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ARTICLE

A prenyl pyrophosphate synthase gene from the zygomycete fungus, *Gilbertella persicaria*

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ABSTRACT In this study, a prenyl pyrophosphate synthase gene was cloned and characterized in the zygomycete fungus *Gilbertella persicaria*. The coding region of the isolated gene comprises a total of 1328 bp determining a 350 amino acids length putative protein and five introns (60, 65, 43, 59, and 48 bp in length) dispersed in the whole coding region. Based on the analysis of the nucleotide and the corresponding amino acid sequence, the gene encodes a farnezyl pyrophosphate synthase, and it was named as *isoA*. In a phylogeny inferred from amino acid sequences of fungal prenyl pyrophosphate synthases, the *Gilbertella* isoA proved to be the most closely related to that of *Mucor circinelloides*. Carotenoid composition of *G. persicaria* was also investigated: it produces β-carotene as the main carotenoid, but it also contains small amounts of β-cryptoxanthin, zeaxanthin and astaxanthin.

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KEY WORDS

Gilbertella
farnezyl pyrophosphate synthase
isoprene biosynthesis
β-carotene
sequence analysis

Isoprenoids are a large group of natural products with diverse structures. They are synthesized from common precursors and their carbon backbones are derived from various numbers of repetitions of five-carbon isopentenyl pyrophosphate (IPP) units: at first, the five-carbon dimethylallyl pyrophosphate (DMAP) and IPP is condensed to form the ten-carbon geranyl pyrophosphate (GPP), and then, the carbon chain is extended by repetitive condensations with further IPP units (Fig. 1). These condensation steps are managed by isoprenyl pyrophosphate synthases, such as farnesyl or geranylgeranyl pyrophosphate (FPP and GGPP, respectively) synthases. Thus, linear, intermediate compounds with various lengths are formed, which serve as precursors in several specific side-routes towards the biosynthesis of many different end-products, such as sterols, carotenoids, the terpenoid moiety of ubiquinones or the prenyl groups of certain proteins (Liang et al. 2002).

Due to their antioxidant properties and other health-related functions, carotenoids are frequently used in the food, pharmaceutical and cosmetic industries and also as feed colour additives. Today, there is an increasing interest in discovering and improving microbial sources of carotenoids (Dufosse 2006). Traditionally, three zygomycetes fungi, belonging in the order Mucorales, *Blakeslea trispora, Phycomyces blakesleeanus* and *Mucor circinelloides*, are used as model organisms to study the carotenoid, in particular the β-carotene, biosynthesis. Moreover, *B. trispora* is currently used as an industrial producer of β-carotene (Rodríguez-Sáiz et al. 2004; Dufossé 2008). Besides carotenoids, these fungi are considered as actual or potential sources of ergosterol, edible oils, and other compounds (Kuzina et al. 2006). A number of other mucoralean species also seem to be promising producers based on their carotenoid and isoprenoid content (Papp et al. 2009). Identification of new producer strains with higher pigment content and/or with different carotenoid composition and their involvement in strain improvement studies would be advantageous.

Here, we report on the carotenoid composition and the cloning and analysis of a FPP synthase gene (*isoA*) of *Gilbertella persicaria*, a fungus closely related to *B. trispora*.

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Materials and Methods

Strains and media

G. persicaria strain SZMC 11089 (Szeged Microbial Collection, Szeged, Hungary) was used in the cloning experiments. Together with this strain, isolates SZMC 11090 and SZMC 11094 were involved in the measurements of the carotenoid content. For nucleic acid extraction, the fungus was cultivated in yeast extract glucose medium (YEG, 5 g yeast extract and 10 g glucose per litre). For carotenoid extraction, strains SZMC 11089 and SZMC 11090 were grown alone or together in mixed cultures on solid minimal medium (YNB, 10 g glucose, 0.5 g yeast nitrogen base without amino acids, 1.5 g $(NH_4)_2SO_4$, 1.5 g sodium glutamate and 20 g agar per litre). In all cases, the fungal cultures were grown for 4 days under continuous light at 25°C. *Escherichia coli* DH5α was

