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THE METHYLOTROPHIC YEAST *OGATAEA (HANSENULA) POLYMORPHA* AS A MODEL ORGANISM FOR STUDYING REPRODUCTIVE ISOLATION

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Summary: The group of methylotrophic yeast *Hansenula (Ogataea) polymorpha* attracts attention as suitable model organism for studying various cellular processes and serves as a producer of medically important heterologous proteins. This group comprises of three independently isolated strains: CBS4732^T, NCYC495 and DL-1. The genomes of all strains are sequenced, but its taxonomic positions are matter of debates. The results of a systematic study aimed to understand the relationship between these strains are presented here. On the basis of sequence homology as well as according to our data from analysis of mating and sporulation abilities CBS4732^T and NCYC495 strains are regarded as the same species. We also attempted to identify the species position of the strain DL-1. Our results suggests that although DL-1 differed in chromosomal organization and sequence divergence from CBS4732^T and NCYC495 strains no clear cases of reproductive isolation have been found. This means that the boundary between these species is hard to define and the group of methylotrophic yeast *O. (H.) polymorpha* is promising model system for elucidating evolutionary processes acting on yeast genomes.

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Keywords: Methylotrophic yeasts; *Hansenula* (*Ogataea*) *polymorpha*; taxonomy; genome evolution.

INTRODUCTION

The group of strains historically designated as *Hansenula polymorpha* is most commonly used among the yeasts able to utilize methanol as a sole carbon and energy source. Recently the former genus *Hansenula* was renamed *Ogataea* but this new name is still not in widespread use and more investigators prefer to use the old name *Hansenula*. In attempt to avoid misunderstanding here we will use both names: *Ogataea* (*Hansenula*).

The three strains of *O.* (*H.) polymorpha* commonly used in research have independent origins and different features. Strain DL-1 (synonymous with ATCC26012, NRRL-Y-7560) was isolated from soil (Levine and Cooney, 1973). Strain NCYC495 (synonymous with

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CBS1976, ATCC14754, NRRL-Y-1789, VKM-Y-1397) was isolated from spoiled orange juice and first described as *Hansenula angusta* (Wickerham, 1951). Strain CBS4732^T (synonymous with CBS1976, ATCC 34438, NRRL-Y-5445, CCY38-22-2) was isolated from soil in Brazil (Morais and Maia, 1959). Cells of all three strains revealed good growth on methanol-containing media and performed very well in large scale fermentations (Gellissen and Melber, 1996). For these and other reasons they are regarded as perfect hosts for industrial production of various foreign proteins (Gellissen and Hollenberg, 1997, van Dijk et al., 2002). In addition these strains are widely used as model systems for various academic investigations such as peroxisome function, biogenesis and degradation (van der Klei and Veenhuis, 2002), genetic control of methanol metabolism (Yurimoto, 2011, Hartner and Glieder, 2006), nitrate assimilation (Siverio, 2002), resistance to heavy metals (Mannazzu et al., 1998) and thermostability (Reinders et al., 1999).

The genomes of all three strains have been sequenced. The CBS4732^T genome was sequenced and analyzed (Ramezani-Rad et al., 2003) but the sequence was never released into the public domain. More recently, the sequences of strains NCYC495 (accession number PRJNA50737) and DL-1 (Ravin et al., 2013) became available. The data showed that strains CBS4732^T and NCYC495 are almost identical in sequence and therefore belong to the same species, which is now called *Ogataea polymorpha* with CBS4732^T as its type strain (Kurtzman et. al., 2011). In contrast, significant sequence divergence was observed between strain DL-1 and the other two strains, indicating that DL-1 is a closely-related but different species than CBS4732^T and NCYC495. For such reasons the species name *Ogataea parapolymorpha* was proposed for DL-1 (Suh and Zhou, 2010; Kurtzman et. al., 2011).

