ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 5, pp. 587–594. © Pleiades Publishing, Ltd., 2015.
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REVIEWS

Towards Reinstatement of the Yeast Genus *Zygowilliopsis* Kudriavzev (1960)

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Received November 18, 2014

Abstract—Experimental data on genetic molecular classification and identification of the yeast genus *Zygowilliopsis* are summarized. The genus is represented by at least five biological species and three varieties of *Z. californica*: var. *californica*, var. *dimennae*, and var. *fukushimae*. Biogeography, ecology and killer activity of *Z. californica* yeasts is considered. Heterogeneity of the taxonomic genus *Barnettozyma* Kurtzman et al. (2008) and the necessity for its revision are discussed.

Keywords: Zygowilliopsis, Barnettozyma, phylogeny, molecular markers, genetic hybridization analysis, hybrid fertility

DOI: 10.1134/S002626171505015X

The yeast originally described as *Zygohansenula californica* Lodder (1932), where *Zygohansenula* was considered as a subgenus of the genus *Hansenula* H. et P. Sydow, has been subjected to multiple taxonomic revisions since its description over 80 years ago. Wickerham and Burton (1948) and Wickerham (1951) assigned this species directly to the genus *Hansenula*: *H. californica* Wickerham (1951). However, the taxon description was based not on the type culture CBS 252 (NRRL Y-17395), but the strain NRRL Y-1680. Later, Lodder and Kreger-van Rij (1952) accepted the assignment of this yeast to the genus *Hansenula*. Attaching great importance to the haploid life cycle and the Saturn-like shape of spores, Kudriavzev (Kudriavzev, 1954; Kudrjawzev, 1960) assigned this yeast to the new monotype genus *Zygowilliopsis* that has a generic priority over the name *Zygohansenula*. Wickerham (1970) did not recognize the genus *Zygowilliopsis* Kudriavzev (1960) and continued to use the generic name *Hansenula* and "the type culture" NRRL Y-1680 for the yeast *Z. californica*. Due to the Saturn-like shape of the spores, von Arx et al. (1977) reclassified the yeast as *Williopsis californica* (Lodder) von Arx et al. (1977). Kurtzman (1984), being Wickerham's successor, continued to use the name *Hansenula californica* (Lodder) Wickerham. Nevertheless, seven years later, Kurtzman (1991) recognized the genus *Williopsis* Zender (1925), to which *Williopsis californica* was assigned. He reconfirmed this later (Kurtzman, 1998). However, ten years later, Kurtzman et al. (2008) created a new genus (*Barnettozyma*) based on the type species *B. populi* (Phaff et al.) Kurtzman et al. (2008) and some other species, including *B. californica* (Lodder) Kurtzman et al. (2008). According to the conventional rules, however, there was no need to create a new genus in such a case; instead, the genus *Zygowilliopsis* Kudriavzev should have been reconstituted, as was done by his predecessors. Moreover, the species *B. californica* of the earliest description was not chosen as the type species of the genus *Barnettozyma*. In the opinion of the authors themselves (Kurtzman et al. 2008), the genus *Barnettozyma* was heterogeneous when it was created and may be subjected to revision when new species are described. In our view, the genus *Komagataea* Y. Yamada et al. (1994) with the type species *K. pratensis* (Bab'eva & Reshetova) Y. Yamada et al. (1994) was unfoundedly included in it with low statistical support. In the latest yeast manual (Kurtzman, 2011), the yeast *Z. californica* is assigned to the genus *Barnettozyma*.