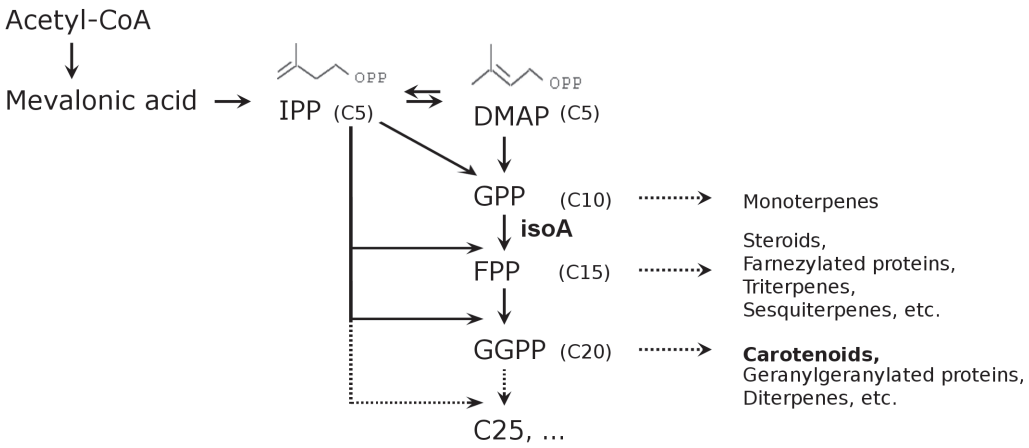

Figure 1. The isoprenoid biosynthetic pathway. IPP, isopentenyl pyrophosphate; DMAP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate.

used in all cloning experiments and plasmid amplifications. *E. coli* was cultivated on Luria-Bertani broth at 37°C; if it was necessary, the medium was supplemented with ampicillin to a final concentration of 50 µg/mL.

Molecular techniques and gene cloning

The genomic DNA of *G. persicaria* was prepared from mycelia disrupted with pestle and mortar in liquid nitrogen and purified in a 0.5 mg/ml bis-benzimide-CsCl density gradient (Iturriaga et al. 1992). General procedures for plasmid DNA preparation, cloning and transformation of *E. coli*, and Southern blotting were performed following standard procedures (Sambrook et al. 1989).

Analysing of known fungal FPP synthase sequences, two degenerated primers, FPP3d and FPP4dr (Table 1) were designed that correspond to the conserved amino acid sequence motifs QDDYFD and QAFF, respectively. A 564-bp fragment was amplified by PCR from the genomic DNA of *G. persicaria* using this primer pair. The entire gene was determined by the inverse PCR (IPCR) method (Ochman et al. 1988) as described previously (Takó et al. 2010). Briefly: to obtain different sets of DNA templates for the IPCR, 10 µg aliquots of the genomic DNA were digested with the restriction enzymes *Pst*I and *Xba*I (Fermentas), each in a total volume of 120 µl. Digested DNA was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) followed by a subsequent extraction with chloroform:isoamyl alcohol (24:1). The purified DNA fragments were self-ligated with T4 ligase (Fermentas) for 18 h at 4°C, precipitated with ethanol and resuspended into distilled water. In the subsequent IPCR reactions, 50 ng of the circularized DNAs was used as templates. IPCR primers were designed in opposite orientation to that of normal PCR (primers designated as GilFPP1 – 4; Table 1). The resulting amplification products were finally cloned into the plasmid pTZ57R/T using the InsT/Aclone PCR Product Cloning Kit (Fermentas) according to manufacturer's instructions and sequenced.

Table 1. Oligonucleotide primers designed for this study.

