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Restriction and Transcription Maps of Mitochondrial DNA of *Trimorphomyces papilionaceus*

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Mitochondrial DNA has been isolated from *Trimorphomyces papilionaceus*. By analyzing DNA fragments digested by restriction enzymes, a restriction site map has been constructed. The mtDNA of *T. papilionaceus* amounts to 48.5 kb in size and is circular in structure. Entire mitochondrial DNA was cloned in *E. coli* plasmids and Northern blot hybridization was done using cloned and subcloned DNAs as probes. Based on hybridization results of mitochondrial RNA transcripts, a transcription map was prepared.

Key words: *Trimorphomyces*, mitochondrial DNA, restriction map, transcription map

Mitochondria contain their own DNA informations of a great diversity in size and organization among organisms. Mitochondrial DNAs (mtDNAs) of fungi are various in size and structure and in the arrangement of genes. The size of fungal mtDNAs mostly ranges from 19 kb to 121 kb and many parts of them consist of introns and noncoding spacer sequences (8). Although most fungal mtDNAs are circular, some linear forms have been observed in *Hansenula mrakii* (22) and *Physarum polycephalum* (21). In spite of the diversity in size and structure of mtDNAs, there exist some common genes in mitochondrial genomes throughout organisms including fungi. These include genes for large subunit ribosomal RNA (LSU rRNA), small subunit ribosomal RNA (SSU rRNA), subunits I, II, III of cytochrome oxidase complex, cytochrome b, and subunit 6 of ATP synthetase complex. The subunit 9 gene of the ATP synthetase complex is found on mtDNA of a yeast, *Saccharomyces cerevisiae* (15), but this gene is found on both nuclear and mitochondrial DNAs in *Neurospora crassa* (16) and *Aspergillus nidulans* (3). A complete set of tRNA genes for mitochondrial protein synthesis is usually encoded on mtDNA in most organisms. In general, there seem to be fundamental similarities of mtDNA among closely related organisms, which are useful to study their relationships.

The authors present here the restriction site map of the mtDNA of *T. papilionaceus*, a basidiomycetous yeast described as a monotypic species under the Tremellaceae (Basidiomycotina) by Oberwinkler and Bandoni (17). The species is a mycoparasitic fungus which parasitizes *Arthrinium sphaerospermum*, a dematiaceous hyphomycete. It is characterized by three phases during life cycle: monokaryotic yeast, dikaryotic yeast, and dikaryotic hyphal phases. During yeast phases, it propagates by budding like common bakery yeasts. The dikaryotic yeast phase is morphologically distinctive because conjugated monokaryotic yeasts form paired conidia of a unique butterfly shape, from which the specific name originates, and then the dikaryotic hyphae develop a fruitbody. The paired conidia are repeatedly and synchronously produced and are readily recognizable under the microscope on the basis of morphology. This kind of conidia is absent in ordinary tremellas and yeasts and has been a taxonomic subject of great interest (17). According to the prepared map, the mtDNA of *T. papilionaceus* is 48.5 kb in size and is circular in structure. Based on the Northern blot hybridization data of RNA transcripts, a transcription map of the mtDNA has been prepared.

Materials and Methods

Strains and culture

A dikaryotic strain UBC 75-7237-d was used in this study. The UBC strain was the gift of R.J. Bandoni (University of British Columbia, Vancouver), which was do-

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nated to K.S. Yoon (Kangwon University, Chuncheon) and then transferred to the authors under the donor's permission. It was grown at 24℃ in shaking cultures of a modified YEPD medium, which was named YEPD-Gal (yeast extract 1.0%, peptone 1.0%, glucose 0.2%, galactose 2.0%) by the authors.

