

Isolation, Characterization and Phylogenetic Analysis of the Cytochrome B Gene (*Cyb*) from the Hypotrichous Ciliate *Pseudourostyla cristata*

Xing-Yin LIU¹, Kam-Len Daniel LEE^{2,3}, Yong-Zhen MAO¹, Li-Pei JIN¹

¹School of Life Sciences, Sun Yat-Sen (Zhongshan) University, Guangzhou, China; ²Department of Applied Biology and Chemical Technology, Hong Kong Polytechnic University, Hong Kong, China; ³State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen, China

Summary. To date, no genes have been characterized from mitochondrial DNA of hypotrichous ciliates. Here we present the complete sequence of the gene (*cyb*) encoding cytochrome *b* in the hypotrichous ciliate *Pseudourostyla cristata*. Analysis of nucleotide and deduced amino acids sequences predicts a protein of 393 amino acids encoded by *cyb*. CYB amino acid similarity and phylogenetic tree analyses strongly support a close evolutionary relationship between *Paramecium*, *Tetrahymena* and *Pseudourostyla*. The heat stress-dependent expression pattern of CYB was verified by semi-quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis.

Key words: ciliate, cytochrome *b*, heat stress, mitochondrion, *Pseudourostyla cristata*.

INTRODUCTION

Ciliates comprise a large and structurally diverse group of unicellular eukaryotes (protists). Phylogenetic reconstructions, inferred from the small subunit rRNA gene and protein-encoding gene sequences demonstrate that ciliates represent a relatively evolved lineage among the protists (Greenwood *et al.* 1991, Bernhard *et al.* 1995, Bernhard and Schlegel 1998, Budin and Philippe 1998, Tourancheau *et al.* 1998). Unlike other eukaryotes, ciliates possess two types of nuclei: poly-

loidy macronuclei (for protein synthesis) and diploid micronuclei (for heritability). Additionally, they contain numerous mitochondria. It is known that the protozoan mitochondrial genomes diverged extremely from all other genomes (Gray *et al.* 2004). This reflects an early branching event and a rapid evolution at the primary sequence level and at the levels of genome content and organization. Furthermore, it represents an evolution of genetic codon usage (Gray *et al.* 2004). The ciliates have among the most rapidly evolving mitochondrial genomes as evidenced by the large number of putative proteins for which function cannot be readily assigned (Brunk *et al.* 2003). Oligohymenophorea and Hypotrichea are distant classes within the Ciliophora phylum. Mitochondrial DNA (mtDNA) data from the ciliates is only limited in *Tetrahymena* and *Paramecium* species (in the

Address for correspondence: Li-Pei Jin, School of Life Sciences, Sun Yat-Sen (Zhongshan) University Guangzhou 510275, Guangdong, People's Republic of China; Fax: +86-020-84036215; E-mail: lssjlp@zsu.edu.cn

class Oligohymenophorea) (Pritchard *et al.* 1990a, Burger *et al.* 2000, Brunk *et al.* 2003), and mtDNA investigations on the thousands of Hypotrichea organisms have not been conducted. Mitochondrial genes are attractive markers for inferring phylogenies of closely related species because of their rapid evolutionary rates compared to those of nuclear DNA (Saccone *et al.* 2000). In particular, among the protein coding genes of mtDNA, cytochrome b (*cyb*) can be used for resolving phylogenetic relationships within a genus and among higher orders, covering a divergence time of roughly 70 million years (Myr) (Irwin *et al.* 1991, Johns and Avise 1998, Callejas and Ochando 2000). Until now, in all ciliate groups, the *cyb* gene has been characterized only in *Paramecium* and *Tetrahymena* (Pritchard *et al.* 1990b, Burger *et al.* 2000, Brunk *et al.* 2003).

The hypotrichous ciliate *Pseudourostyla cristata* is a free-living ciliated protozoan that is characterized by a highly differentiated pattern of the vegetative cell. Recently, we unexpectedly isolated a full-length mt *cyb* cDNA from heat stress-induced phase mRNA of the *P. cristata*. This study represents the first description of CYB in hypotrichous ciliates, so it has allowed us to initiate a molecular phylogenetic comparison with the CYB sequences available from the other species. CYB is an indispensable component of the cytochrome bc₁ respiratory complex, which is responsible for ubiquinol-cytochrome c reductase activity in the mitochondrion (Esposti *et al.* 1993). It has never been reported as an up-regulated gene when responding to heat stress. Thus, our finding is relevant for elucidating the stress signal response role of the bc₁ complex.