Primer	Sequence (5′ to 3′)
FPP3d	AACTGCAGCARGCNTTYTT
FPP4dr	ATCTCGAGTCNARRTARTCRTCYTG
GilFPP1	AGGTTGACCACGACGGGTGATAGA
GilFPP2	CATTGGCCATGCATATGGTA
GilFPP3	TAGAAGCATCCATGATGTCATCAG
GilFPP4	AATGATATACCATAGGTTGGTGTGA

Restriction sites designed in the primers are underlined.

Sequence analysis

DNA sequencing was performed by using an Applied Biosystem 373 DNA sequencer. Blast searches (Altschul et al. 1997) in the GenBank database were performed for prediction of the coding sequence. The programs ProtParam (Gasteiger et al. 2005) was used to characterize the proposed protein. Domain search and prediction were performed using the Motif Scan (MyHits) program (Pagni et al. 2007). These programs were accessed trough the Swiss Expasy Server (www.expasy.ch) and used with default parameters.

To perform phylogenetic analyses, amino acid sequences of several isoprenyl pyrophosphate synthases were downloaded from the NCBI GenBank; sequence data of zygomycetes enzymes and hypothetical proteins were obtained from the genome databases of *M. circinelloides* (DoE Joint Genome Institute; *M. circinelloides* CBS277.49 v1.0; http://genome.jgi-psf.

Table 2. Carotenoid compositions of *G. persicaria* strains and their mixed cultures.

Strain[a]	β-Carotene	γ-Carotene	Lycopene	β-Crypt.[b]	Zeax.	Astax.	Total
11089 (+)	40 ± 6	12 ± 4	19 ± 6	-	10 ± 2	-	90 ± 19
11090 (–)	37 ± 5	11 ± 3	18 ± 7	-	12 ± 3	-	87 ± 22
11094 (+)	33 ± 5	11 ± 3	19 ± 8	0.1 ± 0.5	11 ± 3	4 ± 3	85 ± 23
11089 - 11090	61 ± 7	15 ± 5	21 ± 4	0.5 ± 0.5	8 ± 2	-	112 ± 21
11090 - 11094	52 ± 4	14 ± 6	20 ± 2	1 ± 1	2.5 ± 2	0.5 ± 0.5	106 ± 16

[a] Mating types of the strains are indicated in brackets. [b] Abbreviations: β-Crypt., β-Cryptoxanthin; Zeax., Zeaxanthin; Astax., Astaxanthin. The indicated amounts are average values given in μg/g [dry mass] ± standard deviations, which were calculated from the data of three independent cultures; carotenoid extractions were carried out after cultivation of the strains on YNB for 4 days.

org/Mucci1/Mucci1.home.html); *Phycomyces blakesleeanus* (DoE Joint Genome Institute *P. blakesleeanus* v1.1; http://genome.jgi-psf.org/Phybl1/Phybl1.home.html) and *Rhizopus oryzae* (Broad Institute; *R. oryzae* Database; http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae). Alignment of sequences was computed with the Probalign program (Roshan and Livesay 2006). The resulting alignment was used to infer trees using Bayesian MCMC algorithm implemented in MrBayes 3.2.1 (Ronquist and Huelsenbeck 2003). Markov Chains were run for three million generations sampling every 100th generations. The first 10000 trees were discarded as burn-in, and the remaining trees were used to compute a 50% Majority Rule consensus tree. Bayesian Posterior Probabilities (BPP) ≥0.95 were considered significant.

Carotenoid extraction and analysis

Carotenoids were extracted from 500 mg mycelial powder as described elsewhere (Papp et al. 2006). Samples were analysed by using a modular Shimadzu low-pressure gradient HPLC system. The dried pigments were re-dissolved in 100 μl tetrahydrofuran supplemented with butylated hydroxytoluene (100 μg/mL), and 2 μL was subjected on a Prodigy ODS-3 (4.6 x 150, ODS 3 μm) column (Phenomenex). The separation was performed with a gradient (min/volume of solvent A%/volume of solvent B%: was 0/99/1; 8/60/40; 13/46/54; 15/0/100; 18/0/100; 21/99/1; and 25/99/1), 4% water-96% methanol being used as solvent A and 100% methyl-*tert*-butyl ether as solvent B, at a flow rate of 1 mL/min. The detection wavelength was 450 nm. To identify the carotenoids, the following standards were purchased: astaxanthin, lycopene and β-carotene (Sigma), β-cryptoxanthin and zeaxanthin (Carl Roth), while γ-carotene was purified by HPLC.