The data from genome sequencing are not the ultimate criteria for distinguishing between species. Many authors prefer to use also a complex of genomic and functional data from crossing experiments. According to this view the existence of reproductive isolation is the most important and reliable criterion for distinguishing between species. In the case of yeast species this mean the inability of strains to copulate and resulted hybrids to be sterile or to produce unviable meiotic products (ascospores) in the cases to undergo meiosis (Naumov et al., 2000). In this study we investigate the ability of several strains belonging to various genetic collections to mate and analyzed the obtained hybrids for their viability and marker's segregation during meiosis. Our data confirm that strains from *O. (H.) polymorpha* CBS4732^T and *O. (H.) polymorpha* NCYC495 genetic collections belong to the same yeast species because they are able to copulate normally and the resulted diploids easily enter into meiotic pathway resulting in normal recombination events. Here we present evidences that the strain DL-1 is not in reproductive isolation with *O. (H.) polymorpha* CBS4732^T and NCYC495 strains. In DL-1 clear changes of genome are observed but they cause effects only on pre-mating reproductive isolation. Nevertheless, a large portion of DL-1 genome remains unchanged. This indicates the start of the initial stages

of a process leading to the separation of species. Our results suggests that the strain *Ogataea parapolymorpha* DL-1 can be regarded as a promising model organism for studying the mechanisms involved in development of reproductive isolation. The present paper reveals the potential of methylotrophic yeast from *O. (H.) polymorpha* group for analysis of initial stages of development of reproductive isolation. Studying the mechanisms involved in development of reproductive isolation will help to learn more about the origin of new species and the evolutionary pressures that created them.

MATERIALS AND METHODS

Strains

The isolation and construction of multiply marked strains of *O. (H.) polymorpha* CBS4732^T genetic background were described previously (Lahtchev et al., 2002). Strains from *O. (H.) polymorpha* NCYC495 background were published by Titorenko et al. (1995) and those from *O. (H.) polymorpha* DL-1 by Kang et al. (2002) (Table 1). Auxotrophic mutations *argY*, *ade2-88*, *ade3-5*, *leu2-2(leu1-1)*, *ura3-11*, *met4-220* and *met6,* require arginine, adenine, leucine, uracil and methionine for growth, respectively. Haploids used originate from different genetic collections and the same genes are designated by different symbols. It is important to point that the *ade11* and *leu1-1* mutations in *O. (H.) polymorpha* NCYC495 lines correspond to *ade1* and *leu2* mutations in *O. (H.) polymorpha* CBS4732^T lines. Diploid strains used originate from crosses of strains belonging to the same or different collections.

Media

The principal rich YPD medium (1% w/v yeast extract, 2% w/v peptone, and 2% w/v glucose); was used for cell propagation and strain cultivations. Diploid strains were selected on MIN medium (synthetic minimal medium: 0.67% yeast nitrogen base without amino acids (YNB) and 2% w/v glucose). Synthetic complete SC media is the MIN medium plus the following amino acids and nucleic-acids bases: arginine, adenine, leucine, uracil and methionine, added each to a final concentration of 50 μg ml^{-1}. For determination of auxotrophic phenotypes various DO (Drop out or omission) media which represent SC media lacking one of corresponding amino acids or nucleic-acids bases were used. For induction of mating and sporulation, sporulation (SPOMAL) medium containing 1% D (+) maltose only was used. All solid media were solidified with 2% agar.

Induction of mating ability and copulation protocol.

Multiple crosses on solid media were performed by the plate-to-plate mass hybridization technique. Strains with complementary auxotrophic markers were patched as parallel lines onto two plates with YPD medium and grown overnight at 37°C. Then strains were crossed by velvet replica-plating, transferred onto plates with corresponding sporulation media and incubated at 27°C for 3 days. In the final step, the mated cells were transferred by replica-plating with filter paper onto plates with MIN medium. Growth of the resulting diploids was monitored after one and two days of cultivation at 37°C. The development of

single colonies or confluent growth on crossing squares suggested completion by mating while its absence indicated sterility. The diploid nature of the selected hybrids was confirmed by several criteria: larger size of its colonies and cells, doubled DNA content, segregation of parental auxotrophic markers, and flow cytometry analysis. Resulted diploids were streaked for singe colonies and used for sporulation experiments.

