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Differentiation of *Candida* species and *Candida rugosa* strains with the use of molecular markers in healthy horses

Różnicowanie wybranych *Candida* spp. i szczepów *Candida rugosa* występujących u zdrowych koni z wykorzystaniem markerów molekularnych

Summary. The aim of the study was identification of molecular markers useful in differentiating the species of selected yeast-like fungi on the skin and mucous membranes of horses and in identification of *Candida rugosa* strains. The study material was smears from horse skin and mucous membranes. The isolates were assayed with the use of API 20C AUX (bioMérieux) biochemical tests. The examinations involved *C. lusitaniae*, *C. parapsilosis* and *C. rugosa*. RAPD-PCR analyses included amplification with the use of 20 arbitrary primers (OPG01-OPG20). Genetic distances between the *Candida rugosa* strains were estimated by the Popgen32 programme. The RAPD-PCR analysis yielded amplification products of all the markers used in the study. The number of species-specific DNA fragments ranged from 0 to 6, depending on the primer. At least two specific DNA fragments were found for each of the studied species in the case of primers OPG04, OPG05, OPG06, OPG09, OPG11 and OPG14, which may indicate the possibility of their use for species identification. The present work is the first attempt to describe genetic differences between *Candida rugosa* strains sampled from healthy Hucul horses. Four genotypes within the *Candida rugosa* species, whose frequency ranged from 0.06 to 0.62, were obtained upon combination of DNA profiles of three primers (OPG04, OPG05 and OPG11). The genetic distances identified indicated considerable diversity of the studied strains. The genetic diversity may corroborate different environmental epidemiologic sources of the strains and may be related to their non-pathogenic nature.

Key words: *Candida* spp., *Candida rugosa*, RAPD-PCR, horses

INTRODUCTION

Candida genus fungi are an important cause of nosocomial infections in humans [Banerjee 2005]. Beside *C. krusei* and *C. albicans*, *Candida rugosa* is one of the most frequently isolated yeast-like fungi causing mycotic mastitis in cattle [Santos and Marin 2005, Seker 2010]. *Candida albicans* is the most prevalent agent of candidiasis in humans, although other species are identified equally frequently [Chang *et al.* 2001]. Prompt and detailed diagnosis of fungal infections is vital for immediate and appropriate treatment. Various methods of identification of clinical isolates have been developed, including API 20C or ATB ID 32C tests, but their application requires a few days. A molecular approach has also been employed in human epidemiology for identification of yeast-like fungi from the genus *Candida* [Chang *et al.* 2001, Saville *et al.* 2005]. Characteristic RAPD band patterns have been described for fungi from the genus *Candida*, which renders the RAPD method extremely promising thanks to its easy performance, specificity and sensitivity [Baires-Varguez *et al.* 2007, Colombo *et al.* 2003, Lockhart *et al.* 1997, Saville *et al.* 2005, Steffan *et al.* 1997].

Occurrence of various yeast-like fungi in the horse environment, in which they are opportunistic organisms, is prevalent [own unpublished results]. Still, there are no reports of their molecular identification. Numerous studies demonstrate low or no genetic diversity within particular species of pathogenic fungi, e.g *Candida* spp. [Baires-Varguez *et al.* 2007, Lockhart *et al.* 1997, Taylor *et al.* 1999, Steffan *et al.* 1997]. However, there are no data of opportunistic fungi occurring in horses.

The aim of the study was to indicate RAPD markers that are useful in differentiation of particular yeast-like fungal species sampled from the skin and mucous membranes of horses and in identification of *Candida rugosa* strains.

MATERIAL AND METHODS

The study material consisted of smears from the skin and mucous membranes of horses. All the samples were inoculated on the Sabouraud medium; subsequently, macroscopically homogenous colonies were obtained in selective cultures, which, after growing, were assayed with the API 20C AUX (bioMérieux) biochemical tests. The examinations involved the following yeast-like species: *Candida lusitaniae* (material sampled from Arabian horses); *Candida parapsilosis* and *Candida rugosa* (from Hucul horses). Genetic differences within the *Candida rugosa* species were estimated in material sampled from Hucul horses kept outdoors with the possibility to use a shelter in one of the equestrian centres in Poland.