Results and Discussion

Carotenoid composition of G. *persicaria*

Investigation of *G. persicaria* revealed an interesting carotenoid profile (Table 2). As in other zygomycetes, β-carotene proved to be the main carotenoid product, however, some strains produced astaxanthin in small, but detectable amount and small amounts of zeaxanthin and β-cryptoxanthin (the hydroxylated derivatives of β-carotene) could also be detected. Presence of these hydroxylated forms was earlier demonstrated in another mucoralean fungus, *M. circinelloides* suggesting the existence of a β-carotene hydroxylase activity in these fungi (Papp et al. 2006). Mixed cultures of the *Gilbertella* strains having opposite mating types (*i.e.* opposite sexes) produced significantly more carotenoids, especially β-carotene, than the same strains cultured separately. This feature is known as sexual carotenogenesis (Kuzina and Cerdá-Olmedo 2006) that has been extensively studied in cases of the closely related *P. blakesleeanus* and *B. trispora*. It is induced by trisporic acid and some related compounds, which are β-carotene derivatives and serve as sexual signals for zygomycete fungi. Moreover, the technology for the industrial production of β-carotene by *B. trispora* involves the joint fermentation of strains with opposite mating types (Iturriaga et al. 2005; Dufossé, 2006, 2008).

Cloning of the FPP synthase gene in G. *persicaria*

To isolate the farnezyl pyrophosphate (FPP) synthase gene encoding a key-enzyme of the isoprene biosynthesis, degenerated primer pairs were designed to conserved regions of known fungal FPP synthase genes and a fragment has been amplified by PCR from the genomic DNA of *G. persicaria*. The resulted amplicon was sequenced and the entire gene was subsequently determined by the IPCR technique. The sequence was deposited to the EMBL nucleotide sequence database (accession number: FM209247). As previously the corresponding gene of *M. circinelloides* was named as *isoA* after *iso*prenoid biosynthesis (Velayos et al. 2004), we also applied this designation to the gene determined in this study. The *G. persicaria isoA* gene comprises a total of 1328 bp including the coding sequence of a putative 350 amino acids length protein and 5 introns (60, 65, 43, 59, and 48 bp in length) dispersed in the whole coding region (Fig. 2A). The five introns are at the same position, as the five introns in the *M. circinelloides isoA* (Velayos et al. 2004) and four of those in the proposed FPP synthetase gene of *R. oryzae* (*R. oryzae*

