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TAXONOMIC DESCRIPTION

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Prototheca blaschkeae subsp. *brasiliensis* subsp. nov., isolated from cow milk

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Abstract

A strain of an achlorophyllic alga, named PR24^T, was isolated from cow milk samples from the state of Minas Gerais, Brazil. Based on 18S rDNA, 28S rRNA, D1/D2 region of the LSU rDNA and SSU rRNA gene sequence similarities, this strain was found to be a member of the genus *Prototheca* and closely related to *Prototheca blaschkeae* SAG2064^T. However, the novel strain could easily be distinguished from recognized *Prototheca* species by internal transcribed spacer, species-specific PCR, single-strand conformation polymorphism-PCR analysis and phenotypic characteristics. The inability to grow in Sabouraud broth at pH 4.0 and the different cellular fatty acid composition clearly distinguished PR24^T from the reference strain of *P. blaschkeae*. The combination of genotypic and phenotypic data indicates that strain PR24^T represents a subspecies of *P. blaschkeae*, for which the name *Prototheca blaschkeae* subsp. *brasiliensis* subsp. nov. is proposed. The respective type strain is PR24^T (=DSM 103592^T=IHEM 26958^T).

Prototheca are unicellular achlorophyllous yeast-like microalgae, ubiquitous in nature. They are spherical, oval or even kidney-shaped and closely related to green algae of the genus *Chlorella* [1]. To date, seven species have been well-described into the genus *Prototheca*: *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca stagnora*, *Prototheca ulmea*, *Prototheca blaschkeae*, *Prototheca cutis* and *Prototheca miyajii* [2–5].

Some protothecal species, such as *P. zopfii* and *P. wicherhamii*, are pathogenic in natural conditions and can cause a variety of infections in cattle, dogs, cats and humans. Although *P. wickerhamii*, *P. cutis* and *P. miyajii* have been mostly related to human infections [3, 5], in cows protothecal bovine mastitis is usually caused by *P. zopfii*, *P. wickerhamii* or *P. blaschkeae* [6–9]. *Prototheca* was first linked to mastitis in dairy cows in 1952, but its first description as an agent of bovine mastitis dates from 1969 [10]. At present, the frequency of bovine protothecal mastitis is increasing worldwide [9, 11].

During a study of milk samples collected from cows affected by clinical and subclinical mastitis, atypical strains that appeared to represent a novel *Prototheca* species were isolated [12].

Strain PR24^T was isolated from bovine milk samples collected in a dairy farm located in the state of Minas Gerais (Brazil) using Sabouraud (SB) agar (Biolife) plates. It was routinely maintained at 4 °C after growth at 37 °C for 48 h in SB broth (Biolife). Morphological characteristics of the cells were determined by light microscopy. Growth at different temperatures was observed in SB agar incubated at 10, 25, 30, 37 and 45 °C for 7 days. The influence of pH (4.0, 5.0, 6.0 and 7.0) on *Prototheca* growth was examined in SB broth after 7 days incubation at 37 °C. Initial pH was obtained by adjusting the pH of SB medium with the addition of 1 N HCl or NaOH. Salt tolerance was determined in SB broth containing 2.5, 5, 10 or 20 % (w/v) NaCl. Salt concentrations were chosen according to Marques *et al.* [13]. Biochemical tests were performed using the API ID32A,

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Keywords: protothecal bovine mastitis; cow milk; *Prototheca blaschkeae* subsp. *brasiliensis*.

Abbreviations: ITS, internal transcribed spacer; SB, Sabouraud; SSCP, single-strand conformation polymorphism; ssPCR, species-specific PCR.

The GenBank/EMBL/DDBJ accession numbers for the 18S rDNA gene sequences of strains PR24^T, SAG2064^T and SAG2063^T are KX499482, KX499483 and KX499484, respectively. The accession number for the PR24^T 28S ribosomal RNA gene region of the LSU rRNA partial sequence is KY593250 while the accession number for the SSU rRNA, gene is KY593251. The accession numbers for the 28S rRNA gene partial sequences of strains SAG2021^T, SAG2063^T and SAG2064^T are KY654531, KY654530 and KY654532, respectively.

One supplementary figure is available with the online Supplementary Material.