GENETIC RELATEDNESS OF *ZYGOWILLIOPSIS* YEASTS

Since 1980, *Zygowilliopsis* yeasts have been thoroughly studied using genetic and molecular analyses. Based on the concept of the genetic genus of ascomycetous fungi (Naumov, 1978), according to which a genus is a group of hybridizing species possessing a common mating type system, and taking into account the results of interspecies (in this period) hybridization of the yeasts *Hansenula anomala* (the type species), *H. saturnus, H. beijerinckii, H. mrakii, H. californica*, and *H. dimennae*, these yeasts were divided into three genera: *H. anomala, Williopsis saturnus* (the type species), *W. beijerinckii, W. mrakii*, and *Zygowilliopsis*

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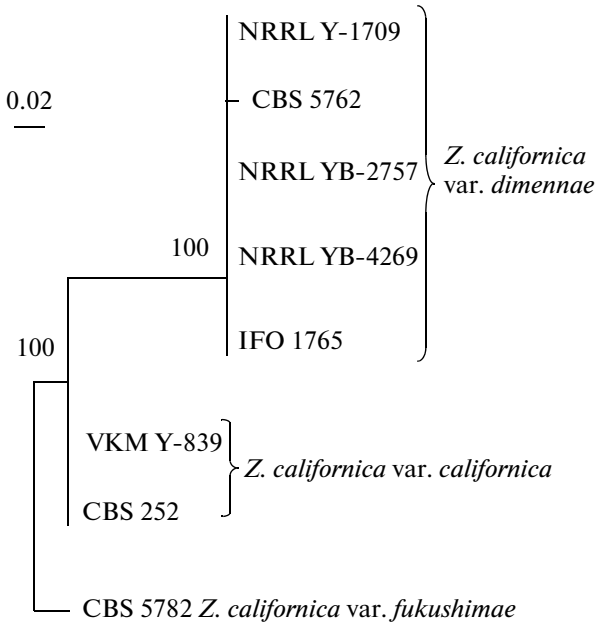

Fig. 1. Phylogenetic tree of the rDNA 5.8S-ITS regions of *Zygowilliopsis californica* (Naumova et al., 2006). The bootstrap values >70% are given. The scale corresponds to 20 nucleotide replacements per 1000 nucleotide positions.

californica/dimennae (Naumov et al., 1980). Later, based on hybrid fertility and recombination of the control markers, the yeast *H. dimennae* was reclassified with reservations as a synonym of *Z. californica* (Naumov et al., 1981). It is noteworthy that *Z. californica* × *H. dimennae* hybrids had decreased ascospore viability (17–21%) compared to intrastrain hybrids (77 and 78%, respectively). This may be indicative of a certain degree of divergence of *Z. californica* and *H. dimennae* genomes.

Taking into account that Wickerham (1970) assigned the yeasts *Endomycopsis fukushimae* Soneda and *Pichia saturnospora* Soneda to the species *Zygowilliopsis* (*Hansenula*) *californica*, these two taxa were subjected to genetic re-identification (Naumov et al., 1985). The results of genetic analysis allowed us to speak about their close relatedness to *Z. californica*. *E. fukushimae* × *Z. californica* and *P. saturnospora* × *Z. californica* hybrids were obtained. Recombination of the control markers was revealed in these hybrids with low spore viability (0–10%). Since the attempts to obtain fertile genetic lines of the parental strains (not considering *Z. californica*) were not successful, the degree of their genetic relatedness was not unambiguously established in that period.

PHYLOGENY OF *ZYGOWILLIOPSIS* YEASTS

A high degree of DNA-DNA hybridization (94–100%) made it possible to assign all the three taxa to one species *Zygowilliopsis* (*Williopsis*) *californica* (Kurtzman, 1991). Nevertheless, considerable heterogeneity of the species *Z. californica* was subsequently revealed by restriction endonuclease analysis of the amplified rDNA fragment, including the 5.8S rRNA gene and the internal transcribed spacers ITS1 and ITS2 (Naumova et al., 2006). Phylogenetic analysis of the ITS1 and ITS2 nucleotide sequences allowed the differentiation of three varieties: *Z. californica* var. *californica*, *Z. californica* var. *dimennae*, and *Z. californica* var. *fukushimae* (Fig. 1). The greatest differences were noted for ITS2: 7–26 nucleotide positions. Note that these varieties form semisterile hybrids between each other with meiotic recombination of the control markers (Naumov et al., 1985). By the example of considerable changes in the ITS2 sequence in *Z. californica* varieties, the limitations of the phylogenetic concept of the species were shown. Yamada et al. (1994), following the authors (Naumov et al., 1980), reinstated the genus *Zygowilliopsis* Kudriavzev, having extended its diagnosis. UP-PCR with the universal primers L45 and N2 and the subsequent dot hybridization with the type strain amplified DNA probe made it possible to carry out the species diagnosis of *Z. californica* (Tokareva et al., 2001). UP-PCR with the universal N2 primer and molecular karyotyping can be used to differentiate *Zygowilliopsis, Williopsis*, and *Komagatae* yeasts.

