Polyphasic taxonomy of the basidiomycetous yeast genus *Rhodosporidium*: *Rhodosporidium kratochvilovae* and related anamorphic species

José Paulo Sampaio,¹ Mário Gadanho,¹ Sofia Santos,²
Filomena L. Duarte,^{2,3} Célia Pais,³ Álvaro Fonseca¹ and Jack W. Fell⁴

Author for correspondence: José Paulo Sampaio. Tel: +351 21 2948300. Fax: +351 21 2948530.
e-mail: jss@mail.fct.unl.pt

¹ Centro de Recursos Microbiológicos, Secção Autónoma de Biotecnologia, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2825-114 Caparica, Portugal

² Estação Vitivinícola Nacional, INIA, 2565-191 Dois Portos, Portugal

³ Centro de Ciências do Ambiente, Departamento de Biologia, Universidade do Minho, 4710-057 Braga, Portugal

⁴ Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Key Biscayne, FL 33419, USA

The phenotypic and genetic heterogeneity of the basidiomycetous yeast species *Rhodosporidium kratochvilovae* was investigated in a group of recent isolates and collection strains. A polyphasic taxonomic approach was followed which included micromorphological studies, nuclear staining, determination of sexual compatibility, physiological characterization, comparison of electrophoretic isoenzyme patterns, PCR fingerprinting, determination of mol% G+C, DNA–DNA reassociation experiments and 26S and ITS rDNA sequence analysis. The results allowed a more natural circumscription of the species, both from the genetic and phenotypic perspectives. The relationships with anamorphic species of the genus *Rhodotorula* were studied and isolates previously identified as *Rhodotorula glutinis* were found to belong to *Rhodosporidium kratochvilovae*. Other isolates included in the study were found to represent members of *Rhodotorula glutinis* var. *dairenensis*. *Rhodosporidium kratochvilovae* was found to include heterothallic strains, besides those already known to be self-sporulating. A total of 17 isolates, which were found to belong to this species, were heterothallic, self-sporulating and anamorphic strains. It is anticipated that integrated polyphasic studies of basidiomycetous yeasts will provide a more coherent classification system and the basis for accurate identification schemes, which in turn are essential for detailed ecological studies.

Keywords: basidiomycetous yeasts, polyphasic taxonomy, *Rhodosporidium kratochvilovae*, *Rhodotorula glutinis*

INTRODUCTION

The heterobasidiomycetous yeast species *Rhodosporidium kratochvilovae* Hamamoto, Sugiyama *et al.* (1988) to accommodate two self-sporulating strains (YK 218 = IGC 4583 and YK 219 = IGC 5579) previously classified in *Rhodosporidium toruloides* Banno. The combination of several taxonomic criteria such as life cycle, electrophoretic mobility of selected enzymes (Yamazaki & Komagata, 1981; Hamamoto *et al.*, 1986a), ubiquinone system (Sugiyama *et al.*, 1985), mol% G+C (Hamamoto *et al.*, 1986b) and DNA–DNA reassociation values (Hamamoto *et al.*, 1987)

supported the proposal of *Rhodosporidium kratochvilovae* as a distinct species. Two other strains (YK 102 = IGC 5580 and YK 103 = IGC 4883) were found to be closely related to *Rhodosporidium kratochvilovae* due to similarities in electrophoretic patterns of enzymes (Yamazaki & Komagata, 1981; Hamamoto *et al.*, 1986a) and high DNA–DNA reassociation values (Hamamoto *et al.*, 1987). These two strains fit the concept of *Rhodotorula glutinis* (Fresenius) Harrison and were regarded as the anamorphic stage of *Rhodosporidium kratochvilovae*, since they were unable to produce mycelium with teliospores (Hamamoto *et al.*, 1988).

Studying recent isolates and collection strains, we investigated the degree of phenotypic and genetic heterogeneity in *Rhodosporidium kratochvilovae* and examined its relationships with anamorphs like *Rho-*

Dedicated to the memory of Dr Cecília Cabeça-Silva, who isolated some of the *Rhodosporidium kratochvilovae* strains studied in this work.

dotorula glutinis. A polyphasic taxonomic approach was followed which included micromorphological studies, nuclear staining, determination of sexual compatibility, physiological characterization, comparison of isoenzyme electrophoretic patterns, PCR fingerprinting, rDNA sequence analysis, determination of mol% G+C and DNA–DNA reassociation experiments.

METHODS

Cultures. A total of 40 strains of *Rhodospiridium kratochvilovae* and related teleomorphic and anamorphic species were investigated. The list of cultures used in this study is presented in Table 1.

Morphological characterization and studies of sexual com-

patibility. For microscopy, cultures were grown on cornmeal agar (CMA) or MYP agar (Bandoni, 1972) at room temperature (20–23 °C) and studied with phase-contrast optics. For determination of sexual compatibility, pairs of 2–4-d-old cultures were crossed on CMA, incubated at room temperature and examined for production of mycelium and teliospores after 1 week. Teliospore germination was investigated after 8–10 weeks. Alternatively, 4–6-week-old teliospores were harvested, soaked in demineralized water for 2 weeks, transferred to water agar and examined after 2–3 d.

