Metschnikowia sinensis sp. nov., *Metschnikowia zizyphicola* sp. nov. and *Metschnikowia shanxiensis* sp. nov., novel yeast species from jujube fruit

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Eight yeast strains were isolated from jujube fruit surfaces collected in Shanxi and Shandong Provinces, China. All eight strains produced needle-shaped ascospores under suitable conditions. Three separate groups, representing three novel species in the genus *Metschnikowia*, were recognized by sequence comparisons of the 26S rDNA D1/D2 domain and internal transcribed spacer (ITS) region. The names *Metschnikowia sinensis* sp. nov. (type strain XY103^T=AS 2.3110^T=CBS 10357^T), *Metschnikowia zizyphicola* sp. nov. (type strain XY201^T=AS 2.3111^T=CBS 10358^T) and *Metschnikowia shanxiensis* sp. nov. (type strain XY801^T=AS 2.3112^T=CBS 10359^T) are proposed for the three novel species. Phylogenetic analysis of the 26S rDNA D1/D2 domain sequence showed that these three novel species are clustered in a clade together with the previously described species *Metschnikowia fructicola*, *Metschnikowia andauensis*, *Metschnikowia pulcherrima* and *Metschnikowia chrysoperlae*.

The surface of fruit is an excellent source of naturally occurring yeast communities (Janisiewicz & Korsten, 2002). The variety of *Metschnikowia* species isolated from fruit surfaces has demonstrated a genetic diversification in the genus *Metschnikowia* under these specific environmental conditions (Davenport, 1976; Kurtzman & Droby, 2001; Péter *et al.*, 2005). Sequence analyses of the 26S rDNA D1/D2 domain and internal transcribed spacer (ITS) region among *Metschnikowia* species isolated from fruits have also indicated that further study on subgenus taxonomy for *Metschnikowia* is necessary (Giménez-Jurado *et al.*, 2003). In the present study, 138 yeast strains were isolated from jujube fruits (*Zizyphus jujuba* cultivars 'Dongzao' and 'Junzao'). Eight of them were distinguished from the other strains by conventional and chemotaxonomic characterization. On the basis of the D1/D2 domain and ITS region sequence comparisons, eight strains were classified into three groups representing three novel species in the genus *Metschnikowia*.

Jujube fruits used in this study were collected from several unmanaged orchards in Shanxi and Shandong Provinces, China, in September and October 2004. Yeast strains were isolated from the surfaces of jujube fruits by the method of Wilson *et al.* (1993). The isolates were purified by streaking and maintained on YM agar (Difco) at 4 °C. The strains studied are described in Table 1.

The morphological, physiological and biochemical characteristics of the yeast strains were determined according to standard methods (Yarrow, 1998). Assimilation of nitrogen compounds was investigated on solid media with starved inocula (Nakase & Suzuki, 1986). Sporulation was induced under the conditions recommended by Pitt & Miller (1968).

Nuclear DNA was extracted from yeast cells by using the method of Makimura *et al.* (1994). The ITS (including 5.8S rDNA) region and 26S rDNA D1/D2 domain sequences were determined according to the method described by Lu *et al.* (2004). Purified PCR products were ligated into the pBluescript SKII(+) vector (Stratagene). Recombined plasmid was transformed into *Escherichia coli* DH5 α and the inserted RNA gene was sequenced by using the ABI BigDye Terminator cycle sequencing kit on an ABI PRISM 377 DNA sequencer. Sequences were aligned with the

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the rRNA operon sequences of strains XY103^T, XY201^T and XY801^T are DQ367881–DQ367883.

Table 1. Origin of strains used in this study

Strains were isolated from fruits of *Zizyphus jujuba* in China. CBS, Centraalbureau voor Schimmelcultures, Fungal and Yeast Collection, Utrecht, The Netherlands; AS, China General Microbiological Culture Collection Center (CGMCC), Academia Sinica, Beijing, China.

