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PRODUCERS, BIOLOGY, SELECTION, AND GENE ENGINEERING

Phylogenetic Analysis of Pectinases from Ascomycetous Yeasts

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Abstract—The paper reports on the search for the superfamily of divergent species-specific *PGU* genes in ascomycetous yeasts in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and the Sanger Institute (http://www.sanger.ac.uk) databases. It has been demonstrated that the species within the *Eremothecium*, *Galactomyces*, *Geotrichum*, *Kluyveromyces*, and *Lachancea* genera contain divergent pectinase genes. Within these genera, we observed the following levels of similarity between the nucleotide sequences of the *PGU* genes: 64.5–98.2% in *Kluyveromyces*, 72.7–81.3% in *Galactomyces/Geotrichum*, and 69–87.9% in *Eremothecium*. Polymeric *PGU* genes capable of interspecies transfer were found in *Galactomyces citri-aurantii*, *Geotrichum klebahnii*, and *Galactomyces candidus*. The importance of *PGU* genes for the diagnosis and selection of yeasts is discussed.

Keywords: pectin, endopolygalacturonase, yeast pectinase, *PGU* gene, *Eremothecium*, *Galactomyces*, *Geotrichum*, *Kluyveromyces*, *Lachancea*

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INTRODUCTION

Until very recently, the role of pectin as the source of carbon in microorganisms has obviously been underestimated. It was only the presence of the methyl group in the pectin molecule that allowed researchers to get an idea of the truly planetary scale of methanol release by plants and the role of pectin in this process. It should also be noted that a large number of methanol-utilizing yeast species associated with plants have been described. The polygalacturonic component of pectin is an important part of carbohydrate nutrition for various microorganisms, including yeasts. It is not without reason that the yeast identification guide [1] would include a differentiating test for the utilization of galacturonic acid, which is the product of the enzymatic destruction of pectin.

Pectin, one of the key plant polysaccharides, is a polymer that consists, to varying degrees, of methylated galacturonic acid residues linked by the α-1,4 glycosidic bond. The molecular weight of pectin varies from 20000 to 50000. The biochemical destruction of pectin is a complex process that involves many different enzymes [2]. The most thoroughly studied enzyme from the genetic point of view is the pectinase from the *Saccharomyces* yeasts [3]. This endopolygalacturonase (EC 3.2.1.15) catalyzes the hydrolysis of α-1,4 glycosidic bonds between the galacturonic acid residues lacking methoxyl groups with the formation of oligogalactouronates. This enzyme is used for tea and coffee processing, the cleaning of plant fiber (e.g., flax), and the clarification and filtration of fruit and berry juices, as well as grape must and wine [2].

Although the pectinolytic activity in yeasts has drawn scientists' attention since the middle of the last century [4–6], there are only a few works reporting the study of pectinase-producing yeasts [7–9]. The only exception, as mentioned above, is the group of pectinolytic yeasts from the *Saccharomyces* genus [3]. It should be noted that Russian research prioritizes the selection of wine yeasts with pectinase activity from the *Saccharomyces* genus [10, 11].

The present work continues the previously published work on the degree of homology between the nucleotide sequence of the *PGU1* gene from *Saccharomyces cerevisiae* S288c and the superfamily of the pectinase *PGU* genes in the *Saccharomyces* yeasts [3].

The goal of the current work was to find the nucleotide sequences of *PGU* genes in the genomes of ascomycetous yeasts deposited in the genomic databases on the basis of their similarity with the known nucleotide sequence of the *PGU1* gene and the corresponding amino acid sequence of the endopolygalacturonase from *S. cerevisiae* S288c and to determine the level of the intraspecies and the interspecies divergence between the identified genes.