Nuclear staining. For nuclei observation, a modified Giemsa staining protocol was used. Cultures were grown on CMA for 1 week and an agar block of approximately 5 mm × 5 mm was cut from a mycelium-containing area. The agar block was placed on a glass slide, and finely cut to small pieces that were allowed to dry for 1 h. Then the specimens were fixed in a mixture of 3:1 ethanol/glacial acetic acid for 30 min,

Table 1 List of strains used in this study

Strains are listed with their original names and those that were renamed during this study are indicated with an asterisk. Their correct designation is listed in Table 2.

Species	Strain no.†	Strain origin‡	Isolation source
<i>Rhodospiridium babjevae</i> Golubev	IGC 5168 ^T	VKM Y-2275 ^T	Herbaceous plant, Moscow, Russia
<i>Rhodospiridium babjevae</i>	IGC 5169	VKM Y-2276	Herbaceous plant, Moscow, Russia
<i>Rhodospiridium diobovatum</i> Newell <i>et</i> Hunter	IGC 4364 ^T	CBS 6085 ^T	Seawater, USA
<i>Rhodospiridium diobovatum</i>	IGC 4640	CBS 6084	Seawater, USA
<i>Rhodospiridium fluviale</i> Fell, Kurtzman, Tallman <i>et</i> Buck	IGC 4701 ^T	CBS 6568 ^T	Brackish water, USA
<i>Rhodospiridium kratochvilovae</i> Hamamoto, Sugiyama <i>et</i> Komagata	IGC 4583 ^T	IAM 13072 ^T	Unknown
<i>Rhodospiridium kratochvilovae</i>	IGC 5579	JCM 8172	Unknown
Anamorph of <i>Rhodospiridium kratochvilovae</i>	IGC 4883	JCM 3772	Unknown
Anamorph of <i>Rhodospiridium kratochvilovae</i>	IGC 5580	JCM 8173	Unknown
<i>Rhodospiridium lusitaniae</i> Fonseca <i>et</i> Sampaio	IGC 4641 ^T		Woodland soil, Lisbon, Portugal
<i>Rhodospiridium lusitaniae</i>	IGC 4599		Dry leaf, Arrábida, Portugal
<i>Rhodospiridium paludigenum</i> Fell <i>et</i> Statzell-Tallman	IGC 4495 ^T	CBS 6566 ^T	<i>Juncus roemerianus</i> marsh, USA
<i>Rhodospiridium paludigenum</i>	IGC 4496	CBS 6567	<i>Rhizophora mangle</i> marsh, USA
<i>Rhodospiridium sphaerocarum</i> Newell <i>et</i> Fell	IGC 4104 ^T	CBS 5939 ^T	Water, Antarctica
<i>Rhodospiridium sphaerocarum</i>	IGC 4105	CBS 5940	Seawater, Antarctica
<i>Rhodospiridium toruloides</i> Banno	IGC 4416	CBS 14	Wood pulp, Sweden
<i>Rhodospiridium</i> sp.*	IGC 4793		Grasshopper, Sintra, Portugal
<i>Rhodospiridium</i> sp.*	IGC 4818		Wood chips, Setúbal, Portugal
<i>Rhodospiridium</i> sp.*	IGC 4819		Moss, Oeiras, Portugal
<i>Rhodotorula araucariae</i> Grinbergs <i>et</i> Yarrow	IGC 4625 ^T	CBS 6031 ^T	Rotting bark of <i>Araucaria araucana</i> , Chile
<i>Rhodotorula glutinis</i> (Fresenius) Harrison var. <i>glutinis</i>	IGC 4177 ^T	CBS 20 ^T	Air
<i>Rhodotorula glutinis</i> var. <i>glutinis</i>	IGC 4706		Reed, Oeiras, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 4776		Leaf, Oeiras, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 4778		Pine cone, Oeiras, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i>	IGC 4780		Blackberries, Sesimbra, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i>	IGC 4781		Dead leaves, Oeiras, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 4783		Polipore fungus, Arrábida, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 4785		Plant litter, Arrábida, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 4787		Seawater, Arrábida, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 4784		Plant litter, Arrábida, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 4897		Flower of <i>Canna indica</i> , Azores, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 5068		Soil, Azores, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 5085		Soil, Azores, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 5244		Culture contaminant, Oeiras, Portugal
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 5591§	CBS 332	Sputum
<i>Rhodotorula glutinis</i> var. <i>glutinis</i> *	IGC 5606	CBS 321	Air, Japan
<i>Rhodotorula glutinis</i> var. <i>dairenensis</i> Hasegawa & Banno	IGC 4885 ^T	JCM 3774 ^T	Unknown
<i>Rhodotorula graminis</i> di Menna	IGC 4842 ^T	CBS 2826 ^T	Grass, New Zealand
<i>Rhodotorula mucilaginoso</i> (Jørgensen) Harrison	IGC 5166 ^T	CBS 316 ^T	Unknown
<i>Rhodotorula mucilaginoso</i>	IGC 4759		Marsh water, Setúbal, Portugal

† IGC, Portuguese Yeast Culture Collection, FCT-UNL, Portugal; ^T, type strain.