MATERIALS AND METHODS

Cell culture and heat stress

Pseudourostyla cristata was originally isolated from a fresh water pond outside Shanghai, China. The cells were cultured in Pringsheim's solution with wheat grains and the food organism *Chilomonas paramecium* at 25–27°C, as described previously (Jin and Ng 1989). The *C. paramecium* was isolated with three cycles of gentle centrifugation (1000 rpm for 1 min.). For the heat stress treatment, the culture dishes containing approximately 10⁶ cells were incubated for 30 min at 42°C.

RNA Isolation and smart cDNA synthesis

Total RNA samples from those cells with two hours post-heat stress were extracted using reagents and protocols described

previously (Liu *et al.* 2004). Smart cDNA was synthesized from the 500 ng total RNA samples using the SMART PCR cDNA synthesis Kit (Clontech) with minor modification: Reverse transcription was conducted in a 10 µl reaction volume at 42°C for 1 h with the 3' BD SMART CDS Primer II A (A modified oligo(dT) primer: 5'-AGCAGTGGTATCAACGCAGAGTACT₃₀VN-3', and ignoring the BD SMART II A oligonucleotide (5'-AGCAGTGGTATCAACGCAGAGTACGCGGG-3') required in manufacture's protocol (Clontech SMART PCR cDNA synthesis Kit User manual PT3041-1). 2.5 µl of this diluted first strand synthesis reaction mixture was added to 98 µl of the Master mix containing 2 µl of the 5' PCR Primer II A (5'-AAGCAGTGGTATCAACGCA GAGT-3'). The mixture was PCR amplified in a PTC-100 thermal cycler (MJ Research, USA) as follow: 95°C, 1 min; 21 × [95°C, 15 s; 65°C, 30 s; 68°C, 6 min]. In order to demonstrate that the template for the LD-PCR product was cDNA and not genomic DNA, a minus-reverse transcriptase control was performed.

Gel electrophoresis, cloning and sequencing

The resulting cDNA products were analyzed in a 1.2% agarose gel. The predominant bands were excised from the gel, and DNA was extracted from the gel slices using the QIAquick Gel Extraction Kit (Qiagen), cloned into the TA cloning vector pMD-T-18 (Takara) and transformed into *E. coli*, DH5a cells. Plasmid DNA samples were prepared from the confirmed recombinant transformants using the EZNA Plasmid Miniprep Kit I (Omega). Both strands of the DNA inserts were sequenced using the 370 DNA Sequencer (Applied Biosystem). The acquired sequences were analyzed with the DNASTar software.

Determination of open reading frames (ORFs) and characteristics of the cloned cDNA

Using the ORF finder at <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>, the cloned cDNA sequence was translated with the protozoan mitochondrial genetic code (Aldritt *et al.* 1989). Similarity searches were performed with the Basic Local Alignment Search Tool X (BLASTX). The protein properties were predicted using ProtParam (Appel *et al.* 1994). The transmembrane (TM) domain of the CYB protein was predicted using TMHMM version 2 software (Sonnhammer *et al.* 1998). The 3D structure of the inferred CYB protein was modeled by the 3D-Pssm Program (Version 2.6.0) (Kelley *et al.* 2000).

Semi-quantitative real time reverse transcriptase (RT)-PCR analysis

Total RNA from the non-heat stress and the 0, 1, 2 and 3 h post-heat stress cells were extracted as described above. cDNA was synthesized from 500 ng of total RNA of each sample using the Omniscript-RT Kit (Qiagen) according to the manufacturer's instructions. Real-time fluorescence signals were measured with SYBR Green I (Roche) in the ABT PRISM 7700 (PE Applied Biosystem). The 17s rRNA (GenBank Accession Number: AY940212) was used as the internal control, and the RT-PCR CYB and 17s rRNA primers were designed with Primer Premier 5.0 software.

All RT-PCR reactions and the minus-reverse transcriptase control were performed in triplicate. The ΔC_T value was determined by

subtracting the target C_T of each sample from its respective 17s rRNA C_T value. The $\Delta\Delta C_T$ was calculated as the difference between ΔC_T of each sample and ΔC_T of the non-heat stress sample. Fold expression differences for CYB were determined by $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen 2001). All statistical analyses were performed with SPSS version 12.0 software (SPSS Inc, IL, USA).

Data and phylogenetic analysis

The amino acid sequence data of CYB were obtained from the Entrez Protein program (<http://ncbi.nlm.nih.gov/entrez/index.htm>). All amino acid sequences were manually optimized and aligned using a computer-assisted procedure, ClustalW, version 1.80 (Thompson *et al.* 1997). The phylogenetic trees were constructed using several members of each major taxon to accurately gauge the CYB global topology rooted with *Rickettsia prowazekii* and *Paracoccus denitrificans*, and the CYB phylogenetic trees were generated with the neighbor-joining (NJ) method implemented in the PHYLIP software package, version 3.6 (Felsenstein 2002). For the NJ analysis, the NEIGHBOR program was used, and the bootstrap proportions were calculated by analyzing 1,000 replications. The distance matrix was computed with PROTDIST; 1,000 bootstrap replications were obtained by the SEQBOOT program.