Table 1. Genetic characteristics and origin of the ascomycetous yeasts studied in this work

Strain (collection)	Genotype	Substrate of isolation, country	GenBank accession no.	Reference
Saccharomyces arboricola H-6	–	*Quercus fabri* oak bark, China	CM001572	[12]
S. bayanus var. *uvarum*	*PGU1b*	*Mesophylax adopersus*, Spain	AACA01000043;	
MCYC 623	*PGU2b*		AACA01000682;	
	PGU3b		AACA01000194	[13]
S. bayanus CBS 395	*PGU1b*	*Ribes nigrum* juice, Netherlands	FR847037	[14]
S. cerevisiae S288c	*PGU1*	Genetic line	BK006943	[15]
S. mikatae IFO 1815	–	Soil, Japan	AABZ01000345	[13]
S. kudriavzevii IFO 1802	–	Decayed leaves, Japan	JH797534	[16]
S. paradoxus CBS 432	–	*Quercus* sp. oak bark, Russia	AABY01000004	[13]
S. pastorianus NRRLY-1551	–	Beer, Denmark	FR847040	[14]
Lachancea kluyveri NRRL Y-12651	–	*Drosophila pinicola*, United States	CM000688	[16]
Kluyveromyces marxianus NBRC1777	–	Soil, Japan	AP014599	[17]
K. marxianus KCTC17555	–	Maize dough, Mexico	KQ039398	[18]
K. marxianus DMKU3-1042	–	Selected strain	AP012213	[19]
K. marxianus CCT 7735	–	Milk factory, Brazil	CP009303	[20]
K. marxianus CECT1043	*EPG1-2*	Cream, United States	AY426825	[21]
K. marxianus BKM Y-719	*EPG1*	Grapes	AJ000076	[22]
K. wickerhamii	*PGW1*	–	AB059425	–
K. wickerhamii UCDFST 54-210	–	*Drosophila* sp., США	AEAV01000501	[23]
Galactomyces citri-aurantii IJ-1	*PG1*	Rotten citrus peel, South Korea	JQ337943	
	PG2		JQ337944	[24]
G. candidus S31	*S31PG1*	Soil near *Citrus unshiu*, Japan	AB099408	[25]
	S31PG2		AB099409	
G. geotrichum S63	*S63PG1*	As above	AB062511	[25]
G. geotrichum Ap2	*Ap2PG1*	Japan	AB083112	[26]
Geotrichum klebahnii B2	*PSE3*	Mutant of SN03 strain	D89650	[27]
Eremothecium gossypii ATCC 10895	–	*Brachynera germarii*, Iran	AE016817	[28]
E. gossypii FDAG1	–	*Oncopeltus fasciatus*, United States	CP002708	[29]
E. cymbalariae DBVPG7215	–	*Brachynera germarii*, Iran	CP002501	[30]
Eremothecium sp.	–	*Boisea trivittata*	CP006024	[31]

The strain numbers in different collections correspond as follows: H-6 = CBS10644, MCYC 623 = CBS7001, KCTC 17555 = CBS 6556, UCD 54-210 = CBS 2745, ATCC 10895 = CBS 1095, DBVPG 7215 = CBS 270.75, and CCT 7735 = UFV-3. The following abbreviations are used for the collection names: VKM—All-Russia Collection of Microorganisms, Moscow; ATCC—American Type Culture Collection, Manassas, United States; CBS—Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands; CECT—Spanish Type Culture Collection, University of Valencia, Spain; DBVPG—Plant Biology Department, University of Perugia, Italy; DMKU— Microbiology Department, Kasetsart University, Bangkok, Thailand; IFO (=NBRC)—Institute for Fermentation, Osaka Japan; KCTC—Korean Collection for Type Cultures, Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea; MCYC—Microbiological Department, Higher Technical School of Agricultural Engineering, Polytechnic University, Madrid, Spain; NBRC (=IFO)—NITE Biological Resource Center, Osaka, Japan; NCYC—National Collection of Yeast Cultures, Norwich, United Kingdom; NRRL – Northern Region Research Center, Peoria, United States; UCDFST—Department of Food Science and Technology, University of California, Davis, California, United States; UFV—Federal University of Viçosa, Brazil. ACM Y-719 is an incorrect number for the *K. marxianus* strain; in fact, the *Torulaspora delbrueckii* strain CBS 158 is deposited under this number in the All-Russia Collection of Microorganisms. (–)—yeast genotype or source is unknown.

MATERIALS AND METHODS

The characteristics of the 26 analyzed ascomycetous yeasts, including their origin, are listed in the table.