‡ CBS, Centraalbureau voor Schimmelcultures, Yeast Division, Delft, The Netherlands; IAM, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan; JCM, Japan Collection of Microorganisms, RIKEN, Japan; VKM, All-Russian Collection of Microorganisms, Moscow, Russia.

§ Type strain of *Torulopsis bronchialis* Ciferri *et* Redaelli.

|| Type strain of *Torula rufula* Saito.

washed in demineralized water, hydrolysed in 1 M HCl at 55–60 °C for 7 min, and washed again in 0.1 M sodium phosphate buffer (pH 7). Specimens were then stained for 2 h in a 1:9 Giemsa (Merck)/phosphate buffer solution. Excess stain was removed by washing with demineralized water. After the slides had dried completely, they were mounted permanently in entellan (Merck).

Physiological characterization. Physiological and biochemical characterization was performed according to the techniques described by Yarrow (1998). Additional assimilation tests were performed using aldaric acids and aromatic compounds, as described by Fonseca (1992) and Sampaio (1999), respectively. The results of the 71 tests selected by Sampaio & Fonseca (1995) were used to calculate the overall similarity of physiological and biochemical profiles, employing numerical taxonomy methods. The complete data matrix is available upon request. The similarity between strains was estimated with the simple-matching coefficient and cluster analysis was performed using the UPGMA (unweighted pair group method using arithmetic averages) algorithm (Sneath & Sokal, 1973). These analyses were computed using the NTSYS-pc software package, version 2.0 (Rolf, 1998).

Comparison of isoenzyme electrophoretic patterns. Cultures were grown in liquid medium containing 0.5% (w/v) Bacto-yeast extract, 1% (w/v) Bacto-peptone and 2% (w/v) glucose, at 25 °C on a rotary shaker (170 r.p.m.), until late exponential phase. Cells were harvested by centrifugation (5000 g, 4 °C, for 7 min), washed twice with 20 ml buffer (3.2 mmol Tris/HCl l⁻¹, pH 7) and stored at -20 °C until further utilization. For protein extraction, cells were disrupted with 1 g 0.5 mm glass beads and 1 ml buffer (60 mmol Tris/HCl l⁻¹, pH 6.8) using a Mini Bead Beater cell homogenizer (Cole Parmer), at 5000 r.p.m. for 30 s, followed by cooling for 4 min on ice. This operation was repeated at least six times, until more than 70% of the cells were disrupted. Insoluble debris and intact cells were removed by centrifugation at 15000 g, 0 °C, for 30 min. Protein extracts were stored at -20 °C. Five enzymes were assayed: esterase (EC 3.1.1.1), acid phosphatase (EC 3.1.3.2), lactate dehydrogenase (EC 1.1.1.27), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and alcohol dehydrogenase (EC 1.1.1.1). Electrophoresis conditions and detection of enzyme activity followed the procedures of Pasteur *et al.* (1987) for alcohol dehydrogenase and of Duarte *et al.* (1999) for the remaining enzymes. The reproducibility of the methods employed was previously checked (Santos *et al.*, 1999). The relative electrophoretic mobilities (R_M) of the enzyme bands were calculated as the ratio of the migration of each band to that of the tracking dye and adjusted by comparison with the values obtained for *Saccharomyces cerevisiae* IGC 4072, electrophoresed on the same gel and used as a reference. The presence or absence of a band with a given R_M was coded with 1 or 0, respectively, and the complete data matrix was analysed with the NTSYS-pc version 2.0 program software, using the Dice coefficient and the UPGMA clustering method.

Determination of mol% G + C and the extent of DNA–DNA reassociation. For DNA isolation the strains were grown for 3–4 d at 22 °C in shaken YM broth (initial pH = 8). Cells were ruptured using a hydraulic cell press and the DNA was purified using the method of Britten *et al.* (1970). The nuclear DNA base composition was determined following the method of Marmur & Doty (1962) with a Guilford Response UV-VIS Spectrophotometer and its Thermal Programming software, with nDNA from *Candida parapsilosis* IGC 2545^T (mol% G + C = 40.2%) as reference.

Values given in this report represent the means of three determinations. For DNA–DNA reassociation experiments the same instrument was used and the methods of Seidler & Mandel (1971), as modified by Kurtzman *et al.* (1980), were followed. Each reassociation experiment was repeated at least three times.