Flow cytometry analysis

For flow cytometry analysis cells were cultivated in 100 ml liquid YPD or MIN medium with vigorous agitation. Cells reaching exponential growth phase were harvested, washed and fixed in 70% ethanol for 12 h at 4°C. The samples were then washed once with 50 mM sodium citrate (pH 7.5) and after treatment with a low ultrasonic dose were resuspended at 8 x 10^8 cells in 0.5 ml 50 mM sodium citrate containing 25 µL 10 mg/ml RNAse. The preparation was incubated for 2 h at 37°C and then 50 µl 20 mg/ml pepsin was added. The incubation was continued an additional 1 h at 37°C. Subsequently, cells were washed with 1 ml of PBS buffer (pH 7.5) and resuspended in 0.5 ml PBS buffer containing 16 µl/ml propidium iodide. The samples then were incubated in the dark for 12-24 h at 4°C and analyzed using a flow cytometer (Becton-Dickinson LSRII FACS analyzer). For each histogram 10,000 cells were analyzed. Two independent determinations were performed for each strain.

Random spore analysis and discrimination of auxotrophic requirements were performed as described in Lahtchev et al., 2002.

RESULTS

Mating capacity and meiotic segregation of hybrids from crosses of strains belonging to the same genetic collections

In our laboratory a lot of multiply marked *O. (H.) polymorpha* strains from three independent genetic collections: CBS4732^T (Lahtchev et al., 2002), NCYC495 (Titorenko et al.,1995) and DL-1 (Kang et al., 2002) are available (Table 1). According to the data from chromosomal pattern and genome sequencing, the strains CBS4732^T and NCYC495 belongs to one yeast species designated as *O. (H.) polymorpha,* but the strain DL-1 is regarded as very close but independent organism and the name *O. parapolymorpha* was proposed (Suh and Zhou, 2010).

The aim of this work was to test these strains for their crossing ability and to analyze segregation patterns of meiotic products. Mating experiments revealed confluent prototrophic growth on crossing squares in strains belonging to the same genetic collections CBS4732^T x CBS4732^T and NCYC495 x NCYC495 crosses (Fig. 1 A, B). These results indicate good mating ability and are in accordance with the vision that both genetic stocks belong to one yeast species *O. (H.) polymorpha*.

Cells from crossing squares were typical diploids as judjed from FACS analysis (Fig. 2 A3, B3). Diploid cells revealed high sporulation ability: 65 – 80% pyramidal asci.

When plated on sporulation SPOMAL medium the isolated diploids developed deep pink color of colonies after 3-5 days of incubations at 27°C. The data from random spore analysis are summarized

Table 1. Strains used in this study.

Strain	Parental strains	Genotype	Reference
A-W1	CBS	*ade3-5 met4-220*	Lahtchev et al., 2002
A-V2	CBS	*leu2-2 ura3-11*	
A-V4	CBS	*ade2-88 met4-220*	
B-AM	NCYC	*ade11 met6*	Titorenko et al., 1995
B-LU	NCYC	*leu1-1 ura3*	
B-L	NCYC	*leu1-1*	
C-LU	DL-1	*leu2 ura3*	Kang et al., 2002
C-ULM	DL-1	*ura3 leu2 argY*	
C-L	DL-1	*leu2*	
C-A	DL-1	*ade2*	
OP15A	A-W1 x A-V2	*ade3-5/ADE3; LEU2/leu2-2; URA3/ura3-11; met4-220/MET4*	This study
OP17B	A-V2 x A-V4	*ade2-88/ADE2; LEU2/leu2-2; URA3/ura3-11; met4-220/MET4*	
OP22	B-AM x B-LU	*ade11/ADE11; LEU1/leu1-1; URA3/ura3; met6/MET6*	
OP26	B-AM x B-L	*ade11/ADE11; LEU1/leu1-1; met6/MET6*	
SS32	B-AM x A-V2	*ade11/ADE11; LEU2/leu2-2 ; URA3/ura3; met6/MET6*	
IS12	B-AM x C-LU	*ade11/ADE11; LEU1/leu1-1; URA3/ura3-11; met6/MET6*	

in Table 3. It is evident that all parental markers being in heterozygous state appeared in meiotic products that ultimately confirm hybrid nature of diploids isolated. The segregation of allelic pairs in meiotic products revealed small deviations from normal monogenic pattern (1^+:1^-) indicating for normal meiotic recombination events. We try to cross many multiply marked strains belonging to *O (H.) prapolymorpha*-DL-1 collection. The same pre-grown and mating induction conditions as in the case of CBS4732^T x CBS4732^T and NCYC495 x NCYC495 crosses have been employed but no appearance of any prototroph growth on mating squares was observed. Additional experiments in other pre-grown media and induction conditions were also without success. This result suggests that in contrast to the NCYC495 and CBS4732^T strains, the auxotrophs isolated from DL-1 genetic stocks (DL-1 x DL-1 crosses) are not able