The study of molecular genetic heterogeneity of the yeast *Z. californica* was continued using the material of a large collection of strains from different parts of the world. New information was obtained from the Japanese strains IFO/NBRC 1771, IFO/NBRC 1880, IFO/NBRC 1881, IFO/NBRC 1882, and IFO 1767 (Naumova et al., 2003). According to the phylogenetic tree of the 26S rDNA D2/D2 sequences of *Zygowilliopsis* yeasts (Fig. 2), strains IFO 1881 and IFO 1882 represented one new species, whereas strain IFO 17671 was a second new species (Gazdiev, 2005). Later, these species were described under the names *Barnettozyma vustinii* Yurkov et al. (2010) and *B. sucrosica* Imanishi et al. (2010). Considering the molecular data (Kurtzman, Robnett, 1998) on the close relatedness of the yeasts *Pichia populi* and *Z. californica*, we performed hybridization analysis of the yeast *P. populi* (Naumov et al., 2009). Simultaneously, we analyzed *Zygowilliopsis* sp. (IFO 1881 and IFO 1882), *Zygowilliopsis* sp. (IFO 1767), and the divergent strain *P. populi* UCD-FS&T 68-603) from North America. According to Phaff et al. (1983), DNA–DNA reassociation of the latter strain with the type culture was 77%. Due to the capacity for hybrid formation, all the five taxa were assigned to the same genus *Zygowilliopsis*, while hybrid sterility indicated the existence of five biological species: *Z. californica, Zygowilliopsis* sp. (= *B. vustinii*), *Zygowilliopsis* sp. (= *B. sucrosica*), *Zygowilliopsis* (*Pichia*) *populi*, and *Zygowilliopsis* sp. (USD-FS&T 68-603).

The genus *Barnettozyma* was established on the basis of phylogenetic analysis of the 18S rRNA, 26S rRNA D1/D2, and the EF-1α translational elonga-

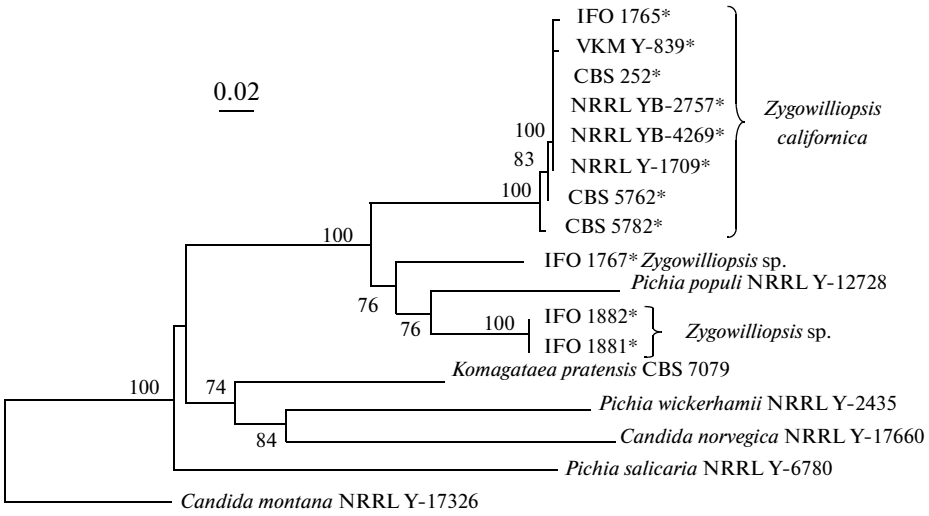

Fig. 2. Phylogenetic tree of the 26S rRNA D1/D2 nucleotide sequences of *Zygowilliopsis* yeasts (Gazdiev, 2005). The strains whose nucleotide sequences were determined in the work (Gazdiev, 2005) are marked with an asterisk. The bootstrap values >70% are given. The scale corresponds to 20 nucleotide replacements per 1000 nucleotide positions.