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API ID32 Staph and API 20 AUX C systems (bioMérieux), according to the manufacturer's instructions. All assays (growth temperature, pH and salt tolerance, and biochemical tests) were performed in triplicate. The following reference strains of the key *Prototheca* species were included in the study: *P. stagnora* ATCC16528^T, *P. wickerhamii* ATCC30395, *P. ulmea* ATCC50112^T (deposited in the American Type Culture Collection, ATCC, USA); *P. zopfii* genotype 2 SAG2021^T, *P. zopfii* genotype 1 SAG2063^T and *P. blaschkeae* SAG2064^T (deposited in the Culture Collection of Algae (SAG) at the University of Göttingen, Germany).

DNA was isolated and purified by using the method of Cremonesi *et al.* [14]. Based on the published 18S rDNA *Prototheca* sequences available in GenBank database, primer pairs were designed using the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/primer3/, December 2015). All the primers used in this study, ranging from 17 to 22 mers, are shown in Table 1 and were designed in order to have similar melting temperatures.

The primers were synthesized by Thermo Fisher Scientific and resuspended to a final concentration of 100 pmol μl^{-1} in sterile double-distilled water. For PCR amplification of 18S rDNA in a total volume of 25 µl, each reaction contained 12.5 µl of 1× HotStarTaq Master Mix (Qiagen GmbH), 0.2 µl of each primer pair and ~30 ng of DNA. The amplification protocol was 95 °C for 15 min, followed by 30 cycles at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 7 min. All PCRs were performed in singleplex on previously extracted DNA, and PCR products were analysed by agarose gel electrophoresis. Unique single bands of the expected size were purified with a commercial kit (Wizard SV Gel and PCR Clean-up System, Promega). PCR amplification of 28S rRNA, D1/D2 region of the LSU rDNA and SSU rRNA were performed as described by Masuda *et al.* [5], Shahid *et al.* [15] and Xiao *et al.* [16] by using primers listed in Table 1.

Sequencing of the 18S rDNA, 28S rRNA, D1/D2 region and SSU rRNA genes was provided by the sequencing service of GATC Biotech AG (Cologne, Germany). Sequence similarity searches were performed using BLAST in the GenBank database, and the sequence information was then imported into CLUSTAL 12.1 for assembly and alignment (www.ebi.ac.uk/Tools/msa/clustalo/). The 18S rRNA, 28S rRNA, D1/D2 region and SSU rRNA genes sequences of strain PR24^T were compared with those of *Prototheca* species retrieved from GenBank. Phylogenetic analysis was performed using the BioNumerics 5.0 software package (Applied Maths) [17]. The aligned sequences were subjected to genetic distance calculation using the neighbour-joining (NJ) method [18]. Data consistency was tested by bootstrapping the

Table 1. List of primers used in the present study

Target	Primer name	Sequence	Amplicon	Reference
18S rRNA	Fragment 1-FOR	GCGAATGGCTCATTACATCA	340 bp	This study
	Fragment 1-REV	GCCTTCCTTGGATGTGGTAG		
	Fragment 2-FOR	TTCTGCCCTATCAACTTTGGA	294 bp	This study
	Fragment 2-REV	TACGCTCTTGGAGCTGGAAT		
	Fragment 3-FOR	AGGAAGGCAGCAGGCGC	460 bp	This study
	Fragment 3-REV	TTCATCATTACTCCGGTCCTG		
	Fragment 4-FOR	TAGCATGGAATAACGGCACA	334 bp	This study
	Fragment 4-REV	TTCAGCCTTGCGACCATACT		
	Fragment 5-FOR	GTAAACGATGCCGACTAGGG	290 bp	This study
	Fragment 5-REV	ATCAACCTGACAAGGCAACC		
	Fragment 6-FOR	CATGGCCGTTCTTAGTTGGT	335 bp	This study
	Fragment 6-REV	AGCTGATGACTTGCGCCTAC		
28S rDNA	28S rDNA (F)	GCTATCAATAAGCGGAGGAAAAG	600 bp	[15]
	28S rDNA (R)	GGTCCGTGTTTCAAGACG		
D1/D2 of LSU	28SF1	AAGCATATCAATAAGCGGAGG	550 bp	[5]
	635	GGTCCGTGTTTCAAGACGG		
SSU rRNA	SSU-F1	AACCTGGTTGATCCTGCCAGTAGTC		[16]
	SSU-R2	TGATCCTTCTGCAGGTTCACCTACG		
ITS-1	ITS5	GGAAGTAAAAGTCGTAACAAGG		[19]
	ITS2	GCTGCGTTCTTCATCGATGC		
ssPCR	Pz-ITS-F	TTCGACCGAACGAAACGA		[11]
	Pz-ITS-R	AATTCCTGGCATTGGCGACA		
	Pb-ITS-F	AAGGCCCTGCGTTCTTCGCA		[11]
	Pb-ITS-R	GCGTGTTCCCGACCGAGAGA		