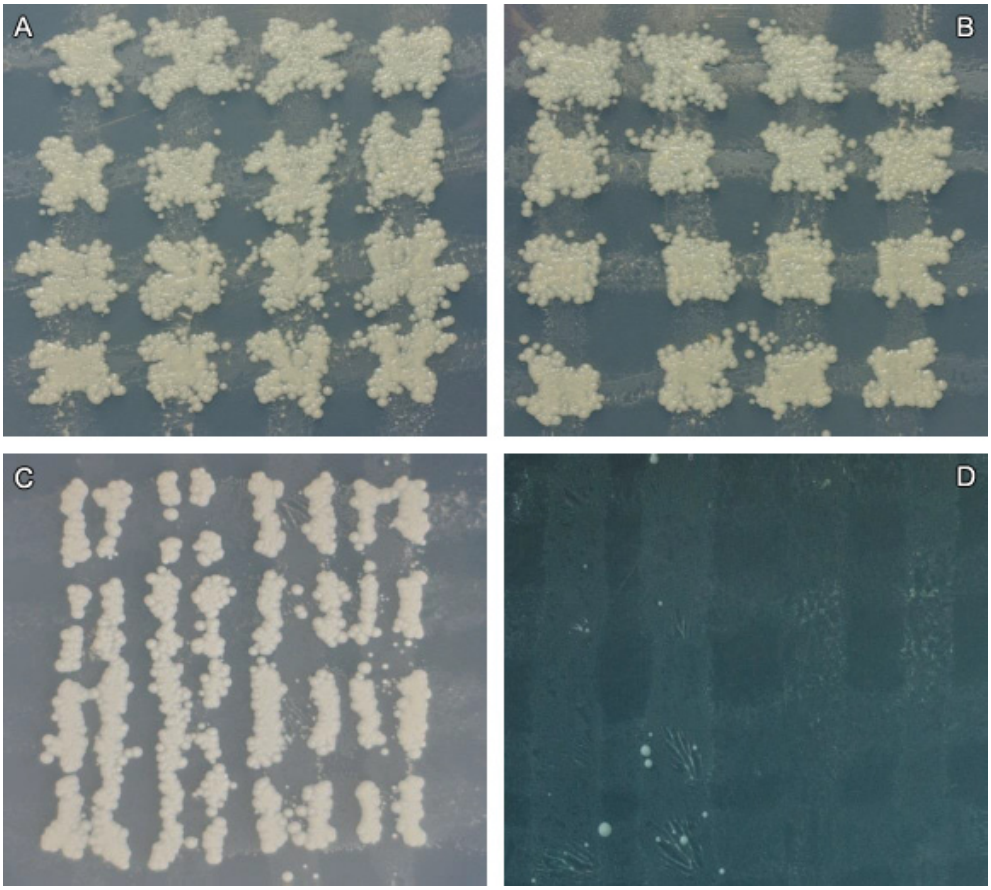

Figure 1. Mating ability of the strains tested. Haploid strains were crossed as described in "Materials and methods". A (cross CBS4732^T x CBS4732^T; strains A-W1, horizontal and A-V2); B (cross NCYC x NCYC; strains B-AM horizontal and B-LU vertical); C (cross CBS4732^T x NCYC; strains B-AM horizontal and A-V2 vertical more narrow lines); D (cross NCYC x DL-1; strains C-LU horizontal and B-AM vertical).

to mate and such sterility is supported with the former reported data (Lahtchev et al., 2002).