Strain	Isolation source
<i>Metschnikowia sinensis</i> sp. nov.	
XY103 ^T (= CBS 10357 ^T = AS 2.3110 ^T)	cv. Junzao, Jiaocheng, Shanxi
XY102	cv. Dongzao, Wudi, Shandong
<i>Metschnikowia zizyphicola</i> sp. nov.	
XY201 ^T (= CBS 10358 ^T = AS 2.3111 ^T)	cv. Junzao, Jiaocheng, Shanxi
XY202	cv. Junzao, Jiaocheng, Shanxi
XY203	cv. Junzao, Jiaocheng, Shanxi
XY204	cv. Dongzao, Wudi, Shandong
XY205	cv. Junzao, Jiaocheng, Shanxi
<i>Metschnikowia shanxiensis</i> sp. nov.	
XY801 ^T (= CBS 10359 ^T = AS 2.3112 ^T)	cv. Junzao, Jiaocheng, Shanxi

CLUSTAL X program (Thompson *et al.*, 1997). A phylogenetic tree was constructed from evolutionary distance data calculated with Kimura's two-parameter model (Kimura, 1980) by using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap values were obtained from 1000 replications. Reference sequences were retrieved from GenBank.

Sequence analysis

Sequence analysis of the D1/D2 domain and ITS region is a useful tool for yeast species identification (Kurtzman & Robnett, 1998; Bai *et al.*, 2002; Scorzetti *et al.*, 2002; Dettman *et al.*, 2003). Previous studies of ascomycetous yeasts have demonstrated that strains with more than 1% substitution in the D1/D2 domain usually represent separate species (Kurtzman & Robnett, 1998).

In this study, three groups represented by strains XY103^T, XY201^T and XY801^T were recognized among the eight strains by D1/D2 and ITS region sequence comparisons. Strains in the same group had identical sequences in the D1/D2 and ITS regions except for strain XY204, which differed from the other strains of the same group by only one nucleotide in the ITS region. Strain XY801^T differed from strains XY103^T and XY201^T by 20 (4%) and 19 (3.8%) substitutions in the D1/D2 domain and by 4 (1.2%) and 27 (7.7%) substitutions in the ITS region, respectively. Strains XY103^T and XY201^T could be differentiated from each other by 9 (1.8%) substitutions in the D1/D2 domain and 21 (6%) substitutions in the ITS region.

A phylogenetic tree was generated from D1/D2 sequences. The phylogenetic positions of the three strains are depicted in Fig. 1. Strains XY103^T, XY201^T and XY801^T formed a statistically well-supported clade with *Metschnikowia chrysoperlae*, *Metschnikowia pulcherrima*, *Metschnikowia andauensis*, *Metschnikowia fruticola* and two undescribed *Metschnikowia* species. Saccharomycete sp. HA1406 was

related most closely to strain XY103^T, its sequence differing by 4 bp. *M. fruticola* was the closest known species to strains XY103^T and XY201^T, differing from XY103^T and XY201^T by 15 (3.0%) and 11 (2.2%) substitutions, respectively, in the D1/D2 domain. *Metschnikowia* sp. NRRL Y-6148 was the closest yeast to strain XY801^T based on its D1/D2 sequence, which was 5 bp different. The closest previously described species to strain XY801^T was *M. chrysoperlae*, which differed from XY801^T by 6 (1.2%) substitutions in the D1/D2 domain and 17 (5.3%) substitutions in the ITS region. Strains XY103^T, XY201^T and XY801^T had approximately 10–19 (2–3.8%) nucleotide substitutions from the other closely related known species, *M. andauensis* and *M. pulcherrima*, in their D1/D2 sequences (Fig. 1). In view of the number of nucleotide substitutions in the D1/D2 domain and ITS region, strains XY103^T, XY201^T and XY801^T were distinct from each other and from any previously described *Metschnikowia* species. Although saccharomycete sp. HA1406 and *Metschnikowia* sp. NRRL Y-6148 were close to strains XY103^T and XY801^T, respectively, based on the D1/D2 domain, detailed morphological and physiological characteristics of HA1406 and Y-6148 are not available (Kurtzman & Robnett, 1998; Wuckowski & Prillinger, 2004). It is difficult to determine whether they are conspecific with strains XY103^T and XY801^T.