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alignments 1000 times with corrections for multiple substitutions [3].

The ribosomal internal transcribed spacer (ITS) and the species-specific (ss) differences of the 18S and 28S rDNA intergenic transcribed spacer were used to highlight genotypic differences among *Prototheca* species. The ITS-1 and ssPCR analysis were performed as reported by White *et al.* [19] and Marques *et al.* [11], all strains were tested using the primer pairs described in Table 1. PCR products were quantified using a BioAnalyzer 2100 applied to the DNA 7500 LabChip kit (Agilent Technologies). DNA from *P. stagnora* ATCC16528^T and *P. ulmea* ATCC50112^T were included in the study.

To highlight the differences among PR24^T and other protothecal species, different strains were subjected to single-strand conformation polymorphism (SSCP)-PCR analysis as previously described by Cremonesi *et al.* [14]. Cellular fatty acid composition was determined in duplicate according to the procedure described by Morandi *et al.* [20].

Microscopically, *P. blaschkeae* subsp. *brasiliensis* cells are spherical and measure 8.6–24 µm in diameter (Fig. 1). Morphologically, cells of PR24^T, *P. zopfii* (SAG2021^T and 2063^T strains) and *P. blaschkeae* (SAG2064^T) looked similar to each other with spherical shape.

Formation of sporangia, which contained two, four, eight or a larger number of sporangiospores, was commonly observed in all three species. The sporangiospores appeared to be arranged in a morula-like shape [1].

Similar results were reported by Masuda *et al.* [5], who showed that the morphology of *P. miyajii* IFM53848^T was indistinguishable from that of *P. wickerhamii* or *P. cutis*. On SB agar at 25, 30 and 37 °C, PR24^T formed white round colonies, smaller than those of *P. zopfii* genotype 2 SAG2021^T, *P. zopfii* genotype 1 SAG2063^T and *P. blaschkeae* SAG2064^T. Growth of PR24^T was not observed at 10 and 45 °C, temperatures at which neither *P. zopfii* nor *P. blaschkeae* strains grew at a detectable level. Differently from the other *Prototheca* species considered, PR24^T strain was not able to grow at pH 4.0 and could not tolerate NaCl concentrations higher than 2.5 % (Table 2). Optimum growth of PR24^T strain occurred at neutral pH and 30–37 °C.

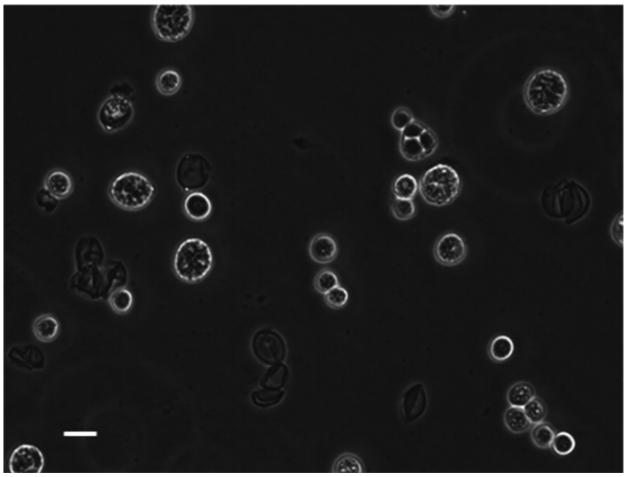

Fig. 1. Culture of PR24^T strain photographed after an incubation of period of 48 h at 37 °C in Sabouraud broth. Bar, 10 µm.