PCR fingerprinting. For DNA extraction, two loopfuls of MYP-agar-grown cultures were suspended in 500 µl lysing buffer (50 mmol Tris l⁻¹; 250 mmol NaCl l⁻¹; 50 mmol EDTA l⁻¹; 0.3%, w/v, SDS; pH 8) and the equivalent to a volume of 200 µl of 425–600 µm glass beads (Sigma) was added. After vortexing for 3 min, the tubes were incubated for 1 h at 65 °C. The suspensions were then centrifuged for 30 min at 4 °C. Nucleic acids were precipitated with 1/10 volume of sodium acetate and two volumes of absolute ethanol for 24 h at -20 °C. Finally, DNA was washed with ethanol (70%, v/v), dried and resuspended in TE (100 mmol Tris/HCl l⁻¹, pH 8, and 100 mmol EDTA l⁻¹).

Seven primers (Pharmacia Biotech) were used: four decamers (Dec. 1, ACGGTCTTGG; Dec. 2, TGCCGAGCTG; Dec. 3, TGCAGCGTGG; and Dec. 4, GGGTAACGCC; all from Prillinger *et al.*, 1997) were employed for RAPD-PCR experiments (Welsh & McClelland, 1990; Williams *et al.*, 1990). The core sequence of the phage M13 (GAGGGTGGCGGTTCT) was used as a single primer in minisatellite-primed PCR experiments (Meyer *et al.*, 1993) and two synthetic oligonucleotides, (GACA)₄ and (GTG)₅, were tested in microsatellite-primed PCR experiments (Meyer *et al.*, 1993). All PCR reactions were performed in 25 µl reaction volumes containing 1×PCR buffer (Pharmacia, Biotech), 2 mmol l⁻¹ of each of the four dNTPs (Promega), 0.8 µmol primer l⁻¹, 10–15 ng genomic DNA and 1 U *Taq* DNA polymerase (Pharmacia, Biotech).

Amplification was performed in a Uno II Thermal Cycler (Biometra), consisting of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 45 s at 93 °C, 60 s at 36 °C (RAPD-PCR) or 50 °C (mini and microsatellite primed PCR) and 60 s at 72 °C and a final extension step of 6 min at 72 °C. A negative control in which DNA was replaced by sterile distilled water was also included. Amplified DNA fragments were separated by electrophoresis in 1.4% (w/v) agarose gels (Gibco-BRL) in 0.5×TBE (Tris-borate-EDTA) buffer at 90 V for 3.5 h, and stained with ethidium bromide. On each gel, a molecular size marker was used for reference (λ DNA cleaved with *Hind*III and Φ X174 DNA cleaved with *Hae*III – Pharmacia, Biotech).

DNA banding patterns were visualized under UV transillumination and images were acquired using a Kodak Digital Science EDA 120 System and the Kodak Digital Science 1D Image Analysis Software. DNA banding patterns were analysed using the GelCompar software package, version 4.1 (Applied Maths, Kortrijk). Similarities among isolates were estimated using the Dice coefficient and clustering was based on the UPGMA method. The distortion introduced by the hierarchic grouping was estimated by comparing the values of the similarity matrix with those of the cluster analysis, to obtain a co-phenetic correlation coefficient (r), based on Pearson's product-moment coefficient (Sneath & Sokal, 1973). Low correlation ($r < 0.7$) indicates that the taxonomic structure is being distorted by the hierarchic clustering.

The reproducibility of PCR patterns was investigated using two repeats of three randomly chosen strains. DNA used in each set of repetitions was extracted from distinct cell

batches and the amplification reactions were performed in different PCR assays. Based on the fingerprint differences observed between each set of repeats, the probability of error was calculated according to Sneath & Johnson (1972). This method was initially defined for phenotypic characters and is based on the binomial distribution. It can be applied when results from a large number of tests are available, in duplicate, for the same strain. In the present study, this approach was used to evaluate PCR fingerprints by considering that each band in a fingerprint is an independent character, expressed in terms of presence or absence. The probability of error (p) associated with each primer was calculated using the formula $p = 1 - \sqrt{1 - 4s^2}$, defined by Sneath & Johnson (1972). The variance (s^2) was calculated as the proportion of unmatched amplified bands in the total number of bands obtained with the two repeats of each strain.

rDNA sequence analysis. DNA was extracted using the methods described for PCR fingerprinting and amplified using primers NS7 (5'-GAG GCA ATA ACA GGT CTG TGA TGC-3') and LR6 (5'-CGC CAG TTC TGC TTA CC-3'). Cycle sequencing of the 600–650 bp region at the 5' end of the 26S rDNA D1/D2 domain employed forward primer F63 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and reverse primer LR3 (5'-GGT CCG TGT TTC AAG ACG G-3'). ITS cycle sequencing primers included the forward primer ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and the reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). Sequences were obtained with a Li-Cor automated sequencer using standard protocols. Alignments were made with MegAlign (DNASar) and visually corrected. Phylogenetic trees were computed with PAUP version 4.0 using parsimony analysis (heuristic search, stepwise addition, random addition sequence, nearest-neighbour interchange, 100 maximum trees). Bootstrap analysis was based on 1000 replicates.