Morphology and physiology

Three groups represented by strains XY103^T, XY201^T and XY801^T were also recognized from morphological characters and physiological tests. All the strains (Table 1) produced needle-shaped ascospores on diluted (1:19) V8 agar after incubation for 14–20 days at 17 °C. Two needle-shaped ascospores formed in a sphaeropedunculate ascus were observed in strains XY103^T, XY201^T and XY801^T (Fig. 2), which is a characteristic typical of some *Metschnikowia* species (Miller & Phaff, 1998).

Fig. 1. Phylogenetic tree derived from neighbour-joining analysis of 26S rDNA D1/D2 domain sequences, depicting the relationships of the three novel *Metschnikowia* species with closely related taxa. Bootstrap percentages over 50 % from 1000 bootstrap replicates are shown. Reference sequences were retrieved from GenBank under the accession numbers indicated.

It has been reported repeatedly that *Metschnikowia* is a physiologically homogeneous genus (Lachance *et al.*, 2001, 2003; Lachance & Bowles, 2002). However, some differential characters are still important. Strains XY103^T, XY201^T and

XY801^T were distinguished from each other by their assimilation reactions with D-xylose, salicin and methyl α -D-glucoside and the fermentation of galactose (Table 2). Strains XY103^T and XY201^T could be differentiated from the

Fig. 2. Micrographs of *Metschnikowia sinensis* sp. nov. XY103^T (a, b), *Metschnikowia zizyphicola* sp. nov. XY201^T (c, d) and *Metschnikowia shanxiensis* sp. nov. XY801^T (e, f). Micrographs show budding yeast cells grown in YM broth for 7 days at 25 °C (a, c, e) and asci with two filiform ascospores and chlamydospores produced on diluted V8 juice agar (1:19) after 2 weeks at 17 °C (b, d, f). Bars, 10 µm.

Table 2. Physiological characteristics of the three novel species and *M. pulcherrima*, *M. fructicola*, *M. chrysoperlae* and *M. andauensis*

Species: 1, *M. pulcherrima*; 2, *M. fructicola*; 3, *M. chrysoperlae*; 4, *M. andauensis*; 5, *M. sinensis* sp. nov.; 6, *M. zizyphicola* sp. nov.; 7, *M. shanxiensis* sp. nov. Data for reference species were taken from Kurtzman & Droby (2001) and Molnár & Prillinger (2005). The following characteristics are negative in all species compared: fermentation of maltose, sucrose, lactose and raffinose, assimilation of L- and D-arabinose, L-rhamnose, DL-lactate, melibiose, lactose, raffinose, inulin, starch, erythritol, methanol, galactitol, inositol and nitrate, starch production and urea hydrolysis. The following characteristics are positive in all species compared: assimilation of D-glucose, D-galactose, sucrose, melezitose, glycerol, ribitol, succinate, D-mannitol and cadaverine and growth at 30 °C. Reactions scored as: +, positive; −, negative; W, weakly positive; V, variable; D, delayed positive; NT, not tested.