The biochemical tests used to differentiate the novel isolate from other *Prototheca* species are shown in Table 2. Three kits were utilized and 55 different nutrient sources in total were tested for protothecal strains. API 20C AUX testing revealed that PR24^T, like *P. blaschkeae*, assimilated glucose, glycerol (after 120 h), galactose and fructose, but not

Table 2. Biochemical tests useful for the differentiation of *P. blaschkeae* subsp. *brasiliensis* from other *Prototheca* species

Strains: 1, *P. stagnora* ATCC16528^T; 2, *P. wickerhamii* ATCC30395; 3, *P. zopfii* genotype 1 SAG2063^T; 4, *P. zopfii* genotype 2 SAG2021^T; 5, *P. ulmea* ATCC50112^T, 6, *P. blaschkeae* SAG2064^T; 7, *P. blaschkeae* subsp. *brasiliensis* PR24^T. Nutrient utilization is scored as: +, utilization; −, no utilization; (−), utilization after 120 h.

Characteristic	1	2	3	4	5	6	7
Growth with:							
pH 4,0	+	+	+	+	+	+	−
5 % NaCl	−	+	−	+	+	−	−
Nutrient utilization:							
Glucose	+	+	+	+	+	+	+
Galactose	(−)	+	−	−	−	+	+
Glycerol	+	+	+	+	(−)	(−)	(−)
Fructose	+	+	+	+	−	+	+
Trehalose	−	+	−	−	−	−	−
Arginine	+	+	+	+	+	(−)	−
Enzymatic activity:							
Arginine Di-hydrolase	+	+	−	+	+	+	−
β-Galactosidase	−	+	+	+	−	+	−

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trehalose and arginine. The API ID32A kit indicated that strain PR24^T was negative for arginine Di-hydrolase and for β-galactosidase, whereas *P. blaschkeae* SAG2064^T was positive for them (Table 2). A detailed explanation of other characteristics is presented in the species description.

Fatty acid composition of strain PR24^T was analysed from cells grown on SB agar at 37 °C for 48 h and was compared with that of *P. blaschkeae* and *P. zopfii* genotype 1 and 2 type strains (Table 3). Since the analyses were carried out in duplicate, no statistical evaluation was performed. Nevertheless, looking at the fatty acid composition, some observations can be made. The main fatty acids in all the strains were $C_{16:0}$, $C_{18:1}$ and $C_{18:2}$, followed by $C_{14:0}$, $C_{16:1}$, $C_{17:0}$, $C_{17:1}$ and $C_{18:0}$. PR24^T is characterized by a very high percentage of unsaturated fatty acids compared with the reference strains. In particular, the amount of $C_{18:1}$ is about 1.5 times that found in *P. blaschkeae* and *P. zopfii*. As regards to fatty acids present in low amounts, $C_{16:1}$, $C_{17:1}$, $C_{17:0}$ and $C_{18:0}$, similarities were observed between PR24^T and *P. blaschkeae* SAG2064^T.

Table 3. Comparison of cellular fatty acid contents (%) of *P. blaschkeae* subsp. *brasiliensis* from other *Prototheca* species (cells grown on SB agar, 48 h, 37 °C)

Strains 1, *P. zopfii* genotype 1 SAG2063^T; 2, *P. zopfii* genotype 2 SAG2021^T; 3, *P. blaschkeae* SAG2064^T; 4, *P. blaschkeae* subsp. *brasiliensis* subsp. nov PR24^T.

Fatty acids (%)	1	2	3	4
$C_{12:0}$	0.2	0.4	0.3	0.5
$C_{14:0}$	0.8	2.2	1.1	1.9
$C_{15:0}$	0.7	0.3	0.7	0.5
$C_{16:0}$	30.5	32.4	24.8	20.1
$C_{16:1}$	0.6	0.7	3.0	2.5
C_{17}	2.5	0.4	1.1	0.9
$C_{17:1}$	1.2	0.1	2.4	2.1
C_{18}	3.2	6.7	2.3	2.8
$C_{18:1}$	27.2	26.9	26.3	44.5
$C_{18:2}$	32.6	29.2	37.7	24.1
$C_{20:0}$	0.6	0.6	0.4	0.2