Isolation of mitochondrial DNA and RNA

Cells were harvested by centrifugation at 4,000×*g* for 5 min and resuspended in cold lysis medium (0.6 M sorbitol, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) and broken with equal weight of glass beads (0.5 mm diam) by repeating 30 sec vortexing and 30 sec cooling 6 times. The mitochondrial pellet was collected from the supernatant by differential centrifugation (18) and purified further on sucrose step gradients of 1.0 M, 1.3 M, 1.6 M, and 2.0 M (11), on which most of mitochondria were obtained from the interlayer of 1.3/1.6 M gradients. For the isolation of mtDNA, the mitochondrial pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and lysed by adding SDS to a concentration of 0.2%. Mitochondrial DNA was extracted with phenol, phenol : chloroform, and chloroform consecutively and precipitated with ethanol, which was redissolved in TE buffer. For the isolation of mitochondrial RNA (mtRNA), the pellet was resuspended and vortexed in equal volume of RNA zol B (Biotecx Lab) and one tenth volume of chloroform for 30 sec. Mitochondrial RNA was recovered from the supernatant and precipitated with ethanol, which was redissolved in TE buffer containing 1 mM aurintricarboxylic acid (9).

Restriction enzyme digestions and molecular cloning

Isolated mtDNAs were digested by 8 restriction endonucleases (*Sal*I, *Bam*HI, *Eco*RI, *Xho*I, *Hin*dIII, *Pvu*II, *Xba*I, and *Eco*RV; Bethesda Research Laboratories, Boehringer Mannheim, New England Biolabs). To localize restriction enzyme sites more precisely, double or triple digestions by various combinations of restriction enzymes were further carried out. Two cloning strategies were employed; 100% cloning of *Eco*RI-digested fragments and cloning of large fragments digested by *Bam*HI-*Sal*I, *Sal*I, and *Xho*I. All the fragments were cloned so that they would overlap each other or one another. The *Eco*RI-, *Sal*I-, and *Xho*I-linearized plasmid vectors [pUC18, pBR 322, and Bluescript KS(+) each] were treated with alkaline phosphatase according to the supplier's instructions (Pharmacia) and the *Bam*HI-*Sal*I-digested plasmid vector (pUC18) was purified by elution of low-melting point agarose gel (19). Restricted fragments and treated vectors were ligated by T4 DNA ligase (Bethesda Research Laboratories) and then transformed (10).

Hybridization techniques

Southern blot hybridization was done following the standard methods (19). Cloned and subcloned DNAs were labelled with [α-^{32}P]dATP (3,000 Ci/mmol, Amersham) using the nick translation kit (Bethesda Research Laboratories) and used as hybridization probes. For Northern blot hybridization, mtRNAs of *T. papilionaceus* were developed with total RNAs of *E. coli* and *T. papilionaceus* as RNA size markers on 6 M urea-1.5% agarose gel using TPE running buffer (40 mM Tris, 36 mM NaH_2PO_4, 1 mM EDTA) (14). Separated gel pieces of *T. papilionaceus* mtRNA were denatured for 10 min by NaOH solution (0.2 M NaOH, 0.6 M NaCl), a new denaturing agent for the urea-agarose gel electrophoresis system, and neutralized for 5 min by the usual neutralization solution (0.5 M Tris HCl, 0.6 M NaCl, pH 7.4). Isolated RNAs were transferred to Hybond-C or Hybond-N (Amersham) and then treated according to the standard methods of Sambrook *et al.* (19). Cloned and subcloned DNAs labelled as probes were hybridized with treated mtRNA transcripts.

Results and Discussion

Restriction map of *T. papilionaceus* mtDNA

Upon the digestion of the mtDNA from *T. papilionaceus* UBC 75-7237-d by 8 restriction endonucleases, numbers and sizes of produced DNA fragments were determined (Fig. 1 and Table 1). According to the results of enzyme digestions, the total estimated size of the digested mtDNA was 48.5 kb, which was a moderate size for a fungal mtDNA. The mtDNA was circular in map structure. The cloned *Bam*HI-*Sal*I, *Eco*RI, *Sal*I, and *Xho*I fragments are listed in Table 2. Based on the analysis of restriction enzyme sites, fragmentation patterns, and Southern blot hybridization data, the relative position of each restriction enzyme site was measured in reference to a *Sal*I site set as a starting point for measurement and a circular restriction site map was constructed (Fig. 2).