DNA was isolated from homogenous colonies with the use of the QIAamp DNA Blood isolation kit (QIAGEN). The RAPD-PCR reaction was performed after quantitative and qualitative evaluation of DNA samples. The reaction mixture (sample volume 30 μl) contained 80 ng DNA, 3 μl PCR buffer, 4, 2 μl Q solution, 200 μM of each nucleotide, 0.2 μM of arbitrary primer, 25 mM $MgCl_2$, and 1 U *Taq* polymerase. In order to select the primers useful in differentiation of the particular species of the genus *Candida*, preliminary examinations were performed on 3 yeast-like species: *C. parapsilosis*, *C. rugosa* and *C. lusitaniae* with the use of 20 primers OPG01-OPG20 (*Operon Technologies, Inc.* KIT G; Tab. 1). Three (OPG04, OPG05 and OPG1) of the twenty arbitrary

primers were used in further tests performed on *Candida rugosa* yeast-like microorganisms isolated from the skin and mucous membranes of three Hucul horses (the fungi were sampled from the smears taken from the nostrils, mouth, ear, groin and collateral groove, and from the foreskin of one of the horses).

The amplification reaction consisted in initial denaturation (94°C, 5 min); 46 cycles of: denaturation (94°C, 1 min), annealing of the primers (in the preliminary tests – separate amplification reactions for 36°C, 2 min) and extension of DNA strands (72°C, 1 min); terminal extension of primers (72°C, 10 min) and cooling to temperature 4°C. The RAPD-PCR products were fractioned in 2.5% agarose gel (using bromophenol blue loading buffer). The gels were analyzed in UV light (Transilluminator) and archived with the use of the Scion Image programme (Syngen Biotech). GeneRuler 50bp DNA Ladder and GeneRuler 100bp DNA Ladder, Fermentas, were used for identification of the size of DNA fragments obtained.

The genetic distances between the identified strains of the genus *Candida rugosa* were assessed based on the frequency of DNA fragments in the polymorphic *loci* using the Popgen32 programme (Yeh and Yang 2000). The genetic distances obtained were used for construction of a phylogenetic tree with the method of neighbour-joining (NJ) [Saitou and Nei 1987] using the Mega v. 4.0 computer programme [Tamura *et al*. 2007].

The experimental horses, from which smears were taken, received permanent zootechnical and veterinary care. All the animals underwent a prophylaxis programme of parasitic invasion control and vaccinations. All the horses were healthy during the investigations. None of them received any treatment before the study (at least 1 month earlier) or afterwards (within maximum 1 month).

RESULTS AND DISCUSSION

The RAPD-PCR analysis yielded amplification products of all the markers used in the study. They met the criteria of the band pattern and efficiency (Fig. 1).

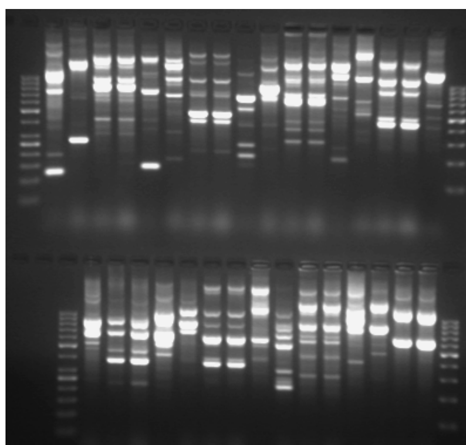

Fig. 1. Electrophoregram of the RAPD-PCR analysis of the genus *Candida* performed with the use of OPG09 – OPG16 primers (the first and last lane – size markers)
Rys. 1. Elektroforegram analizy RAPD-PCR u gatunków z rodzaju *Candida* z wykorzystaniem starterów OPG09 – OPG16 (pierwsza i ostatnia ścieżka – marker wielkości)

The primers analysed in the study generated varied numbers of DNA fragments characteristic for the three species. The numbers ranged from 3 (OPG06 and OPG20) to 9 (OPG01 and OPG7) for *C. parapsilosis*, from 2 (OPG20) to 11 (OPG02, OPG04 and OPG05) for *C. Rugosa*, and from 3 (OPG13, OPG15, OPG19, OPG20) to 12 (OPG02) for *C. lusitaniae* (Tab. 1). Not all of the primers investigated generated specific bands for each of the study species. 30% of the primers failed to distinguish all the three *Candida* spp. (Tab. 1). The number of specific DNA fragments for the particular species ranged from 0 (in some species within 6 markers) to 6 (for *C. lusitaniae* and *C. rugosa* for primers OPG05 and OPG10, respectively). At least two specific DNA fragments for each of the study species were reported in the case of OPG04, OPG05, OPG06, OPG09, OPG11 and OPG14.