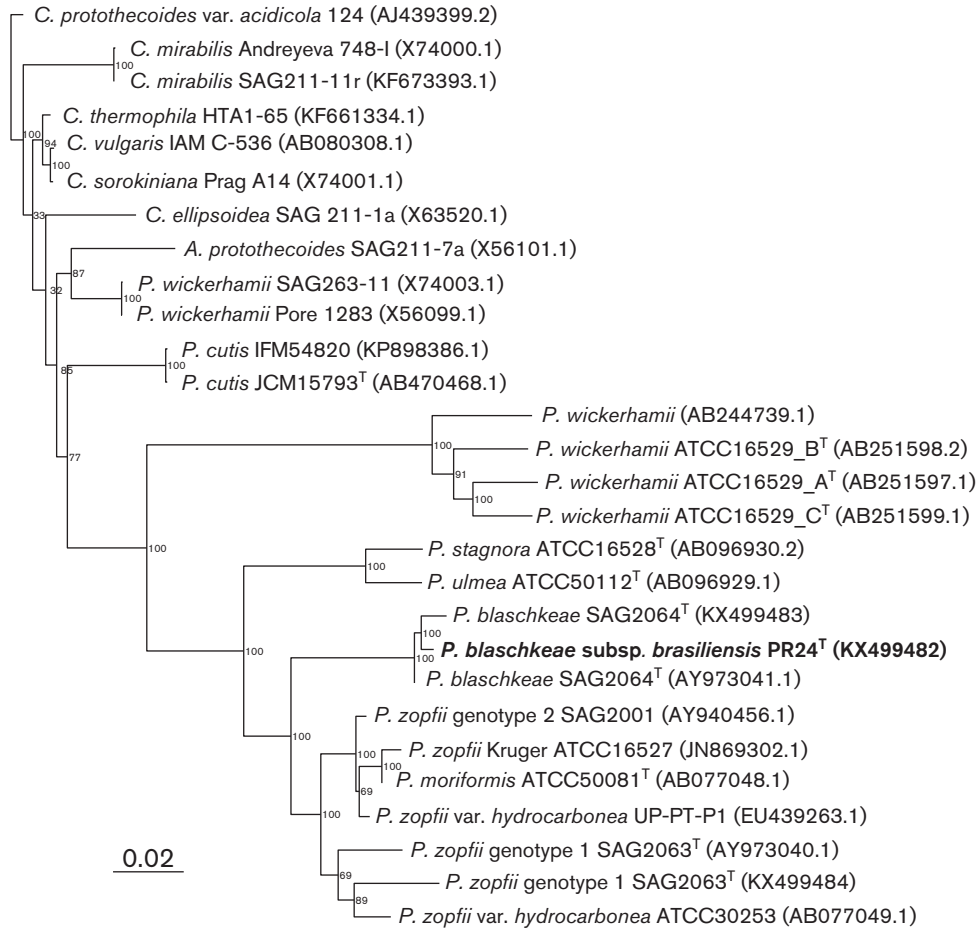

Fig. 2. Phylogenetic relationships of *Prototheca blaschkeae* subsp. *brasiliensis* and other closely related species, based on 18S rRNA gene sequences. The trees were reconstructed using the NJ method. Numbers at nodes are percentage bootstrap frequencies derived from 1000 resamplings. GenBank accession numbers are shown in parentheses. Bar, genetic distance between the strains analysed.

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The almost-complete 18S rDNA gene sequence of PR24^T obtained in this study (1452 bp) indicated that the strain belonged to the genus *Prototheca*. Phylogenetic analysis with the NJ method revealed that the novel strain clustered with *P. blaschkeae* and was related to *P. zopfii* (Fig. 2). Furthermore, the PR24^T strain showed 98.8, 98.4, 96.6 and 96.4 % 18S rDNA gene sequence similarity with the type strains of *P. blaschkeae* SAG2024^T (GenBank accession no. AY973041.1 and KX499483), *P. zopfii* genotype 2 SAG2001^T (X63519.2) and *P. zopfii* genotype 1 SAG2063^T (KX499484), respectively. Low sequence similarities (<93.0 %) were found with other recognized species such as: *P. ulmea* ATCC50112^T (92.1 % – AB096929.1), *P. stagnora* ATCC16528^T (91.6 % – AB096930.2), *P. wicherhamii* 263-11 (83.0 % – X74003.1) and *P. cutis* JCM15793^T (81.7 % – AB470468.1). The 18S rDNA gene sequences of PR24^T, SAG2063^T and SAG2064^T are available in the online Supplementary Material.

