Curr Microbiol (2015) 71:268–273
DOI 10.1007/s00284-015-0847-1

Molecular Phylogeny and Taxonomy of *Yamadazyma dushanensis* f.a., sp. nov., a Cellobiose-Fermenting Yeast Species from China

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Received: 14 February 2015 / Accepted: 15 April 2015 / Published online: 3 June 2015

Abstract Three yeast strains of *Yamadazyma dushanensis* f.a., sp. nov. were isolated from rotten wood samples collected in the Dushan Forest Park, Nanyang, Henan Province, China. Sequence analyses of the D1/D2 domains of the large subunit rRNA gene and the internal transcribed spacer (ITS) regions revealed that this new species is located in the *Yamadazyma* clade (Debaryomycetaceae and Saccharomycetales), with three closely related species, namely, *Yamadazyma terventina*, *Yamadazyma mexicana* and *Candida trypodendroni*. The novel species differed from these three described species by 5–6 nt substitutions in the D1/D2 sequences. However, the ITS sequences of the new species were quite divergent from those of *Y. terventina*, *Y. mexicana* and *C. trypodendroni* with 12–18 nt substitutions. This new yeast species could assimilate cellobiose and other compounds related to rotting wood. The fermentation of cellobiose in Durham tubes was observed for the strains of this new yeast. The new species could also be distinguished from its closely related species, *Y. terventina*, *Y. mexicana* and *C. trypodendroni*, based on a number of morphological and physiological characteristics. The type strain is *Y. dushanensis* sp. nov. NYNU 14668 ^{T} (=CICC 33051^{T} = CBS 13914^{T}).

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Introduction

The genus *Yamadazyma* was described by Billon-Grand (1989) to accommodate 16 species that had previously been assigned to the genus *Pichia* and formed CoQ-9 as their major ubiquinone, as well as hat-shaped ascospores [1, 2]. Unfortunately, the genus has not been generally accepted as the polyphyletic nature of *Yamadazyma* became evident from the D1/D2 domains of the large subunit (LSU) rRNA gene sequence analysis after a few years [2]. In 2010, some of the species that had been assigned to this genus by Billon-Grand (1989) were transferred to the newly described genera *Babjeviella*, *Meyerozyma*, *Millerozyma* and *Priceomyces* based on the phylogenetic analysis of the D1/D2 domains of the LSU rRNA and the nearly complete small subunit (SSU) rRNA gene [3]. Thereafter, the genus *Yamadazyma* became a well-supported clade and a generally accepted genus in the family Debaryomycetaceae of the order Saccharomycetales [3, 4]. In the fifth edition of "The Yeasts, A Taxonomic Study", *Yamadazyma philogaea*, the type species, as well as *Yamadazyma akitaensis*, *Yamadazyma mexicana*, *Yamadazyma nakazawae*, *Yamadazyma scolyti*, *Yamadazyma triangularis* and 23 *Candida* species are placed in the *Yamadazyma* clade [4, 5]. Since then, a few novel *Candida* species in this clade have been described, including *Candida kanchanaburiensis* [6], *Candida khaothaluensis*, *Candida vaughaniae*, *Candida tallmaniae* [7] and *Candida oceani* [8]. Recently, several additional members of the genera *Yamadazyma* have been proposed, namely, *Yamadazyma terventina* [9], *Yamadazyma siamensis*, *Yamadazyma phyllophila*, *Yamadazyma paraphyllophila* [10] and *Yamadazyma ubonensis* [11].

During an investigation of the yeast species diversity associated with rotten wood in Central China, three asexual

cellobiose-fermenting yeast strains have been obtained. The sequences of the D1/D2 domains of the LSU rRNA gene have shown that these isolates are closely related to *Y. mexicana*, *Y. terventina* and *Candida trypodendroni*, belonging to the *Yamadazyma* clade. However, the internal transcribed spacers (ITS) regions sequences of the isolates are quite divergent from these three known species, which represent a new species of the genus *Yamadazyma*. In this study, we propose the description of this new species as *Yamadazyma dushanensis* f.a., sp. nov. based on the morphology, physiology and phylogenetic analysis of the D1/D2 domains of the LSU rRNA gene and the ITS regions.

Materials and Methods

Yeast Isolation and Culture

The strains belonging to the proposed novel species, NYNU 14656, NYNU14668^{T} and NYNU14669, were isolated from three different rotten wood samples collected in the Dushan Forest Park located near Nanyang (33°3′38″N and 112°34′44″E) in Henan Province, Central China. This area is a warm, temperate zone, with monsoon-influenced semi humid continental climate. All necessary permits were obtained from Dushan Forest Park Administration of Nanyang, Henan Province, China.