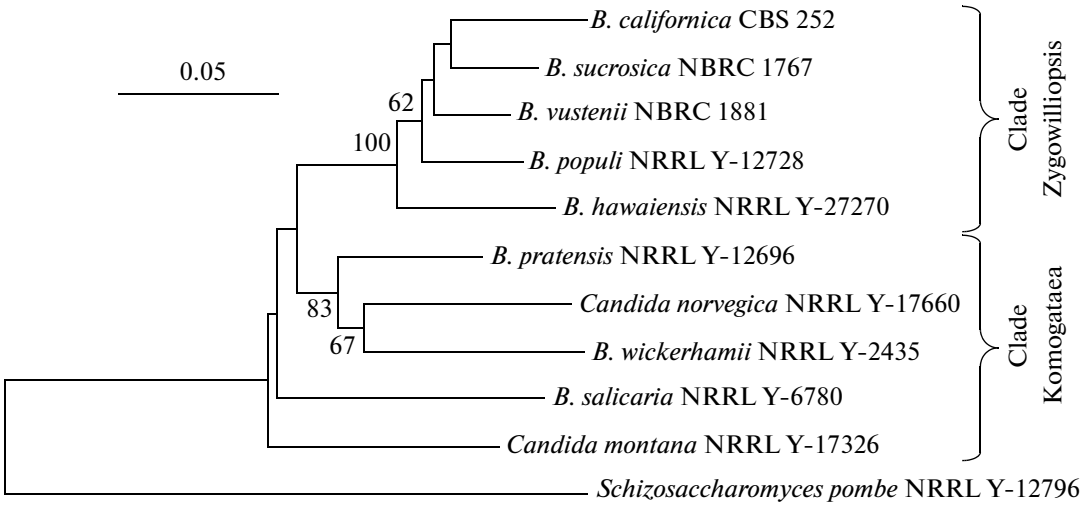

Fig. 3. Phylogenetic analysis of the 18S rRNA, 26S rRNA D1/D2, and the EF-1α translational elongation factor nucleotide sequences of *Barnettozyma* yeasts. The type culture of the yeast *Schizosaccharomyces pombe* NRRL Y-12796 was used as the outgroup. The scale corresponds to 50 nucleotide replacements per 1000 nucleotide positions. The bootstrap values >50% are given. The phylogenetic tree was constructed using the Neighbor-Joining method implemented in the MEGA 5 software package.

tion factor nucleotide sequences (Kurtzman, 2011; Kurtzman et al., 2008). Figure 3 shows the results of our phylogenetic analysis carried out on the basis of the same three markers for *Barnettozyma* yeasts, including the recently described species *B. vustinii* and *B. sucrosica* (the GenBank sequences of the three markers used are shown in Table 1). The clade Zygowilliopsis, including the species *B. californica, B. sucrosica, B. vustinii, B. populi*, and *B. hawaiensis*, is identified with 100% significance in the phylogenetic tree. In the case of phylogenetic analysis of a heterogeneous genus, the addition of new species is known to be able to change the tree topology. This is what occurred to the species *B. wickerhamii*, that was assigned to the clade Komagatae. The latter clade was divided into two groups of species: *B. pratensis, B. wickerhamii, C. norvegica* and *B. salicaria, C. montana*. It should be noted that when the genus *Barnettozyma* was proposed, Kurtzman et al. (2008) did not rule out that the discovery of new species could lead to its division into several new genera.