Sequence analysis of the 28S rRNA and D1/D2 region of the LSU rDNA restated that PR24^T strain (KY593250) was closely related to *P. blaschkeae* (Fig. 3). In particular, the D1/D2 region of PR24^T showed 99.3 % similarity *P. blaschkeae* UP-PT-P2 (HG515045.1). Moreover, the result of SSU rDNA gene sequence of this strain (KY593251) indicated 97.4 % similarity to *P. blaschkeae* SAG2024^T (AY973041.1).

As previously described by Hirose *et al.* [21], ITS-PCR analysis is a useful tool for protothecal species identification, since the sizes of ITS amplicons are variable among the *Prototheca* species (*P. wickerhamii*>*P. blaschkeae*>*P. zopfii*>*P. cutis*). Moreover, Wang *et al.* [19] used the ITS methods in order to distinguish eukaryotic species that are very close genetically.

In our study, the ITS-1 of PR24^T (629 bp) was found to be smaller than that of *P. blaschkeae* SAG2064^T (640 bp) (Fig. S1, available with the online Supplementary Material); moreover, the ssPCR [11] confirmed the separation of strain PR24^T from other protothecal species. Using primer pair Pb-ITS, no differences were detected between PR24^T and *P. blaschkeae* SAG2064^T (PCR products of about 716 bp), but with primers Pz-ITS the novel isolate produced a