RESULTS AND DISCUSSION

During a programme of isolating yeasts able to utilize low-molecular-mass aromatic substrates, several strains of pink yeasts were collected and identified as *Rhodotorula glutinis* (Sampaio, 1994). Later, two of those strains (IGC 4818 and IGC 4819) were found to be sexually compatible, producing mycelium with true clamp connections and teliospores which, upon germination, originated phragmobasidia and sessile basidiospores (Fig. 1). The sexually compatible strains IGC 4818 and IGC 4819 shared more physiological similarities with the self-sporulating species *Rhodospiridium kratochvilovae* than with the remaining *Rhodospiridium* species. Furthermore, the only relevant morphological difference that could be detected between the crossing of IGC 4818 and IGC 4819 and the strains of *Rhodospiridium kratochvilovae* was the absence of clamp connections in the latter. The hypothesis that the species *Rhodospiridium kratochvilovae* includes hitherto undescribed heterothallic strains, as suggested by both the physiological and micromorphological comparisons, was tested using different approaches.

Based on the overall similarity of physiological data, Sampaio & Fonseca (1995) observed that strains of

Fig. 1. Line drawing of hyphae, teliospores, basidium and basidiospores resulting from the crossing of IGC 4818 and IGC 4819 on CMA after 1 month at room temperature. The teliospore on the left germinated and originated a four-celled basidium. The teliospore on the right is still attached to the mycelium (note the complete clamp connections). Bar, 5 μ m.

Rhodotorula glutinis clustered in two distinct main groups, one including the type strain of the species and the other including IGC 4883, an anamorphic strain of *Rhodospiridium kratochvilovae*. Some of the strains investigated in that study were also included in the present report in order to check their identity.

PCR fingerprinting and isoenzyme analysis

Reproducibility of PCR fingerprinting was evaluated in preliminary experiments by estimating the probability of error associated with each primer. Decamer 4 and (GACA)₄ were rejected, given their poor reproducibility in repeated PCR experiments ($p = 0.237$ and 0.259 , respectively). The remaining five primers showed clear and reproducible patterns and the values of p were 0 for primer M13, 0.032 for primer (GTG)₅ and varied between 0.061 and 0.075 for the remaining RAPD primers. Therefore their ability to produce species-specific profiles was investigated with a selected group of strains. The dendrogram resulting from the combined numerical analysis of the PCR profiles obtained with the five primers is shown in Fig. 2. Besides the two self-sporulating strains (IGC 4583^T and IGC 5579) reported in the original description, the *Rhodospiridium kratochvilovae* cluster contained a recent isolate of a self-sporulating *Rhodospiridium* sp. (IGC 4793), two strains considered as anamorphs of *Rhodospiridium kratochvilovae* (IGC 4883 and IGC 5580), two other strains (IGC 5591 and IGC 5606) reported as related to the type strain of *Rhodospiridium kratochvilovae* based on the analysis of whole-cell protein patterns (Vancanneyt *et al.*, 1992), the two above-mentioned sexually compatible strains (IGC 4818 and IGC 4819), and IGC 4776, IGC 4778, IGC 4785 and IGC 4787, which had been previously identified as *Rhodotorula glutinis* (Sampaio & Fonseca, 1995). A similar delimitation of the *Rhodospiridium*

Fig. 2. Dendrogram of selected strains of *Rhodosporidium* and *Rhodotorula* based on the combined analysis of the PCR fingerprints obtained with primers Decamer 1, Decamer 2, Decamer 3, (GTG)₅ and M13, using the Dice coefficient and the UPGMA cluster method (r , co-phenetic correlation coefficient). The black circle indicates the node corresponding to the *Rhodosporidium kratochvilovae* cluster.

Fig. 3. Dendrogram of selected strains of *Rhodosporidium* and *Rhodotorula* based on the combined analysis of the electrophoretic patterns of the enzymes esterase, acid phosphatase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase and alcohol dehydrogenase, using the Dice coefficient and the UPGMA cluster method (r , co-phenetic correlation coefficient). The black circle indicates the node corresponding to the *Rhodosporidium kratochvilovae* cluster.

kratochvilovae cluster was observed using the electrophoretic patterns of five isoenzyme systems (Fig. 3). These results support the hypothesis that all the strains in this group belong to a single species. In spite of the agreement between the PCR fingerprinting (Fig. 2) and isoenzyme analysis (Fig. 3), a discrepant result was observed for *Rhodotorula glutinis* IGC 4784, if previously published data are taken into consideration. This strain was characterized as a member of the *Rhodosporidium kratochvilovae* group based on physiological data (Sampaio & Fonseca, 1995), but clustered outside this group and close to the type strain of *Rhodotorula glutinis* var. *dai* Hasegawa et Banno in both analyses of the present study.

