoid (3–6×3–6 μm) and occur singly or in pairs (Fig. 2a). Budding is multilateral. After a month at 25 °C, a delicate ring may be formed and sediment is present. On YM agar after 7 days at 25 °C, colonies are white to cream, butyrous and smooth with an entire margin. In Dalmau plate culture on corn meal agar after 12 days at 25 °C, pseudohyphae are present, but no true hyphae are observed (Fig. 2b). Ascospores form on 5 % malt extract agar, corn meal agar and YCBS agar after 12 days at 25 °C; asci are unconjugated, persistent and each contain a single coiled ascospore (Fig. 2c, d).

Glucose, galactose, maltose, trehalose (weakly) and D-xylose are fermented, but not methyl α-D-glucoside, sucrose, melibiose, lactose, cellobiose, melezitose, raffinose or inulin. Glucose, galactose, trehalose, maltose, soluble starch, salicin, D-xylose, ethanol (weakly or negative), glycerol, D-mannitol, D-glucitol, succinate, D-gluconate (weakly), D-glucosamine, 2-keto-D-gluconate, arbutin and D-glucono1,5-lactone are assimilated. No growth occurs in inulin, sucrose, raffinose, melibiose, lactose, melezitose, methyl α-D-glucoside, cellobiose, L-sorbose, L-rhamnose, L-arabinose, D-arabinose, D-ribose, methanol, erythritol, ribitol, galactitol, myo-inositol, DL-lactate, citrate, 5-keto-D-gluconate, L-arabinitol or xylitol. Ethylamine, L-lysine and cadaverine (weakly) are assimilated. No growth occurs in nitrate, nitrite, creatine, creatinine, glucosamine, imidazole or D-tryptophan. Growth occurs at 37 °C, but not at 42 °C. No growth occurs in 10 % (w/v) NaCl plus 5 % (w/v) glucose, or in 0.01 % cycloheximide, or 1 % (v/v) acetic acid. Starch-like compounds are not produced. Urea hydrolysis and DBB reactions are absent.

Strain NYNU 14914^T, the holotype, was isolated from the gut of the insect *Anomala corpulenta* collected from Baotianman Mountain in Henan Province, PR China.The MycoBank number of the type strain is MB 816795.

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