The methods for yeast isolation from decayed wood samples were detailed by Cadete et al. [12]. Approximately 1 g of each sample was added to 20 mL of sterile yeast extract-malt extract (YM) broth (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone and 1 % glucose; adjusted to pH 4.0–4.5 with 1 M HCl) supplemented with 0.025 % sodium propionate and 200 mg/L chloramphenicol in a 150 ml Erlenmeyer flask and incubated at 25 °C for 3 days on a rotary shaker. The enrichment culture was spread out on YM agar supplemented with 0.025 % sodium propionate and 200 mg/L chloramphenicol, and then incubated at 25 °C for 3–4 days. The different yeast morphotypes were purified at least twice, and then stored on YM agar slants at 4 °C and in 15 % glycerol at −80 °C.

Morphological, Physiological and Biochemical Characteristics

The methods used to determine the morphological, physiological and biochemical properties were performed according to established methods [13, 14]. 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 % 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. The cultures were observed for ascospore production every week for a month at 15 and 25 °C.

DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted using the Ezup Column Yeast Genomic DNA Purification Kit according to the protocol of the manufacturer (Sangon Biotech, Shanghai, China). The D1/D2 domains of the LSU rRNA gene and ITS regions were amplified by PCR, and sequenced using primers NL1 and NL4 [2], and ITS1 and ITS4 [15], respectively. Both DNA strands were sequenced, and the reactions were carried out using a Dye Terminator cycle sequencing kit (Applied Biosystems, Warrington).

Phylogenetic Analysis

The sequences were compared pairwise using BLAST search [16] and then aligned with type strain sequences of the related species acquired from GenBank using the multiple alignment program CLUSTAL_X version 1.81 [17]. The alignment covered 1096 characters, of which 694 were constant, 125 were phylogenetically uninformative and 277 were informative. Phylogenetic and molecular evolutionary analyses were conducted using the MEGA software version 5.0 [18]. The evolutionary distance data were calculated from Kimura's two-parameter model in the neighbour-joining analyses [19, 20]. The heuristic search (close-neighbour-interchange) was used in the maximum parsimony analyses. Gaps and missing data were treated as complete deletion. *Yamadazyma triangularis* NRRL Y-5714 ^{T} was set as the outgroup. Confidence limits were estimated from bootstrap analysis (1000 replicates) [21], and only values above 50 % were recorded on the resulting tree. Reference sequences were retrieved from GenBank under the accession numbers indicated in the tree.

Results and Discussion

Yeast Isolation and Diversity

In this work, 53 yeast strains were isolated from 17 rotten wood samples collected in the Dushan Forest Park. By comparison of the D1/D2 domains of the LSU rRNA gene and ITS sequences for all isolates for rapid identification, 50 isolates present in the samples were identified as 13 known species, wherein *Candida chauliodes*, *Candida fructus*, *Candida maltosa*, *Hanseniaspora uvarum*,