Table 1. Strains of the genera *Barnettozyma*, *Candida*, and *Schizosaccharomyces* used for multigenic phylogenetic analysis of the nucleotide sequences of the 18S rRNA gene, the 26S rRNA D1/D2 domain, and the EF-1α translational elongation factor

Species identity and the type strain numbers	GenBank accession no. of the sequences		
	18S	D1/D2	EF-1α
B. californica CBS 252	EF550276	U75957	EF552500
B. hawaiensis NRRL Y-27270	EF550416	AF153675	EF552502
B. populi NRRL Y-12728	EF550415	U75427	EF552501
B. pratensis NRRL Y-12696	EF550412	U75964	EF552498
B. salicaria NRRL Y-6780	EF550410	U75420	EF552496
B. sucrosica NBRC 1767	KT247988	AB525768	KT247986
B. vustenii NBRC 1881	KT247987	AB525766	KT247985
B. wickerhamii NRRL Y-2435	EF550409	U75419	EF552495
C. montana NRRL Y-17326	EF550413	U62305	EF552499
C. norvegica NRRL Y-17660	EF550411	U62299	EF552497
Sch. pombe, NRRL Y-12796	EF550486	U40085	EF552572

Abbreviated collection names: CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, United States; NBRC (ex-IFO), NITE Biological Research Center, Chiba, Japan. The species *B. sucrosica* and *B. vustinii* are not represented by the type strains.

MOLECULAR BIOGEOGRAPHY, ECOLOGY AND KILLER ACTIVITY OF *ZYGOWILLIOPSIS* YEASTS

Molecular identification of *Z. californica* strains (Naumova et al., 2003, 2006; Tokareva et al., 2001; Liu et al., 2007; Naumov et al., 2011) allows us to judge about their biogeography and ecology (Table 2). These yeasts were found in at least four regions of the world: Europe, North America, Australia, and Asia, including isolated islands (Japan, Taiwan, and New Zealand). The main source of their isolation and, probably, habitation, is the upper layer of soil and, to a lesser degree, mushrooms and the excrements of insects. *Z. californica* yeasts were often associated with the rhizosphere of cultivated plants (Vustin and Bab'eva, 1981). Note the distribution of *Z. californica* varieties (Table 2). The yeast *Z. californica* var. *californica* was usually isolated in Europe, rarer in the United States; single isolates are known from Japan, Australia, and New Zealand. The variety *Z. californica* var. *dimennae* is typical of Taiwan, and of the United States to a lesser degree; single isolates originate from Japan, India, and New Zealand, and none have been found in Europe. As for *Z. californica* var. *fukushimae*, only two isolates are known: one from Japan, the other from Taiwan. Having identified *Z. californica* strains by the 26S rRNA D1/D2 marker, we did not have an opportunity to analyze the internal transcribed ITS2/ITS2 spacers for all the strains in order to identify the varieties. The status and prevalence of *Z. californica* varieties cannot be considered to be a conclusively solved problem. The results of hybridization analysis of *Z. californica* yeasts (Naumov et al., 2009) showed normal recombination of the control markers in interstrain hybrids; hybrid fertility was decreased, but there was no essential difference in the viability of the hybrid ascospores obtained by hybridization of the yeasts of one variety or of different ones. Since the strains were isolated in different regions of the world (in Europe, North America, Far Eastern Asia, and New Zealand), it is evident that they represent divergent geographic populations of the species *Z. californica*. The high genetic relatedness of these populations allows us to consider them as only the early stages of species formation.

