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ARTICLE

Genetic variability of astaxanthin-producing yeasts: random amplified polymorphic DNA (RAPD) analysis of *Phaffia rhodozyma* and *Xanthopyllomyces dendrorhous*

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ABSTRACT Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione)-accumulating yeasts are of great biotechnological interest. Random amplified polymorphic DNA (RAPD) analysis involving 5 primers and 13 astaxanthin-producing yeast strains was performed. Cluster analysis based on RAPD markers differentiated isolates of *Xanthopyllomyces dendrorhous* (self-sporulating) and *Phaffia rhodozyma* (asexual) at an intraspecific level. Strains considered to be derived from the same isolate, but which had had different strain histories, revealed significant differences in their RAPD patterns. The applicability of RAPD analysis for the species-level differentiation of these yeasts is discussed.
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KEY WORDS

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Phaffia rhodozyma was described by Miller et al. (1976). The accumulation of astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) as primary pigment in this red yeast created considerable biotechnological interest (Johnson and An 1991; Johnson and Schroeder 1996). Although astaxanthin is found in nature in several organisms (e.g. certain marine fish, crustaceans and birds), the number of natural sources of practical value is very limited. At the same time, both the aquaculture industry (as a feed supplement for salmon and trout) and various other applications connected with the excellent antioxidant properties of astaxanthin (Schroeder and Johnson 1993) demand substantial amounts of this carotenoid.

Golubev (1995) described the sexual cycle of this yeast and of the teleomorph *Xanthopyllomyces dendrorhous*, proposed to be con-specific with the anamorph *P. rhodozyma*. However, a number of data have accumulated that contradict this assumption. Kucsera et al. (1998) studied 6 isolates and suggested that *X. dendrorhous* and *P. rhodozyma* are different yeast species: the type strain of *P. rhodozyma* (CBS 5905) differed from all the other investigated isolates as it was able to reproduce only asexually and it produced respiratory-deficient petite mutants. Similarly, when the same 6 isolates were screened for the presence of extrachromosomal genetic elements, all proved to harbour double-stranded DNA plasmids, with the exception of *P. rhodozyma* type strain CBS 5905 (Pfeiffer et al. 1994). The most convincing evidence for the separation of the two proposed yeast species has been obtained through the analysis of their rDNA IGS (intergenic spacer) and ITS (internal transcribed spacer) sequences (Fell and Blatt 1999). The phylogentic trees based on these data suggested the species-level separation of the CBS 5905 isolates (*P. rhodozyma*) and 4 other isolates (*X. dendrorhous*).

Random amplified polymorphic DNA (RAPD) analysis has proved to be a rapid and very sensitive molecular method when genetic polymorphism is to be detected (Williams et al. 1990). This assay has been employed for the characterization of many fungi, e.g. *Rhizomucor* (Vastag et al. 2000), *Gilbertella* (Papp et al. 2003) and *Rhizopus* (Vágvölgyi et al. 2004).

The aim of the present study was to investigate the utility of DNA polymorphisms detected by RAPD analysis for astaxanthin-producing yeasts. Five random primers were tested for their ability to detect variability among 13 yeast strains, and cluster analysis was performed on the basis of these data.

Materials and Methods

Microorganisms, media and culture conditions

The names and origins of the 13 yeast strains examined are listed in Table 1. On the basis of their collection codes, some of the strains (1, 7, 8 and 10; 2 and 4) were regarded as originating from the same isolate, but they were obtained independently from different culture collections. Three strains of *Cryptococcus sp.* (designated Cr1, Cr69 and Cr76) were used as outgroups for numerical analysis. The strains were maintained on yeast-malt extract agar (YM: 0.5% malt extract, 0.25% yeast extract, 1% glucose, 0.25% peptone and 1.5% agar) slants at 4°C. For nucleic acid extractions, the strains were cultivated in liquid YM medium at 18°C with continuous shaking (200 rpm).

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Table 1. List of the astaxanthin-producing yeast strains investigated.