BLAST was used to search for sequences homologous to the open reading frame (1086 bp) of the known nucleotide sequence of the *PGU1* gene from the *S. cerevisiae* S288c yeast (GenBank accession no. BK0069431) in the GenBank ((http://www.ncbi.nlm.nih.gov/genbank/) and Sanger Institute (http://www.sanger.ac.uk) databases. Nucleotide and amino acid sequences were aligned manually with the aid of the BioEdit software (http://www.mbio.ncsu.edu/BiEdit/bioedit.html). Phylogenetic trees were reconstructed by the neighbor-joining method with the MEGA6 software [31].

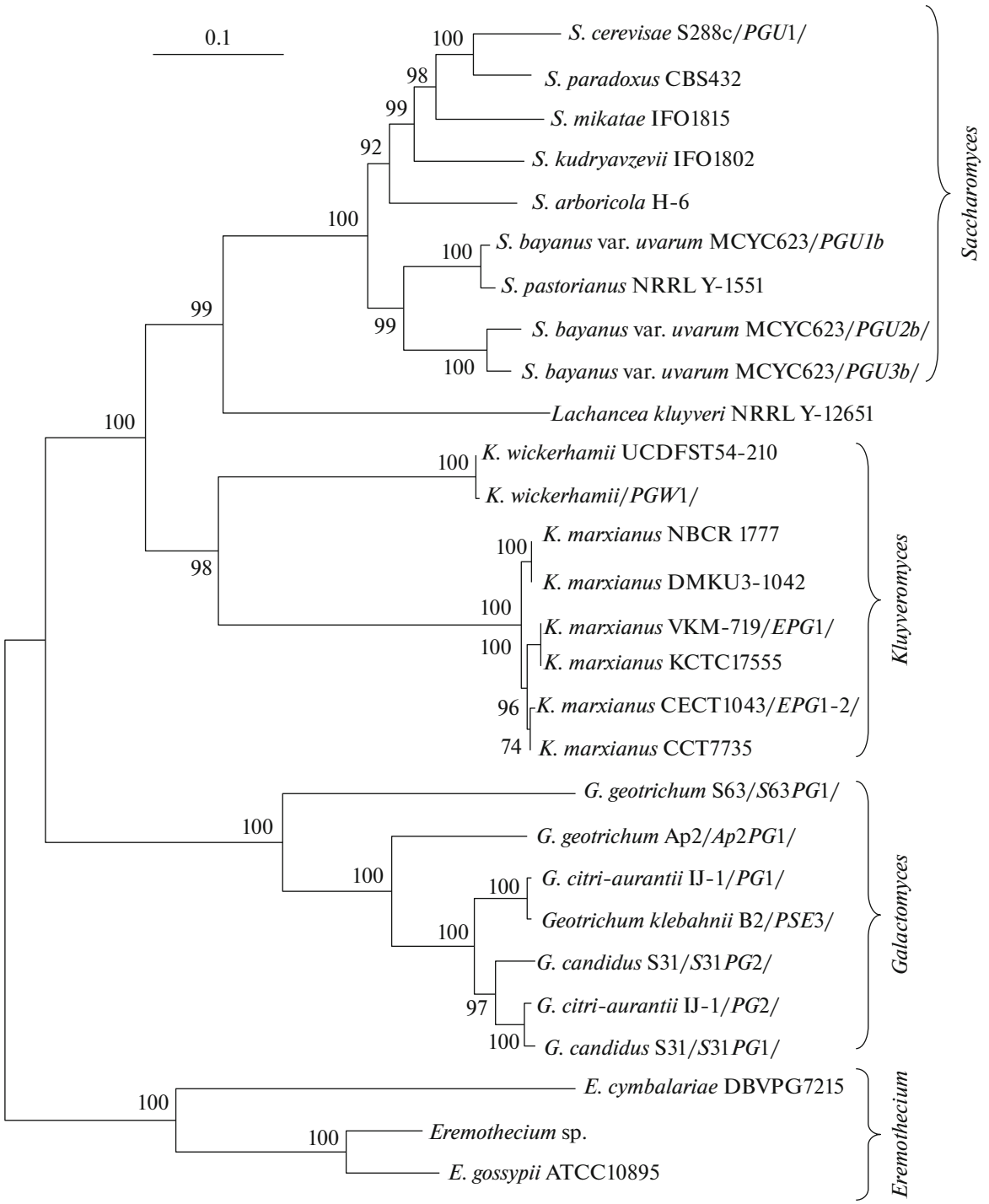

Fig. 1. Phylogenetic tree of ascomycetous yeasts reconstructed on the basis of the nucleotide sequences of the *PGU* genes. Bootstrap values greater than 70% are shown. Scale bar corresponds to 100 substitutions per 1000 nucleotides. Endopolygalacturonase genes from the *E. gossypii* strains FDAG1 and ATCC10895 are identical. In certain strains, the names for the identified *PGU* genes are provided according to the original classification in slant brackets.