RESULTS AND DISCUSSION

Isolation and sequence of the cytochrome b gene

Agarose gel electrophoresis of the RT-PCR products illuminates a strong band at approximately 1.65 kb for the two hours post-heat stressed RNA samples (Fig. 1A: Lane 1). 3'BD SMART CDS primer II A primers sequences was found in the 5' and 3' ends. The minus-reverse transcriptase control (Fig. 1B: Lane 2) demonstrated that the RT-PCR template for the product was cDNA and not genomic DNA. The unexpected result was a surprise and was reconfirmed, but when we used the two hour post-heat stress sample for RT-PCR per Clontech's SMART system instructions with 3' BD SMART CDS primer II A. and BD SMART II A oligonucleotide, the above result can not be repeated and a smear of cDNA without the characteristic bright band was found (Fig. 1B: Lane 1). Brunk *et al.* (2003) found that the *Tetrahymena thermophila* mitochondrial genome appears to have two transcriptional units, transcribed from a nearly central bi-directional promoter set. Therefore, we hypothesized that this product may be up-regulated under heat stress, and that it may be a bidirectional transcription. It will be very interesting to investigate whether a similar phenomenon occurs in other ciliates, for example, among *Tetrahymena* and *Paramecium* species. Furthermore, constructing a heat-

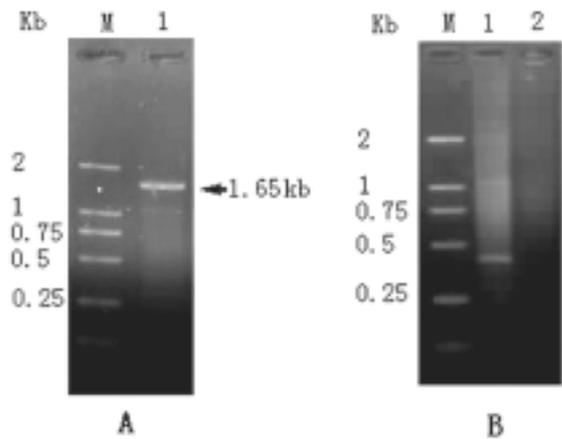


Fig 1. Agarose gel electrophoresis of RT-PCR products. RT-PCR products of RNA from 2 h post-heat stress were analyzed by electrophoresis in a 1.2 % agarose gel. **A** - lane M: Marker (DL 2000, Takrara); lane 1: cDNA was synthesized as described in the material and method. The arrow indicates a strong band at 1.65kb. **B** - lane M: Marker (DL 2000, Takrara); lane 1: cDNA was synthesized as described in Clontech SMART PCR cDNA synthesis Kit User manual; lane 3: A minus-reverse transcriptase control.

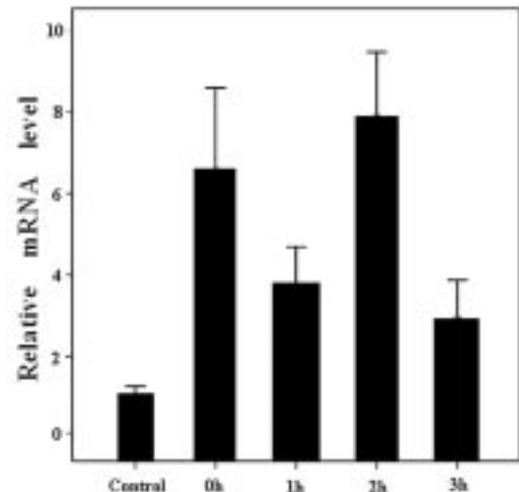


Fig. 2. CYB expression patterns in *Pseudourostyla cristata* under the condition of heat stress. The relative CYB mRNA expression level in the non-heat stress control was regarded as 1.000. Relative to the non-heat stress control, a 6.37-fold increase in the CYB mRNA was found immediately (0 h) post-heat stress treatment. One and two hours post-heat stress treatment, 3.58 and 7.76-fold increases were observed, respectively. Even at three hours post- heat stress treatment, a 2.53-fold increase remained.

inducible gene expression system in these simple organisms is recommended.