One of the leading factors of yeast competitiveness is killer toxins (mycocins), which are capable of changing the composition of both natural and industrial populations. The reports on the killer activity of *Zygowilliopsis* yeasts are contradictory in contrast to the ecologically and physiologically close *Williopsis* yeasts (Naumov et al., 2012). For example, one killer strain *Z. californica* SBD 399 was found (Vustin et al., 1988), whereas no killer activity was observed in 18 strains of this species from the DBVPG collection (Rosini, 2001; Industrial Yeasts Collection DBVPG, 2001). Possessing a large collection of *Z. californica* strains and the well-selected (Vustin et al., 1988) sensitive tester *Candida nitratophila* VKPM Y-740 (=VKM Y-1300=CBS 2027), we attempted to reveal the role of their killer activity (Naumov et al., 2011; Naumov, Lee, 2011). Of the 93 *Z. californica* strains of different geographic and ecological origin used, almost all were capable of synthesizing a toxin to a certain degree. The behavior of some of the strains is shown on Fig. 4. Taking into account that simultaneously with the isolation of *Z. californica* yeasts in

Table 2. Origin and the killer activity of the *Zygowilliopsis (Barnettozyma) californica* strains used

Strain no.	Source and site of isolation	Phenotype	Strain no.	Source and site of isolation	Phenotype
North American and European strains					
CBS 252[a]	Leaves, United States	K	VKM Y-168[a]	Soil, Portugal	N
NRRL Y-1709[b]	Soil, Minnesota, United States	K	VKM Y-839[a]	Blueberry juice, Kola Peninsula	K
NRRL YB-1863[b]	Forest soil, United States	K	VKM Y-1918[a]	Berries, Kola Peninsula	K
NRRL YB-1873[b]	Soil, Michigan, United States	K	SBD 398[a]	Beetroot rhizosphere, Moscow district	K
NRRL Y-1999[a]	Soil, California, United States	N	SBD 399	Carrot rhizosphere, Moscow district	K
NRRL Y-3178[a]	Elm wormhole dust, United States	K	SBD 440[a]	Cabbage rhizosphere, Moscow district	K
NRRL YB-4713[b]	Oat soil, Canada	K	SBD 3708[a]	Gut of the millipede *Pachyiulus flavipes*, Gurzuf, Russia	K
NRRL Y-1681[a]	Soil, Sweden	Kw	SBD 3712[a]	Southern chernozem soils, Russia	Kw
NRRL YB-2998[a]	Beech wormhole dust, Sweden	K			
Australian, New Zealand, and Asian strains					
CBS 5760[a]	Soil, South Australia	ND	NBRC 1765[b]	Soil, Japan	ND
CBS 5782[c]	Bear dung, Japan	ND	VKPM Y-1177	Soil, Altai, Russia	K
CBS 5762[b]	Soil, India	ND	NRRL YB-2757[b]	Fir wormhole dust, Japan	ND
NBRC 1764[a]	Soil, Japan	ND	NRRL YB-4269[b]	Soil, New Zealand	ND
NBRC 1766	Soil, Japan	ND	NRRL Y-5861[a]	Soil, New Zealand	ND
Taiwanese strains					
FN21S02	Soil, Wufeng, Hsinchu	K	GG4S04	Soil, Lugu, Nantou	
SM9S05	Soil, Jianshih, Hsinchu	K	GA5M01	Fungus *Coprinus* sp., Guosing, Nantou	K
SA4S12	Soil, Sanyi, Miaoli	N	GY1S04[b]	Soil, Jiasian, Kaohsiung	Kw
SA8S04[b]	Soil, Sanyi, Miaoli	K	GY8S02[c]	Soil, Taoyuan, Kaohsiung	Kw
SA17S04[b]	Soil, Emei, Hsinchu	K	GY8S10	Soil, Taoyuan, Kaohsiung	K
SU1S01[b]	Soil, Renai, Nantou	K	GY10S04	Soil, Taoyuan, Kaohsiung	K
SU3S01	Soil, Renai, Nantou	K	GY12S02[b]	Soil, Taoyuan, Kaohsiung	K
SU13S01[b]	Soil, Renai, Nantou	K	GY46S11	Soil, Alishan, Chia-I	K
SU20S02[b]	Soil, Renai, Nantou	K	GY47S09	Soil, Alishan, Chia-I	K
SU25S01[b]	Soil, Renai, Nantou	K	GE5S06	Soil, Rueisuei, Hualein	N
SC1S02[b]	Soil, Dasi, Taoyuan	K	GE6S05	Soil, Rueisuei, Hualein	K
SC4S01	Soil, Dasi, Taoyuan	K	GE7S06	Soil, Rueisuei, Hualein	N
SD2S17[b]	Soil, Jianshih, Hsinchu	K	GE10S03	Soil, Jhuosi, Hualein	N
SD3S05	Soil, Jianshih, Hsinchu	K	GE12S06	Soil, Jhuosi, Hualein	K

Table 2. (Contd.)

