Full Paper

Starmerella caucasica sp. nov., a novel anamorphic yeast species isolated from flowers in the Caucasus

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Taxonomic analysis of budding yeast strains isolated from flowers of *Wisteria sinensis* (Fabales, Fabaceae) abundantly visited by flying insects, mainly bees in city parks of Baku is described. The isolates forming slightly pink colonies and propagating by budding represent a hitherto unknown yeast species for which the name *Starmerella caucasica* is proposed. The sequences of the D1/D2 domains of the large subunit rRNA genes and the ITS1-5.8S-ITS2 regions were highly similar in the isolates and indicated a close relationship with *Candida kuoi* and *Starmerella bom-bicola* in the phylogenetic analysis. *S. caucasica* can be separated from these species by its growth on glucosamine and D-tryptophan, in vitamin-free medium and at 37°C, and its inability to grow on citrate, ethylamine, cadaverine and in media supplemented with 0.01% of cycloheximide. The type strain is 11-1071.1^T. It has been deposited in Centraalbureau voor Schimmelcultures (Utrecht, the Netherlands) as CBS 12650^T, the National Collection of Agricultural and Industrial Microorganisms (Budapest, Hungary) as NCAIM Y.02030^T and the Culture Collection of Yeasts (Bratislava, Slovakia) as CCY 90-1-1^T. The GenBank accession numbers for nucleotide sequences of *S. caucasica* are JX112043 (D1/D2 domain of the 26S rRNA gene) and JX112044 (ITS1-5.8S-ITS2). Mycobank: MB 800536.

Key Words—flower-borne; osmotolerant; *Starmerella caucasica* sp. nov.; taxonomy; yeast

Introduction

Flower-visiting insects can infect the flower organs and the flower nectar with yeasts carried on their bodies or in their digestive tracts (e.g. Pozo et al., 2011; Sandhu and Waraich, 1985). The association of flowers and pollinating or non-pollinating insects feeding on pollen and nectar with yeasts has been demonstrated by numerous works over the past three decades (e.g. Lachance et al., 2001b; Limtong et al., 2012; Pozo et al., 2012; Sandhu and Waraich, 1985;

Sipiczki, 2010). Species of *Metschnikowia*, *Starmerella* and related anamorphic *Candida* species seem to be frequent colonists of insect-visited flowers (e.g. Lachance et al., 2001b; Nakase et al., 2010; Pimentel et al., 2005; Rosa et al., 2007). Most species of the *Starmerella* clade have been reported from bees or sugar-rich microhabitats associated with bees (Pimentel et al., 2005; Rosa et al., 1999, 2003, 2007; Spencer et al., 1970; Teixeira et al., 2003). This report describes a novel species of the *Starmerella* clade isolated from flowers of leguminous plants in East Caucasus.

Materials and Methods

Yeast isolation. Yeasts were isolated from aseptically dissected flowers of *Wisteria sinensis*. The excised internal parts of the flowers were macerated in

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0.5 ml sterile water and samples of the macerates were streaked on YPGA (1% yeast extract, 1% peptone, 2% glucose, 2% agar, w/v) plates without dilution. Yeast colonies were isolated from the plates after 6 days of incubation at 25°C. The isolates were purified by streaking samples of their cultures on YPGA plates and selecting individual colonies. The isolates were maintained at –80°C.

Amplification and sequencing of chromosomal regions. Genomic DNA was extracted from overnight cultures of the strains grown in YPGL broth at 25°C as described previously (Sipiczki, 2003). The extracted DNA was used for the amplification of the D1/D2 domains of the large subunit (LSU) 26S rRNA genes and the ITS1-5.8S-ITS2 regions. The primers used were NL-1 and NL-4 for the D1/D2 domains (O'Donnell, 1993), ITS1 and ITS4 for the ITS region (White et al., 1990). The amplified DNA was purified and sequenced using the amplification primers.

Sequence similarity search and phylogenetic analysis. The D1/D2 and ITS sequences of the isolates were compared with each other using the bl2seq algorithm of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). To identify similar sequences deposited in databases, a MEGABLAST similarity search was performed in the NCBI database. The sequences of the most similar hits were extracted from the database and used for phylogenetic analysis. The corresponding sequences of the type strains of the *Starmerella* clade and related species were downloaded from the CBS database (<http://www.cbs.knaw.nl/collections/BioloMICS.aspx>). For multiple alignment of sequences, the CLUSTAL W 1.7 (Thompson et al., 1994) and the MAFT version 6 (Kato and Toh, 2008) algorithms were used. The aligned sequences were analyzed with neighbor-joining and DNA parsimony methods of the PHYLIP software package, version 3.67 (Felsenstein, 2007). Confidence limits for phylogenetic trees were estimated from bootstrap analysis (1,000 replications; SEQBOOT and CONSENSE of the PHYLIP package). The trees were visualized with the TREEVIEW programme (Page, 1996).