After deleting the BD SMART CDS Primer II A sequence, the sequence was 1538 bp long and encompassed a full-length ORF with a protein sequence of 393

Table 1. Codon usage in *Pseudourostyla cristata cyb* gene

aa	Codon	#	%	aa	Codon	#	%	aa	Codon	#	%	aa	Codon	#	%		
Phe	TTT	37	88.1	Ser	TCT	3	15.8	Tyr	TAT	29	90.6	Cys	TGT	4	66.7		
	TTC	5	11.9		TCC	3	15.8		TAC	3	9.4		TGC	2	33.3		
Leu	TTA	38	84.4	Pro	TCA	7	36.8	Ter	TAA	*	100	Trp	TGA	14	93.3		
	TTG	3	6.7		TCG	6	31.6		TAG	0	0		TGG	1	6.7		
	CTT	2	4.4		CCT	5	38.5		His	CAT	14		87.5	Arg	CGT	3	50
	CTC	1	2.2		CCC	0	0			CAC	2		12.5		CGC	1	16.7
	CTA	1	2.2		CCA	5	38.5		Gln	CAA	6		100	CGA	2	33.3	
	CTG	0	0		CCG	3	23.1			CAG	0		0	CGG	0	0	
Ile	ATT	28	77.8	Thr	ACT	8	40	Asn	AAT	17	85	Ser	AGT	2	40		
	ATC	3	8.3		ACC	0	0		AAC	3	15		AGC	3	50		
	ATA	5	13.9		ACA	5	25		Lys	AAA	7		87.5	Arg	AGA	4	100
Met	ATG	11	100	ACG	7	35	AAG	1		12.5	AGG	0	0				
	Val	GTT	14	60.9	Ala	GCT	5	41.7	Asp	GAT	9	75	Gly	GGT	8	36.4	
GTC		3	13.0	GCC		0	0	GAC		3	25	GGC		0	0		
GTA		6	26.1	GCA		1	8.3	Glu	GAA	16	88.9	GGA		10	45.4		
GTG		0	0	GCG		6	50		GAG	2	11.1	GGG		4	18.2		

Percentage (%) indicates the proportion of codons used for each amino acid. Asterisk (*) denote stop codon, excluding ATT as initiation codon.

amino acids (GenBank accession Number: AY940211). AUU is used as the initiation codon, and the first AUG codon is 114 nt downstream. The termination codon is UAA. This codon usage is consistent with that of *Tetrahymena pyriformis* mtDNA (Edqvist *et al.* 2000). Continuity of the ORF and normal-length protein sequence rule out the presence of introns. BLASTx search shows the protein sequences with the highest homologies or similarities were the CYB sequences.

Expression pattern of CYB under heat stress

To determine the CYB mRNA expression levels in the heat stress-induced *P. cristata*, a semi-quantitative real-time RT-PCR analysis was performed (Fig. 2). Relative to the non-heat stress control, a six-fold increase in the CYB mRNA immediately (0 h) post heat-treatment for 30 min was observed. Following one h and two h post-heat stress, the cells exhibited four- and seven-fold differences respectively. Even at three h post-heat stress, a near two half-fold increase was maintained (Fig. 2). We hypothesized that the dynamic expression pattern may adapt to the heat stress response. The high level of cellular CYB expression may reflect a high demand for mitochondrial electron transport and respiratory activities to compensate for the imposed heat stress. However, because the mitochondrial complex was not isolated from the organism, it is impossible to establish an experimental link between

respiration and heat stress. Since similar findings in other unicellular organisms have not been reported, we only can speculate CYB protein as the locus of regulation of electron transport in *P. cristata* mitochondria under the conditions of heat stress. Thus the heat stress-induced up-regulation of CYB mRNA may reflect a concurrent increase in CYB protein levels, which could increase respiratory rates and thus ATP synthesis for survival and ciliary movement.

Analysis and Comparison of CYB amino acid sequence

The deduced amino acid sequence is a 47-kDa protein with an isoelectric point of 5.68. The total number of negatively charged residues (Asp + Glu) is 30, and the total number of positively charged residues (Arg + Lys) is 18. The ratio of negative to positive residues is 1.76. The grand average of hydropathicity (GRAVY) is 0.419. The aliphatic index is 100.41. The deduced protein contains ten putative TM domains, and thus the TM profile is similar to that of the *Paramecium aurelia* CYB (Brunk *et al.* 2003). The results of 3D structure prediction is most similar to that of the *Gallus gallus* cytochrome bc1 transmembrane subunit (PSSM E-value of: 1.66e-08, = 95% certainty).

