Detection of Dehydrogenase Genes from Ciliates Using the Polymerase Chain Reaction Technique

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INTRODUCTION

Water pollution is a threat to human existence due to the presence of ever increasing levels of toxic pollutants. Daily consumption of dirty, contaminated water is the greatest cause of worldwide death everyday (Mason, 1996).

The use of natural organisms to solve environmental problems is increasingly becoming popular and is viewed as a most cost-effective approach. Since the identification of different pollutants in the environment, a number of pollutant-degrading microorganisms, primarily bacteria, have been studied, identified and isolated.

However, unicellular eukaryotic organisms such as ciliated protozoa can be tapped as alternatives in abating pollution. More than fifty percent of the population in organically polluted rivers and streams are ciliates, indicating their potential as good indicators of organic pollution (Krishnamoorthi, 1979 and Mason, 1996). They play a significant role in wastewater treatment and natural process of water purification by consuming bacteria and particulate organic matter in suspension, thereby reducing turbidity and BOD (Curds, 1982).

Landis (1988) reported that ciliates contain enzymes which can hydrolyze certain pollutants and can play an important role in communities

engineered to degrade such chemical pollutants as polychlorinated biphenyls (PCBs), organophosphates and herbicides either *in situ* or as part of a waste treatment process. Enzyme assays can be done to detect these enzymes. However, direct detection of genes encoding pollutant-degrading enzymes from ciliates can now be done by the application of polymerase chain reaction or PCR.

PCR is a DNA-cloning technique that directly amplifies a DNA segment flanked between two known sites. It is rapid, highly sensitive, specific and an *in vitro* method of DNA amplification. It has been used in clinical diagnostics, forensic analysis and identification of individuals. The technique has been used to detect genes that encode important proteins and enzymes from ciliates like telomerases (Lingner *et al.*, 1994), heat shock proteins (Budin *et al.*, 1998), DNA polymerase (Hoffman *et al.*, 1997), alpha-tubulins (Torres *et al.*, 1997), and histones (Bernhard *et al.*, 1998). Likewise, in this study, PCR will be used to detect dehydrogenase genes from ciliates.

Dehydrogenases have been involved in degradation pathways of xenobiotics like PCBs. They are involved in the catabolism of toluene (Zeng, 1999), 1,1,1-Trichloro-2,2-bis-bis(4'chlorophenyl)ethane or DDT (Feng, 1999), naphthalene (Zeng, 1999), and phthalate family (Burke *et al.*, 1999).

This study aims to detect dehydrogenase genes from ciliates through PCR amplification. It also aims to clone and sequence the amplified products and to use bioinformatics as a means of analyzing and confirming the products.

Since ciliates are ubiquitous in any organically-polluted freshwater, they could be of potential help in abating pollution. This study, therefore, may provide a basis for the evaluation of such potential.

MATERIALS AND METHODS

Ciliates Used in the Study

Six different ciliate species (Figures 1 and 2) were used in the study. Three reference ciliate strains, *Tetrahymena thermophila* B2698 (T1), *Paramecium caudatum* (P1) and *P. tetraurelia* (P2) were obtained from Japan.[1] Three local ciliate isolates were also used in the study; one *Tetrahymena* sp. (Tm) from UP Lagoon,[2] and two predominant ciliates *Tetrahymena* sp. (T3) and *P. aurelia complex* (L)[3] from UP Pump lift station, Diliman, Quezon City.

Extraction of Genomic DNA

Genomic DNA from ciliates was isolated by phenol-chloroform/ethanol precipitation method (modified from Ausubel *et al.,* 1988). The cells were harvested by centrifugation at 2,500rpm for 5 minutes at 4°C from a 3-day old 20mL suspension culture. The pellets were washed with sterile PBS, suspended in digestion buffer and proteinase K and incubated at 55°C overnight with shaking. The digested cells were extracted twice with phenol-chloroform-isoamyl alcohol. Genomic DNA was precipitated with 3M sodium acetate and absolute ethanol, washed with 70% ethanol, dried in speed vacuum (Automatic Environmental SpeedVac 2000) and resuspended in sterile distilled water. DNA was quantified by spectrophotometry and visualized in ethidium bromide-stained 0.9% agarose gel under UV light. One microliter (μL) of these genomic DNA samples was used as template for PCB-gene detection per 50μL-PCR reaction tube.