To determine the stability of the *Rhodosporidium kratochvilovae* cluster, a larger set of strains was studied, including representatives of all the other *Rhodosporidium* species (Table 1). The PCR fingerprinting method was employed but only two primers were used. The selected primers were M13 and (GTG)₅ due to their higher reproducibility and their ability to produce homogeneous and species-specific profiles. The DNA fingerprints and the dendrogram resulting from the combined numerical analysis of the PCR profiles obtained with the two primers are shown in

Fig. 4. All species of the genus *Rhodosporidium* originated distinct patterns and therefore *Rhodosporidium kratochvilovae* was clearly separated from the other members of the genus. Moreover, four additional strains (IGC 4783, IGC 5068, IGC 5085 and IGC 5244), previously identified as *Rhodotorula glutinis*, originated *Rhodosporidium kratochvilovae*-type PCR fingerprints (Fig. 4).

Species delimitation

The results from PCR fingerprinting and isoenzyme approaches suggested that several teleomorphic and anamorphic strains might represent additional members of *Rhodosporidium kratochvilovae*. To test this hypothesis, a selected group of strains was investigated with other methodologies. The methods used at this stage were determination of mol% G + C, assessment of the degree of DNA–DNA hybridization, determination of the relatedness of 26S rDNA sequences and investigation of sexual compatibility.

The mol% G + C values (thermal denaturation method) determined in this study were 70.2 ± 0.18 and 69.9 ± 0.15 for the self-sporulating strains IGC 4583^T

Fig. 4. DNA banding patterns and resulting dendrogram of selected strains of *Rhodosporidium* and *Rhodotorula* based on the combined analysis of the PCR fingerprints obtained with primers (GTG)₅ and M13, using the Dice coefficient and the UPGMA cluster method (r , co-phenetic correlation coefficient). The black circle indicates the node corresponding to the *Rhodosporidium kratochvilovae* cluster.

and IGC 5579, respectively, and 68.7 ± 0.17 and 68.9 ± 0.12 for the anamorphic strains of *Rhodosporidium kratochvilovae* IGC 4883 and IGC 5580, respectively. The mol% G+C values obtained for IGC 4793 (self-sporulating) and IGC 4818 (mating type) were 70.9 ± 0.14 and 71.3 ± 0.25 , respectively. Since the mol% G+C values of IGC 4793 and IGC 4818 were similar to the values determined for the reference strains, the hypothesis that both strains

belong to *Rhodosporidium kratochvilovae* could not be rejected. On the other hand, *Rhodotorula glutinis* IGC 4784, the strain clustering close to *Rhodotorula glutinis* var. *daiensis* in the PCR fingerprinting and isoenzyme analysis, had a mol% G+C of 64.9 ± 0.22 . This value was similar to the one obtained for the type strain of *Rhodotorula glutinis* var. *daiensis* (mol% G+C of IGC 4885^T = 64.9 ± 0.18). To determine the conspecificity of IGC 4793 and IGC 4818 with *Rhodo-*

Fig. 5. Results of DNA–DNA reassociation experiments involving strains of *Rhodospiridium kratochvilovae* and *Rhodotorula glutinis* var. *dairenensis*.

sporidium kratochvilovae and to clarify the relationship of IGC 4784 with *Rhodotorula glutinis* var. *dairenensis*, DNA–DNA reassociation experiments were performed as depicted in Fig. 5. Considering the homology values higher than 70% as an evidence of conspecificity, we confirmed that IGC 4793 and IGC 4818 belong to *Rhodospiridium kratochvilovae* whereas IGC 4784 is a member of *Rhodotorula glutinis* var. *dairenensis*. Therefore, IGC 4818 is designated mating type A1 of *Rhodospiridium kratochvilovae* and IGC 4819 mating type A2. The two compatible strains were then used to detect additional heterothallic strains among the anamorphic strains found in the *Rhodospiridium kratochvilovae* cluster (Figs 2, 3 and 4). The strains IGC 4818 and IGC 4819 were crossed, separately, with the remaining non self-sporulating

strains. Results were scored positive when mycelium with clamp connections and teliospores was produced. The currently recognized strains of *Rhodospiridium kratochvilovae* and their type of sexuality are depicted in Table 2. Most of the *Rhodotorula glutinis* strains included in the *Rhodospiridium kratochvilovae* cluster were found to belong to one of the two mating types of *Rhodospiridium kratochvilovae*. The strain IGC 4883, previously characterized as an anamorphic stage of *Rhodospiridium kratochvilovae* (Hamamoto *et al.*, 1988), is in fact a heterothallic strain, although positive mating reactions were infrequent and difficult to detect due to the scarcity of mycelium with teliospores. Weak mating reactions also occurred with strain IGC 4776. Moreover, when the self-sporulating strain IGC 4793 was crossed with strain IGC 4819 (mating type A2), besides the self-sporulating mycelium with incomplete clamp connections, some branches of mycelium with true clamp connections were detected. This observation suggests that IGC 4793 behaves as either a self-sporulating or a heterothallic strain. This phenomenon did not occur when crossings were performed with the opposite mating type or in crossings involving other heterothallic and self-sporulating strains. Absence of mating reactions was recorded for four strains (Table 2). Since these strains repeatedly failed to demonstrate any type of sexual compatibility and as a consequence no sexual state has been observed, we consider that they should not be designated *Rhodospiridium kratochvilovae*. On the other hand, it seems redundant to propose a new *Rhodotorula* species to accommodate these isolates since, according to currently used taxonomic criteria, namely DNA–DNA reassociation values, the sexual and asexual strains of the *Rhodospiridium kratochvilovae* cluster are conspecific. Therefore we propose the designation ‘anamorphic state of