Fig. 3 shows an alignment of CYB sequences from CYB amino acid sequences from *P. cristata*, *Tetrahymena pyroformis*, *Paramecium tetraurelia*, *Plasmo-*

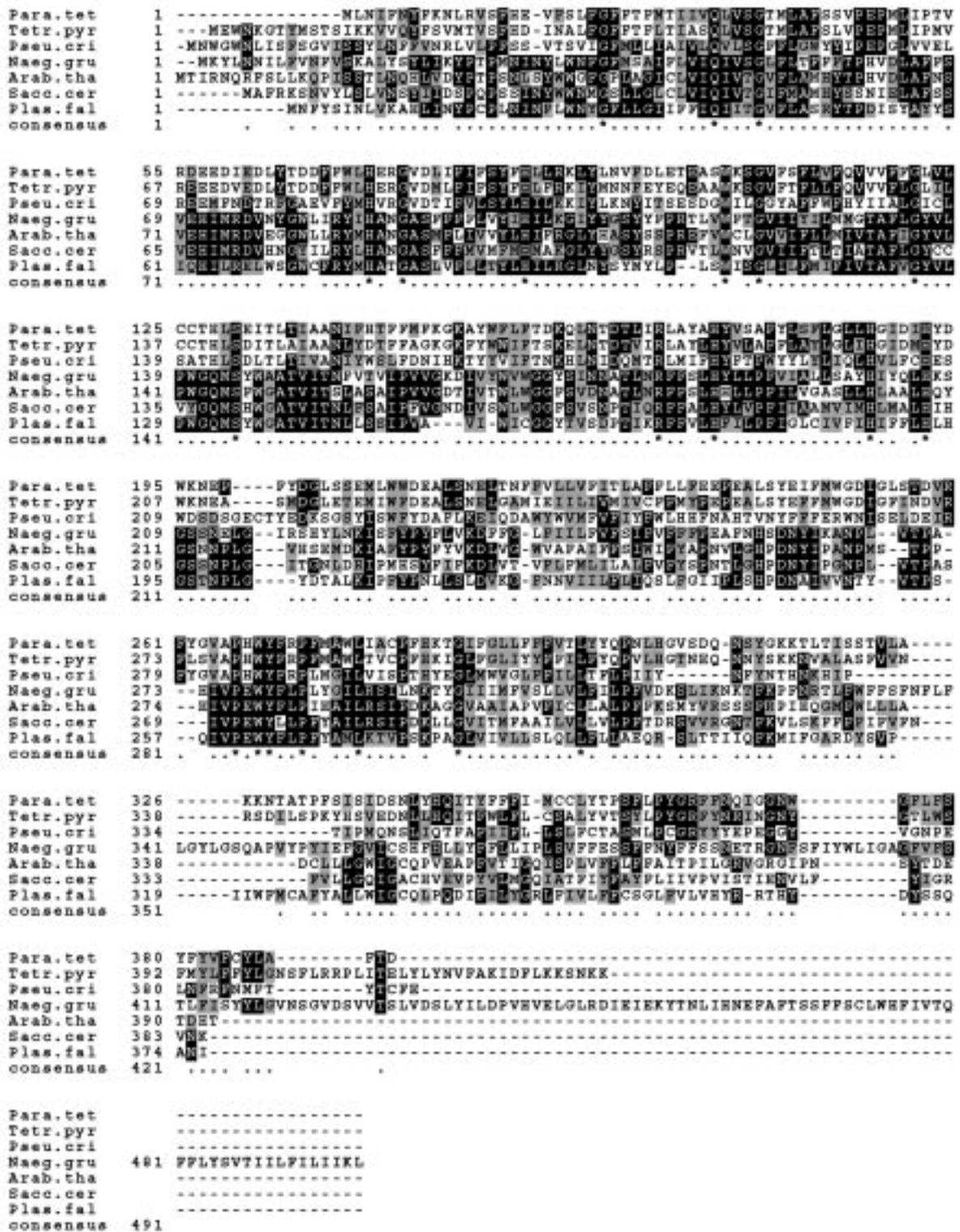


Fig. 3. CYB amino acid sequence alignment from various organisms. CYB amino acid sequences from *Pseudourostyla cristata* (Pseu. cri), *Tetrahymena pyriformis* (Tetr. pyr), *Paramecium tetraurelia* (Para. tet), *Plasmodium falciparum* (Plas. fal), *Naegleria gruberi* (Naeg. gru), *Arabidopsis thaliana* (Arab. tha), and *Saccharomyces cerevisiae* (Sacc. cer) were aligned by CLUSTALW. Identical amino acids are darkly shaded and similar amino acids are lightly shaded. Positions with the highest consensus, i.e. identical amino acid sequences, are denoted by asterisks (*) along the bottom line (consensus line). Other positions with consensus are denoted by dots (.). Gaps were introduced to improve alignment and these are marked with dashes (-) along the individual sequences.