Mating capacity and meiotic segregation of hybrids from crosses of strains belonging to different genetic collections

A large numbers of crosses between strains from CBS4732^T genetic stock with strains from NCYC495 collection were performed. The aim of most crosses was complementation tests of mutations in genes controlling methanol metabolism or peroxisome biogenesis. Confluent prototrophic growth on crossing squares was observed in such experiments suggesting good mating between strains belonging to these two collections. A typical example for such crosses is presented on Fig. 1C. The observed prototroph confluent growth indicates good mating ability of strains belonging to different genetic collections. Besides,

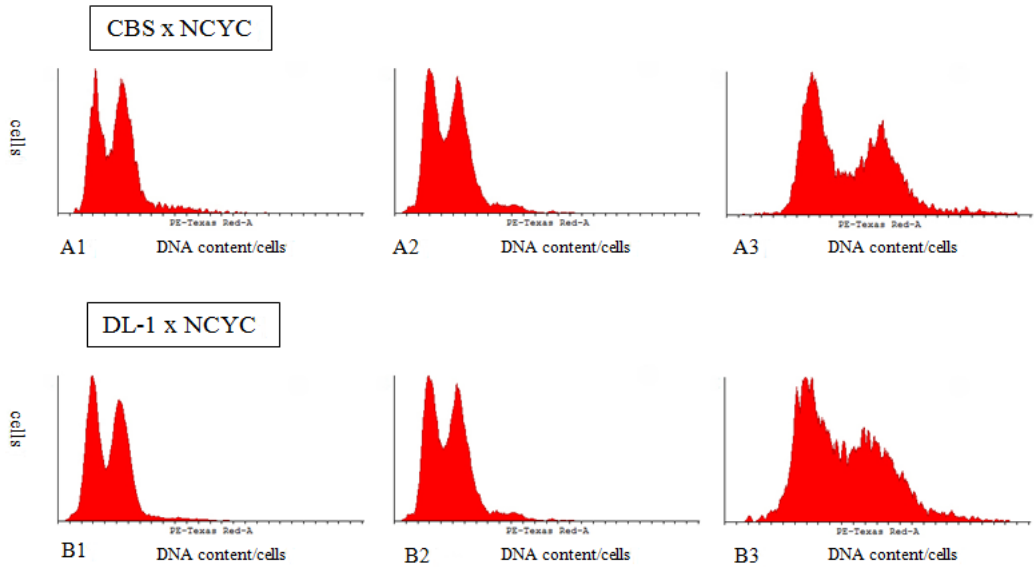

Figure 2. Histograms of DNA content per cell measured by flow cytometric analysis of *O. (H.) polymorpha* haploid (A1 and A2; B1 and B2) and diploid strains (A3 and B3).
(A) Strains involved in crosses CBS x NCYC: A1 Haploid B-AM; A2 Haploid A-V2; A3 Diploid SS32.
(B) Strains involved in crosses NCYC x DL-1: B1 Haploid B-AM; B2 Haploid C-LU; B3 Diploid IS12.

the performed ascospore analysis revealed segregation of the genetic markers matching those typical for the monogenic segregation (Table 2) and thus confirming the same genetic nature of the strains. Such mating compatibility supports the vision that strains from both collections belongs to one yeast species.

The data from analysis of allele pair segregation in meiotic products of hybrid SS32 (cross CBS4732^T x NCYC495) are presented in Table 3. In these "heterozygous" crosses normal monogenic segregations (1^+:1^-) were obtained for all of the allele pairs analyzed. Recombination tests of many other auxotrophic mutations used in our collection were also performed and similar segregation patterns were observed. These data suggests normal meiotic recombination between homologous chromosomes of strains belonging to CBS4732^T and NCYC495 collections and is additional confirmation for identity of both genomes.

We tried to cross several multiply marked stains belonging to DL-1 stock with the strains from CBS4732^T and NCYC495 stocks. The results from crosses between strains C- LU (from DL-1 collection) and B-AM (from NCYC495 stock) are presented in Fig. 1D. Very low amount of single prototrophic colonies appeared on crossing squares. The absence of confluent growth is in sharp contrast with mating obtained in crosses (CBS4732^T x CBS4732^T, NCYC495 x NCYC495 and CBS4732^T x NCYC495). This result suggests a very low mating compatibility between the strains tested. Similar results were obtained when strains from CBS4732^T stock were crossed with

Table 2. Segregation of allele pairs in meiotic products of hybrids obtained from crosses between strains from the same origin: OP15A and OP22.