RESULTS AND DISCUSSION

In our previous works [3, 32, 23], we described a superfamily of divergent species-specific *PGU* genes in 115 *Saccharomyces* strains belonging to the following yeast species: *S. arboricola*, *S. bayanus* (var. *uvarum*), *S. cariocanus*, *S. cerevisiae*, *S. kudriavzevii*, *S. mikatae*, and *S. paradoxus*, as well as to the hybrid taxon *S. pastorianus* (syn. *S. carlsbergensis*). We also demonstrated the naturally occurring interspecies *PGU* gene transfer from *S. cerevisisae* to *S. bayanus* and from *S. paradoxus* to *S. cerevisiae*.

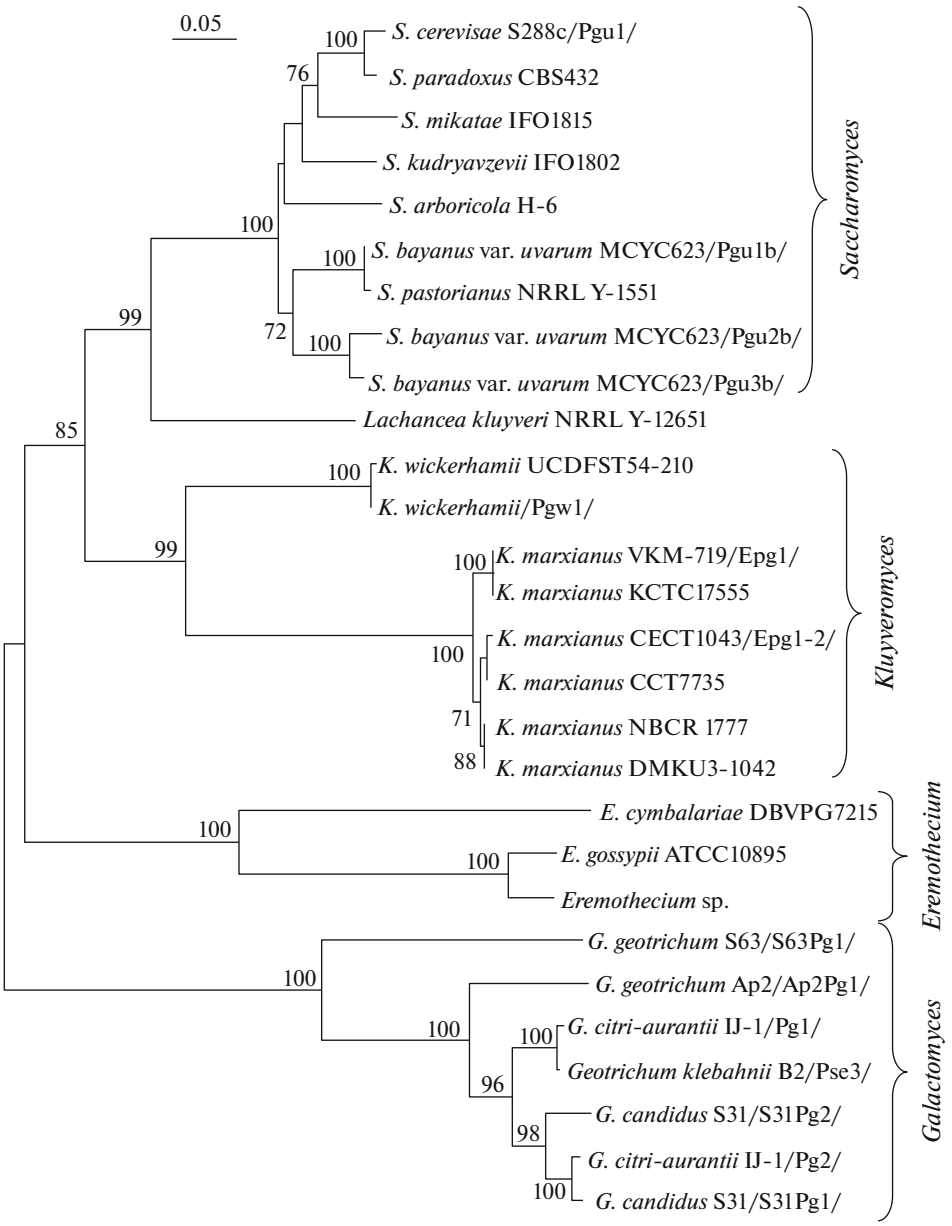

Fig. 2. Phylogenetic tree of ascomycetous yeasts reconstructed on the basis of the amino acid sequences of their Pgu endopolygalacturonases. Bootstrap values greater than 70% are shown. Scale bar corresponds to 50 substitutions per 1000 amino acid residues. Endopolygalacturonases from the *E. gossypii* strains FDAG1 and ATCC10895 are identical. For certain strains, the abbreviated names for different Pgu endopolygalacturonases are provided in slant brackets.

The phylogenetic analysis performed in the current work revealed a number of distinct clusters of *PGU* genes, which strictly follow the subdivision of the analyzed ascomycetous yeasts into the genera (Fig. 1).

The first gene cluster found in *Saccharomyces* yeasts was analyzed in detail earlier (see above). For this reason, for comparison, the phylogenetic tree presented in Fig. 1 contains only the *PGU* genes from the type strains and reference strains of the species belonging to this genus.

The individual branch representing the *PGU* gene from *Lachancea kluyveri* NRRL Y-12651 adjoins the first cluster. It seems possible that, if additional species from this polytypic genus [1] are taken into analysis, a full-fledged second cluster could be obtained.

The third cluster contains the *PGU* genes from the two *Kluyveromyces* genus species, namely *K. marxianus* and *K. wickerhamii*. We should note the high similarity level (97.7–98.2%) between the *PGU* alleles in five *K. marxianuis* strains, while the level of similarity within the entire genus is much lower (64.5%).

The fourth cluster is represented by the yeast-like fungi from the *Galactomyces* and *Geotrichum* genera, with the identity level between the corresponding