Table 1. Characteristics of primers OPG01-OPG20 in the species from the genus *Candida*
Tabela 1. Charakterystyka markerów OPG01-OPG20 u gatunków z rodzaju *Candida*

Primer Starter	Nucleotide sequence 5'→3' Sekwencja nukleotydowa 5'→3'	Species Gatunek	Size range of amplified bands (bp)** Zakres wielkości amplifikowanych fragmentów (pz)	Tnb*	Spec*	RAPD fragments differentiating *Candida* spp.** Fragmenty RAPD różnicujące *Candida* spp.
1	2	3	4	5	6	7
OPG01	CTACGGAGGA	*C. parapsilosis*	150–2000	9	4	500, 900, 1000, 1200
		C. rugosa	150–800	7	2	600, 700
		C. lusitaniae	370–2000	6	1	1400
OPG02	GGCACTGAGG	*C. parapsilosis*	250–1600	7	4	250, 270, 700, 1200
		C. rugosa	300–1100	11	0	-
		C. lusitaniae	300–1600	12	0	-
OPG03	GAGCCCTCCA	*C.parapsilosis*	500–2000	7	2	600, 700
		C. rugosa	400–1500	6	3	400, 1400, 1500
		C. lusitaniae	270–2000	4	1	270
OPG04	AGCGTGTCTG	*C. parapsilosis*	300–950	5	3	300, 400, 450
		C. rugosa	200–1800	11	4	500, 570, 1000***, 1200, 1800***
		C. lusitaniae	330–1500	5	3	330, 620, 1500
OPG05	CTGAGACGGA	*C. parapsilosis*	550–2000	5	4	550, 600, 1200, 2000
		C. rugosa	220–1300	11	5	220, 360, 420, 570, 950***
		C. lusitaniae	380–1700	7	6	380, 400, 900, 1000, 1400, 1700
OPG06	GTGCCTAACC	*C. parapsilosis*	800–1500	3	2	800, 900
		C. rugosa	200–1500	9	5	200, 250, 700, 1000, 1300
		C. lusitaniae	300–1400	5	2	350, 1200
OPG07	GAACCTGCGG	*C. parapsilosis*	300–1400	9	2	500, 1400
		C. rugosa	200–1300	10	2	200, 700
		C. lusitaniae	300–1300	7	1	1000
OPG08	TCACGTCCAC	*C. parapsilosis*	450–1200	7	1	500
		C. rugosa	250–1500	6	2	250, 380
		C. lusitaniae	170–1500	7	2	170, 210
OPG09	CTGACGTCAC	*C. parapsilosis*	450–1400	5	2	700, 1400
		C. rugosa	450–1500	5	4	500, 900, 1100, 1500
		C. lusitaniae	550–1700	4	2	750, 1700

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cd. tab. 1

1	2	3	4	5	6	7
OPG10	AGGGCCGTCT	*C. parapsilosis*	250–1600	6	2	250, 280
		C. rugosa	300–1600	9	6	300, 400, 500, 700, 800, 1200
		C. lusitaniae	750–1700	4	1	1700
OPG11	TGCCCGTCGT	*C. parapsilosis*	200–1100	6	3	270, 550, 1100
		C. rugosa	200–1000	7	4	250***, 500***, 700***, 1700***
		C. lusitaniae	200–1500	7	3	370, 830, 1200
OPG12	CAGCTCACGA	*C. parapsilosis*	300–1300	8	3	300, 800, 1100
		C. rugosa	650–1300	9	3	650, 900, 1000
		C. lusitaniae	500–1300	4	1	500
OPG13	CTCTCCGCCA	*C. parapsilosis*	170–1500	6	3	170, 200, 1500
		C. rugosa	600–1000	3	1	600
		C. lusitaniae	500–1200	3	2	500, 1200
OPG14	GGATGAGACC	*C. parapsilosis*	400–2000	5	2	400, 1000
		C. rugosa	250–2000	7	3	250, 450, 600
		C. lusitaniae	200–1200	6	4	200, 300, 500, 1200
OPG15	ACTGGGACTC	*C. parapsilosis*	400–2000	7	0	-
		C. rugosa	350–2000	9	1	350
		C. lusitaniae	700–1000	3	1	700
OPG16	AGCGTCCTCC	*C. parapsilosis*	450–900	6	2	450, 900
		C. rugosa	250–800	5	3	250, 400, 480
		C. lusitaniae	500–800	4	0	-
OPG17	ACGACCGACA	*C. parapsilosis*	190–1900	5	1	190
		C. rugosa	450–1900	6	1	800
		C. lusitaniae	450–1900	6	0	-
OPG18	GGCTCATGTG	*C. parapsilosis*	300–1900	8	3	300, 400, 600
		C. rugosa	200–1900	6	3	200, 250, 350
		C. lusitaniae	500–1900	4	1	1000
OPG19	GTCAGGGCAA	*C. parapsilosis*	300–1100	6	0	-
		C. rugosa	300–1100	6	0	
		C. lusitaniae	500–1000	3	1	500
OPG20	TCTCCCTCAG	*C. parapsilosis*	700–1900	3	1	
		C. rugosa	900–1900	2	0	-
		C. lusitaniae	700–1900	3	0	

