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EXPERIMENTAL ARTICLES

Molecular Genetic Analysis of the Yeast *Komagataea (Williopsis) pratensis* Strains Isolated from the Caucasian and Tien Shan Soils

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Abstract—The analysis of sixteen *Komagataea (Williopsis) pratensis* isolates from Caucasian and Tien Shan soils by the PCR, blot hybridization, and isoenzyme electrophoresis techniques showed that fifteen of them do belong to the species *K. pratensis*. The isolates from the two geographic areas differed in some physiological characteristics and in the PCR product profiles obtained with the microsatellite primers $(CAC)_5$ and $(GACA)_4$.

Key words: geographic populations, *Williopsis*, *Komagataea pratensis*, PCR analysis, isoenzymes.

The species *Williopsis pratensis* Bab'eva et Reshetova was first described based on the morphological and physiological analyses of 16 strains [1]. Unlike other species of the genus *Williopsis*, which have 3–4 saturn-like ascospores in one ascus, this species has one (rarely two) saturn-like ascospore in one ascus. Physiologically, the species *W. pratensis* is distinguished by its ability to ferment maltose but not glycerol. The type strain of this species, KBP 2692 (CBS 7079), has a unique rRNA gene sequence. Based on these results, a new genus *Komagataea* with the type species *K. pratensis* (Bab'eva et Reshetova) Yamada *et al.* was proposed [2]. The data of Yamada *et al.* and other authors [3, 4] give grounds to believe that the species *K. pratensis* is phylogenetically different from other species of the genus *Williopsis*. This belief is supported by the data of Drissi *et al.* [5], who showed that the mitochondrial DNAs of different *Williopsis* species hybridize with the ORF1 of *W. mrakii* (Wickerham) Krasil'nikov and related species *W. suaveolens* (Klocker) Naumov *et al.*, *W. beijerinckii* (van der Walt) Naumov et Vustin, and *W. sargentensis* (Wickerham et Kurtzman) Naumov. At the same time, this ORF1 did not hybridize with the mitochondrial DNA of *K. pratensis*. Furthermore, the DNA–DNA reassociation rates between *K. pratensis* and the species of the genus *Williopsis* were as low as 5–17% [6].

In spite of this ample evidence, the genus *Komagataea* has not yet been officially recognized [7]. Nevertheless, in this paper, we will adhere to the classification of *K. pratensis* proposed by Yamada *et al.* [2].

A recent trend in yeast taxonomy is to use, along with traditional morphological and physiological analyses, the molecular genetic methods of analysis and classification, such as the polymerase chain reaction (PCR) technique with nonspecific primers possessing random nucleotide sequences, PCR with randomly amplified polymorphic DNA (RAPD-PCR), PCR with universal primers (UP-PCR) [8–12], and isoenzyme analysis [13–15]. These approaches, however, have not yet been used for the analysis of *K. pratensis* except for the type strain *K. pratensis* CBS 7079, which was analyzed earlier by the DNA reassociation and ribosomal gene sequencing techniques.

The aim of the present study was to conclusively identify the *K. pratensis* isolates with the aid of the universal N21 primer and to analyze the intraspecies polymorphism of this species by the PCR techniques and isoenzyme electrophoresis.

MATERIALS AND METHODS

Experiments were carried out with 16 *K. pratensis* strains (table), of which 11 strains (KBP 2678 through KBP 2688) were isolated from dark gray mountain soil, which was collected at an altitude of 1500 m in the deciduous forest belt of the Trans-Ili Alatau of the northern Tien Shan [16] and five strains (KBP 2689, KBP 2690, KBP 2691, KBP 2694, and the type strain *K. pratensis* KBP 2692 (= CBS 7079)[1], were isolated from mountain-meadow soil collected at an altitude of 2200 m in the subalpine zone of the Kavkazsky Nature Reserve in Dagestan (the Russian Federation) [17]. *W. saturnus* (Klocker) Zender CBS 5761 and the type

[1] KBP, collection of yeasts of the Department of Soil Biology of Moscow State University; and CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands.