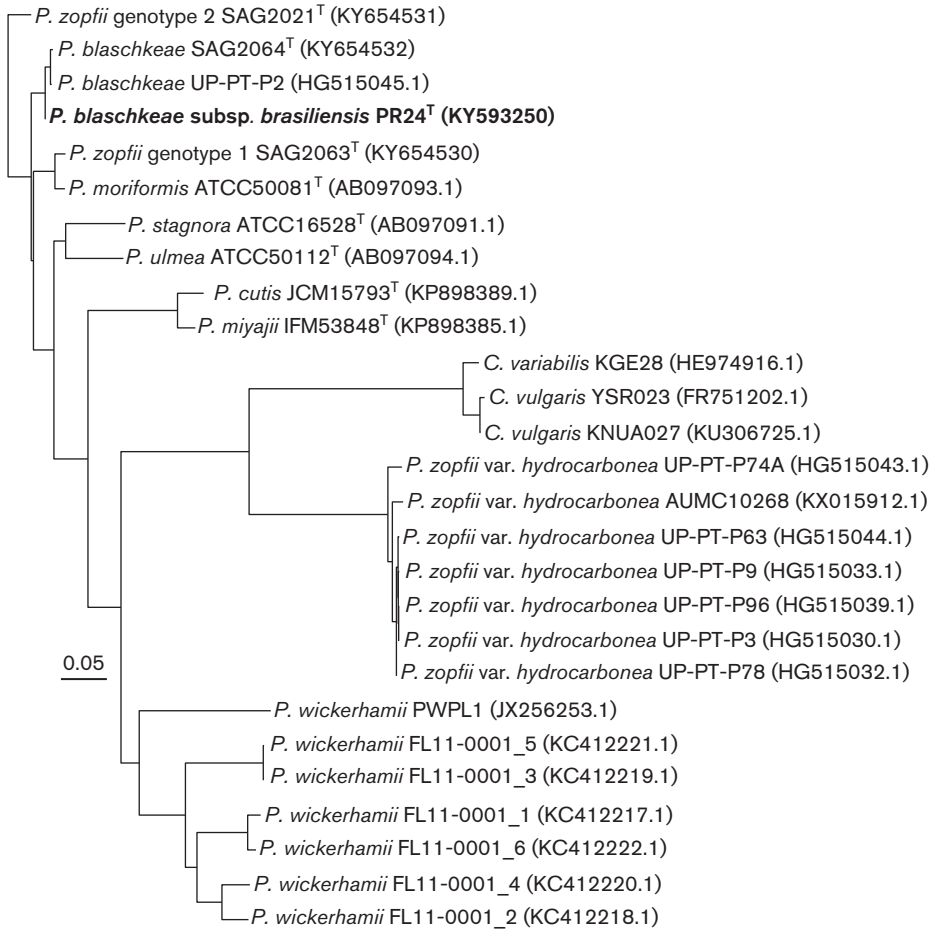

Fig. 3. Phylogenetic relationships of *Prototheca blaschkeae* subsp. *brasiliensis* and other closely related species, based on 28S rRNA gene sequences. The phylogenetic tree was reconstructed and drawn as described in the legend of Fig. 2. GenBank accession numbers are shown in parentheses. Bar, genetic distance between the strains analysed.

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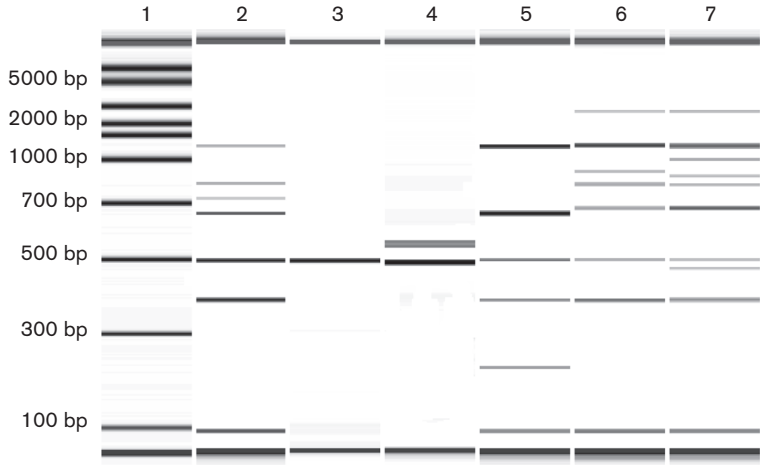

Fig. 4. ssPCR analysis of *P. blaschkeae* subsp. *brasiliensis* and other protothecal species. Lanes: 1, DNA ladder (DNA 500 LabChip Kit; Agilent Technologies); 2, *P. blaschkeae* subsp. *brasiliensis* PR24^T; 3, *P. zopfii* genotype 2 SAG2021^T; 4, *P. zopfii* genotype 1 SAG2063^T; 5, *P. blaschkeae* SAG2064^T; 6, *P. stagnora* ATCC16528^T; 7, *P. ulmea* ATCC50112^T.

characteristic pattern (seven bands of 1229, 809, 726, 656, 495, 390 and 96 bp) that confirms the subspecies status of PR24^T (Fig. 4). These findings were also confirmed by SSCP-PCR analysis, an accurate method for *Prototheca* identification [14], which highlighted that PR24^T strain had a different SSCP pattern from *P. blaschkeae*, *P. zopfii*, *P. stagnora*, *P. ulmea* and *P. wickerhamii* reference strains (Fig. 5). Moreover, randomly amplified polymorphic DNA-PCR analysis conducted with three different primers (M13, OPA-4 and OPA-18) showed that the potential new subspecies was clearly separated from other protothecal species [12].