* Tnb – total number of bands – całkowita liczba prążków
* Spec – number of specific bands – liczba specyficznych prążków
** Approximate values – wartości przybliżone
*** bands yielded by some *Candida rugosa* isolates – prążki występujące tylko w części izolatów *Candida rugosa*

According to various studies, the RAPD profile patterns are consistent within the species due to low genetic diversity in the populations of several pathogenic yeast species, including *Candida* spp. [Baires-Varguez *et al*. 2007, Lockhart *et al*. 1997, Steffan *et al*. 1997, Taylor *et al*. 1999].

Bautista-Muñoz *et al*. [2003] performed a differentiation analysis of 9 yeast species: *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. kefyr*, *C. krusei*, *C. lusitaniae* and *C. guilliermondii* with the use of the RAPD technique and OPE-18, OPE-04 and OPA-18 primers. Their study results confirmed the usefulness of RAPD analyses for identification of *Candida* spp. Patterns generated by different arbitrary primers were consistent within particular species and contained several fragments that were unique for each species.

Baires-Varguez *et al*. [2007] employed the RAPD technique using primer OPE-18 for identification of fungal species from the genus *Candida*. They found the method to be specific and sensitive in identification of *Candida glabrata, Candida guilliermondii, Candida tropicalis, Candida pelliculosa, Candida albicans, Candida krusei,* and *Candida lusitaniae*.

The results of the aforementioned investigations provided basis for identification of DNA fragments characteristic for the particular fungal species found in horses (Tab. 1). However, given these results (Tab. 2, Fig. 2–4) and genetic diversity in the *Candida rugosa* species, it was assumed that the study material was not sufficiently abundant for formulating far-fetched conclusions about bands that were characteristic for the respective study species (Tab. 1).

Primers generating the largest number of species-specific bands (not fewer than 3 species-specific bands – OPG04, OPG05 and OPG11) were used for identification of *Candida rugosa* strains. It was assumed in the study that primers that met these criteria would be suitable for an intraspecific analysis of strains.

The electrophoregrams (Fig. 2–4) present the results of the RAPD-PCR analysis performed using three primers (OPG04, OPG05 and OPG11, respectively).

In the analyses with primer OPG04 (Fig. 2), in horse 1 and 3, a different *Candida rugosa* genotype was isolated from the groin and collateral groove than that from the nostrils, mouth and ear. In horse 2, a different genotype was found only on the ear mucosa membrane. The yeast genotypes determined in the groin and collateral groove in horse 1 and 3 and in the ear in horse 2 were characterised by absence of 1000 and 1800 bp DNA fragments (Fig. 2).