Northern blot hybridization of *T. papilionaceus* mtDNA

In Northern blot hybridization, 7 fragments of Table 2 and 4 fragments subcloned from them were used as hybridization probes. Treatment of RNA by the NaOH solution as a denaturing agent gave good resolution and even the specific activity of 1×10^6 cpm/μg of DNA probes could detect as many transcripts as in conventional methods and maintained used nitrocellulose papers in

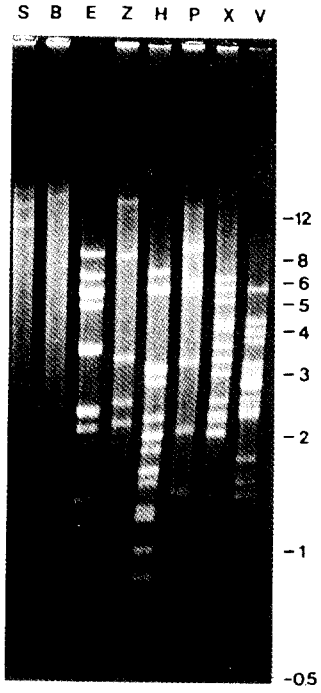

Fig. 1. Restriction fragment patterns of the mtDNA from *T. papilionaceus* UBC 75-7237-d on single digestions. The mtDNA was digested by restriction enzymes *Sal*I (S), *Bam*HI (B), *Eco*RI (E), *Xho*I (Z), *Hin*dIII (H), *Pvu*II (P), *Xba*I (X), and *Eco*RV (V) and the resulting fragments were separated using 0.6% horizontal agarose gel electrophoresis. Numbers on the right of the picture indicate the size of the fragments in kb developed by a 1 kb linear DNA ladder size standard (Bethesda Research Laboratories).

good enough condition to be reused three times or more. LSU and SSU rRNAs of *T. papilionaceus* were identified by comparing with sizes and positions of 23S and 16S rRNAs of *E. coli*. Along with these RNAs, 8 RNA transcripts were detected using 11 cloned and subcloned fragments as probes. By locating encoding regions of RNA transcripts, a transcription map was prepared (Fig. 3).

Transcription map of *T. papilionaceus* mtDNA

When Northern hybridization analysis was performed, 8 transcripts including SSU rRNA and LSU rRNA were identified. and their sizes were calculated in reference to those of 23S rRNA (2094 bases; 2) and 16S rRNA (1541 bases; 1) of *E. coli* and 5S rRNA (118 bases; 12) of *T. papilionaceus*. The LSU rRNA was estimated to be about 3500 bases, the SSU rRNA 1700 bases, and other RNAs from A and A′ to E were 2900 bases, 2900 bases, 1300 bases, 1200 bases, 700 bases, and 300 bases each (Fig. 3). The LSU rRNA detected by probes 2, 3, 4, and 5 seemed to reside in the region of probes 3 and 4. The transcript D detected by the probe 2 and by the pTPmt202 clone of Fig. 1, which clone ranges from the right of the probe 2 to the left of the probe 5 (data not shown), was predicted to reside within the region of LSU rRNA.

Transcripts A and A′ were detected by probes 1, 5, 8, and 9 at a same position. Probes 1 and 5 are distantly separated by about 15 kb and are apparently intervened by some other genes. Thus, it is believed that transcripts A and A′ are encoded by two independent genes, even though they were not differentiated from each other in Northern blot hybridization. The transcript A may ex-

Table 1. Restriction fragments of *T. papilionaceus* UBC 75-7237-d mtDNA.

*Sal*I	*Bam*HI	*Eco*RI	*Xho*I	*Hin*dIII	*Pvu*II	*Xba*I	*Eco*RV
21.8	29.5	8.1	15.7	6.4	13.3	6.1	5.6
15.0	18.0	6.5	15.0	5.7*	8.4	5.6	4.3
11.7	1.0	5.8	8.0	3.1*	8.0	5.1	4.0
		5.7	3.4	2.9	5.9	4.3	3.8
		5.0	2.5	2.3	5.6	4.2	3.2
		3.5*	2.2	2.0*	3.2	3.7	3.1
		2.4*	0.7	1.8	2.1	3.3	2.9
		2.1		1.6*	1.4	3.0	2.8*
		1.4		1.5	0.7	2.5	2.5
		1.1		1.2	0.6	2.3	2.3*
		1.0		1.1		2.2	1.7
				0.9		2.0*	1.5
				0.7		1.0	1.4
				0.5		0.6*	0.5
						0.5	
48.5	48.5	48.5	47.5	45.3	49.2	49.0	44.7

Restriction fragment sizes are given in kb and asterisks indicate two overlapped fragment bands. Total 88 fragments are listed here but there were some more fragments which were lost below the 0.5 kb marker at the bottom of agarose gels and did not show in Fig. 1.