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strain *W. mrakii* CBS 1707 were used as the reference strains.

The strains were cultivated at 28°C on a complete YPG agar medium containing (g/l) glucose, 20; peptone, 10; yeast extract, 10; and agar, 20. Physiological tests were performed by standard methods [7].

Yeast susceptibility to the killer toxin of strain *Pichia punctispora* VKM Y-1363 was assayed at 18°C on glucose–peptone agar with glycerol (50 ml/l) prepared using sodium-succinate buffer (pH 4.5) [18].

PCR-based analyses. PCR analysis with the N21 primer (5'-GGATCCGAGGGTGGCGGTTCT-3') was performed using a Techne PHC-2 thermal cycler under the conditions described earlier [11].

PCR analyses with the microsatellite primers $(CAC)_5$, $(GACA)_4$, and $(GTG)_5$ and with the universal M13 primer (5'-GAGGGTGGCGGTTCT-3') were carried out in 20 μl of a reaction mixture containing 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.1 U/μ Super-Taq DNA polymerase, 20–200 ng of template DNA, and 20–30 ng of particular primer. Each of the 30 PCR cycles with the primers $(GACA)_4$, $(CAC)_5$, and $(GTG)_5$ was a standard three-step reaction with DNA denaturation at 94°C for 30 s, primer annealing at 42, 52, and 50°C ($(GACA)_4$, $(GTG)_5$, and $(CAC)_5$, respectively) for 30 s, and DNA synthesis at 72°C for 60 s. Each of the 40 PCR cycles with the M13 primer included the DNA denaturation step at 94°C for 60 s, the primer annealing step at 52°C for 120 s, and the DNA synthesis step at 74°C for 180 s.

RAPD-PCR analysis with the OPA-01 primer (5'-CAGGCCCTTC-3') was carried out in 20 μl of a reaction mixture containing 0.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.05 U/μl Dynasyme II DNA polymerase (Finnzymes, Finland), 20–200 ng of template DNA, and 15 ng of the primer. Each of the 45 PCR cycles included the DNA denaturation step at 94°C for 60 s, the primer annealing step at 36°C for 60 s, and the DNA synthesis step at 72°C for 120 s.

PCR products were analyzed by electrophoresis in 1.2% agarose gel run at 65 V for 4–5 h. The developed gel was stained with ethidium bromide and photographed under UV light.

The homology of the DNA amplified with the N21 primer was determined by hybridizing its PCR products with the amplified DNA of strain KBP 2692 as the probe. The PCR products were transferred to a nitrocellulose membrane (Sigma) by the Southern procedure. The amplified DNA of strain KBP 2692 was eluted from the agarose gel with the aid of the GeneClean kit (Bio 101, Inc., the United States) according to the manufacturer's instruction. The products were marked with the nonradioactive label digoxigenin-11-dUTP (Boehringer Mannheim, Germany). Hybridization and the analysis of hybridization products were performed as recommended by the Boehringer Mannheim firm.

Isoenzyme analysis. Cell lysates were prepared for electrophoresis as described by Naumov *et al.* [15] and analyzed by electrophoresis on (76 × 76 mm) cellulose acetate plates (Helena Laboratories, the United States) carried out under the following conditions: room temperature; electrophoresis buffer contains 0.025 M Tris and 0.052 M glycine; sample volume is 1–2 μl; electrophoresis voltage and run times are 60 V and 30 min for glucose-6-phosphate dehydrogenase and 110 V and 15–20 min for all other enzymes. Enzyme staining was carried out as described earlier [15]. The seven enzymes studied were alcohol dehydrogenase (ADH, EC 1.1.1.1), glucose-6-phosphate isomerase (GPI, EC 5.3.1.9), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), isocitrate dehydrogenase (IDH, EC 1.1.1.42), lactate dehydrogenase (LDH, EC 1.1.1.27), mannose-6-phosphate isomerase (MPI, EC 5.3.1.8), and phosphoglucomutase (PGM, EC 5.4.2.2).