Morphological and taxonomic examination. Colony morphology was examined on YPGA plates after 1 month of incubation at 25°C. The morphology of yeast cells was examined microscopically using overnight cultures grown at 25°C in YPGL broth and on YPGA plates. The ability to form mycelium or pseudomycelium was checked using cultures growing in thin

YPGA films sandwiched between glass slides (Sipiczki, 2011). Sporulation was tested on yeast-extract-glucose agar, malt-extract agar, cornmeal agar and Goro-dkova agar (van der Walt and Yarrow, 1984) in pure and mixed cultures (incubation at 16°C and 25°C for 3 weeks). For taxonomically relevant physiological properties, the isolates were tested using the standard taxonomic methods described by van der Walt and Yarrow (1984).

Results and Discussion

Flowers of blooming *Wisteria sinensis* plants frequently visited by bees and other flying insects were collected from 3 parks of the city of Baku (Azerbaijan) and used for yeast isolation. Most flowers contained yeasts. Although various yeast colonies emerged on the plates, yeasts showing slightly pink, smooth colonies (Fig. 1a) appeared to be dominant in 7 plant samples. One colony of this type was isolated from each plant sample for further examination. The isolates propagated by budding (Fig. 1b) and formed neither hyphae nor pseudohyphae.

The D1/D2 domains of the large subunit (LSU) 26S rRNA genes of the isolates were amplified and sequenced. Out of 7 isolates, six had identical D1/D2 do-

Fig. 1. Morphology of *Starmerella caucasica* 11-1071.1^T. (a) Colony morphology on YPGA after 1 month of incubation at 25°C. (b) Yeast cells in overnight culture growing in YPGL at 25°C. Scale bar is 5 µm.

mains that differed in one nucleotide from the corresponding sequence of the seventh isolate. The isolate 11-1071.1 representing the larger group was selected for further examination and tests. The GenBank accession numbers of the the D1/D2 sequences are JX112043 and JX481889 for 11-1071.1 and 11-1071.2, respectively.

The MEGABLAST similarity search with these sequences found no identical sequences in the NCBI database. The most similar D1/D2 hits were sequences of taxonomically uncharacterized *Candida* and *Starmerella* isolates from various substrates, including flowers. To determine the position of the isolates in the system of validly described species, the D1/D2 sequence of 11-1071.1 was compared with the corresponding sequences of the type strains of the *Starmerella* clade and related species. The Blast comparisons identified *Candida kuoi* (11 nucleotide differences from CBS 7267^T in the overlapping parts of the sequences) and *Starmerella bombicola* (23 substitutions when compared to CBS 6009^T) as the most closely related species. This close relationship is also supported by the phylogenetic analysis shown below. When the ITS regions were compared, the isolate 11-1071.1 (JX112044) differed from *S. kuoi* CBS 7267^T (HQ111058) at 38 positions (92% identity) and from *S. bombicola* CBS 6366 (HQ111054; the sequence of the type strain is not available) at 41 positions (91% identity). The ITS sequences of the other Caucasian isolates were either identical to that of 11-1071.1 or showed single nucleotide substitutions or deletions. The GenBank accession numbers of the latter sequences are JX910132 (strain 1071.2), JX910133 (strain 1071.3) and JX910134 (strain 1071.8). These results indicate that the Caucasian isolates represent a hitherto undescribed budding yeast species. This conclusion is supported by the previous studies showing that yeast strains with >1% substitution in the D1/D2 domains (Kurtzman and Robnett, 1998) and >3% substitution in the ITS regions (Nilsson et al., 2008) usually represent separate species.

Besides the revealed differences in the rRNA chromosomal regions, the Caucasian isolates turned out to differ from the type strains of any species in numerous taxonomically relevant physiological properties (Table 1). No variability of taxonomic traits was detected among the seven isolates. In spite of the close relationship with the teleomorph species *S. bombicola*, none of the isolates formed spores. They were also

mixed in pairs but conjugation was not observed.