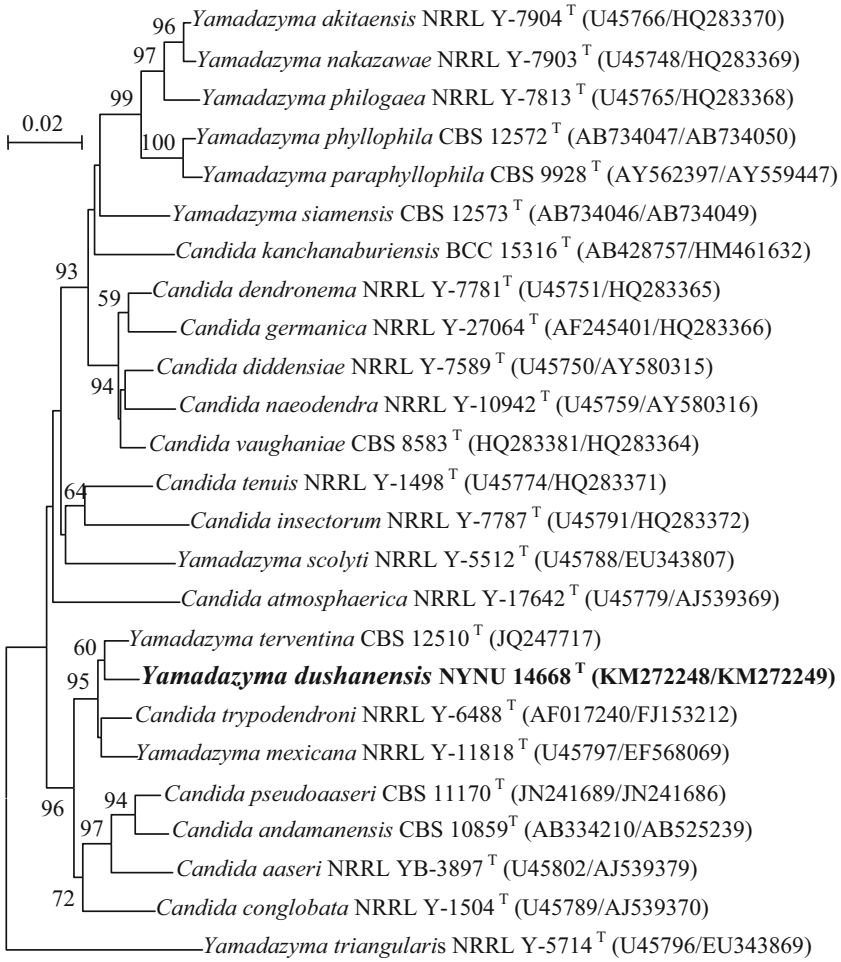

Fig. 1 Phylogenetic tree derived from neighbour-joining analysis based on the combined sequences of the D1/D2 domains of the LSU rRNA gene and ITS regions, depicting the relationships of *Yamadazyma dushanensis* sp. nov. with its nearest phylogenetic relatives. *Yamadazyma triangularis* NRRL Y-5714 T was used as an outgroup. Bootstrap values of above 50 % are given at nodes based on 1000 replications. The bar represents two substitution per 100 nucleotides

Kluyveromyces lactis, *Lachancea kluyveri*, *Pichia membranifaciens*, *Prototheca zopfii*, *Saccharomyces cerevisiae*, *Saccharomycopsis selenospora*, *Scheffersomyces lignicola*, *Torulaspora delbruecki*, and *Trichosporon siamense*. The remaining three strains, including NYNU 14656, NYNU14668^{T} and NYNU14669, which could ferment cellobiose, were distinct from any previously described species based on the sequence comparisons of the D1/D2 domains of the LSU rRNA gene and the ITS regions.

Novel Species Delineation, Identification and Ecology

The three strains of *Y. dushanensis* sp. nov. shared identical sequences in both D1/D2 and ITS regions. Sequence analyses of the D1/D2 domains of the LSU rRNA gene revealed that this new species was closely related to species in the *Yamadazyma* clade (Debaryomycetaceae and Saccharomycetales). In terms of pairwise sequence similarity, the close relatives of *Y. dushanensis* sp. nov. include *Y. terventina*, *Y. mexicana* and *C. trypodendroni*. The D1/D2 sequences of the novel species differed by five nt substitutions and two gaps from *Y. terventina*, by six nt substitutions from *Y. mexicana*, and by five nt substitutions and one gap from the later species. In general, the strains of a species show no more than 0–3 nt differences (0–0.5 %). Strains showing six (1 %) or greater substitutions are usually separate species, whereas strains with an intermediate number of nucleotide substitutions are also generally considered as separate species [2, 22–24]. For further assessment of genetic separation, the ITS sequences of the three strains of novel species were analysed. In the ITS regions, the sequence of this new species significantly differed with 12 nt substitutions and three gaps from *Y. terventina*, 17 nt substitutions and six gaps from *Y. mexicana*, and 18 nt substitutions and 10 gaps from *C. trypodendroni*, respectively. Groenewald et al. also observed that only 2 nt substitutions in the D1/D2 domain may represent different taxa of the *Yamadazyma* clade, where 9 or more nucleotide differences can be found between the ITS regions of these species [7]. This study clearly indicated that the D1/D2 sequence alone is insufficient for species delimitation in the *Yamadazyma* clade, and that the ITS sequence is a good alternative marker to obtain