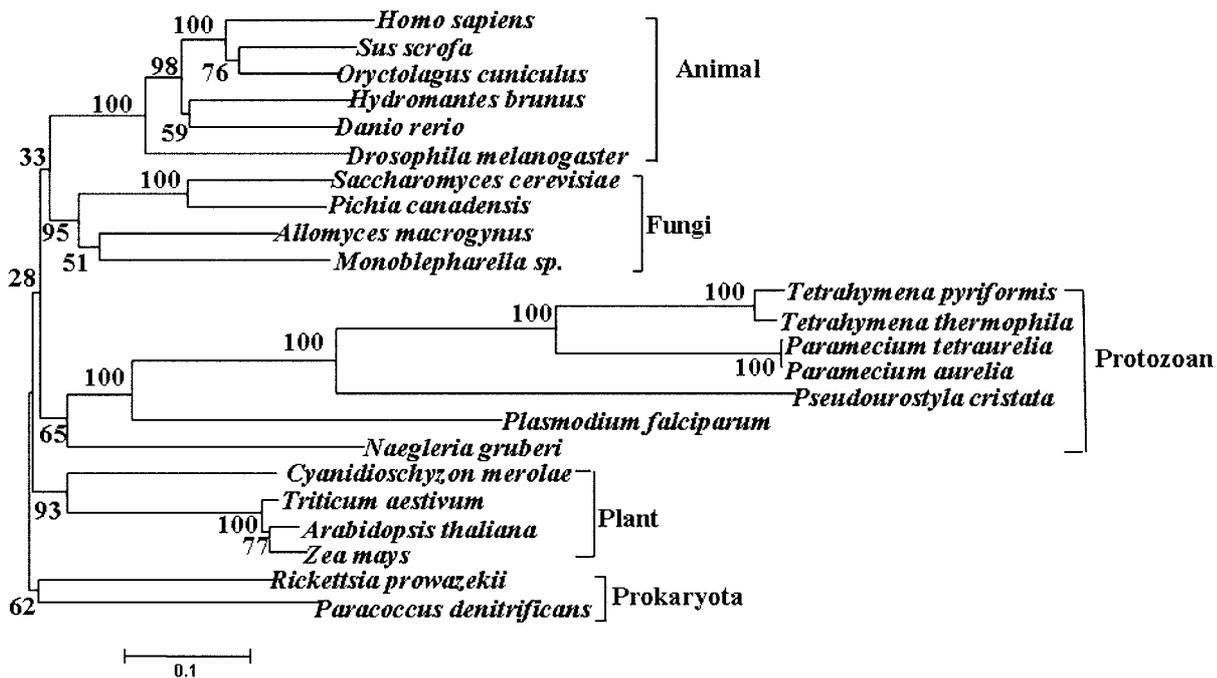


Fig. 4. Neighbor-joining (NJ) phylogenetic tree based on CYB amino acid sequence alignment. Bootstrap support, calculated from 1,000 replicates, is shown at each branch (in %, only values > 50% are listed). The sequences used in this study are available from the GenBank/EMBL databases with the following accession numbers: *Pseudourostyla cristata* (AY940211), *Paramecium aurelia* (NP_059398), *Paramecium tetraurelia* (P15585), *Tetrahymena thermophila* (NP_149395), *Tetrahymena pyriformis* (NP_049598), *Naegleria gruberi* (AAG17790), *Monoblepharella sp.* (NP_803529), *Hydromantes brunus* (YP_097270), *Danio rerio* (NP_059343), *Saccharomyces cerevisiae* (CAA58861), *Drosophila melanogaster* (NP_008288), *Arabidopsis thaliana* (NP_178804), *Plasmodium falciparum* (AAT00449), *Oryctolagus cuniculus* (AAS54913), *Sus scrofa* (AAU04561), *Zea mays* (CAA25367), *Rickettsia prowazekii* (CAA14733), *Paracoccus denitrificans* (AAA25572), *Homo sapiens* (AAB58955), *Pichia canadensis* (BAA06572), *Allomyces macrogynus* (AAC49221), *Triticum aestivum* (P07747), *Cyanidioschyzon merolae* (BAA34655). *Rickettsia prowazekii* and *Paracoccus denitrificans* are selected as the outgroup species.

dium falciparum, *Naegleria gruberi*, *Arabidopsis thaliana*, and *Saccharomyces cerevisiae*. CYB from the ciliates *T. pyriformis* and *P. tetraurelia* show higher amino acid similarity (41.5 % and 43.7 % respectively) to *P. cristata* CYB. Notably, CYB from the more distantly related organisms *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Plasmodium falciparum*, and *Naegleria gruberi* show lower level of similarity (31.0%, 27.7%, 30.7% and 26.1% respectively) to *P. cristata* CYB. These results are consistent with the idea that ciliate mt DNA sequence branched from other groups early in evolution and evolved very rapidly (Brunk *et al.* 2003).