	Allele pair	Number of meiotic segregants obtained		Expectation		Deviation
		(+)	(-)			
A*	*ade3-5:ADE3*	108	116	112^{+}	112	4
	leu2-2:LEU2	107	117	112^{+}	112	5
	ura3-11:URA3	106	118	112^{+}	112	6
	met4-220:MET4	100	124	112^{+}	112	12
B**	*ade11 : ADE11*	211	225	218^{+}	218^{-}	7
	leu2-2 : LEU2	221	215	218^{+}	218^{-}	3
	ura3-11: URA3	224	212	218^{+}	218^{-}	6
	met6 : MET6	213	223	218^{+}	218^{-}	5

*Hybrid OP15A ($CBS4732^{T}$ x $CBS4732^{T}$)
**Hybrid OP22 (NCYC x NCYC)

strains from DL-1 collection (data not shown).

Cells from rare colonies were streaked on solid rich medium and the single colonies obtained were tested for their abilities. They revealed good growth on rich and synthetic media and large sized colonies were formed. Microscopic inspections revealed that cells of these colonies are larger in size then parental haploids. The data from FACS analysis indicated double DNA content per cell characteristic for diploids. (Figs. 2 A3 and B3) After 4-5 days incubation at 27°C on sporulation medium these diploids revealed pink color and 45% of four spore asci. After treatment with diethyl ether a large majority of single colonies were obtained which indicated good spore viability. The results from the analysis of

Table 3. Segregation of allele pairs in meiotic products of hybrid SS32 obtained by crosses between strains from different origins: B-AM (NCYC) and A-V2 (CBS).

Allele pair	Number of meiotic segregants obtained		Expectation		Deviation
	(+)	(-)			
ade11:ADE11	108	116	112^{+}	112^{-}	4
leu2-2:LEU2	107	117	112^{+}	112^{-}	5
ura3-11:URA3	106	118	112^{+}	112^{-}	6
met6:MET6	100	124	112^{+}	112^{-}	12

Table 4. Segregation of auxotrophic markers in meiotic progeny of interspecies hybrid IS12 obtained by crossing DL-1(*leu2 ura3*) and NCYC (*ade11 met6*).

Allele pair	(+)	(-)	Deviation	Number of segregants tested
ADE11(+)*:ade11*(-)	223	9	+107	232 or 116(+); 116(-)
LEU2(+)*:leu2*(-)	115	117	+1	232 or 116(+); 116(-)
URA3(+)*:ura3*(-)	222	10	+106	232 or 116(+); 116(-)
MET6(+)*:met6*(-)	103	129	13	232 or 116(+); 116(-)

allelic pairs in segregants of hybrid IS12 (cross NCYC495 x DL-1) are presented in Table 4. These data indicate almost perfect segregation of *leu2*:*LEU2* allele pair and very close to normal segregation for *met6*:*MET6* alleles. In contrast to them big deviations were observed for *ade11:ADE11* and *ura3-11:URA3* pairs. The appearance of all parental markers in heterozygous state is strong confirmation for the hybrid nature of the isolated diploids.

DISCUSSION

The aim of our work was to make functional analysis of strains from three independent genetic stocks in attempts to elucidate their taxonomic positions. Here the results from many experiments dealing with complementation and recombination tests of genes encoding for auxotrophic markers are summarized and presented. According to the first taxonomic understanding, all three strains are members of one yeast species. Recently new data based on molecular methods have made revision of this concept and strains CBS4732^T and NCYC495 are regarded as the same species, whereas the strain DL-1 is individual organism called *O. (H.) parapolymorpha* DL-1 (Suh and Zhou, 2010; Kurtzman et. al., 2011). In our experiments irrespective of strain progeny almost similar and reproducible data were obtained concerning mating and sporulation abilities of the used strains as well as segregation patterns of investigated allele pairs. These results serve as good positive controls and are in perfect agreement with the data from full genome sequencing because they confirm the vision that both CBS4732^T and NCYC495 strains belong to the same yeast species, *O. (H.) polymorpha*.