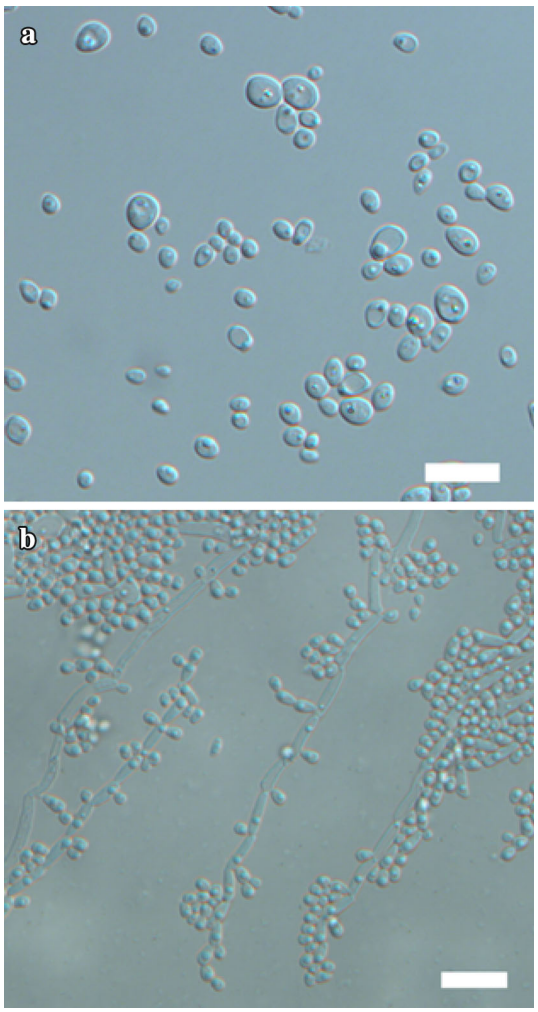

Fig. 2 Photomicrographs of *Yamadazyma dushanensis* sp. nov. NYNU 14668^T. **a** Budding cells grown on YM broth for 3 days at 25 °C. **b** Pseudohyphae formed on cornmeal agar after 12 days at 25 °C. *Bar* 10 μm

better understanding of the relatedness of the different *Yamadazyma* species, including its anamorphs.

In the neighbour-joining phylogenetic tree constructed from the combined sequences of the D1/D2 domains of the LSU rRNA gene and ITS regions, *Y. dushanensis* sp. nov. occupies a basal position with respect to *Y. terventina*, *Y. mexicana* and *C. trypodendroni* (Fig. 1). Similar result was obtained from the Maximum Parsimony analysis (results not shown). These results lend further support to the conclusion that *Y. dushanensis* sp. nov., *Y. terventina*, *Y. mexicana* and *C. trypodendroni* are regarded as closely related but different species.

Cells of *Y. dushanensis* sp. nov. were ovoid (Fig. 2a) and proliferated by multilateral budding. Pseudohyphae were formed, but true hyphae were not (Fig. 2b). The new species is not able to ferment D-xylose, but weak fermentation of cellobiose in Durham tubes was observed after 3 days. However, the strains of the new species have not been observed to produce ascospores or exhibit conjugation on the most common sporulation media (YM agar, 5 % malt extract agar, corn meal agar and YCBAS agar), either alone or in pairwise mixtures, at 15 and 25 °C for 1–4 weeks. In spite of this result, the novel species has been named *Y. dushanensis* and not *C. dushanensis*, according to the 'one fungus—one name' recommendation [25].

Yamadazyma dushanensis sp. nov. could be distinguished physiologically from *Y. terventina* with respect to its ability to assimilate D-arabinose, melibiose and raffinose, and grow at 37 °C, as well as its inability to assimilate DL-lactate. The new species also differed from *Y. mexicana* by positive assimilation of L-sorbose, growth in 10 % NaCl plus 5 % glucose, and lack of lactose assimilation. The new species was easily separated from

Table 1 Physiological characteristics that differentiate *Yamadazyma dushanensis* sp. nov. from closely related taxa

Characteristic	Yeast species			
	Y. dushanensis	*Y. terventina*[a]	*Y. mexicana*[a]	*C. trypodendroni*[a]
Assimilation of				
L-Sorbose	+	+	−	−
D-Arabinose	+	−	v	+
Melibiose	+	−	w/−	−
Lactose	−	−	+	−
Raffinose	+	−	+	+
DL-Lactate	−	+	−	−
Growth tests				
10 % NaCl 5 % glucose	−	−	+	v
37 °C	+	−	+	−

+ Positive, − negative, *d* delayed, *w* weakly positive, *v* variable

[a] Data from [4, 5, 9]

Table 2 Physiological characteristics of *Yamadazyma dushanensis* sp. nov.