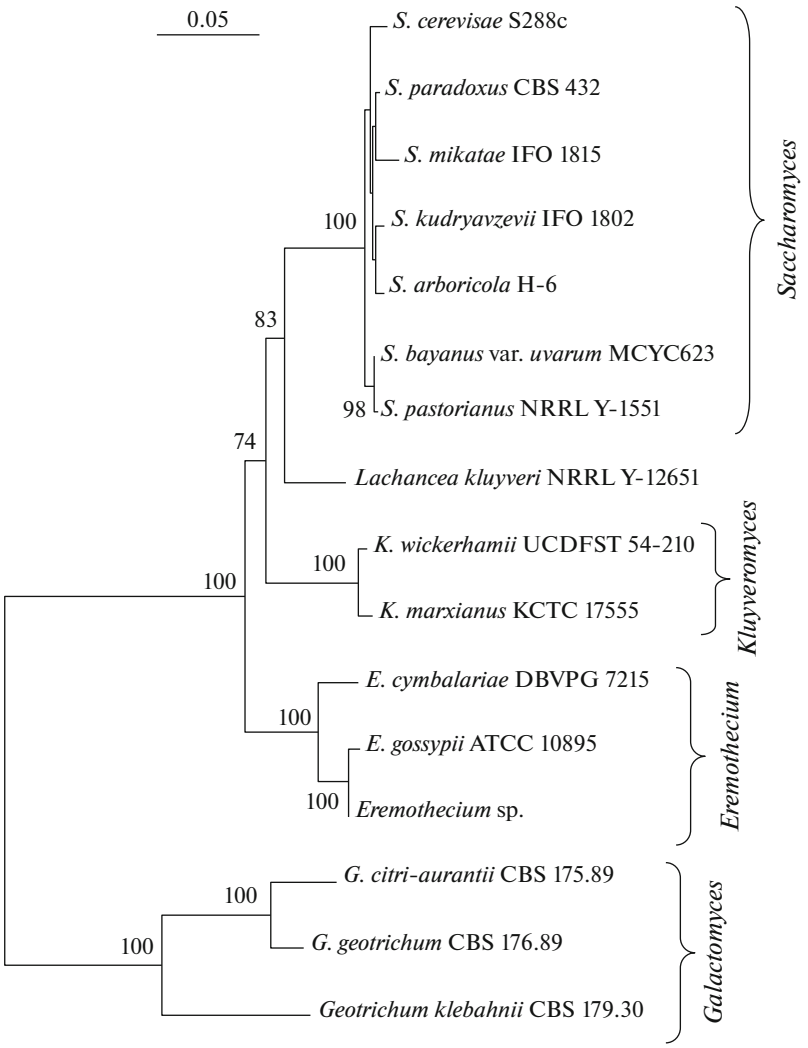

Fig. 3. Phylogenetic tree of the ascomycetous yeasts based on the nucleotide sequences of the D1/D2 region of the 26S large ribosome subunit. Bootstrap values greater than 70% are shown. Scale bar corresponds to 50 substitutions per 1000 nucleotides. The following strains are characterized by the identical D1/D2 nucleotide sequences: (1) *S. bayanus* var. *uvarum* MCYC623 and CBS 395; (2) *K. marxianus* KCTC 17555, NBRC 1777, VKM Y-719, CECT 1043, and CCT 7735.

PGU genes reaching 72.7–81.3%. Here, we should point out the appearance of divergent *PGU* genes (the presence of superfamilies) within the *Galactomyces geotrichum*, *Galactomyces citi-aurantii*, and *Galactomyces candidus* species, as well as the high similarity of the *PGU* genes observed in the following species pairs: *Galactomyces citi-aurentii* and *Geotrichum klebahnii* and *Galactomyces citi-aurentii* and *Galactomyces candidus*. The latter observation indicates the possible interspecies transfer of the *PGU* genes within the *Galactomyces/Geotrichum* genera, similar to that shown by us for *Saccharomyces* species. It should be mentioned here that *Galactomyces* yeasts basically meet the genetic concept of the genus [34, 35], according to which the species of the same genus share a common mating-type system allowing them to cross in any interspecific combinations [36, 37]. This apparently may result in the interspecies transfer of the *PGU* genes within the *Galactomyces* genus and the anamorphic *Geotrichum* species.

The fifth cluster is composed by the *PGU* genes (69–87.9% similarity) of different species of the recently expanded *Eremothecium* genus [1], namely *E. cymbalariae*, *E. gossypii*, and *Eremothecim* sp.

Figure 2 presents the results of phylogenetic analysis of the amino acid sequences of pectinases from the analyzed ascomycetous yeasts; the obtained phylogenetic tree corresponds well with that obtained on the basis of the nucleotide sequences of the *PGU* genes (see Fig. 1).