The most unsolved and unclear question remained refers to the taxonomic position of strain DL-1. The key question concerns its species status: is this an individual organism, or does it belong to the group of CBS4732^T and NCYC495 strains? The concept of a species is central to the biological sciences but the definition of a species is controversial (Mallet 1995; Coyne and Orr 1998). The independence of DL-1 from CBS4732^T/ NCYC495 strains is supported by several molecular criteria. Determination of chromosomal number: 6 chromosomes in *O. (H.) polymorpha* CBS4732^T and NCYC495 (Waschk et al.,2002) and 7 chromosomes in *O. (H.) parapolymorpha* DL-1 (Ravin et al., 2013) indicate species independence. Such a vision is supported by data obtained from full genome sequencing suggesting 86% homology

between DL-1 and CBS4732^T/NCYC495 strains (Ravin et al., 2013). These criteria suggest that DL-1 is individual organism close to CBS4732^T/NCYC495 twins. However, for full agreement with such a statement it is necessary to satisfy the other important criterion, namely existence of reproductive isolation.

Reproductive isolation preventing gene flow between diverging populations is crucial for the process of speciation. One of the general reproductive isolation mechanisms is genetic incompatibility (Dobzhansky-Muller incompatibility), which is caused by improper interactions between genetic loci that have functionally diverged in two different species (Dobzhansky, 1937; Muller, 1942). Two forms of reproductive barriers (premating and post mating) have been identified between ascomycetous yeasts. Premating reproductive isolation is a relatively weak barrier and usually leads to reduction of hybridization rates, but hybrids still form readily when the nearest available mate is another species. Postmating reproductive isolation is stronger and caused sterility of F1 hybrids. Most gametes produced by F1 hybrids are not viable and fail to form colonies in a laboratory fertility assay.

Our data indicated strong reduction of mating ability in DL-1 x CBS4732^T/NCYC495 crosses. This can be regarded as clue for premating reproductive isolation. However, it is possible that the observed rare mating could be due to inability of MAT switching or the expression of only one of the MAT gene (MATa1/MATa2 or MATα1/MATα2) pairs (Hanson et al., 2014). Nevertheless the existence of mating ability (even very decreased!) suggests partial complementation of products encoded by the MAT genes of crossed strains. Further analysis of rare diploids obtained indicated their real hybrid nature. Both mating and sporulation processes were possible because the involved partners use the same induction and sporulation media and environmental conditions. Such similarity indicates also the absence of reproductive isolation. The isolated hybrids were viable and capable of dividing asexually in various rich and synthetic media. Our data showed that DL-1 x CBS4732^T/NCYC495 hybrids revealed normal sporulation and spore viability suggesting the absence of post meiotic reproductive isolation. In our case ascospores were viable and a normal monogenic segregation for two allele pairs (*LEU2* located in chromosome IV and *MET6* in still unidentified chromosome) was observed. This result suggests that at least these two chromosomes are collinear in both strains. For the other two makers analyzed (*URA3* chromosome I and *ADE11* in still unidentified chromosome) very big deviations from normal segregation were observed indicating low sequence homology between these chromosomes as a consequence of changes in chromosome arrangement. Our data suggests that sequence divergency did not ultimately lead to reproductive isolation in DL-1 x CBS4732^T/NCYC495 hybrids. The observed decreased mating ability can be explained by genetic complementation of mutations in genes encoding MAT switching apparatus (Maekawa and Kaneko, 2014; Hanson et al., 2014) Normal meiotic segregation of some markers indicates partial and initial steps of dividing of the DL-1 strain from *O. (H.) polymorpha* group and starting a process of reproductive isolation. This means that a boundary between these

species is hard to define and that the group of methylotrophic yeast *O.* (*H.) polymorpha* can be regarded as model system for elucidating initial steps of reproductive isolation. Such a process is still not completed and the strain DL-1 is a very suitable and promising model system. Yeasts give more advantages for such kind of studies: simple life cycle, rapid and easy cultivations in cheap media, well developed methods of classical and molecular genetics, full genome sequence data available, etc. Till now the number of such model systems is limited primary to several species belonging to *Saccharomyces cerevisiae sensu stricto* complex. This paper reveals the potential of methylotrophic yeast from *O. (H.) polymorpha* group for analysis of the initial stages of development of reproductive isolation.

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