PCR Primers

Six pairs of PCR primers used for gene detection were designed and patterned after the bph operon of *Pseudomonas pseudoalcaligenes* KF707. Different genes including the dehydrogenase gene in this operon, have been identified and sequenced (Furukawa *et al.,* 1992). PCR primers were generated by using OLIGO 4.0-s (Mac) software and were chemically synthesized in Japan.[4]

PCR Amplification, Purification and Optimization

To optimize the PCR conditions of each primer (i.e. when a single distinct band is consistently amplified for a given sample), a 50μL reaction mixture was prepared for n+1 tubes. Different concentrations of the reagents were tried and changed in each reaction as PCR conditions were optimized.

In a premix preparation, taq polymerase was added last. A fixed volume of the premix was transferred into each microfuge tube. The DNA sample was added last to complete the master mix. It was spun briefly, overlaid with mineral oil and placed in a programmed automated thermocycler (Minicycler, MJ Research) for amplification. The temperatures and duration of each step in the PCR profile were changed one factor at a time until the PCR conditions were optimized (modified from Erb *et al.,* 1993; Innis *et al.*, 1990; and Davis *et al.*, 1994). The PCR products were analyzed in 2% agarose gel electrophoresis.

As soon as PCR conditions were optimized, a 100-μL PCR reaction mix was prepared for cloning. The amplified products (100μL) mixed with 20μL gel loading buffer were loaded into a 1% low melting agarose and purified using the GFX Band Purification Kit (Pharmacia, Biotech).

Cloning of the Purified PCR Products

The purified PCR products were cloned using an Original TA Cloning Kit (Invitrogen), which involved ligation of the products into pCR 2.1 vector and transformation of competent cells using the ligated vectors by brief heat shock treatment. *Echerichia coli* XL1-Blue was used as competent cells. Preparation of competent cells involved chemical treatment using $CaCl_2$ and RbCl to *E. coli* cells.

The transformed cells were then spread on previously prepared Luria-Bertani (LB) agar plates with Ampicillin ($^{Amp+}$) and X-gal and incubated for 37°C for at least 18 hours. The ampicillin-resistant vectors had been engineered to allow one step screening for host cells carrying the inserts. The host cells that carried the vectors with the PCR inserts grew in the presence of ampicillin and produced white colonies when plated on LB agar containing a compound called Xgal. Restriction sites for the insertion of PCR products was in lacZ gene of the vector, responsible for the formation of blue colonies in the presence of Xgal. Insertion of PCR products disrupted and inactivated lacZ gene, which resulted in white colonies.

Screening of Clones

Ten white colonies were chosen from the incubated agar. Each colony was inoculated and streaked into 3mL LB broth^{Amp+} and LB agar^{Amp+}, respectively. As negative control, at least 2 blue colonies were likewise inoculated and streaked. The LB plates were incubated at 37°C for 18 hours, while the broth cultures were incubated in an environmental shaker (B. Braun Biotech International) at 120rpm for 18 hours at 37°C.

Plasmid DNA (pDNA) was extracted from the cultures using the small scale LiCl Method. The pDNA were then digested with restriction enzyme EcoR1 to verify PCR inserts.

Preparation of Plasmids for DNA Sequencing

A loopful of each bacterial colony having the inserts was inoculated into 40mL LB broth^{Amp+} for medium scale pDNA isolation. Back up streaking of the same colonies on agar plate was also done. The LB plates were incubated at 37°C for 18 hours while the broth cultures were incubated in an environmental shaker at 120rpm for 18 hours at 37°C. The cells were harvested and used for extraction of pDNA using Alkaline Lysis Method (Maniatis *et al.*, 1989) and Midi-prep Concert Kit (Gibco). The purified pDNAs were sent to Japan Gene Bank, National Institute of Infectious Diseases for nucleotide sequencing.[5]

Sequence Analysis

Consensus sequence was determined for each amplified product using Manual Multiple Sequence Alignment function of DNASIS-Mac (3.6 Version). The consensus sequences were compared with the bph genes and against all gene sequences from gene banks using DNASIS, BLASTN search, which compares a DNA sequence to nucleotide sequences in NCBI Genbanks (Altschul *et al.*, 1997) and other related bioinformatics queries for sequence homologies. FASTXY search which compares a DNA sequence to protein sequence databanks in EMBL (Pearson *et al.*, 1997) was also done.