Some physiological characteristics of the *Komagataea pratensis* strains

Strains	Growth on		Susceptibility to the *Pichia punctispora* mycocin
	L-Rhamnose	citric acid	
Tien Shan strains:			
2678, 2681, 2685, 2686	+	+	+
2688	+	±	+
2679, 2680, 2682	+	+	–
2683, 2684, 2687	+	±	–
Caucasian strains:			
2690, 2691, 2692(T)	–	±	+
2694	–	–	+

Note: All strains were obtained from the Collection of Yeasts of the Department of Soil Biology of the Moscow State University. The letter T marks the type strain. "+" and "–" stand for "good growth" or "high susceptibility to mycocin," "no growth" or "resistance to mycocin," and "poor growth," respectively.

Data processing. Strain relatedness was evaluated by comparing their PCR products and isoenzyme patterns according to the formula $D = 1 - (a + b)/n$, where D is the difference coefficient; a is the sum of the nucleotide fragments present in both compared strains; b is the sum of the fragments that are absent in the both compared strains; and n is the total number of nucleotide fragments. The difference matrix was calculated based on the PCR product patterns obtained with four primers ($(GTG)_5$, $(GACA)_4$, $(CAC)_5$, and M13) and on the analysis of six enzymes, GPI, G6PDH, IDH, LDH, MPI, and PGM. Phylogenetic analysis was performed using the PHYLIP 3.5 software package [19]. Phylogenetic tree was constructed with the aid of the UPGMA program (version 3.572).