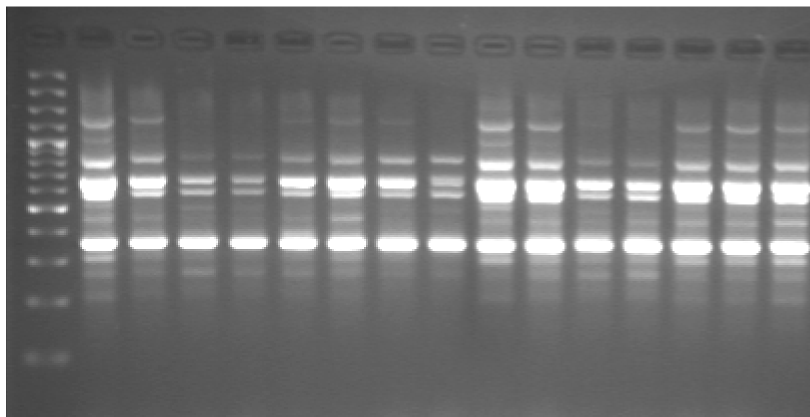

Fig. 2. Electrophoregram of the RAPD-PCR analysis with the OPG04 primer of *Candida rugosa* yeast-like organisms isolated from various parts of horses' bodies (the first lane on the left – the size marker, subsequently from left to right: smears sampled from the individual horses – lanes 1–4 (mouth, ear, groin, collateral groove, respectively); lanes 5–10 (collateral groove, foreskin, groin, ear, mouth, nostrils); lanes 11–15 (groin, collateral groove, ear, mouth, nostrils)
Rys. 2. Elektroforegram analizy RAPD-PCR z mikoorganizmów drożdżopodobnych *Candida rugosa* izolowanych z różnych miejsc ciała koni z wykorzystaniem startera OPG04 (pierwsza ścieżka od lewej – marker wielkości, kolejno od lewej: wymazy pobrane od różnych koni – ścieżki 1–4 (odpowiednio: jama ustna, ucho, pachwina rowek), ścieżki 5–10 (rowek przystrzałkowy, napletek, pachwina, ucho, jama ustna, nozdrza), ścieżki 11–15 (pachwina, rowek przystrzałkowy, ucho, jama ustna, nozdrza)

Like in the case of primer OPG04, primer OPG05 allowed identification of two genotypes. The fungus isolated from the collateral groove in one of the horses only was characterised by a different alignment of DNA fragments, i.e. there were no 800, 950, 1200 and 1300 bp fragments in this strain (Fig. 3). The other samples analysed displayed identical band alignment, which indicated absence of differences in DNA amplification in the isolated fungi. This may point to limited usefulness of OPG05 used individually for differentiating strains of the species *Candida rugosa*.

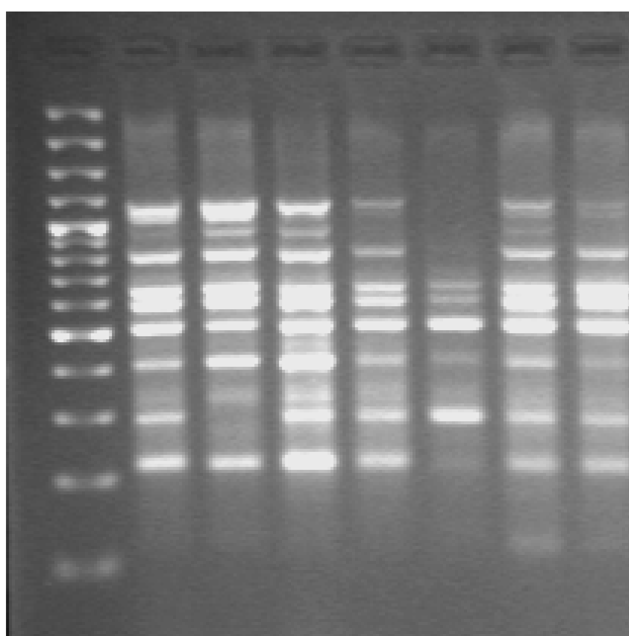

Fig. 3. Electrophoregram of the RAPD-PCR analysis with the OPG05 primer of *Candida rugosa* yeast-like organisms isolated from various parts of horses' bodies (the first lane on the left – the size marker; the sixth lane from the left – the different strain isolated from the collateral groove in one of the horses – absence of bands 800, 950, 1200 and 1300)
Rys. 3. Elektroforegram analizy RAPD-PCR z mikoorganizmów drożdżopodobnych *Candida rugosa* izolowanych z różnych miejsc ciała koni z wykorzystaniem startera OPG05 (pierwsza ścieżka od lewej – marker wielkości, szósta ścieżka od lewej – odmienny od pozostałych szczep wyizolowany z rowka przystrzałkowego jednego z badanych koni – brak prążków 800, 950, 1200 i 1300)

Analyses of the material performed with the use of primer OPG11 revealed three *Candida rugosa* genotypes (Fig. 4). As in the case of OPG04, additional 350 bp DNA fragments were found in the fungal isolates from the groin and collateral groove in horse 1, and the ear mucous membrane in horse 2. A different genotype, where only two 350 and 400 bp DNA fragments were shown (Fig. 4), was found in the collateral groove in horse 2 (as in the case of primer OPG05).