Fermentation			
D-Glucose	+	Inulin	–
D-Galactose	+	Cellobiose	w
Sucrose	+	Methyl-α-D-glucoside	w
Maltose	w	Melibiose	+
Lactose	–	Melezitose	w
Raffinose	+	Starch	–
α,α-Trehalose	+	D-Xylose	–
Carbon assimilation			
D-Glucose	+	Raffinose	+
D-Galactose	+	Melezitose	+
L-Sorbose	+	Inulin	–
D-Glucosamine	+	Soluble starch	–
D-Ribose	+	Glycerol	+
D-Xylose	+	Erythritol	+
L-Arabinose	+	Ribitol	+
D-Arabinose	+	Xylitol	+
L-Rhamnose	+	L-Arabinitol	–
Sucrose	+	D-Glucitol	+
Maltose	+	D-Mannitol	+
Trehalose	+	Galactitol	–
Methyl-α-D-glucoside	+	*myo*-Inositol	–
Cellobiose	+	DL-Lactate	–
Salicin	+	Succinate	+
Arbutin	+	Citrate	+
Melibiose	–	Methanol	–
Lactose	–	Ethanol	w
Nitrogen assimilation			
Nitrate	–	Creatine	–
Nitrite	–	Creatinine	–
Ethylamine	+	Glucosamine	–
L-Lysine	+	Imidazole	–
Cadaverine	+	D-Tryptophan	+
Growth tests			
0.01 % Cycloheximide	–	Growth at 37 °C	+
10 %NaCl/5 % glucose	–	Growth at 40 °C	–
1 % Acetic acid	–		
Additional tests			
Starch formation	–	Urea hydrolysis	–
Acetic acid production	–	Diazonium blue B reaction	–

+ Positive, − negative, *w* weakly positive

Candida sergipensis based on its ability to assimilate L-sorbose and melibiose and grow at 37 °C (Table 1).

Species in the *Yamadazyma* clade are very common and could be isolated from diverse habitats, such as water, plants, animals and guts of insects and termites [4–10, 26, 27]. Many of these species have been isolated from plants, such as *Y. akitaensis*, *Y. mexicana*, *Candida diospyri*, *Candida buinensis*; whereas *Candida michaelii*, *Candida gorgasii*, *Candida lessepsii*, *Candida cerambycidarum*, *Candida endomychidarum*, *Candida amphixiae*, *Candida diddensiae*, *Candida naeodendra* and *Candida dendronema* exhibited an association with the gut of beetles and other insects [4, 5]. Until the present, no members of this clade have been reported to have been isolated from rotten wood. The presence of this novel species from rotting wood in this study may be attributed to the idea that these yeasts are being carried to rotten wood by visiting insects. Therefore, the rotten wood may be an interesting subject for further investigation of yeast in this clade.

Description of *Yamadazyma dushanensis* Hui, Wang, Ren and Li sp. nov

In YM broth after three days at 25 °C, cells are ovoid and variable in size (2–5 × 2.5–7 μm) and occur singly or in pairs (Fig. 2a). Budding is multilateral. Sediment formed after 1 month, but no pellicle was observed. On the YM agar after 3 days at 25 °C, the streak culture was butyrous, white, raised with a smooth surface and had an entire margin. In Dalmau plates after 12 days on cornmeal agar at 25 °C, pseudohyphae were formed, but true hyphae were not (Fig. 2b). No asci or signs of conjugation were observed after growth on the most common sporulation media. A summary of the physiological and other growth characteristics of *Y. dushanensis* is given in Table 2. The GenBank/EMBL/DDBJ accession numbers for the sequences of the D1/D2 domains of the LUS rRNA gene and the ITS regions of *Y. dushanensis* sp. nov. NYNU 14668^T are KM272248 and KM272249, respectively. The Mycobank deposit number is MB 811446.

The type strain NYNU 14668^T was isolated from rotten wood collected in June 2014 from Dushan Forest Park in Nanyang, Henan Province, Central China. The living culture from type was deposited at China Center of Industrial Culture Collection (CICC), Beijing, China as CICC 33051^T and Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands as CBS 13914 ^{T}.

The species name *dushanensis* (du.shan.en′sis. N.L. fem. adj.) refers to Dushan, Nanyang, Henan Province, central China, the geographical origin of the species.

Acknowledgments This work was supported by the National Natural Science Foundation of China (31370073) and the Research Planning Project of Basic and Advanced Technology of Henan Province, China (122300410032).