	Original code[a]	Other code[b]	Source[c,d]
1	CBS 5905	ATCC24202	ex *Fagus crenata*, Japan
2	CBS 5908	ATCC24203	ex *Alnus japonica*, Kiso, Japan
3	CBS 6938	CCRC22365	ex sap on stump of *Betula* sp., Finland
4	ATCC 24203	CBS 5908	ex *Alnus japonica*, Kiso, Japan
5	ATCC 24229	-	ex *Cornus brachypoda*, Hiroshima, Japan
6	ATCC 24261	-	ex *Betula maximowicyiana*, Yamagata, Japan
7	CBS 5905	ATCC24202	ex *Fagus crenata*, Japan
8	CBS 5905	ATCC24202	ex *Fagus crenata*, Japan
9	ATCC 24228	-	ex *Betula papyrifera*, Rainbow Lake, Alaska
10	CCY 77-1-1	CBS 5905	ex *Fagus crenata*, Japan
11	ATCC 24230	UCD 67-385	ex *Betula tauschii*, Kiso, Japan
12	W/IMP-UB	-	u.s., Norway
13	Z/IMP-UB	-	u.s., Norway

[a]The code which is used throughout this paper for clarity.
[b]Abbreviations: ATCC: American Type Culture Collection, Manassas, VA, USA. CBS: Centralalbureau voor Schimmelcultures, Utrecht, The Netherlands. CCRC: Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan. CCY: Czechoslovak Collection of Yeasts, Bratislava, Slovakia. IMP-UB: Institute of Microbiology and Plant Physiology, University of Bergen, Norway. UCD: H. J. Phaff Yeast Culture Collection, University of California Davis, Davis, CA, USA.
[c]Strains regarded as the same, but obtained independently from different sources: 1, 7, 8 and 10; 2 and 4.

RAPD analysis

Total DNA was isolated by a modification of the method of Leach et al. (1986). Yeast DNA sequences were amplified by the 10-mer primers OPC-02 (5′-GTGAGGCGTC-3′), OPC-04 (5′-CCGCATCTAC-3′), OPC-05 (5′-GATGACCGCC-3′), OPC-07 (5′-GTCCCGACGA-3′) and OPC-08 (5′-TGGACC-GGTG-3′) from Operon Kit C (QIAGEN Operon, Alameda, CA, USA).

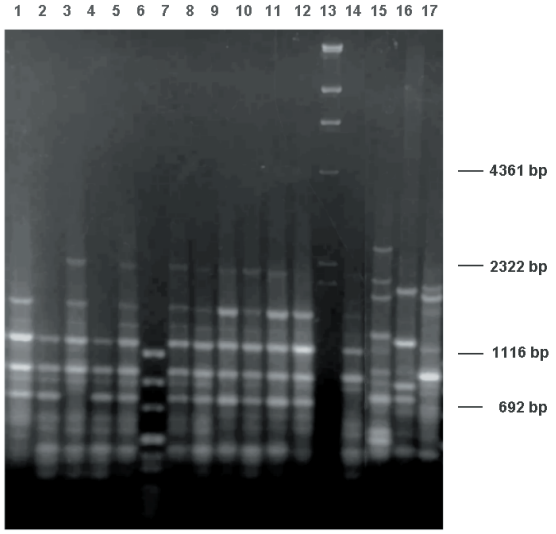

Figure 1. Representative amplification patterns of *Phaffia*, *Xanthophyllomyces* and *Cryptococcus* strains obtained with OPC-02 used as primer. Lane 6, pUC Mix Marker DNA as size standard (Fermentas). Lane 13, *Hind*III-digested λ DNA as size standard (Fermentas). Lanes 1-5, strains 1, 2, 3, 4 and 5, respectively. Lanes 7-12, strains 6, 8, 9, 10, 11 and 12, respectively. Lane 14, strain 13. Lanes 15-17, *Cryptococcus* strains Cr1, Cr69 and Cr76, respectively.

Amplifications were performed as described earlier (Vastag et al. 2000), with slight modifications. The reaction mixtures (25 μl) contained 1 x PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton X-100), 200 μM each of the dNTPs (Pharmacia, Peapack, NJ, USA), 2.5 mM $MgCl_2$, 0.2 μM primer, 0.5 U of Taq DNA polymerase (Zenon, Oakville, ON, Canada) and 125 ng of genomic DNA. Samples were overlayered with 40 μl of sterile mineral oil (Sigma, Budapest, Hungary). Control reactions, without genomic DNA extract, were also run.

PCR was carried out with a PTC-100-60 DNA programmable thermal controller (MJ Research, Waltham, MA, USA) set for a denaturation step at 93°C for 1 min, followed by 45 cycles at 92°C for 1 min, at 37°C for 1 min and at 72°C for 1 min. An extension step at 72°C for 6 min was applied after the final amplification cycle.