Strain no.	Source and site of isolation	Phenotype	Strain no.	Source and site of isolation	Phenotype
ES20S01[b]	Soil, Sinyi, Nantou	K	GY5M06	Fungus *Coprinus* sp., Taoyuan, Kaohsiung	K
ES24S04[a]	Soilà, Sinyi, Nantou	N	NN2S73	Soil, Yamingshan, Taipei	K
ES26S10[b]	Soil, Sinyi, Nantou	K	NN19S71[b]	Soil, Jianshih, Hsinchu	K
EN5S06[b]	Soil, Meishan, Chia-I	K	NN21S71	Soil, Jianshih, Hsinchu	K
EN6S24	Soil, Meishan, Chia-I	K	NY1S01[b]	Soil, Sinyi, Nantou	K
EN11S01	Soil, Sinyi, Nantou	K	NY2S01	Soil, Sinyi, Nantou	K
EN12S20	Soilà, Sinyi, Nantou	K	NY8S01	Soil, Sinyi, Nantou	K
EN13S02	Soil, Sinyi, Nantou	K	NY3W01	Decaying wood pulp, Sinyi, Nantou	K
EN14S04[c]	Soil, Sinyi, Nantou	K	NU10L03	Leaves *Acer kawakamii*, Taoyuan, Kaohsiung	K
EN15S02	Soil, Sinyi, Nantou	K	NU1S71	Soil, Liouguei, Kaohsiung	K
EN19S11[b]	Soil, Sinyi, Nantou	K	NU17S71	Soil, Beinan, Taitung	Kw
EN22S01	Soil, Sinyi, Nantou	K	NU3W72	Decaying wood pulp, Caotun, Nantou	K
EN23S01	Soil, Sinyi, Nantou	K	NU3M71	Fruit body of fungus, Beinan, Taitung	K
EN24S08	Soil, Sinyi, Nantou	K	NU21S02	Soil, Beinan, Taitung	K
EN27S09	Soil, Sinyi, Nantou	K	ND4S71	Soil, Taian, Miaoli	K
EN31S03	Soil, Sinyi, Nantou	K	ND5S72	Soil, Taian, Miaoli	K
GG4S14[b]	Soil, Lugu, Nantou	K	TJ15M01	Fungus *Pleurotus* sp., Sioulin, Hualein	K
GG5S03	Soilà, Lugu, Nantou	K	TJ14M03	Fungus *Auricularia polytricha*, Sioulin, Hualein	K
GG2S03	Soil, Lugu, Nantou	K	TJ11S09	Soil, Sioulin, Hualein	Kw

Phenotypes: K and Kw, the presence of strong and weak killer activity, respectively; N, the absence of killer activity; ND, no data. *Z. californica* varieties: a, var. *californica*; b, var. *dimennae*; c, var. *fukushimae*. Abbreviated collection names: VKM, All-Russian Collection of Microorganisms, Moscow; VKPM, All-Russian Collection of Industrial Microorganisms, Moscow; SBD, Soil Biology Department, Moscow University, Moscow; the names of other collections are given in the note to Table 1. The Taiwanese strains originate from the yeast collection of the National Hsinchu University of Education, Hsinchu, Taiwan.