The protein alignment also reveals a conserved peptide (VAPHWYFRP) among three ciliate taxa. Four invariant histidine residues (Fig. 4: Asterisks (*)) indicated H86, H100, H187 and H 201) proposed as the

heme-binding sites in CYB (Edqvist *et al.* 2000) were present in the *P. cristata* CYB sequence.

Codon usage

The overall deduced codon usage for *cyb* gene of *P. cristata* is summarized in Table 1. Like *T. pyriformis*, TGA is used preferentially to encode Trp. The *P. cristata cyb* (72% A+T) also exhibits a preference for A or T in the third position of codons (80% A+T), whereas first (excluding Trp and Met) and second position A+T biases are lower (60% and 68%, respectively). First position preferences for A or T in Leu codons contribute to the high A+T content of the gene. Of 40 Leu residues, 33 are specified by TTA, whereas TTG, CTA and CTC, CTT together account for only 7 codons, and CTG is not used at all. Changes in mitochondrial codon frequencies can occur as a result of AT selection pressure. Such

pressure is manifested by an increase in the A+T content of spacer regions or the third positions relative to first. Table 1 shows that 12 of 13 Trp residues are encoded using the TGA codon (a universal stop codon). Only one Trp residues was encoded using the universal TGG codon. As in *P. aurelia* mtDNA, TGA and TGG appear with almost equal frequency, but *T. pyriformis* mt DNA uses TGA almost exclusively (Brunk *et al.* 2003). The variant assignment of TGA among three kinds of ciliates further illustrates the point that codon usage is highly variable and basically uninformative with respect to understanding global phylogenetic relationship (Lang *et al.* 1987, Paquin and Lang 1996).

Phylogenetic reconstruction based on CYB sequence comparisons

A CYB phylogeny based on neighbor-joining (NJ) analyses (Fig. 4) shows four major clades: protozoa, plants, fungi and animals with moderate to very high bootstrap support. Animals and fungi are each robustly monophyletic. Regarding CYB evolutionary trends, protozoan CYB was closest to plant CYB. Within the protozoan clade, the apicomplexan species (*Plasmodium falciparum*) and the ciliate species (*Paramecium*, *Tetrahymena* and *Pseudourostyla*) form a well-supported (100% bootstrap) monophyletic group, in agreement with nuclear rRNA trees, although overall support for the protozoan clade is low (bootstrap value 63%). Within the class Oligohymenophorea of ciliates, *Paramecium* and *Tetrahymena* are sister species, forming a monophyletic grouping with long branches. Since *P. cristata* belongs to the class Hypotrochea, the tree shows Oligohymenophorea and Hypotrochea branching together to form a basal clade with 100% bootstrap support. This is in agreement with morphological data and other protein coding gene trees (Tourancheau *et al.* 1998). The long branches indicate that the common ancestor of ciliates must have possessed a highly divergent *cyb* gene; moreover their *cyb* genes are obviously evolving at an accelerated and unequal rate. The extreme divergence in ciliates complicates phylogenetic reconstruction and assessment of relationships. It also highlights the importance of isolating and characterizing other ciliate mitochondrial genes, which should provide additional information into codon usage and insights about the evolution of protozoan.