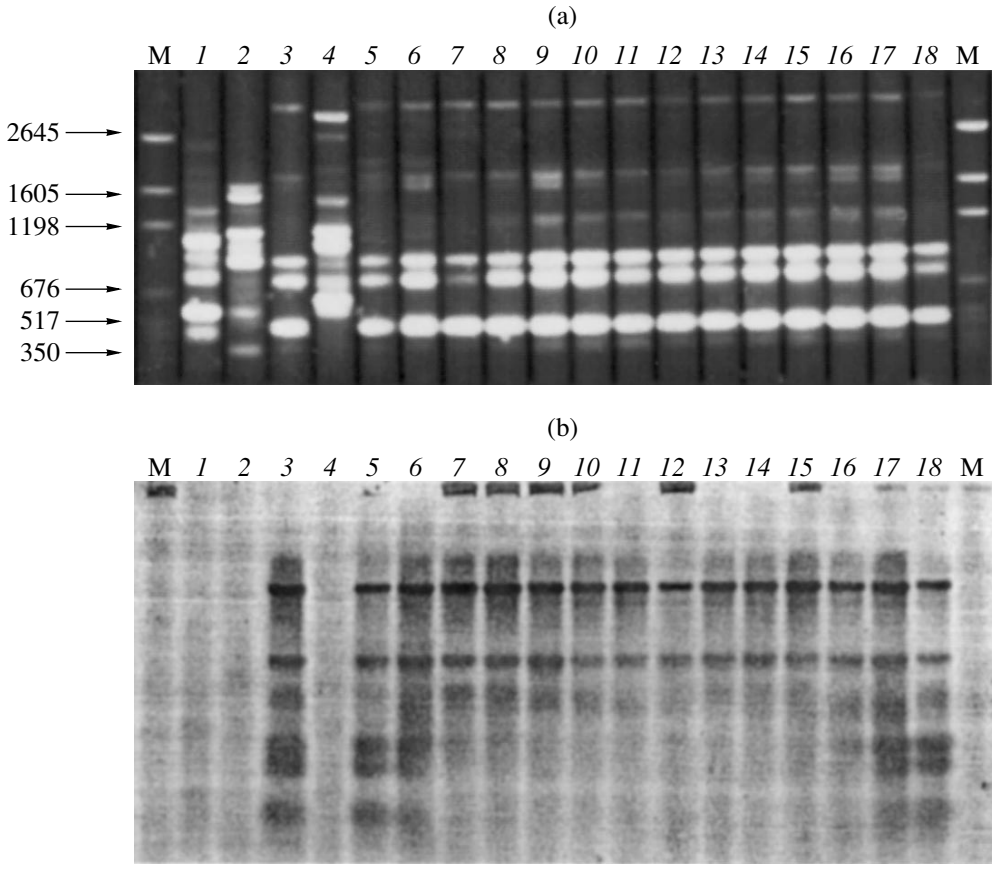

Fig. 1. (a) PCR analysis of *K. pratensis* strains with the universal N21 primer and (b) subsequent hybridization of their PCR products with the amplified DNA of the type strain KBP 2692. Lanes: *1*, *W. saturnus* CBS 5761; *2*, *W. mrakii* CBS 1707; *K. pratensis 3*, KBP 2692; *4*, KBP 2689; *5*, KBP 2690; *6*, KBP 2691; *7*, KBP 2694; *8*, KBP 2678; *9*, KBP 2679; *10*, KBP 2680; *11*, KBP 2681; *12*, KBP 2682; *13*, KBP 2683; *14*, KBP 2684; *15*, KBP 2685; *16*, KBP 2686; *17*, KBP 2687; *18*, KBP 2688; and M, molecular weight markers (bp).

RESULTS

Reevaluation of the taxonomic affiliation of the isolates. Based on the results of morphological and physiological analyses, all of the sixteen isolates under study were assigned earlier to the species *K. (Williopsis) pratensis* [1]. In this study, we reevaluated their affiliation to this species by the PCR technique with the universal N21 primer. As can be seen from Fig. 1a, the fifteen isolates, including the type strain *K. pratensis* KBP 2692, had identical UP-PCR profiles (lanes *3* and *5* through *17*) with the characteristic major nucleotide fragment about 550 bp in size, two 680-bp fragments, and three minor 2700-, 1600-, and 1200-bp fragments. The UP-PCR profiles of these isolates appreciably differed from those of the reference species *W. saturnus* and *W. mrakii* (Fig. 1a, lanes *1* and *2*, respectively). One of the isolates, KBP 2689, considerably differed from the other *K. pratensis* isolates in its UP-PCR profile (Fig. 1a, lane *4*).

The PCR products of all of the isolates were then transferred from the agarose gel onto a nitrocellulose membrane by the Southern blot technique and subjected to hybridization with the amplified DNA products of the type strain KBP 2692 (Fig. 1b). The PCR products of the fifteen isolates that had identical UP-PCR profiles in Fig. 1a underwent strong hybridization (Fig. 1b, lanes *3*, *5–17*). At the same time, the PCR products of the reference species *W. saturnus* and *W. mrakii* did not hybridize with those of strain KBP 2692 (lanes *1* and *2*). It should be noted that the weak hybridization bands of some strains around 600 bp (lanes *7–16*) were probably due to the incomplete transfer of the respective PCR products to the nitrocellulose membrane. Thus, the data of both the UP-PCR analysis and subsequent hybridization indicated that the fifteen isolates, KBP 2678 through KBP 2688, KBP 2690 through KBP 2692, and KBP 2694, do belong to the species *K. pratensis*. At the same time, strain KBP 2689 does not obviously belong to this species, since it has a different UP-PCR profile; does not hybridize with the type strain *K. pratensis* KBP 2692 (Fig. 1b, lane *4*); and, as physiologic tests showed, is able to assimilate L-sorbose, L-rhamnose, inulin, and soluble starch and fails to grow on 2-ketogluconate, which is not typical of the other *K. pratensis* strains. For this reason, strain KBP 2689 was excluded from further studies.