Taiwan, the yeasts of the genus *Saccharomyces* were isolated from the same sources (Naumov and Lee, 2011; Naumov et al., 2013), we studied the action of *Z. californica* killer toxins on *Saccharomyces* yeasts. The results obtained allowed us to speak about the species specificity of *Z. californica* toxins. Although the toxins of the Taiwanese strains studied killed the test culture *C. nitratophila*, only some of them acted upon *S. cerevisiae* strains. No *Z. californica* toxins were found to which *S. kudriavzevii* were sensitive. All this considered, we assume that *S. kudriavzevii* is associated with leaf fall and soil where they can resist the action of the killer toxins of the soil *Z. californica* yeasts. It is evident that *S. cerevisiae* displaying sensitivity to *Z. californica* toxins are not able to inhabit soil and end up there accidentally. Indeed, the greater part

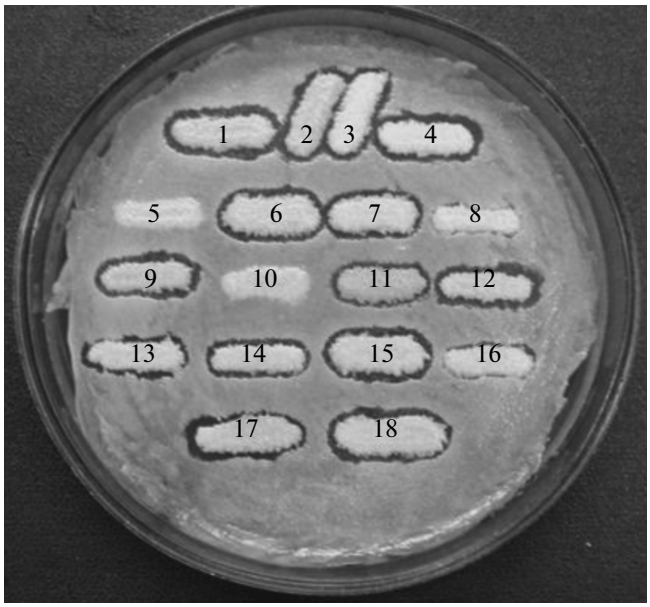

Fig. 4. Killer activity of different Zygowilliopsis californica strains (Naumov et al., 2011): (1) CBS 252; (2) NRRL Y-1709; (3) NRRL YB-1863; (4) NRRL YB-1873; (5) NRRL Y-1999; (6) NRRL Y-3178; (7) NRRL YB-4713; (8) NRRL Y-1681; (9) NRRL YB-2998; (10) VKM Y-168; (11) VKM Y-839; (12) VKM Y-1918; (13) SBD 398 (=CBS 8862); (14) SBD 440 (=CBS 8864), (15) SBD 3708; (16) SBD 3712; (17) SBD 399 (=CBS 8863); (18) VKPM Y-1177 (=SBD 3028).

of the Taiwanese strains of *S. cerevisiae*, as distinct from *S. kudriavzevii*, was isolated from the phyllosphere of plants and fungi (Naumov and Lee, 2011).

Summing up the foregoing, we want to note that under the generic name *Zygowilliopsis* Kudriavzev (1960), the species name *Z. californica* (Lodder) Kudriavzev (1960), and its sibling species, an array of data on the genetics, taxonomy, biogeography, ecology, and killer activity of these yeasts was published. Taking into account phylogenetic differentiation of the genus *Barnettozyma* Kurtzman et al. (2008) into the clades Zygowilliopsis and Komagataea, the genus *Barnettozyma* will have to be rejected and the genus *Zygowilliopsis* with numerous species will have to be reinstated. For the genus *Zygowilliopsis* to be formally reinstated, we are presently studying its new species. As far as the clade Komagataea having a low statistical support is concerned, it can formally be differentiated as a genus (a number of modern yeast genera are phylogenetically very heterogeneous). However, in order to establish the boundaries of the putative genus *Komagataea*, we need to discover new constituent species.

ACKNOWLEDGMENTS

We are grateful for fruitful cooperation to V.I. Kondratieva, N.G. Tokareva, M.M. Vustin, and D.O. Gazdiev. The study was supported by the joint Russian-Taiwan grant of the Russian Foundation for Basic Research, project no. 14-04-92001-NNS_a, and NSC, project no. 103-2923-B-134-001-MY3.

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Translated by E. Babchenko