Table 2 Sexuality and physiological traits of *Rhodospiridium kratochvilovae* strains

Data from *Rhodotorula glutinis* are included for comparison. Sex., sexuality; Ss, self-sporulating; Mt, mating type; An, anamorphic; Xil., xylitol; Cx 100, cycloheximide 100 p.p.m.; Cx 1000, cycloheximide 1000 p.p.m.; Ga., gallic acid; Cat., catechol; Vit., growth without vitamins; 35 °C, growth at 35 °C.

Species	Strain	Sex.	Xil.	Cx 100	Cx 1000	Ga.	Cat.	Vit.	35 °C
<i>Rhodospiridium kratochvilovae</i>	IGC 4583 ^T	Ss	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 5579	Ss	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 4793	Ss, Mt A1	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 4818	Mt A1	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 5244	Mt A1	–	–	–	+	+	+	+
<i>Rhodospiridium kratochvilovae</i>	IGC 4776	Mt A2	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 4778	Mt A2	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 4783	Mt A2	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 4785	Mt A2	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 4819	Mt A2	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 4883	Mt A2	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 5068	Mt A2	–	–	–	+	+	+	–
<i>Rhodospiridium kratochvilovae</i>	IGC 5085	Mt A2	–	–	–	+	+	+	+
Anamorph of <i>Rhodospiridium kratochvilovae</i>	IGC 4787	An	–	–	–	+	+	+	–
Anamorph of <i>Rhodospiridium kratochvilovae</i>	IGC 5580	An	–	–	–	+	+	+	–
Anamorph of <i>Rhodospiridium kratochvilovae</i>	IGC 5591	An	–	–	–	+	+	+	+
Anamorph of <i>Rhodospiridium kratochvilovae</i>	IGC 5606	An	–	–	–	+	+	+	+
<i>Rhodotorula glutinis</i> var. <i>glutinis</i>	IGC 4177 ^T	An	+	+	+	–	–	+	–
<i>Rhodotorula glutinis</i> var. <i>dairenensis</i>	IGC 4885 ^T	An	+	+	+	–	–	–	+
<i>Rhodotorula glutinis</i> var. <i>dairenensis</i>	IGC 4784	An	+	+	+	–	–	–	+
<i>Rhodotorula glutinis</i> var. <i>dairenensis</i>	IGC 4897	An	+	+	+	–	–	–	+

Fig. 6. Giemsa's staining micrographs of strains of *Rhodosporidium kratochvilovae* grown on CMA for 1 week at room temperature. (a)–(d) IGC 4583^T; (e)–(i) IGC 4793; (j)–(l) IGC 4785 × IGC 4818. Bar, 10 µm.

Rhodosporidium kratochvilovae' to be applied in such cases.

The nuclear behaviour of *Rhodosporidium kratochvilovae* was studied with Giemsa staining and selected micrographs are presented in Fig. 6. Giemsa staining revealed that the mycelium resulting from the crossing of heterothallic strains was dikaryotic (Fig. 6j). The mycelium of the self-sporulating strains was also dikaryotic, one of the nuclei usually being retained in the incomplete clamp connection. In some cases, three nuclei could be observed per mycelial compartment (Fig. 6i). This could be the result of the division of the free nucleus and suggests that the mycelium is functionally homo-dikaryotic. In the heterothallic strains, teliospores were dikaryotic immediately after formation (Fig. 6k) but, when mature, only one nucleus was observed (Fig. 6l). In the self-sporulating strains IGC 4583^T and IGC 4793, nuclear behaviour in the teliospore was difficult to interpret. Based on micrographs of teliospores in different developmental stages, we tentatively propose the following sequence of events: (i) young teliospores have a single nucleus (if present, the incomplete clamp connection at the base of the teliospore has no nucleus at this stage) (Fig. 6a, e and f); (ii) the nucleus divides in the teliospore (Fig. 6b, g); (iii) one of the resulting two nuclei migrates to the incomplete clamp connection at the base of the teliospore (Fig. 6c); the nucleus that remained in the teliospore divides again (Fig. 6d, h); (iv) the two nuclei in the teliospore fuse and the teliospore becomes uninucleate but diploid (micrographs not shown but identical to Fig. 6l). In conclusion, *Rhodosporidium kratochvilovae* seems to be dikaryotic and then diploid, either in the heterothallic or in the self-sporulating condition. This suggests that meiosis takes place as usual, and would explain why the basidia of the self-sporulating strains have the typical four compartments instead of two. The main difference between heterothallic and self-sporulating life cycles would be, respectively, the heterokaryotic versus homokaryotic condition of the dikaryon.