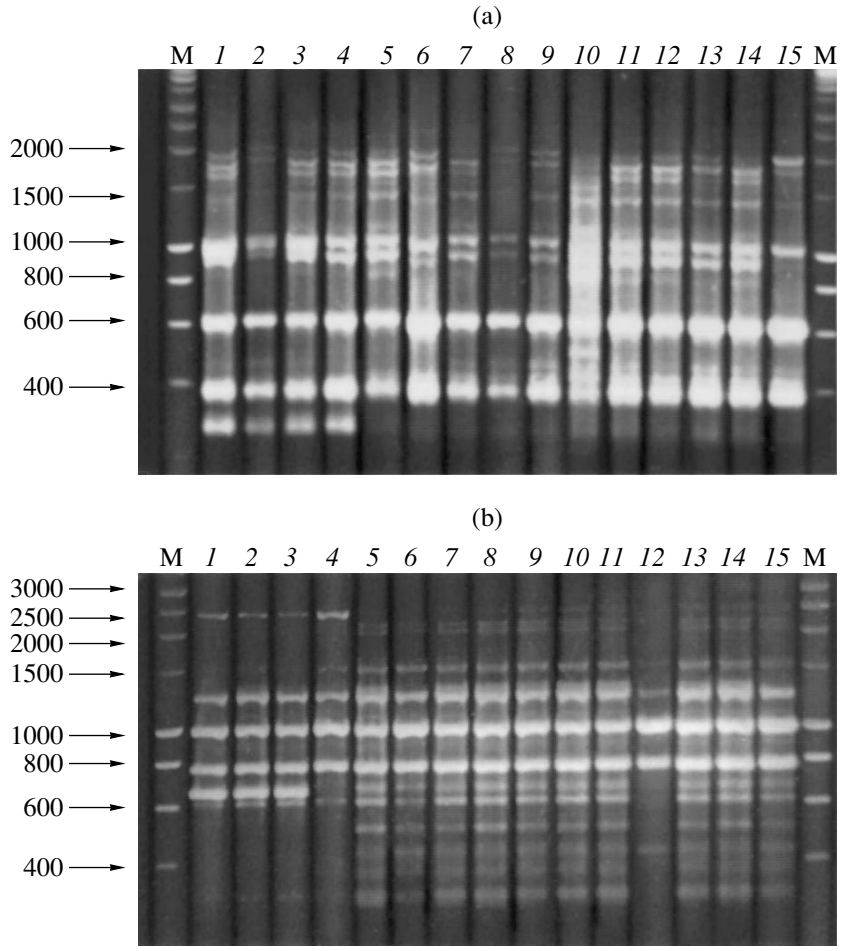

Fig. 2. PCR analysis of *K. pratensis* strains with the (a) $(CAC)_5$ and (b) $(GACA)_4$ primers. Lanes: *1*, KBP 2692; *2*, KBP 2690; *3*, KBP 2691; *4*, KBP 2694; *5*, KBP 2678; *6*, KBP 2679; *7*, KBP 2680; *8*, KBP 2681; *9*, KBP 2682; *10*, KBP 2683; *11*, KBP 2684; *12*, KBP 2685; *13*, KBP 2686; *14*, KBP 2687; *15*, KBP 2688; and M, molecular weight markers (bp).

The intraspecies polymorphism of *K. pratensis* isolates was studied by isoenzyme electrophoresis and RAPD-PCR techniques. Of the seven enzymes studied, six enzymes (alcohol dehydrogenase, glucose-6-phosphate isomerase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, mannose-6-phosphate isomerase, and phosphoglucomutase) were found to be monomorphic: each of the fifteen *K. pratensis* strains contained only one respective isoenzyme (data not presented). It should be noted that polymorphism, with respect to alcohol dehydrogenase, was studied only for strains KBP 2680–2683, KBP 2690–2692, and KBP 2694. The seventh enzyme, isocitrate dehydrogenase, turned out to be polymorphic. Two isolates from the Caucasus, KBP 2690 and KBP 2691, and five isolates from the Tien Shan, KBP 2678–2680, KBP 2682, and KBP 2688, possessed only one allel, Idh-1. A second allel, Idh-*2*, was revealed in the type strain KBP 2692, in one isolate from the Caucasus (KBP 2694), and in six isolates from the Tien Shan (KBP 2681 and KBP 2683–2687).