We have already mentioned above the taxonomic importance of *PGU* genes, at least for the identification of the yeast genera. The evolutionary tree of the analyzed ascomycetous yeasts based on the D1/D2 ribosome sequences presented in Fig. 3 shows good

correspondence with the divergence of the *PGU* genes in these yeast strains. When yeasts are identified based on the phenotype, it seems reasonable, along with an assessment of their ability to utilize galacturonic acid, to use Petri dish test for the presence of active pectinase, especially since it would allow primary selection of producers of this enzyme.

Another conclusion that can be made from our study, in addition to the ascertainment of the taxonomic value of *PGU* genes, is the importance of the identification of *PGU* genes. From our point of view, the analysis of pectinase genes will also allow primary screening for producers of this enzyme. It was demonstrated previously that, in filamentous fungi with high pectinase activity, such as for example *Aspergillus niger* [38] and *Sclerotina sclerotiorum* [39], as well as in the yeasts *G. citri-aurantii* [24], *G. geotrichum* [25], and *S. bayanus* var. *uvarum* [3], the genome of a single strain may contain not a single, but several, polymeric *PGU* genes. Polymeric genes normally show a cumulative effect that enhances the corresponding trait. On the other hand, only one *PGU* gene in the highly efficient pectinolytic strains, such as *K. marxianus*, may indicate that the overexpression of this gene is the result of some regulatory mechanisms, in particular, the strong promoter. The identification of such strains is important for subsequent genetic engineering work.

CONCLUSIONS

To summarize, phylogenetic analysis of the pectinase genes has great importance for the evolutionary genetics and selection of ascomycetous yeasts.

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