The phylogenetic analysis of D1/D2 domain sequences confirmed the association of the Caucasian isolates with the *Starmerella* clade. The trees obtained (example in Fig. 2) had slightly different bootstrap values but identical topologies. Isolates 11-1071.1 and 11-1071.2 formed a branch with *S. bombicola*, *C. kuoi* and numerous uncharacterized isolates. Within the branch, it was separated from both species with strong statistical support. A similar analysis with the ITS1-5.8S-ITS2 sequences could not be performed because these sequences were not available for all species shown in the D1/D2 tree. To accommodate the Caucasian isolates in the taxonomic system of yeasts, the species name *Starmerella caucasica* sp. nov. Sipiczki is proposed. The alternative possibility could be to act according to the hitherto applied general rule of naming of anamorphic species with ascomycetous affinity and assign the new species to the anamorphic genus *Candida*. However, it is much more distantly related to *C. tropicalis*, the type strain of *Candida*, than to the *Starmerella* species. Since under the new International Code of Nomenclature for algae, fungi and plants “all legitimate fungal names are now treated equally for the purposes of establishing priority, regardless of the life history stage of the type” (Norvell, 2011), priority is given in this paper to a name that best reflects the phylogenetic relationships.

Although most species of the *Starmerella* clade have been described from flowers and flower-associated insects (e.g. Lachance et al., 2001a; Nakase et al., 2010; Rosa et al., 2003, 2007), their strains also occur in wine-related environments and substrates with high sugar concentration. For example, *S. bombicola* is quite common in fermenting grape must (Csoma and Sipiczki, 2008; Sipiczki et al., 2005) where its presence can favorably affect wine quality (Milanovic et al., 2012). *C. kuoi* was isolated from concentrated grape juice (Kurtzman, 2012). The association of these yeasts both with flowers and with wine-making may be attributed to their ability to tolerate high concentrations of sugar characteristic to both nectar and grape must. All three species can grow in up to 60% glucose (see below).

Somewhat surprisingly, the taxonomically uncharacterized strain AS2.4033, most closely located to *S. caucasica* on the D1/D2 phylogenetic tree (Fig. 2), was isolated neither from flower- nor from wine-related habitats but from the intestine of a mantis (Shi-An

Table 1. Physiological properties that differentiate *Starmerella caucasica* 11-1071.1^T from *Starmerella bombicola* and *Candida kuoi*.

Property	<i>Starmerella caucasica</i> 11-1071.1 ^T	<i>Starmerella bombicola</i> CBS 6009 ^{T,*}	<i>Candida kuoi</i> CBS 7267 [*]
Fermentation of			
Raffinose	–	w, d	–
Assimilation of			
L-Sorbose	+	d	d
D-Ribose	w	–, d, w	+
Maltose	w	–	–
Trehalose	w	–	–
Salicin	–	–	d
Arbutin	–	–	d
Raffinose	w	–, d, w	+
Melezitose	w	–	–
Meso-Erythritol	–	–	d
Xylitol	–, w	–	d
D-Glucitol	+	+, d, w	+
Succinate	–	d	–
Citrate	–	+	+
Ethanol	+	+	–
Quinic acid	w	–	–
Nitrate	w	–	–
Ethylamine	–	+	+
Cadaverine	–	+	+
Glucosamine	+	–	–
D-Tryptophan	+	–	–
Growth with 0.01% cycloheximide	–	+	d
Growth in vitamin-free medium	+	–	–
Acid production	+	–	–
Growth at 37°C	+	–	–

+ : growth; – : no growth; w: weak growth; d: delayed growth, ^T: type strain.

* Data from CBS (Centraalbureau voor Schimmelcultures): <http://www.cbs.knaw.nl/collections/BioloMICS.aspx>

Wang, personal communication). As mantises are exclusively predatory insects that feed on pests and other small animals, the presence of AS2.4033 cells in the intestine microflora might be interpreted as demonstrating that the yeasts of this clade can also adapt to very different habitats. However, the most plausible explanation is that the mantis had simply eaten flower-visiting insects carrying AS2.4033 cells. A detailed taxonomic examination should be performed with this strain to find out if it is conspecific with *S. caucasica*.

Numerous species of the *Starmerella* clade, including *S. bombicola* and *C. kuoi*, produce sophorolipids (Kurtzman, 2012; Kurtzman et al., 2010). Although the production of these carbohydrate-based, amphiphilic biosurfactants is not a distinctive, taxonomically relevant property of the *S. bombicola* subclade (e.g. Chen

et al., 2006; Tulloch et al., 1968), it might be useful from a biotechnological point of view to test cultures of the *S. caucasica* strains by MALDI-TOFF MS for sophorolipid production.

Latin diagnosis of *Starmerella caucasica* Sipiczki sp. nov.

In medio liquido dextrosum et peptonum et extractum levidensis continente (YPGL) post dies 3 ad 25°C, cellulae ovoidae, ellipsoideae aut globosae, 1–3 × 2–4 µm, singulae et binae, per gemmationem multipolarem reproducentes. Coloniae in agarō YPGA post unum mensem ad 25°C, pallide roseae, glabrae, butyrosae, cremae, cum margina integer. Pseudohyphae nullae; hyphae verae non fiunt. Ascosporae non fiunt post 30 dies ad 16 et 25°C in agarō acetico, agarō fa-

Fig. 2. Phylogenetic position of *Starmerella caucasica* revealed with the neighbor-joining distance method based on sequences of the D1/D2 domains of the LSU rRNA genes.