All five of the RAPD primers used gave rise to PCR profiles with a great number of distinct bands for all of the fifteen *K. pratensis* strains. The PCR profiles obtained with the primers $(GTG)_5$, M13, and OPA-01 were nearly identical (data not presented). At the same time, the PCR profiles with the two other primers, $(CAC)_5$ and $(GACA)_4$, allowed the fifteen strains under study to be divided into two groups, which corresponded to the geographic areas from which they were isolated. The first group included strains KBP 2690, 2691, 2694, and the type strain KBP 2692 from the Caucasus, and the second group included the eleven strains from Tien Shan (KBP 2678–2688). The PCR profiles with the $(CAC)_5$ primer had two major nucleotide fragments 400 and 600 bp in size and three minor fragments about 1000, 1500, and 2000 bp in size. The isolates from the Caucasus differed from the Tien Shan isolates in having an additional 300-bp band in their RAPD-PCR profiles (Fig. 2a, lanes *1–4*). The Tien Shan isolates slightly differed from each other in the number and position of the minor bands (Fig. 2a, lanes *5–15*). More significant differences between the two groups of the isolates were revealed in their RAPD-PCR profiles obtained with the $(GACA)_4$ primer. In addition to the

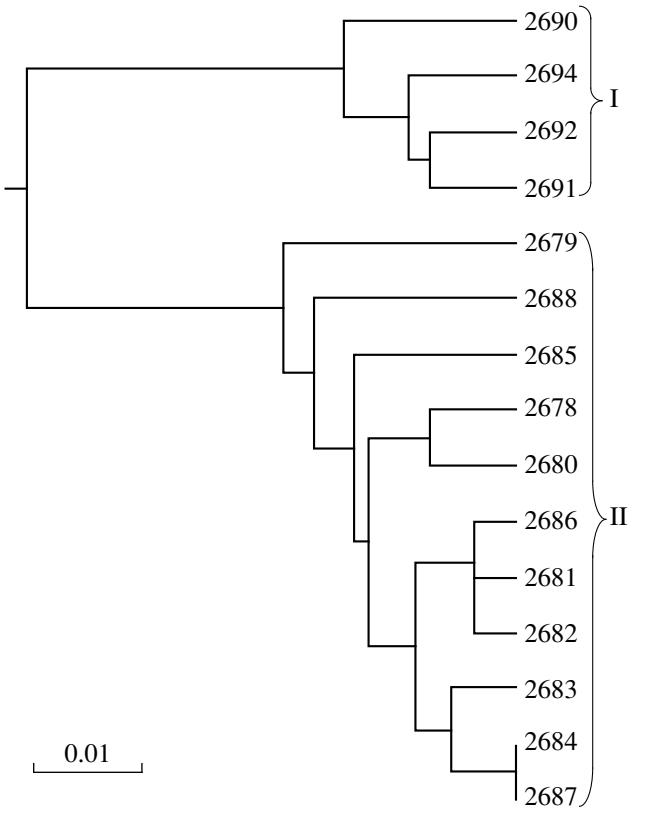

Fig. 3. Dendrogram showing the population structure of *K. pratensis* strains isolated from the (I) Caucasian and (II) Tien Shan soils. The dendrogram was constructed on the basis of the polymorphism of four PCR product patterns with different primers and on the electrophoretic analysis of six isoenzymes with the aid of the UPGMA program (version 3.572) from the PHYLIP 3.5 software package [19].

major 800-, 1000-, and 1300-bp nucleotide fragments present in all isolates, the isolates from the Caucasus had a 2500-bp band, whereas the Tien Shan isolates had three minor bands in this size range. On the other hand, the Tien Shan isolates had an additional major 1500-bp band and three minor bands from 300 to 550 bp in size, which were absent in the isolates from the Caucasus. All the isolates studied had minor fragments about 600 bp in size.