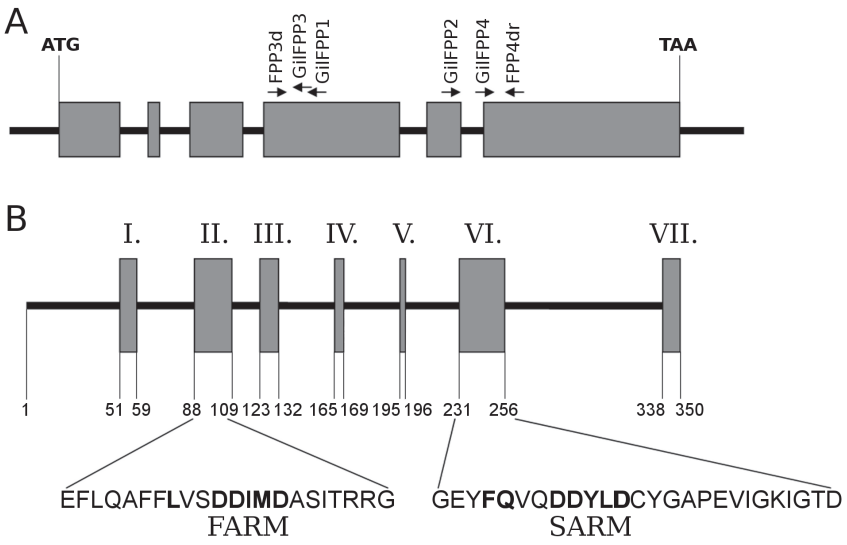

Figure 2. Schematic representation of the *G. persicaria isoA* gene (A) and the main regions of the encoded protein (B). Arrows indicate the positions of the primers for the IPCR amplifications and cloning. Bold characters indicate conserved amino acids important for the catalytic activity.

Database, RO3G_14682) indicating a conservative intron positioning in these organisms.

The G. *persicaria* FPP synthase

The polyprenyl synthase catalytic domain (40 – 311 aa) contains the seven conserved regions revealed in FPP synthases of several organisms (Koyama et al. 1993; Fig. 2B). In the regions II and VI, the first (98 – 102 aa) and the second (238 – 242 aa) aspartate rich motifs, FARM and SARM, respectively, also could be identified. These motifs are characteristic to the prenyl pyrophosphate synthases and necessary to the substrate binding and the catalysis (Wang and Ohnuma 2000; Szkopinska and Plochocka 2005; Poulter 2006). The conserved FQ motif (234 – 235 aa) in the region VI, which is responsible for holding of the substrate in a proper conformation for the condensation with IPP, was also found in the amino acid sequence (Fig. 2B).

Similarity searches in the genome sequence databases of *M. circinelloides*, *P. blakesleeanus* and *R. oryzae* with the *Gilbertella* isoA sequence revealed one FPP synthases in each database, one GGPP synthase in *M. circinelloides*, which was already isolated and characterized by Velayos et al. (2003), one putative GGPP synthase and one hypothetical protein in *P. blakesleeanus* and two GGPP synthase-like hypothetical proteins in *R. oryzae.* To compare the putative FPP synthase of *G. persicaria* with other fungal isoprenyl pyrophosphate synthases, a phylogeny was inferred from the amino acid sequences of zygomycete FPP, GGPP and solanesyl pyrophosphate (SPP) synthases known or predicted until today and several further FPP and GGPP synthases representing the two other major fungal groups, Ascomycetes and Basidiomycetes. GGPP synthases of *Deinococcus radiodurans* and *Thermus thermophilus* (GGPP synthase type II) were also involved in the analysis as outgroup. Members of the Reversible-Jump MCMC analysis selected the Wag model implemented in MrBayes as the best fit model to the data. The resulting consensus tree is presented on Figure 3; all clades received significant BPPs (≥0.95). On this tree, four large clades can be recognized, the fungal GGPP synthases, a clade containing the solanesyl pyrophosphate synthase of *M. circinelloides* and closely related zygomycete sequences, the fungal FPP synthases including *Gilbertella* isoA and the two type II GGPP synthases involved as outgroup. Fungal FPP and GGPP synthases belong in the type I FPP synthases and type III GGPP synthases, respectively (Wang and Ohnuma 2000; Szkopinska and Plochocka 2005); within the corresponding clades, zygomycete isoprenyl pyrophosphate synthases form groups clearly separated from Ascomycetes and Basidiomycetes. The *M. circinelloides* isoA (Velayos et al. 2004) proved to be the most closely related to the FPP synthase of *G. persicaria*.

In this study, isolation and characterization of the *G. persicaria isoA* gene was performed. As we know, this is the first complete structural gene that has been identified in this species.