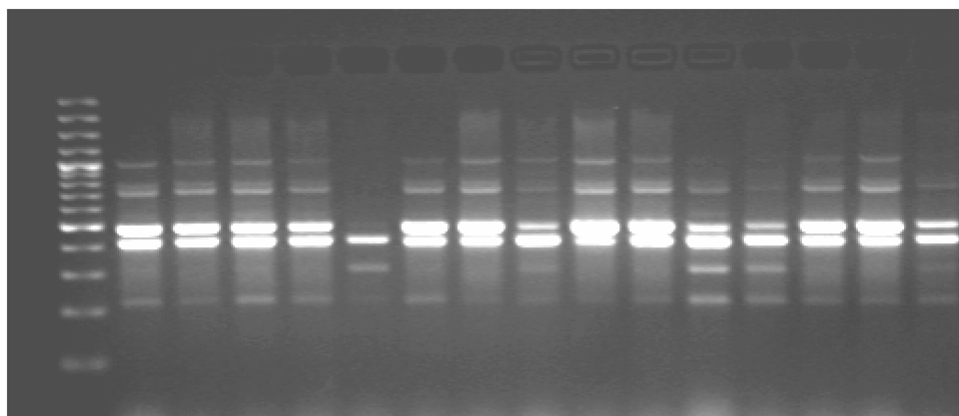

Fig. 4. Electrophoregram of the RAPD-PCR analysis with the OPG11 primer of *Candida rugosa* yeast-like organisms isolated from various parts of horses' bodies (the first lane on the left – the size marker, subsequently from left to right: smears sampled from the individual horses – lanes 1–4 (mouth, ear, groin, collateral groove, respectively); lanes 5–10 (collateral groove, foreskin, groin, ear, mouth, nostrils); lanes 11–15 (groin, collateral groove, ear, mouth, nostrils)
Rys. 4. Elektroforegram analizy RAPD-PCR z mikoorganizmów drożdżopodobnych *Candida rugosa* izolowanych z różnych miejsc ciała koni z wykorzystaniem startera OPG11 (pierwsza ścieżka od lewej – marker wielkości, kolejno od lewej: wymazy pobrane od różnych koni – ścieżki 1–4 (odpowiednio: jama ustna, ucho, pachwina, rowek przystrzałkowy), ścieżki 5–10 (rowek przystrzałkowy, napletek, pachwina, ucho, jama ustna, nozdrza), ścieżki 11–15 (pachwina, rowek przystrzałkowy, ucho, jama ustna, nozdrza)

The primers generated 29 DNA fragments, while the band pattern in the analyses performed with the use of primers OPG04, OPG05 and OPG11 consisted of 11, 11, and 7 various length DNA fragments, respectively. A combination of the DNA profiles obtained using the three primers generated 4 genotypes within the *Candida rugosa* species. The first one (*C. rugosa* 1) exhibited frequency of 0.62. It was found on the mouth and ear mucous membrane of all the experimental horses, on the ear mucous membrane of two horses, and on the groin skin and foreskin mucous membrane of the stallion. *C. rugosa* 2 and *C. rugosa* 3 strains displayed frequency of 0.19 and 0.13, respectively. *C. rugosa* 4 strain was only identified in the collateral groove of one horse. The highest *C. rugosa* genotype diversity was found in the collateral groove. A different strain of the study species (*C. rugosa* 2, 3 and 4) was present in each of the experimental horses (Tab. 2).

The present report is the first attempt at description of the genetic differences between *Candida rugosa* strains occurring in Hucul horses. The study horses did not display disease symptoms, which may testify to differences between human pathogenic strains and non-pathogenic strains found in horses. The *Candida rugosa* pathogen rarely infects humans; however, candidemia caused by it resulted in high mortality rates despite administration of antifungal therapy with amphotericin B [Colombo et al. 2003]. Investigations aiming at identification of *C. rugosa* genotypes isolated from several clinical samples were conducted by Colombo *et al*. [2003] with the use of RAPD primers M2, B14 and CDU. Although numerous DNA fragments were generated by each primer, the authors did not report differences in the number of bands between the particular clinical samples.

In cattle, *Candida rugosa* was the second frequently occurring fungus isolated from clinical or subclinical mastitis and represented 16.4% of all identified fungi [Seker 2010]. Beside *C. krusei* and *C. albicans*, it was the most frequently isolated yeast in mycotic mastitis [Santos and Marin 2005]. These reports were, however, not confirmed in investigations carried out in Poland [Krukowski *et al*. 2006], in which species that were most frequently isolated from clinical and subclinical mastitis included *C. kefyr*, *C. humicola*, *C. rugosa* and *C. inconspicua*.

Table 2. The occurrence site and frequency of the individual strains of the *Candida rugosa* species
Tabela 2. Miejsce występowania i frekwencja poszczególnych szczepów w obrębie gatunku *Candida rugosa*