Characteristic	1	2	3	4	5	6	7
Fermentation of:							
D-Glucose	V	+	+	+	+	+	+
D-Galactose	W/−	W	D/−	−	W	−	W
Assimilation of:							
L-Sorbose	V	+	+/D	+	+	+	+
D-Glucosamine	D/−	−	−	D	−	−	−
D-Ribose	V	−	W/−	−	−	−	−
D-Xylose	V	+	−	D	D/W	+	+
Maltose	V	+	+	+	+	+	+
Methyl α-D-glucoside	V	+	+/D	+	+	+	D
Cellobiose	V	+	+	+	+	+	+
Salicin	V	+	+/D	+	W	+	W
Trehalose	+	−	+	+/D	+	+	+
Citrate	V	−	D/W	−	−	−	−
Ethanol	V	+	+	+	+	+	+
Hexadecane	NT	+	NT	NT	−	−	−
Nitrite	−	NT	−	−	−	−	−
Ethylamine	V	NT	+	−	+	+	+
L-Lysine	V	NT	+	+	+	+	+
Growth:							
Without vitamins	−	−	−	−	W	+	W
At 35 °C	V	+	W	+	+	+	+
At 37 °C	−	+	NT	−	−	−	−
On 50 % D-glucose	+	NT	+	+	+	+	+

closely related species *M. fructicola* by their assimilation of trehalose and their negative reactions for assimilation of hexadecane and fermentation of galactose and their ability to grow on medium without vitamins. Strain XY801^T differed from the closely related species *M. chrysoperlae* by its ability to assimilate D-xylose, its inability to assimilate citrate and its growth on medium without vitamins. Strains XY103^T, XY201^T and XY801^T differed from *M. andauensis* and *M. pulcherrima* in assimilation reactions with D-glucosamine and ethylamine and in growth on medium without vitamins.

Molecular, morphological and physiological comparisons demonstrated that strains XY103^T, XY201^T and XY801^T represent three novel species in the genus *Metschnikowia*, for which we propose the names *Metschnikowia sinensis* sp. nov., *Metschnikowia zizyphicola* sp. nov. and *Metschnikowia shanxiensis* sp. nov.

Latin diagnosis of *Metschnikowia sinensis* Xue et Zhang sp. nov.

In medio liquido YM (Difco) *post dies* 7 *ad* 25 °C, *cellulae vegetativae globosae aut ovoidae* (4·5–7·5 × 3·7–7·5 µm), *singulae vel binae*. *Per gemmationem multipolarem reproductentes*. *Annulus et sedimentum formantur*. *Post unum mensem ad* 25 °C, *annulus et sedimentum formantur*. *In agar* YM *post unum mensem ad* 25 °C, *butyrosa, brunneusacremea, glabra, convexa*. *In agar* *farinae Zea mays* *post dies* 7 *ad* 25 °C, *pseudohyphae et hyphae verae non fiunt*. *Ascosporae fiunt in agar* V8 *post dies* 14–20 *ad* 17 °C. *Glucosum et galactosum (infirme) fermentatur at non sucrosus, maltosus, lactosus nec raffinosis*. *Glucosum, galactosum, L-sorbosum, sucrosus, maltosus, cellobiosus, trehalosus, melezitosus, D-xylosus (lente et infirme), ethanolus, glycerolus, ribitolus, D-mannitolus, methyl α-D-glucosidus, salicinus (infirme) et acidus succinicus (infirme) assimilantur at non lactosus, melibiosus, raffinosis, amylos solubilis, L-arabinosus, erythritolus, D-arabinosus, D-ribosus, L-rhamnosus, inulinus, D-glucosaminus, methanolus, galactitolus, acidus DL-lacticus, acidus citricus, inositolus nec hexadecanus*. *Ammonium sulfatum, L-lysinus, ethylaminus et cadaverinus assimilantur at non kalium nitricus nec natrium nitrosus*. *Ad crescentiam vitaminum non necessarium est (infirme)*. *Maxima temperatura crescentiae*: 35 °C. *Materia amyloidea iodophila non formantur*. *Ureum non hydrolysat*. *Typus: isolatus ex jujuba*, XY103^T, *depositus in collectione* China General Microbiological Culture Collection Center, Academia Sinica (AS 2.3110^T).

Description of *Metschnikowia sinensis* Xue & Zhang sp. nov.

Metschnikowia sinensis (sin.en'sis. N.L. fem. adj. *sinensis* pertaining to China, referring to the geographical origin of the type strain).