To compare the information given by the different approaches aiming at species recognition, the D1/D2 region of the 26S rDNA of a selected group of strains was sequenced and compared with available sequences from related species (Fig. 7). No nucleotide differences were found among the *Rhodosporidium kratochvilovae* strains or among the three strains of *Rhodotorula glutinis* var. *dairenensis* studied. Due to their usefulness in the discrimination of closely related taxa (Fell *et al.*, 2000), the ITS sequences of a restricted number of strains of *Rhodosporidium kratochvilovae* were determined. No base pair differences were found between IGC 4583^T (self-sporulating), IGC 4818 (mating type) and IGC 5591 (anamorph). Therefore, sequence data concur with the other taxonomic criteria used in this work and point to the presence of a single species in the *Rhodosporidium kratochvilovae* cluster, irrespective of the different types of sexuality found.

Fig. 7. Phylogenetic tree of *Rhodosporidium kratochvilovae* and related species, obtained by parsimony analysis (heuristic search, random addition, nearest neighbour interchange, 100 maximum trees) of the D1/D2 region of the 26S rDNA. Branch lengths are proportional to the number of nucleotide differences and the numbers given on the branches are the bootstrap values (> 50%) of 1000 replicates. GenBank accession numbers are indicated after species and strain designation.

To improve the phenotypic recognition of *Rhodosporidium kratochvilovae*, the physiological characterization of the species was re-evaluated using conventional and non-conventional physiological tests. All tests that previously gave variable responses for the strains of the *Rhodosporidium kratochvilovae* cluster, as defined by molecular methods, were repeated. In some cases, the variable results were confirmed but in other cases, uniform responses were detected, which contributed to a better phenotypic circumscription of the species. The physiological profiles of IGC 4783 and IGC 4784 were also checked due to the discrepancies found between previously published data (Sampaio & Fonseca, 1995) and the present study. For these two strains, some of the former results were found to be incorrect. The revised physiological data were analysed using the overall similarity approach (Fig. 8). The results were in accordance with the data derived from the other approaches presented above (Figs 2, 3, 4, 5 and 7). Salient physiological characteristics of *Rhodosporidium kratochvilovae*, allowing the discrimination from *Rhodotorula glutinis*, are listed in Table 2. The reinvestigation of physiological characteristics indicates that a relationship with *Rhodosporidium kratochvilovae* can be presumed for anamorphic strains falling in the general delimitation of *Rhodotorula*

glutinis (i.e. pink-coloured, inositol-negative, melezitose-positive and nitrate-positive), but unable to grow in the presence of cycloheximide, unable to utilize xylitol and capable of using the aromatic compounds gallic acid and catechol.

Based on sequence data from the D1/D2 region of the 26S rDNA, Fell *et al.* (1998) observed that the type strain of *Rhodotorula glutinis* var. *dairenensis* was clearly distinct from the type strain of *Rhodotorula glutinis* var. *glutinis*. These results, as well as those presented in our study, indicate that *Rhodotorula glutinis* var. *dairenensis* should be placed in a species of its own. However, a detailed characterization of additional isolates of *Rhodotorula glutinis* var. *dairenensis* should be accomplished prior to any formal taxonomic proposal.

Integrated polyphasic studies of basidiomycetous yeasts, such as the one presented in this investigation, contribute to expanding our knowledge on the genetic and phenotypic boundaries of species. Another important aspect is the unveiling of new anamorph–teleomorph connections in order to reduce the recognized heterogeneity of taxa such as *Rhodotorula glutinis*. Moreover, these studies allow the evaluation of different taxonomic approaches currently available.

Fig. 8. Phenogram of *Rhodosporidium* and related *Rhodotorula* species based on the overall similarity (simple matching coefficient) and cluster analysis (UPGMA) of 78 physiological tests (r , co-phenetic correlation coefficient). The black circle indicates the node corresponding to the *Rhodosporidium kratochvilovae* cluster.

In the present case, the microsatellite-primed PCR fingerprinting method using the M13 and (GTG)₅ primers proved to be suitable for the discrimination of the species under study. Since it allows a rapid and accurate investigation of a large number of strains, this procedure is currently used in our laboratory for the molecular identification of environmental isolates. In the future, the study of other species in the genus *Rhodosporidium* will allow a thorough reassessment of the traditional morphological and physiological criteria currently used in the taxonomy of this genus.

These efforts will conceivably result in a more natural classification system and will improve our understanding of the ecology of the different *Rhodosporidium* species.

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