Horse no. Nr konia	Smear sampling area (mucous membrane/skin) Miejsce wymazu (błona śluzowa/ skóra)	Genetic group Grupa genetyczna				Total (frequency) Łącznie (frekwencja)
		OPG04	OPG05	OPG11	upon combination of-DNA profiles from 3 primers po połączeniu profili DNA 3 markerów	
1	nostrils/nozdrza	1	1	1	*C. rugosa* 1	
1	mouth/jama ustna	1	1	1	*C. rugosa* 1	
1	ear/ucho	1	1	1	*C. rugosa* 1	
2	nostrils/nozdrza	1	1	1	*C. rugosa* 1	
2	mouth/jama ustna	1	1	1	*C. rugosa* 1	
2	groin/pachwina	1	1	1	*C. rugosa* 1	
2	foreskin/napletek	1	1	1	*C. rugosa* 1	
3	ear/ucho	1	1	1	*C. rugosa* 1	
3	mouth/jama ustna	1	1	1	*C. rugosa* 1	
3	nostrils/nozdrza	1	1	1	*C. rugosa* 1	10 (0.62)
1	groin/pachwina	2	1	2	*C. rugosa* 2	
1	collateral groove/ rowek przystrzłkowy	2	1	2	*C. rugosa* 2	
2	ear/ucho	2	1	2	*C. rugosa* 2	3 (0.19)
3	collateral groove/ rowek przystrzłkowy	2	1	1	*C. rugosa* 3	
3	groin/pachwina	2	1	1	*C. rugosa* 3	2 (0.13)
2	collateral groove/ rowek przystrzłkowy	1	2	3	*C. rugosa* 4	1 (0.06)

The genetic distance between *Candida rugosa* 4 and the other genetic groups exhibited relatively high values ranging from 0.4 to 0.5. The lowest distance was found between strain 2 and 3 (Tab. 3).

Table 3. Similarities (above the diagonal) and standard genetic distances (below the diagonal) between the individual strains of the *Candida rugosa* species
Tabela 3. Podobieństwa (nad przekątną) i standardowe dystanse genetyczne (pod przekątną) pomiędzy poszczególnymi szczepami w obrębie gatunku *Candida rugosa*

	C. rugosa 1	*C. rugosa* 2	*C. rugosa* 3	*C. rugosa* 4
C. rugosa 1	-	0.8966	0.9310	0.6552
C. rugosa 2	0.1092	-	0.9655	0.6207
C. rugosa 3	0.0715	0.0351	-	0.5862
C. rugosa 4	0.4229	0.4769	0.5341	-

Yeast-like fungi constitute over 40% of all fungi identified in the living environment of Hucul horses (own unpublished results).

Although the yeast-like fungi were found in Hucul horses, the genetic distances determined indicated considerable genetic diversity of the study strains. Own (unpublished) studies have indicated occurrence of *C. rugosa* on the skin of the back, groins, mucous membrane of nostrils, mouth and ear, and in the collateral groove in primitive horses, including Hucul horses. The strain diversity reported in this paper may indicate their different environmental epidemiological sources, i.e. feed, water, stable equipment, and bedding. A human source should not be excluded either, since *C. rugosa* occurred on stable doors and bolts.

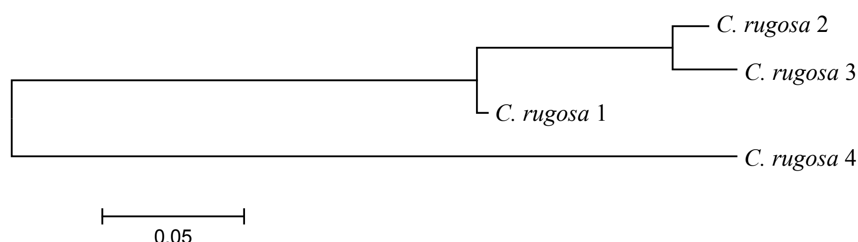

Fig. 5. Dendrogram indicating hypothetical phylogenetic relationships between the respective strains of the *Candida rugosa* species
Rys. 5. Dendrogram wskazujący na hipotetyczne filogenetyczne relacje poszczególnych szczepów w obrębie gatunku *Candida rugosa*

Based on the genetic distances, a tree was constructed to present the phylogenetic relationships between the four *Candida rugosa* strains (Fig. 5). This is an unrooted dendrogram which does not contain external groups, but the phylogenetic separateness of *Candida rugosa* 4 from the other strains seems most evident. The results obtained (Fig. 5) indicate that three genetic groups of *Candida rugosa* belong to one clade, whereas the *C. rugosa* 4 strain constitutes a separate phylogenetic branch, thus suggesting its genetic separateness. Strain 4 was found in the collateral groove in one of the horses; therefore, it cannot be excluded that it originated from the bedding. Furthermore, outdoor breeding may account for the presence of the genetically different strain as well. It should be emphasized that *Candida rugosa* in the collateral groove belonged to different genetic groups in each of the experimental horses (Tab. 2, Fig. 5). Similarly, different *C. rugosa* strains were found in horses' groins (Tab. 2, Fig. 5). This indicates a pos-

sibility of risk posed to horses by fungi occurring in the bedding. Unambiguous confirmation of this thesis requires continuation of the research based on representative sampling of the bedding in horses' shelters and paddocks.