The amplification products (10 μl of each reaction) were analysed by electrophoresis on 0.9% agarose gels in TAE

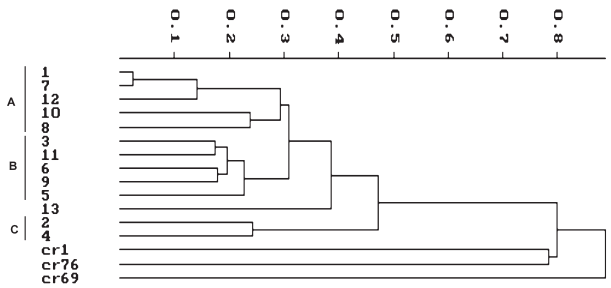

Figure 2. Dendogram obtained by UPGMA linkage with clustering of Jaccard coefficients calculated from RAPD data. The scale represents dissimilarity (squared distance). The strain numbers on the left are those listed in Table 1. The cophenetic correlation coefficient of the similarity matrix and the resulting dendogram was 0.9825.

buffer (40 mM Tris-acetic acid pH 7.6, plus 1 mM Na_2EDTA) containing 0.5 μg/ml ethidium bromide. Banding patterns were visualized by UV fluorescence. *Hind*III-digested λ DNA (Fermentas, Vilnius, Lithuania) and pUC Mix Marker (Fermentas) were used as size standards. Each isolate was analysed by RAPD-PCR at least 3 times.

Numerical analysis of the RAPD patterns

The RAPD banding patterns were analysed in order to determine the genetic relatedness of the isolates. A matrix based on the presence or absence of amplicons observed after electrophoretic separation was created. From these data, similarity matrix of Jaccard coefficients were calculated and used with the UPGMA (unweighted pair-group method using arithmetic averages) linkage (Sneath and Sokal 1973) to produce a dendogram. Numerical analysis was performed with the SYNTAX 5.0 software package (Podani 1993).

Results and Discussion

All 5 primers used efficiently amplified various regions of the investigated yeast genomes. They revealed different levels of variability, but in general a substantial level of polymorphism was detected among the 13 strains. As an example, results of the RAPD experiment with the OPC-02 primer are shown in Figure 1.

The data derived from the RAPD experiments were used for cluster analysis. A dendrogram was generated by using unweighted pair group average linkage clustering of the Jaccard coefficients. The dendrogram obtained is shown in Figure 2. Three *Cryptococcus* strains were used as outgroups during this analysis. The dendrogram revealed 3 clusters (A, B and C) and 4 unclustered strains. Among these, we found the 3 *Cryptococcus* strains used as outgroups. They differed at a high level both from all *Phaffia* and *Xanthophyllomyces* strains and from each other. Cluster A contains 5 *P. rhodozyma* strains (strain 1 is the type strain of *P. rhodozyma*). Among them, 4 strains (1, 7, 10 and 8) are known to be the same origin, but had undergone prolonged maintenance in different culture collections. Suprisingly, their RAPD patterns displayed characteristic differences. Members of this cluster are not able to sporulate, but are able to produce a respiratory-deficient petite mutant (Kucsera et al. 1998) and have a genome size higher than 20 Mbp (Nagy et al. 1994). In contrast, cluster B involves *Xanthophyllomyces* strains with sporulation capability, a petite-negative character and a genome size around 16 Mbp (Nagy et al. 1994). Cluster C contains 2 *Xanthophyllomyces* strains with a common origin (ATCC and CBS maintenance), but with several different RAPD markers. Strain 13 was situated as an unclustered strain in the dendrogram.

These results prove the applicability of the RAPD method for investigations of intraspecific variability and for determinations of strain-specific markers (*Phaffia* and *Xanthophyllomyces* strains separate well when RAPD markers are evaluated), but they also demonstrate some limits of this approach. Particularly as concerns culture collection strains which have undergone prolonged maintenance, special care is required in assessments of natural genetic variability. Interestingly, some of the *Xanthophyllomyces* strains (strains 2, 4 and 13) also revealed very high, nearly species-level differences from the other *Xanthophyllomyces* strains in this RAPD analysis.

This rather high intraspecific genetic variability might originate from the aneuploid or polyploid state of these yeasts. An earlier study relating to the electrophoretic karyotyping of some wild-type and mutagenized strains of *Xanthophyllomyces* raised questions concerning their haploid character (Nagy et al. 1997). A recent investigation (Hermosilla et al. 2003) concluded that *X. dendrorhous* is diploid. A similar result was obtained when the ploidy of the *P. rhodozyma* type strain (ATCC 24202) was evaluated via flow cytometric analyses of propidium iodide-stained cells and mutagenic inactivation kinetics (Medwid 1998): the findings suggested that *P. rhodozyma* is polyploid.