References

1. Billon-Grand G (1989) A new ascosporogenous yeast genus: Yamadazyma gen. nov. Mycotaxon 35:201–204

2. Kurtzman CP, Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie van Leewenhoek 73:331–371
3. Kurtzman CP, Suzuki M (2010) Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, and *Scheffersomyces*. Mycoscience 51:2–14
4. Kurtzman CP (2011) *Yamadazyma* billon-grand. In: Kurtzman CP, Fell JW, Boekhout T (eds) The yeasts, a taxonomic study, vol 2, 5th edn. Elsevier, Amsterdam, pp 919–925
5. Lachance MA, Boekhout T, Scorzetti G, Fell JW, Kurtzman CP (2011) *Candida* Berkhout. In: Kurtzman CP, Fell JW, Boekhout T (eds) The yeasts, a taxonomic study, vol 2, 5th edn. Elsevier, Amsterdam, pp 987–1279
6. Nakase T, Jindamorakot S, Ninomiya S, Imanishi Y, Kawasaki H, Potacharoen W (2008) *Candida kanchanaburiensis* sp. nov., a new ascomycetous yeast species related to *Pichia nakazawae* isolated in Thailand. J Gen Appl Microbiol 54:259–265
7. Groenewald M, Robert V, Smith M (2011) The value of the D1/D2 and internal transcribed spacers (ITS) domains for the identification of yeast species belonging to the genus *Yamadazyma*. Persoonia 26:40–46
8. Burgaud G, Arzur D, Sampaio JP, Barbier G (2011) *Candida oceani* sp. nov., a novel yeast isolated from a mid-atlantic ridge hydrothermal vent (−2300 meters). Antonie Van Leeuwenhoek 100:75–82
9. Ciafardini G, Zullo BA, Antonielli L, Corte L, Roscini L, Cardinali G (2012) *Yamadazyma terventina* sp. nov., a yeast species of the *Yamadazyma* clade from Italian olive oils. Int J Syst Evol Microbiol 63:372–376
10. Kaewwichian R, Yongmanitchai W, Kawasaki H, Wang PH, Yang SH, Limtong S (2013) *Yamadazyma siamensis* sp. nov., *Yamadazyma phyllophila* sp.nov. and *Yamadazyma paraphyllophila* sp. nov., three novel yeast species isolated from phylloplane in Thailand and Taiwan. Antonie Van Leeuwenhoek 103:777–788
11. Junyapate K, Jindamorakot S, Limtong S (2014) *Yamadazyma ubonensis* f.a., sp. nov., a novel xylitol-producing yeast species isolated in Thailand. Antonie Van Leeuwenhoek 105:471–480
12. Cadete RM, Santos RO, Melo MA, Mouro A, Gonçalves DL, Stambuk BU, Gomes FC, Lachance MA, Rosa CA (2009) *Spathaspora arborariae* sp. nov., a d-xylose-fermenting yeast species isolated from rotting wood in Brazil. FEMS Yeast Res 9:1338–1342
13. Barnett JA, Payne RW, Yarrow D (2000) Yeasts: characteristics and identification, 3rd edn. Cambridge University Press, Cambridge
14. Kurtzman CP, Fell JW, Boekhout T, Robert V (2011) Methods for isolation, phenotypic characterization and maintenance of yeasts. In: Kurtzman CP, Fell JW, Boekhout T (eds) The yeasts, a taxonomic study, 5th edn. Elsevier, Amsterdam, pp 87–110
15. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide for methods and applications. Academic Press, New York, pp 315–322
16. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
17. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DJ (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
18. 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. Mol Biol Evol 28:2731–2739
19. Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120
20. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
21. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
22. Kurtzman CP (2006) Yeast species recognition from gene sequence analyses and other molecular methods. Mycoscience 47:65–71
23. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, The Barcoding Consortium (2012) The internal transcribed spacer as a universal DNA barcode marker for Fungi. Fungal Barcoding Consortium. Proc Natl Acad Sci USA 109:6241–6246
24. Nisiotou AA, Nychas GJ (2008) *Kazachstania hellenica* sp. nov., a novel ascomycetous yeast from a Botrytis-affected grape must fermentation. Int J Syst Evol Microbiol 58:1263–1267
25. Hawksworth DL (2011) A new dawn for the naming of fungi: impacts of decisions made in Melbourne in July 2011 on the future publication and regulation of fungal names. IMA Fungus 2:155–162
26. Suh SO, Nguyen NH, Blackwell M (2005) Nine new *Candida* species near *C. membranifaciens* isolated from insects. Mycol Res 109:1045–1056
27. Ganter PF (2006) Yeast and invertebrate associations. In: Rosa CA, Peter G (eds) The yeast handbook, biodiversity and ecophysiology of yeasts, 1st edn. Springer, Heidelberg, pp 303–370