Table 2. Designation and sizes of cloned fragments of *T. papilionaceus* UBC 75-7237-d mtDNA.

Designation	Vector	Cloned fragment	
pTPmt101	pUC18	*Bam*HI-*Sal*I	7.0 kb
pTPmt102	PUC18	*Bam*HI-*Sal*I	8.0 kb
pTPmt111	pBR322	*Bam*HI-*Sal*I	11.0 kb
pTPmt201	pUC18	*Eco*RI	2.3 kb
pTPmt202	pUC18	*Eco*RI	5.5 kb
pTPmt203	pUC18	*Eco*RI	5.9 kb
pTPmt204	pUC18	*Eco*RI	6.5 kb
pTPmt205	pUC18	*Eco*RI	3.7 kb
pTPmt206	pUC18	*Eco*RI	3.5 kb
pTPmt208	pUC18	*Eco*RI	2.4 kb
pTPmt209	pUC18	*Eco*RI	2.1 kb
pTPmt210	pUC18	*Eco*RI	1.0 kb
pTPmt211	PUC18	*Eco*RI	1.1 kb
pTPmt212	PUC18	*Eco*RI	1.4 kb
pTPmt213	PUC18	*Eco*RI	5.0 kb
pTPmt214	PUC18	*Eco*RI	8.0 kb
pTPmt301	pBR322	*Sal*I	15.0 kb
pTPmt401	Bluescript KS(+)	*Xho*I	15.7 kb
pTPmt402	Bluescript KS(+)	*Xho*I	15.0 kb

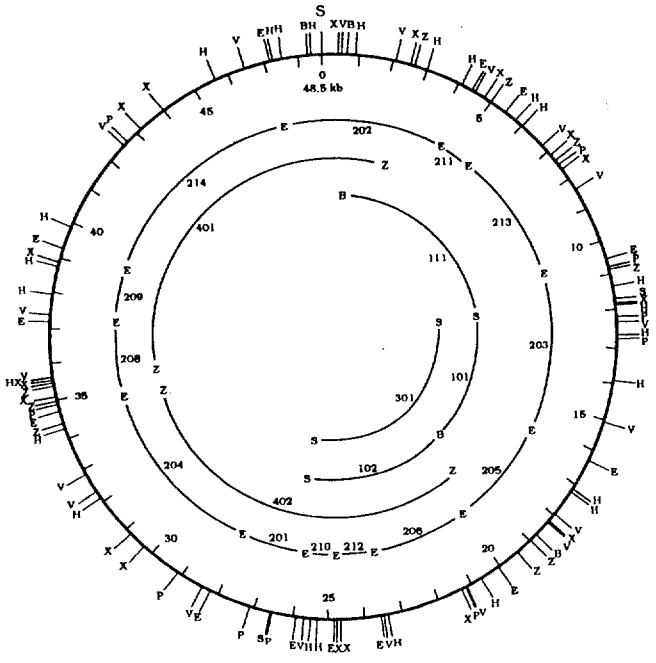

Fig. 2. Circular composite restriction site map of the mtDNA from *T. papilionaceus* UBC 75-7237-d. Total 100 sites were recognized. The outer circle shows pointed restriction enzyme sites where the letters correspond to restriction enzymes denoted in Fig. 1 and starts at a *Sal*I site in the middle of the upper half circle. The inner circle shows the restricted sites, locations, and extents of cloned *Eco*RI fragments and the innermost incomplete circles those of cloned *Bam*HI-*Sal*I, *Sal*I, and *Xho*I fragments. Numbers around inside circles indicate designated ones of cloned fragments listed in Table 2.