The results of the present study provide further information concerning the genetic make-up of the two astaxanthin-producing yeasts *P. rhodozyma* and *X. dendrorhous*, and help to identify genetic markers for the species delimitation which is not allowed by simple morphological traits.

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References

Fell JW, Blatt GM (1999) Separation of the strains of the yeasts *Xanthophyllomyces dendrorhous* and *Phaffia rhodozyma* based on rDNA IGS and ITS sequence analysis. J Ind Microbiol Biotechnol 23:677-681.

Golubev WI (1995) Perfect state of *Rhodomyces dendrorhous* (*Phaffia rhodozyma*). Yeast 11:101-110.

Hermosilla G, Martínez C, Retamales P, León R, Cifuentes V (2003) Genetic determination of ploidy level in *Xanthophyllomyces dendrorhous*. Anton Leeuw Int J G 84:279-287.

Johnson EA, An G-H (1991) Astaxanthin from microbial sources. CRC Crit Rev Biotechnol 11:297-326.

Johnson EA, Schroeder W (1996) Astaxanthin from the yeast *Phaffia rhodozyma*. Stud Mycol 38:81-90.

Kucsera J, Pfeiffer I, Ferenczy L (1998) Homothallic life cycle in the diploid red yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). Anton Leeuw Int J G 73:163-168.

Leach J, Finkelstein DB, Rambosek JA (1986) Rapid miniprep of DNA from filamentous fungi. Fung Genet Newslett 33:32-33.

Medwid RD (1998) *Phaffia rhodozyma* is polyploid. J Ind Microbiol Biotechnol 21:228-232.

Nagy Á, Garamszegi N, Vágvölgyi Cs, Ferenczy L (1994) Electrophoretic karyotypes of *Phaffia rhodozyma* strains. FEMS Microbiol Lett 123:315-318.

Nagy Á, Palágyi Zs, Ferenczy L, Vágvölgyi Cs (1997) Radiation-induced chromosomal rearrangement as an aid to analysis of the genetic constitution of *Phaffia rhodozyma*. FEMS Microbiol Lett 152:249-254.

Palágyi Zs, Ferenczy L, Vágvölgyi Cs (2001) Carbon-source assimilation pattern of the astaxanthin producing yeast *Phaffia rhodozyma*. World J Microbiol Biotechnol 17:95-97.

Papp T, Vastag M, Nagy Á, Michailides TJ, Vágvölgyi Cs (2001) Genetic variability of the postharvest pathogen *Gilbertella persicaria*: identification of randomly amplified polymorphic DNA (RAPD) markers correlating with (+) and (-) mating types. Anton Leeuw Int J G 80:301-309.

Pfeiffer I, Kucsera J, Varga J, Ferenczy L (1994) Regulatory effect of linear DNA plasmids in *Phaffia rhodozyma*. 7th Int Congr Mycol Div IUMS, Prague, Abstracts p 364.

Podani J (1993) SYN-TAX-pc. Computer programs for multivariate data analysis in ecology and systematics. Version 5.0. User's Guide. Sciencia Publishing, Budapest, Hungary.

Schroeder WA, Johnson EA (1993) Antioxidant role of carotenoids in *Phaffia rhodozyma.* J Gen Microbiol 139:907-912.

Sneath PHA, Sokal RR (1973) Numerical Taxonomy. W.H. Freeman, San Francisco, CA, USA.

Vágvölgyi Cs, Heinrich H, Ács K, Papp T (2004) Genetic variability in the species *Rhizopus stolonifer*, assessed by random amplified polymorphic DNA analysis. Anton Leeuw Int J G 86:181-188.

Varga J, Vágvölgyi Cs, Nagy Á, Pfeiffer I, Ferenczy L (1995) Isoenzyme, restriction fragment length polymorphism and random amplified polymorphic DNA characterization of *Phaffia rhodozyma* Miller et al. Int J Syst Bacteriol 45:173-177.

Vastag M, Papp T, Kasza Zs, Vágvölgyi Cs (2000) Intraspecific variation in two species of *Rhizomucor* assessed by random amplified polymorphic DNA analysis. J Basic Microbiol 40:269-277.

Williams JGK, Kubelik AR Livak KJ, Rafalsky JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531-6535.