RESULTS AND DISCUSSION

Genomic DNA Extraction

Figure 3 shows the extracted genomic DNA of ciliates as run in 2% agarose gel electrophoresis. The harvest of 20-ml ciliate cultures containing 2.30 x 10^5 to 5.6 x 10^5 cells yielded a spectrophometric DNA quantity ranging from 400.5 ng/μL to 1,190 μg/μL at OD260.

Primers and Optimization Conditions

The sequences of the six primers (bphA1, bphA2, bphA3, bphA4, bphB and bphC) for the sense and antisense, respectively are given below: bphA1, 5' AAG GAG ACG TTG AAT CAT GAG 3' (21-mer) and 5' AGC CCA CCA TGT TGA ATT CT 3' (20-mer); bphA2, 5' AAG AAT TCA ACA TGG TGG GCT G 3' (22-mer) and 5' GTG CTG GGC TAG AAG AAC ATG CTC 3' (24-mer); bphA3, 5' ACA AGA AGC GTT ATG AAA TTT ACC AG 3' (25-mer) and 5' CCA CGT CAC CTT CAA GAT AGC CGC CA 3' (26-mer); bphA4, 5' TCT GGC GCC ATG ATC GAC AC 3' (20-mer) and 5' CGG TCA TGC GCA TCA ATT CGG 3' (21-mer); bphB, 5' TGG AAA AAT GAA ACT GAA AGG TG 3' (23-mer) and 5' ACT GTC TCC TTC GTC GAT GCT TA 3' (23-mer); and bphC, 5' AAG CAT CGA CGA AGG AGA CAG TA 3' (23-mer) and 5' CCC GTG CTA TTC AGG TTG TTG TT 3' (23-mer).

Only 3 of the 6 primer pairs (bphA1, B and C) amplified PCR products after optimization. The optimized PCR profile for the three pairs is shown in Tables 1a-b. These primers shared similar profile except in primer concentrations and annealing temperatures. A primer concentration of 2 μM was for bphB and C and 1 uM only for bphA1. The annealing temperatures observed were 40°C for

bphA1 and B and 45°C for C. The empirical annealing temperatures were lower by 15.5°C to 18.8°C compared to the suggested annealing temperatures set by OLIGO (Table 2). This difference is possible since there is no single PCR protocol appropriate to all situations. Consequently each new PCR application is likely to require optimization (Innis *et al.* 1990).

The three primers worked at much lower annealing temperatures than the suggested optimum annealing temperatures for each primer. No bands were observed when the annealing temperature was higher than 50°C. A similar study conducted by Erb *et al.* (1993) which aimed to detect bphC genes by PCR amplification from a microbial population of a river soil sediment also selected low annealing temperatures at 35^0C-40^0C. The low annealing temperature is very crucial in gene detection by PCR since it allows amplification of bph genes that may have divergent base pair compositions.

Amplified PCR Products

Only 3 ciliate species (or their genomic DNA extracts) yielded four 4 amplified PCR products (amplicons) as shown in Table 3. The amplified products were run in 2% agarose gel electrophoresis as shown in Figures 4a-c. From these figures the approximate size of each amplicons was computed. The figures revealed that BphA1 primer amplified a product size of 650 base pairs (bp) from *P. aurelia complex* and *Tetrahymena* sp. (Fig. 4a), while bphB primer amplified a 400bp-product from *T. thermophila* (Fig. 4b). BphC primer amplified a 940bp-product from from *T. thermophila* (Figure 4c). The expected size of the amplified products set by OLIGO for primers bphA1, B and C are 1445bp, 861bp and 942bp, respectively. Most of the PCR products, except for CT1 from *T. thermophila,* had much lower molecular weights than expected. The results clearly show that the amplicons from the ciliates are not exactly the same as the pseudomonads bph genes.