Growth in YM broth: after 7 days at 25 °C, cells are globose to oval, 4·5–7·5 × 3·7–7·5 µm, single or in pairs. Budding is multilateral (Fig. 2a). Spherical, heavy-walled chlamydospore-like 'pulcherrima' cells are abundant (Fig. 2b). Sediment and a ring are formed. After 1 month at 25 °C, sediment and a ring are present. Growth on YM agar: after 1 month at 25 °C, colonies are butyrous, brownish-cream, smooth and convex. Dalmau plate culture on cornmeal agar: after 7 days at 25 °C, pseudohyphae or true hyphae are not formed. Formation of ascospores: asci arising from chlamydospores are formed on diluted V8 agar (1:19) after incubation for 14–20 days at 17 °C. Asci are sphaeropedunculate, usually 19–33 µm in length, containing one

or two needle-shaped ascospores (Fig. 2b). A summary of physiological and other characteristics is given in Table 2.

The type strain, XY103^T (= AS 2.3110^T = CBS 10357^T), was isolated from jujube fruit (*Zizyphus jujuba* 'Junzao') collected in Jiaocheng, Shanxi Province, China, in September 2004.

Latin diagnosis of *Metschnikowia zizyphicola* Xue et Zhang sp. nov.

In medio liquido YM post dies 7 ad 25 °C, cellulae vegetativae globosae aut ovoidae (3·0–7·5 × 2·5–7·0 µm), singulae vel binae. Per gemmationem multipolarem reproducentes. Annulus et sedimentum formantur. Post unum mensem ad 25 °C, annulus et sedimentum formantur. In agarō YM post unum mensem ad 25 °C, butyrosa, brunneusa-cremea, glabra, convexa. In agarō farinae *Zea mays* post dies 7 ad 25 °C, pseudohyphae et hyphae verae non fiunt. Ascosporae fiunt in agarō V8 post dies 14–20 ad 17 °C. Glucosum fermentatur at non galactosum, sucrosum, maltosum, lactosum nec raffinotum. Glucosum, galactosum, L-sorbozum, sucrosum, maltosum, cellobiosum, trehalosum, melezitotum, D-xylozum, ribitolum, ethanolum, glycerolum, D-mannitolum, methyl α-D-glucosidum, salicinum et acidum succinicum (infirmum) assimilantur at non lactosum, melibiosum, raffinotum, amyllum solubile, erythritolum, inulinum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, D-glucosaminum, methanolum, galactitolum, DL-acidum lacticum, acidum citricum, inositolum nec hexadecanum. Ammonium sulfatum, L-lysinum, ethylaminum et cadaverinum assimilantur at non kalium nitricum nec natrum nitrosum. Ad crescentiam vitaminum non necessarium est. Maxima temperatura crescentiae: 35 °C. Materia amyloidea iodophila non formantur. Ureum non hydrolysat. Typus: isolatus ex jujuba, XY201^T, depositus in collectione China General Microbiological Culture Collection Center, Academia Sinica (AS 2.3111^T).

Description of *Metschnikowia zizyphicola* Xue & Zhang sp. nov.

Metschnikowia zizyphicola (zi.zy'phi.co.la. N.L. n. *Zizyphus* generic name of jujube; L. suff. -cola from L. n. *incola* inhabitant; N.L. n. *zizyphicola* inhabitant of *Zizyphus*, referring to the isolation of the type strain from jujube, *Zizyphus jujuba*).

Growth in YM broth: after 7 days at 25 °C, cells are globose to oval, 3·0–7·5 × 2·5–7·0 µm, single or in pairs. Budding is multilateral (Fig. 2c). Spherical, heavy-walled chlamydospore-like 'pulcherrima' cells are abundant (Fig. 2d). Sediment and a ring are formed. After 1 month at 25 °C, sediment and a ring are present. Growth on YM agar: after 1 month at 25 °C, colonies are butyrous, brownish-cream, smooth and convex. Dalmau plate culture on cornmeal agar: after 7 days at 25 °C, pseudohyphae or true hyphae are not formed. Formation of ascospores: asci arising from chlamydospores are formed on diluted V8 agar (1:19)

after incubation for 14–20 days at 17 °C. Asci are sphaeropedunculate, usually 20–34 µm in length, containing one or two needle-shaped ascospores (Fig. 2d). A summary of physiological and other characteristics is given in Table 2.