Outgroup: *Saccharomyces cerevisiae*. GenBank accession numbers are given in parentheses. Bootstrap values are from 1,000 re-samplings and are given at branch nodes. Values less than 50% are not shown. Bar, 0.1 substitutions per nucleotide position. T: type strain. NT: neotype. X: *Torulopsis bacillaris* var. *obesa* (MB353896) currently classified as *C. apicola*. Y: *Torulopsis bacillaris* var. *bacillaris* (MB429813) currently classified as *C. stellata*. Q: Type of *Candida halonitratophila* currently assigned to *C. etchellsii*. Z: Type of *Candida nodensis* currently assigned to *C. etchellsii*.

rina maydis confecto, YPGA seu medio Gorodkowae. Glucosum et sucrosus fermentantur. Non fermentantur galactosum, maltosum, trehalosum, melibiosum, lactosum, cellobiosum, melezitotum et raffinotum. Assimilantur D-glucosum, D-galactosum, L-sorbotum, D-ribosum (infirme), sucrosus, trahalosum (infirme), maltosum (infirme), raffinotum (infirme), melezitotum (infirme), glycerolum, xylitolum (variabiliter), D-glucitolum, D-mannitolum, ethanolum, acidum quinicum (infirme), D-tryptophanum, L-lysinum, D-glucosaminum et nitratum (infirme). Non assimilantur D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, cellobiosum, salicinum, arbutinum, melibiosum, lactosum, inulinum, amyllum solubile, erythritolum, ribitolum, L-arabinitolum, galactitolum, myo-inositolum, D-glucuronum, acidum DL-lacticum, acidum succinicum, acidum citricum, methanolum, nitritum, ethylaminum, cadaverinum, creatininum et imidasolum. Crescit in medio cum 60% (w/v) glucosum, medio sine vitamino et in 37°C. Non crescit in medio 100 µg cycloheximido

ml⁻¹ addito et in medio cum 1% (w/v) acidum aceticum.

Typus 11-1071.1^T (=CBS 12650^T=CCY 90-1-1^T=NCAIM Y.02030^T), isolatus ex floris *Wisteria sinensis* in Baku, Azerbaijan.

Description of *Starmerella caucasica* Sipiczki sp. nov.

Starmerella caucasica (cau'ca.si.ca N.L. nom. fem. adj. *caucasica* referring to the Caucasus, where the type strain was isolated).

After 3 days of incubation at 25°C in the liquid medium YPGL, cells are ovoid, ellipsoid or round to elongate, 1–3 × 2–4 µm, occur singly or in pairs and propagate by budding (Fig. 1b). On YPGA agar medium, after 1 month at 25°C, the colonies are pinkish, glabrous, butyrous, dull, and soft with an entire margin (Fig. 1a). Neither pseudohyphae nor true hyphae are formed. No sporulation was observed on YPGA, acetate agar, malt extract agar, corn meal agar or Gorodkova agar after 30 days of incubation at 16°C and 25°C. D-Glucose and sucrose are fermented; D-galac-

tose, maltose, trehalose, melibiose, lactose, cellobiose, melezitose and raffinose are not fermented. D-Glucose, D-galactose, L-sorbose, D-ribose (weakly), sucrose, trehalose (weakly), maltose (weakly), raffinose (weakly), melezitose (weakly), glycerol, xylitol (variably), D-glucitol, D-mannitol, ethanol, quinic acid (weakly), D-tryptophan, L-lysine, D-glucosamine (as nitrogen source) and nitrate are assimilated; D-xylose, L-arabinose, D-arabinose, L-rhamnose, cellobiose, salicin, arbutin, melibiose, lactose, inulin, starch, erythritol, ribitol, L-arabinitol, galactitol, myo-inositol, D-glucuronate, DL-lactate, succinate, citrate, methanol, nitrite, ethylamine, cadaverine, creatinine and imidazole are not assimilated. Growth in medium supplemented with 0.01% cycloheximide or with 1% acetic acid is negative. Growth in vitamin-free medium and at 60% glucose is positive. Acid production on chalk agar is positive. Maximum growth temperature is 37°C. The physiological characteristics presented in Table 2 show that *S. caucasica* and the most closely related species differ in numerous properties which allow their differentiation by conventional taxonomic tests.

The type strain is 11-1071.1^T (=CBS 12650^T=CCY 90-1-1^T=NCAIM Y.02030^T), which was isolated from a *Wisteria sinensis* flower in Baku, Azerbaijan.

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