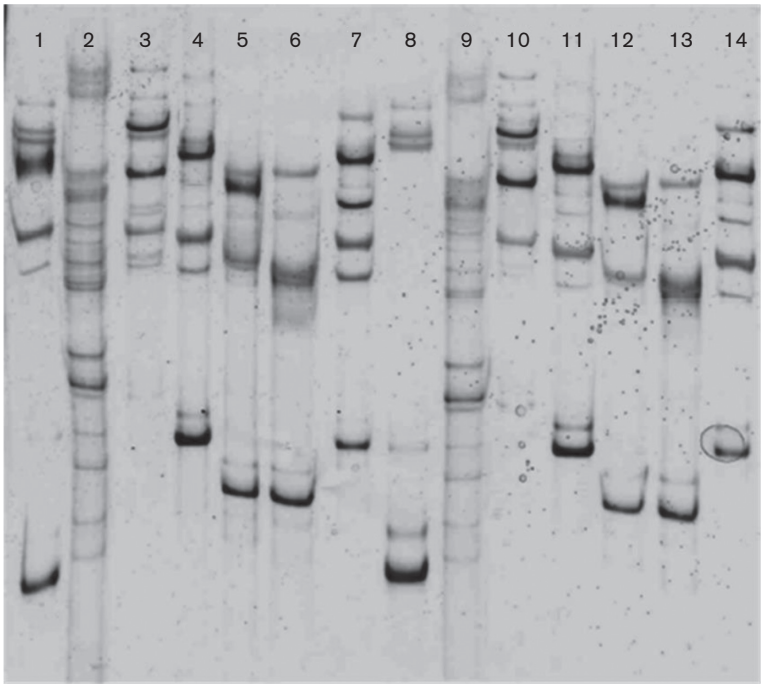

Fig. 5. Polymerase chain reaction-single strand conformation polymorphism (SSCP-PCR) analysis of the two 18S ribosomal DNA fragments amplified. Lanes: 1 and 8 *P. ulmea* ATCC50112^T; 2 and 9 *P. wickerhamii* strain; 3 and 10 *P. stagnora* ATCC16528^T; 4 and 11 *P. blaschkeae* subsp. *brasiliensis* PR24^T; 5 and 12 *P. zopfii* genotype 2 SAG2021^T; 6 and 13 *P. zopfii* genotype 1 SAG2063^T; 7 and 14 *P. blaschkeae* SAG2064^T.

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Usually, *Prototheca* isolates are classified based on phenotypic characteristics, such as macroscopic and microscopic morphologies and carbon assimilation profiles. Although genotypic characterization of the rDNA has been explored in addition to these traditional methods, systematic criteria for classification of species of the genus *Prototheca* have not been established yet [5].

In conclusion, this study shows that strain PR24^T, isolated from cow milk samples, has phenotypic and genotypic differences from *P. blaschkeae* SAG2064^T despite sharing high 18S rDNA, 28S rRNA and D1/D2 region of the LSU rDNA gene sequence similarity. On the evidence presented, we consider that strain PR24^T merits separate subspecies status, and for which the name *Prototheca blaschkeae* subsp. *brasiliensis* subsp. nov. is proposed.

DESCRIPTION OF *PROTOTHECA BLASCHKEAE* SUBSP. *BRASILIENSIS* SUBSP. NOV.

Prototheca blaschkeae subsp. *brasiliensis* (bra.si.li.en′sis. N.L. fem. adj. *brasiliensis* of or pertaining to Brazil, where the type strain was isolated).

After 48 h at 37 °C in Sabouraud broth, *P. blaschkeae* subsp. *brasiliensis* cells are arranged in a morula-like shape measuring 8.6–24 μm in diameter.

After 3 days on Sabouraud agar at 37 °C, the streak culture is white to ivory, smooth and glistening with an entire margin. It grows from 25 to 37 °C, at 2.5 % NaCl, pH 5–7, but not at 10 and 45 °C, with 5 % NaCl or at pH 4. Adequate growth occurs at 30 and 37 °C.

The following carbon compounds are assimilated: glucose, D-galactose, D-fructose, glycerol and mannose. The following elements are not assimilated: D-adonitol, L-arabinose, cellobiose, lactose, maltose, D-mannitol, melezitose, raffinose, D-ribose, D-sucrose, D-sorbitol, trehalose, turanose, D-xylose, inositol, xylitol, methyl α-D-glucopyranoside, N-acetylglucosamine and 2-keto-D-gluconate. Aesculin, arginine, ornithine and urea are not hydrolysed and arginine Dihydrolase and β- galactosidase are not produced. The predominant cellular fatty acids are $C_{18:1}$, $C_{18:2}$ and $C_{16:0}$. The type strain, PR24^T (=DSM 103592^T=IHEM 26958^T), was isolated from cow milk sample in the state of Minas Gerais, Brazil. PR24^T is also deposited in the collections of Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER, Brescia; www.ibvr.org) and the Institute of Sciences of Food Production (CNR ISPA, Milan).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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