The isolates from the Caucasus and Tien Shan were also found to differ in some physiological characteristics (see table). For instance, unlike the Tien Shan isolates, the isolates from the Caucasus were unable to assimilate L-rhamnose and citric acid (or could only weakly assimilate the latter). In accordance with the data of other authors [18], all the isolates from the Caucasus were found to be sensitive to the mycocin of *Pichia punctispora* VKM Y-1363, whereas some of the Tien Shan isolates were sensitive to this mycocin and some were resistant to it (see table).

DISCUSSION

The UP-PCR and RAPD-PCR techniques are similar, differing only in the primers and the reaction conditions used [9]. Due to the universality of UPs, they can amplify species-specific PCR products. At the same time, differences in their PCR products allow particular strains within one species to be differentiated [20]. The data of UP-PCR analysis and the subsequent blot hybridization presented in this paper convincingly demonstrated that fifteen of the sixteen *K. pratensis* strains taken for analysis do belong to this species. Earlier, Golubev and Blagodatskaya [18] found that these strains slightly differed in some physiological characteristics and in the sensitivity to yeast mycocins. They suggested that the *K. pratensis* strains from the Caucasian soils do belong to the species *K. pratensis*, whereas the isolates from the Trans-Ili Alatau soils represent a separate species. The species status of the strains under discussion cannot be determined by genetic hybridization analysis, since they contain only one ascospore in the ascus. The PCR product patterns of the fifteen *K. pratensis* strains obtained with the universal N21 primer were identical and differed appreciably from those of the *Williopsis* strains. It should be noted that the N21 primer also allows the sibling species *W. saturnus* and *W. suaveolens* to be easily differentiated [11].

According to the data of Yamazaki and Kimagata [13], the four *Williopsis* species, *W. beijerinckii*, *W. saturnus*, *W. subsufficiens* (Wickerham) Vustin *et al.*, and *W. mrakii*, and the species *Zygowilliopsis californica* Kudriavzev can easily be differentiated based on the electrophoretic patterns of seven enzymes: malate dehydrogenase, glutamate dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, lactate dehydrogenase, esterase, and catalase. The isoenzyme analysis performed in this study confirmed the genetic homogeneity of *K. pratensis*: the fifteen strains of this species exhibited some differences only in isocitrate dehydrogenase, i.e., in one of the seven enzymes analyzed. Some polymorphism of these strains can hardly be related to their geographic location, since the PCR product patterns of these species with the $(GTG)_5$, M13, and OPA-01 primers were identical. At the same time, the strains isolated from the Caucasian and Tien Shan soils somewhat differed in their PCR product patterns obtained with the $(CAC)_5$ and $(GACA)_4$ primers.

To estimate the degree of divergence between the two geographic groups of *K. pratensis* strains, we calculated the difference matrix using the RAPD-PCR data (the intensity of the PCR product bands was not taken into account) and the results of isoenzyme analysis. This matrix was then used to construct a dendrogram with the aid of the UPGMA program from the PHYLIP 3.5 software package (Fig. 3). The dendrogram exhibited two distinct clusters, one of which comprised the type strain and the three isolates from the Caucasus and the other cluster included the eleven Tien Shan isolates. The divergence of *K. pratensis* strains was 0.78–1.59% within the cluster of Caucasian strains and 0–2.13% within the cluster of Tien Shan strains.

Some differences in the physiological characteristics (the assimilation of carbon sources) and in the PCR product patterns of the two geographic *K. pratensis* populations obtained with the $(CAC)_5$ and $(GACA)_4$ primers indicate their nascent genetic divergence.

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