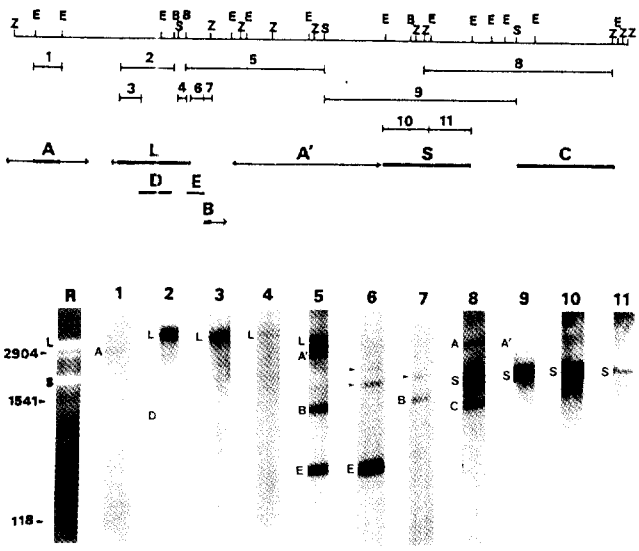

Fig. 3. Northern blot hybridization of transferred RNAs with cloned and subcloned fragments of the mtDNA from *T. papilionaceus* 75-7237-d. Transcription map (upper figure) linearized at a *Xho*I site of the 35.4 kb clockwise position from the starting point of Fig. 2. Letters above the map are abbreviations of four restriction enzymes, *Sal*I (S), *Bam*HI (B), *Eco*RI (E), and *Xho*I (Z), denoted in Fig. 1 and indicate their restriction sites which were set to the map scale in a row. Locations of probes are indicated below the map and their numbers represent 7 cloned (numbers 1, 4, 5, 8, 9, 10, and 11) and 4 subcloned (numbers 2, 3, 6, and 7) mtDNA fragments. Solid lines and arrows below the map correspond to the locations and possible extents of genes coding for detected RNAs. Autoradiographs (lower figure) showing the hybridized positions of specific cloned and subcloned fragments of mtDNA. Numbers above lanes are the same as the probe numbers from No. 1 to 11 in the upper figure. The lane R is mtRNAs of *T. papilionaceus* developed on 6 M urea-1.5% agarose gel. On the left of the lane R, sizes and positions of 23S rRNA (2904 kb) and 16S rRNA (1541 kb) of *E. coli* and 5S rRNA (118 kb) of *T. papilionaceus* are given in kb as markers. LSU rRNA (L) and SSU rRNA (S) of *T. papilionaceus* were identified by comparing with 23S and 16S rRNAs of *E. coli*, and other RNAs are indicated in lanes as letters from A and A' to E. And the arrows indicate nonspecific or unknown hybridization.

tend to the left of the probe 8, and A' may be possibly located somewhere in the region between probes 5 and 9 of Fig. 3. Genes for transcripts B, E, and LSU rRNA are clustered around the pTPmt202 region of mtDNA, but SSU rRNA, A', B, and C RNA genes are scattered throughout the remaining genome. Judging from gene locations of the map, large and small subunit rRNA genes are comparatively distant and are set 18 kb apart. Even though no structural patterns are categorized in transcription maps yet, LSU and SSU rRNA genes are known to be located separately in most fungi (7). Taking transcript locations into account, mitochondrial genes are

distributed all over the genome and most RNA genes are concentrated on the 9 kb size of mtDNA around the LSU rRNA gene. Considering that the smallest mtDNA size to accommodate mitochondrial genes is about 15 kb as in animal cells (6), the largest portion of the mitochondrial genome of *T. papilionaceus* can be non-coding spacers or intronic regions.

The fact that the transcript D gene resides in the region of LSU rRNA can be interpreted in two ways. One is that this gene overlaps the LSU rRNA gene in the same or opposite DNA strand. The other is that the transcript D can be an intermediate or end product of splicing of LSU rRNA intron. There are several reports about the presence of introns in LSU rRNA genes, found from *Neurospora crassa* (4), *Podospora anserina* (5), *Aspergillus nidulans* (13), and *Saccharomyces cerevisiae* (20), which positively supports the present presumption. But this presumption needs to be confirmed through final sequencing analysis for LSU rRNA.

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