Cloning and Nucleotide Sequencing

The amplicons were successfully cloned and sequenced. Figures 5a-d show the 2% gel agarose electrophoresis of EcoRI digests of the clonal plasmid DNA that carry the cloned amplicons. Comparing Figures 4a-c and Figures 5a-d revealed that the size of PCR products and their respective clonal plasmid digest are approximately the same, thus confirming the clones to carry the amplicon inserts.

The amplicons were successfully sequenced and, by using the Manual Multiple Sequence Alignment function of DNASIS-Mac (3.6 Version), consensus sequence was determined for each amplicon (see Appendices 1-4).

Consensus sequences were shorter than the actual PCR products, as shown in Table 4.

Analysis of the Sequenced Amplicons

BLASTN search and DNASIS showed that the A1Tm sequence from *Tetrahymena* sp. and A1L sequence from *Paramecium* sp. are different sequences. A1L fragment 405-432bp is homologous with BphA1 sequences of species of *Pseudomonas* (Table 5). In FASTXY search, translated A1L yielded homology with probable alcohol dehydrogenase of *Pseudomonas sp.* (Table 6). FASTXY search for translated A1Tm aligned with NADH dehydrogenase of *Bodo saltans.* Best scores also include NADH-ubiquinone oxidoreductases of *P. tetraurelia, Phytophthora infestus* and *Globodera pallida* (Table 7).

A1Tm sequence and A1L sequence were both amplified by the same primer bphA1. However, the two products are not homologous when aligned against each other using DNASIS. Only fragment 405-432bp from A1L showed homology with BphA1 genes (dioxygenase genes) of pseudomonads by BLAST and DNASIS search. This fragment is the annealing site of the antisense primer implying that the primer worked by annealing perfectly to its specific site and that the terminal end of the A1L sequence is a dioxygenase sequence. In the case of A1Tm sequence, it has no homologies with bph genes and bphA1 primers have no annealing sites in the sequence itself. These results suggest that the A1Tm and A1L are two different sequences. However, in FASTXY the search from EMBL databanks revealed that translated A1L and A1Tm are in some ways related since they commonly aligned to dehydrogenases. A1L aligned to a probable alcohol dehydrogenase of *Pseudomonas sp.* and A1Tm with NADH dehydrogenase and NADH-ubiquinone oxidoreductase.

BLASTN search revealed that fragments of BT1 sequence (annealing sites of the primers) are homologuous to all BphB genes from different *Pseudomonas* strains, either to the terminal or the initial site of the Bph genes. Interestingly, BT1 also aligned with human sorbitol dehydrogenase and L-iditol-2-dehydrogenase genes (Table 8). Moreover, FASTXY search revealed that BT1 showed protein homology with NADH dehydrogenase of a honeybee (Table 9). Theoretically, BT1 should be a dehydrogenase gene since the primer bphB was designed to amplify a dehydrogenase gene, and such is confirmed since sequence analysis results strongly suggest that BT1 is a dehydrogenase gene.

BLASTN search revealed that the CT1 sequence matches perfectly with the *Tetrahymena thermophila* dynein heavy chain gene. FASTXY search also revealed that translated CT1 is a heavy dynein chain (Table 10) and has higher homology scores with *Tetrahymena thermophila.* It also aligns with the dynein

chains of *Paramecium tetraurelia*, sea urchins and fruit flies. Dynein, an ATPase (an enzyme that hydrolyzes ATP) belongs to a family of motor enzymes involved in microtubule sliding in cilia and flagella and contribute to microtubule-based transport inside cells (Xu *et al.* 1999).

When bphC primers were aligned with the dynein sequences, no homologies were found. The amplification of the dynein heavy chain gene can only be explained if the primers found annealing sites in the dynein heavy chain sequence. Considering the secondary structure of the DNA, the primers probably annealed at a separate but linearly-linked DNA conformation escaping intervening sequences at the coiled portion and thus amplifying a very different product .The identity of CT1 sequence with dynein heavy chain is an interesting result. The usefulness of bioinformatics in confirming the identity of the amplified products is clearly demonstrated.