The type strain, XY201^T (= AS 2.3111^T = CBS 10358^T), was isolated from jujube fruit (*Zizyphus jujuba* 'Junzao') collected in Jiaocheng, Shanxi Province, China, in September 2004.

Latin diagnosis of *Metschnikowia shanxiensis* Xue et Zhang sp. nov.

In medio liquido YM post dies 7 ad 25 °C, cellulae vegetativae globosae aut ovoidae (3·7–7·5 × 2·5–7·5 µm), singulae vel binae. Per gemmationem multipolarem reproducentes. Annulus et sedimentum formantur. Post unum mensem ad 25 °C, annulus et sedimentum formantur. In agarō YM post unum mensem ad 25 °C, butyrosa, brunneusa-cremea, glabra, convexa. In agarō farinae *Zea mays* post dies 7 ad 25 °C, pseudohyphae et hyphae verae non fiunt. Ascosporae fiunt in agarō V8 post 14–20 dies ad 17 °C. Glucosum et galactosum (infirmum) fermentatur at non sucrosum, maltosum, lactosum nec raffinotum. Glucosum, galactosum, L-sorbozum, sucrosum, ribitolum, maltosum, cellobiosum, trehalosum, melezitotum, D-xylozum, ethanolum, glycerolum, D-mannitolum, methyl α-D-glucosidum (lente), salicinum (infirmum) et acidum succinicum (infirmum) assimilantur at non lactosum, erythritolum, melibiosum, raffinotum, amyllum solubile, L-arabinosum, D-arabinosum, D-ribosum, inulinum, L-rhamnosum, D-glucosaminum, methanolum, galactitolum, acidum DL-lacticum, acidum citricum, inositolum nec hexadecanum. Ammonium sulfatum, L-lysinum, ethylaminum et cadaverinum assimilantur at non kalium nitricum nec natrum nitrosum. Ad crescentiam vitaminum non necessarium est (infirmum). Maxima temperatura crescentiae: 35 °C. Materia amyloidea iodophila non formantur. Ureum non hydrolysat. Typus: isolatus ex jujuba, XY801^T, depositus in collectione China General Microbiological Culture Collection Center, Academia Sinica (AS 2.3112^T).

Description of *Metschnikowia shanxiensis* Xue & Zhang sp. nov.

Metschnikowia shanxiensis (shan.xi.en'sis. N.L. fem. adj. *shanxiensis* pertaining to Shanxi, referring to the geographical origin of the type strain).

Growth in YM broth: after 7 days at 25 °C, cells are globose to oval, 3·7–7·5 × 2·5–7·5 µm, single or in pairs. Budding is multilateral (Fig. 2e). Spherical, heavy-walled chlamydospore-like 'pulcherrima' cells are abundant (Fig. 2f). Sediment and a ring are formed. After 1 month at 25 °C, sediment and a ring are present. Growth on YM agar: after 1 month at 25 °C, colonies are butyrous, brownish-cream, smooth and convex. Dalmau plate culture on cornmeal agar: after 7 days at 25 °C, pseudohyphae or true hyphae are not formed. Formation of ascospores: asci arising from chlamydospores are formed on diluted V8 agar (1:19)

after incubation for 14–20 days at 17 °C. Asci are sphaeropedunculate, usually 22–35 µm in length, containing one or two needle-shaped ascospores (Fig. 2f). A summary of physiological and other characteristics is given in Table 2.

The type strain, XY801^T (= AS 2.3112^T = CBS 10359^T), was isolated from jujube fruit (*Zizyphus jujuba* 'Junzao') collected in Jiaocheng, Shanxi Province, China, in September 2004.

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