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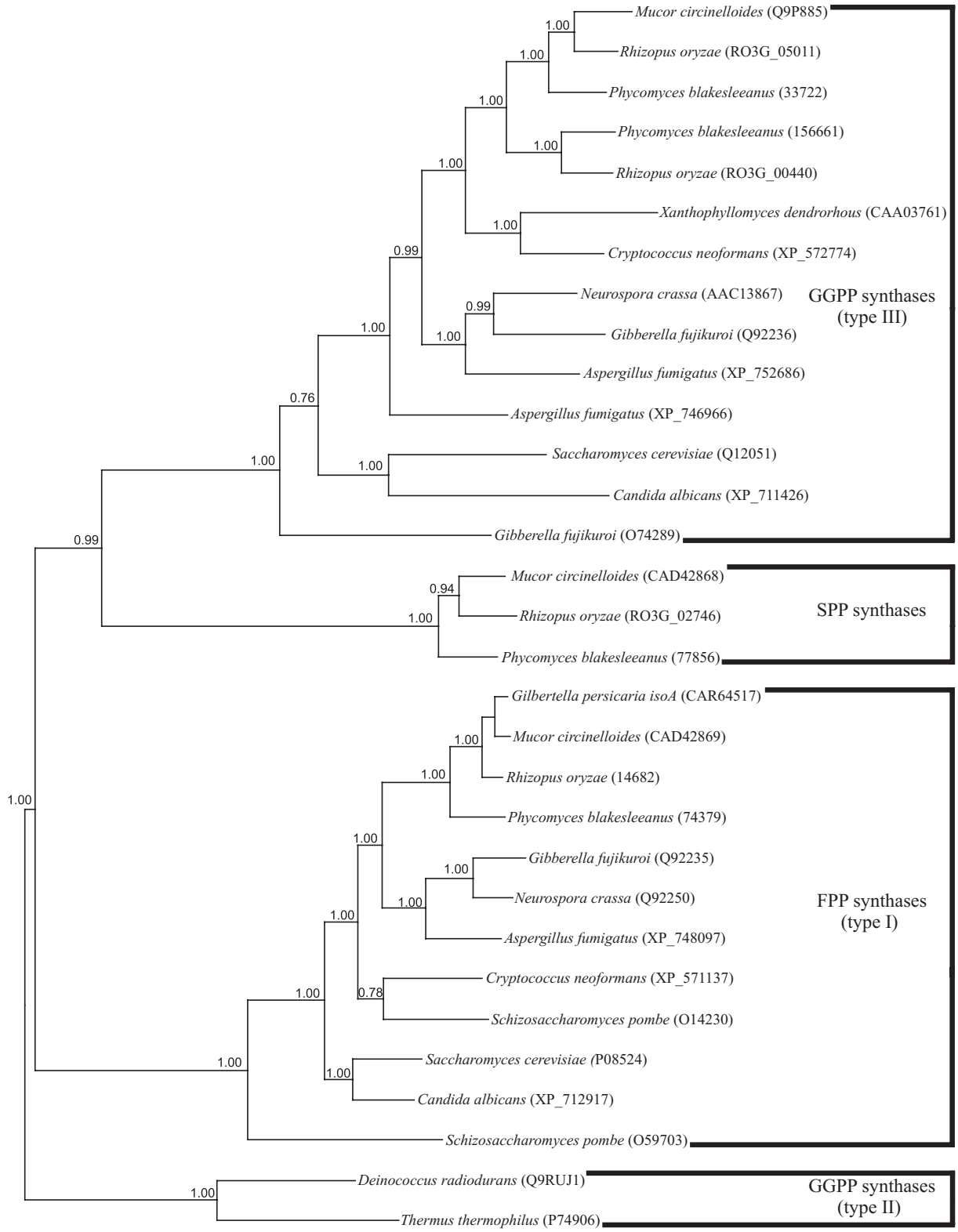

Figure 3. Phylogenetic analysis of fungal isoprenyl pyrophosphate synthases: Bayesian 50% Majority Rule consensus phylogram; numbers indicate posterior probabilities (BPP). Amino acid sequences of *M. circinelloides, P. blakesleeanus* and *R. oryzae* were obtained by similarity search in genome databases (protein IDs of the corresponding database are indicated on the tree). Other sequences were obtained from the NCBI database (accession numbers are indicated on the tree).

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