Literature results of investigations conducted with the use of the RAPD technique for differentiation of *Candida* spp. yeasts demonstrate band patterns that are unique for each species [Bautista-Muñoz *et al*. 2003, Lockhart *et al*. 1997, Steffan *et al*. 1997]. However, the results obtained in this study do not corroborate the possibility of RAPD technique use for species identification due to the diversity within *Candida rugosa*. This is confirmed by primer OPG11, which, in initial analyses of *Candida* spp., generated 4 bands that were characteristic for *C. Rugosa* and absent in other *Candida* spp.; after a detailed analysis, however, none of the band was characteristic for all the *C. rugosa* isolates (Tab. 1). This may hold true only to the study species, but it cannot be excluded that only pathogenic species, rather than the opportunistic and non-pathogenic species from our study, do not show genetic diversity. It is therefore possible that the RAPD technique may be employed for differentiation of pathogenic fungal species [Bautista-Muñoz *et al*. 2003, Lockhart *et al.* 1997, Steffan *et al*. 1997]. In the case of opportunistic yeast-like fungi found in horses, more material from various sources should be examined in order to determine the length of DNA fragments typical of particular *Candida* spp.

CONCLUSIONS

1. Six of the twenty primers tested (OPG04, OPG05, OPG06, OPG09, OPG11 and OPG14) may be used for differentiation of the study species of yeast-like fungi, as each of them generated at least two species-specific DNA fragments.
2. OPG04, OPG05 and OPG11 primers have been recognized as useful in identification of strains of the *Candida rugosa* species. The 4 genetic groups identified confirm the high diversity of the aforementioned opportunistic fungi in Hucul horses.
3. Due to the genetic diversity of *Candida rugosa*, one should be cautious when employing the RAPD technique for differentiation of *Candida* spp.
4. *Candida* spp. differentiation tests should be performed using numerous fungal isolates sampled from various body parts, with samples from the collateral groove, groin, nostrils or mouth and ear as the key elements of the examinations.
5. The genetic diversity of *Candida rugosa* in may be related to their non-pathogenic nature. Literature provides only reports of absence of genetic diversity of various pathogenic fungal species. Therefore, it seems advisable to explore genetic differences between pathogenic (including opportunistic pathogens) and non-pathogenic strains.

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Streszczenie. Celem pracy było wskazanie markerów genetycznych do różnicowania gatunków wybranych grzybów drożdżopodobnych występujących na skórze i błonach śluzowych koni, jak też rozróżniania ich szczepów w obrębie gatunku *Candida rugosa*. Materiał do badań stanowiły wymazy ze skóry i błon śluzowych koni. Izolaty oceniano testami biochemicznymi API 20C AU-X. Badaniami objęto: *C. lusitaniae*, *C. parapsilosis* i *C. rugosa*. Analizy RAPD-PCR obejmowały amplifikację z użyciem 20 arbitrary primer (OPG01-OPG20). Odległości genetyczne pomiędzy szczepami *Candida rugosa* oszacowano przy wykorzystaniu programu Popgen 32. W wyniku przeprowadzonej analizy RAPD-PCR otrzymano produkty amplifikacji wszystkich użytych w pracy markerów. Liczba gatunkowo specyficznych fragmentów DNA w zależności od markera wahała się od 0 do 6. Minimum dwa specyficzne fragmenty DNA dla każdego z badanych gatunków odnotowano w przypadku markerów: OPG04, OPG05, OPG06, OPG09, OPG11 i OPG14, co może wskazywać na możliwość ich wykorzystania w identyfikacji gatunkowej. Niniejsze opracowanie stanowi pierwszą próbę opisania genetycznych różnic pomiędzy szczepami *Candida rugosa* występującymi u zdrowych koni huculskich. Po połączeniu wyników profili DNA trzech markerów (OPG04, OPG05 i OPG11) otrzymano 4 genotypy w obrębie gatunku *Candida rugosa*, których frekwencja wynosiła od 0,06 do 0,62. Określone odległości genetyczne wskazywały na znaczną odrębność badanych szczepów. Różnorodność genetyczna szczepów może świadczyć o ich różnych środowiskowych źródłach epidemiologicznych i może być związana z ich niechorobotwórczym charakterem.

Słowa kluczowe: *Candida* spp., *Candida rugosa*, RAPD-PCR, konie