The failure of primers bphA2, bphA3, and bphA4 to amplify products from any of the ciliates in the study indicates that the DNA samples do not contain the corresponding complementary sequences. The same may be inferred for the absence of amplified products from other ciliates by the primers that worked in other samples. For instance, the bphA1 primer amplified products from *Tetrahymena sp.* and *P. aurela complex* only but not from the rest of the *Tetrahymena* and *Paramecia* samples. Primers bphB and bphC amplified products from *T. thermophila* (T1) but not from *Tetrahymena spp.* (Tm and T3) which are all *Tetrahymena* species. Since they all belong to the same genus, different responses are indicative of genetic variation at species level.

SUMMARY, CONCLUSION AND RECOMMENDATIONS

Six primer pairs were designed from the bph operon of *Pseudomonas pseudoalcalogenes* KF707. Optimum PCR conditions were obtained empirically. All primers had much lower annealing temperatures than what were suggested by the OLIGO program.

Four PCR products were obtained from 3 ciliates out of 6 species. Two amplicons A1L and A1Tm amplified by bphA1 primers are from *P. aurelia complex* and from *Tetrahymena sp.* respectively, and the other two amplicons BT1 by bphB and CT1 by bphC are both from *T. thermophila.*

A1L, A1Tm and BT1 were much shorter than expected, indicating that these are not the same as the bph genes of Pseudomonads. Analysis of the

sequenced amplicons using bioinformatics (BLASTN and FASTXY search) strongly suggests that A1L, A1Tm and BT1 sequences are dehydrogenase genes (summarized in Table 11). These are not only potential PCB-degrading genes, but may also be involved in the degradation pathway of other xenobiotics.

The CT1 sequence differs from the other products as was shown by BLASTN and FASTXY search that it is 100% homologous with the dynein heavy chain of *T. thermophila.* Although the bphC primers had no homology with the latter sequence, possible annealing sites may have been formed as a consequence of the secondary structure of the DNA template.

The results indicate that some ciliates species may have PCB-degrading potential and that there is genetic variation in the species used in the study.

It is recommended that ciliates potentially useful in organically polluted water can be studied further in order to understand their specific role in degrading and abating water pollutants. The use of a positive control in optimizing the PCR conditions is also recommended. Other molecular techniques may be used to further confirm the expression of dehydrogenase genes in ciliates.

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[1]Prof. Toshiro Sugai and Prof. Isoji Miwa, both from Ibaraki University, Mito, Japan, gave the reference ciliates.

[2]Mark Pierre S. Dimamay (Ph.D. student of the National Institute of Molecular Biology and Biotechnology, UP Diliman, Quezon City.) isolated the ciliate from UP Lagoon.

[3]Prof. Kazuyuki Mikami, EEC, Miyagi University of Education, Aza-Aoba, Arakami, Sendai, Japan identified the local *Paramecium* species.

[4]Prof. Ronald R. Matias, senior professor, Biology Institute, College of Science, UP Diliman, Q.C. designed and synthesized the PCR primers in Japan.

[5]Dr. Yosuke Kameoka, National Institute of Infectious Diseases, Japan Gene Bank, sequenced the amplicons.

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Tables

Table 1a. PCR Profile: Optimized Reagent Concentrations

Reagents	Final Concentration	Volume/Reaction Tube
Premix		
a) PCR buffer (Pharmacia)	1X	5.00 µL
b) dNTP mix (Pharmacia)	0.20 mM	0.50 µL
c) Primer 1 (sense)	1.00 µM (2.00 µM)	0.50 µL
d) Primer 2 (antisense)	1.00 µM (2.00 µM)	0.50 µL
e) Taq polymerase (Pharmacia)	0.05 U/µL	0.50 µL
	Subtotal	7.00 µL
Master Mix		
a) Premix		7.00 µL
b) DNA Sample		1.00 µL
c) Sterile distilled water		42.00 µL
	TOTAL	50.00 µL

Table 1b. PCR Profile: Optimized Cycling Conditions

Steps	Temperature ^{0}C	Time	
a) Initial denaturation	94	5 min	
b) Denaturation	95	30 sec	*
c) Annealing	40 (45)	1 min	*
d) Extension	72	3 min	*
e) Final Extension	72	10 min	

*35 no. of cycles

Table 2. Comparison between annealing temperatures suggested by Oligo 4.0-s and the empirical optimum annealing temperatures