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REFERENCES

- Aldritt S. M., Joseph J. T., Wirth D. F. (1989) identification of cytochrome b in *Plasmodium gallinaceum*. *Mol. Cell Biol.* **9**: 3614-20
- Appel R. D., Bairoch A., Hochstrasser D. F. (1994) A new generation of information retrieval tools for biologists: the example of the ExPASy WWW server. *Trends Biochem. Sci.* **19**: 258-260
- Bernhard D., Schlegel M. (1998) Evolution of histone H4 and H3 genes in different ciliate lineages. *J. Mol. Evol.* **46**: 344-354
- Bernhard D., Leipe D. D., Sogin M. L., Schlegel M. (1995) Phylogenetic relationships of the Nassulidia within the phylum ciliophora inferred from the complete small subunit rRNA gene sequences of *Furgasonia blochmanni*, *Obertriumia georgiana*, and *Pseudomicrothorax dubius*. *J. Euk. Microbiol.* **42**: 126-131
- Brunk C. F., Lee, L. C., Tran A. B., Li J. (2003) Complete sequence of the mitochondrial genome of *Tetrahymena thermophila* and comparative methods for identifying highly divergent genes. *Nucl. Acids Res.* **31**: 1673-1682
- Budin K., Philippe H. (1998) New insights into the phylogeny of eukaryotes based on ciliate Hsp70 sequences. *Mol. Biol. Evol.* **15**: 943-56
- Burger G., Zhu Y., Littlejohn T. G., Greenwood S. J., Schnare M. N., Lang B. F., Gray M. W. (2000) Complete sequence of the mitochondrial genome of *Tetrahymena pyriformis* and comparison with *Paramecium aurelia* mitochondrial DNA. *J. Mol. Biol.* **297**: 365-380
- Callejas C., Ochando M. D. (2000) Recent radiation of Iberian barbell fish (Teleostei, Cyprinidae) inferred from cytochrome b genes. *J. Hered.* **91**: 283-288
- Esposti M., Devries D. S., Crimi M., Ghelli A., Patarnello T., Meyer A. (1993) Mitochondrial cytochrome b evolution and structure of the protein. *Biochem. Biophys. Acta* **1143**: 243-271
- Edqvist J., Burger G., Gray M. W. (2000) Expression of mitochondrial protein-coding genes in *Tetrahymena pyriformis*. *J. Mol. Biol.* **297**: 381-93
- Felsenstein J. (2002) PHYLIP (Phylogeny Inference Package) Version 3.6. Distributed by the author, Washington: Department of Genome Sciences, University of Washington, Seattle
- Gray M. W., Lang B. F., Burger G. (2004) Mitochondria of Protists. *Ann. Rev. Genetics* **38**: 477-524
- Greenwood S. J., Sogin M. L., Lynn D. H. (1991) Phylogenetic relationships within the class Oligohymenophora, phylum Ciliophora, inferred from the complete small subunit rRNA gene sequences of *Colpidium campylum*, *Glaucoma chattoni* and *Opisthnecta heneguyi*. *J. Mol. Evol.* **33**: 163-174
- Irwin D. M., Kocher T. D., Wilson A. C. (1991) Evolution of the cytochrome b gene of mammals. *J. Mol. Evol.* **32**: 128-144
- Jin L. P., Ng S. F. (1989) The somatic function of the germ nucleus in *Pseudourostyla cristata*: asexual reproduction and stomatogenesis. *J. Protozool.* **36**: 315-326
- Johns G. C., Avise J. C. (1998) A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome b gene. *Mol. Biol. Evol.* **15**: 1481-1490
- Kelley L. A., MacCallum R. M., Sternberg M. J. E. (2000) Enhanced genome annotation using structural profiles in the program 3D-PSSM. *J. Mol. Biol.* **299**: 501-522
- Lang B. F., Cedergren R., Gray M. W. (1987). The mitochondrial genome of the fission yeast, *Schizosaccharomyces pombe*. Sequence of the large-subunit ribosomal RNA gene, comparison of potential secondary structure in fungal mitochondrial large-subunit rRNAs and evolutionary considerations. *Eur. J. Biochem.* **3**: 527-537
- Livak K. J., Schmittgen T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* **25**: 402-408
- Liu X. Y., Mao Y. Z., Chen J. H., Lee K. L.-D., Jin L. P. (2004) Total RNA Extraction from Large Ciliates. *Chinese J. Zool.* **39**: 44-47
- Paquin B., Lang B. F. (1996) The mitochondrial DNA of *Allomyces macrogynus*: the complete genomic sequence from an ancestral fungus. *J. Mol. Biol.* **5**: 688-701

- Pritchard A. E., Seilhamer J. J., Mahalingam R., Sable C. L., Venuti S. E., Cummings D. J. (1990a) Nucleotide sequence of the mitochondrial genome of *Paramecium*. *Nucl. Acids Res.* **18**: 173-180
- Pritchard A. E., Sable C. L., Venuti S. E., Cummings D. J. (1990b) Analysis of NADH dehydrogenase proteins, ATPase subunit 9, cytochrome b, and ribosomal protein L14 encoded in the mitochondrial DNA of *Paramecium*. *Nucl. Acids Res.* **18**: 163-71
- Saccone C., Gissi C., Lanave C., Larizza A., Pesole G., Reyes A. (2000) Evolution of the mitochondrial genetic system: an overview. *Gene* **261**: 153-159
- Sonnhammer E. L., Heijne G., Krogh A. (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **6**: 175-182
- Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G. (1997) The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* **24**: 4876-4888
- Tourancheau A. B., Villalobo E., Tsao N., Torres A., Pearlman R. E. (1998) Protein coding gene trees in ciliates: comparison with rRNA-based phylogenies. *Mol. Phylogenet Evol.* **10**: 299-309

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