Primers	Suggested Annealing Temperatures By Oligo, ^{o}C	Empirical Optimum Annealing Temperatures ^{o}C
bphA1	58.8	40
bphB	59.7	40
bphC	60.1	45

Table 3. The primers that consistently amplified PCR products from ciliates' genomic DNA

Primers	Size of the PCR Products in base pairs (bp)					
	Tetrahymena spp.			*Paramecium* spp.		
	T1	Tm	T3	P1	P2	L
bphA1			650			650
bphA2						
bphA3						
bphA4						
bphB	400					
bphC	940					

Table 4. The primers, their respective amplified PCR products (amplicons) and source of genomic DNA from which the products are amplified; and the comparison between the size of the amplicons and consensus sequences

Primers	Amplicons	Genomic DNA Source	Size of Amplicons (bp)	Size of Consensus Sequence (bp)
bphA1	A1L	*P. aurelia complex*	650	425
bphA1	A1Tm	*Tetrahymena* sp.	650	586
bph B	BT1	*T. thermophila*	400	380
BphC	CT1	*T. thermophila*	940	426

Table 5. Results of homology search for A1L consensus sequence (425bp) of *P. Aurelia complex* using BLASTN from Genbank, NCBI (Altschul *et al.*, 1997)

A1l Fragments (bp)	Organism	Genbank Sequences	Identities
405-423	*Pseudomonas* sp.	BphA1 gene biphenyl dioxygenase	19/19 (100%)
405-423	*P. pseudoalcaligenes*	Dioxygnease gene	19/19 (100%)

Table 6. Results of protein homology search for A1L consensus sequence of *P. Aurelia complex* using FASTXY from EMBL Protein Databanks (Pearson *et al.*, 1997)

Organism	Protein	Identities (Position of Overlap)
Pseudomonas sp.	Probable alcohol dehydrogenase (319 aa)	27% in 87% aa overlap (261-7:126-212)

Table 7. Results of protein homology search for A1Tm consensus sequence of *Tetrahymena* sp. using FASTXY from EMBL Protein Databanks (Pearson *et al.*, 1997)

Organism	Protein	Identities (position of overlap)
Bodo saltans	NADH Dehydrogenase subunit (212 aa)	30% in 133 aa overlap (146-529:25-148)
P. tetraurelia	NADH-Ubiquinone Oxidoreductase (193 aa)	27% in 166 aa overlap (125-580:33-191)
Phytophthora infestans	NADH-Ubiquinone Oxidoreductase (664 aa)	26% in 166 aa overlap (74-532:499-659)
Globodera pallida	NADH-Ubiquinone Oxidoreductase (287 aa)	27% in 168 aa overlap (41-523:70-226)

Table 8. Results of homology search for BT1 consensus sequence (380bp) of *Tetrahymena thermophila* using BLASTN from Genbank, NCBI (Altschul *et al.*, 1997)

BT1 Fragments (bp)	Organism	Genbank Sequences	Identities
1-23 357-380	*P. Pseudoalcaligenes*	Dihydrodiol dehydrogenase gene	23/23 (100%) 23/23 (100%)
1-23 357-380	*Pseudomonas sp.*	Bph gene	23/23 (100%) 23/23 (100%)
1-23 357-380	*Pseudomonas aeruginosa*	Dehydrogenase gene	23/23 (100%) 23/23 (100%)
1-23	*Pseudomonas sp. LB400*	Dihydrodiol dehydrogenase gene	23/23 (100%)
66-91	*Homo sapiens*	Sorbitol dehydrogenase gene	23/24 (95%)
68-91	*Homo sapiens*	Sorbitol dehydrogenase gene	23/24 (95%)
68-91	*Homo sapiens*	L-iditol-2-dehydrogenase gene	23/24 (95%)

Table 9. Results of protein homology search for BT1 consensus sequence of *T. thermophila* using FASTXY from EMBL Protein Databanks (Pearson *et al.*, 1997)

Organism	Protein	Identities (Position of Overlap)
Apis cerana (Indian honeybee)	NADH Dehydrogenase subunit (265 aa)	25% in 118 aa overlap (34-354:91-208)

Table 10. Results of protein homology search for CT1 consensus sequence of *T. thermophila* using FASTXY from EMBL Protein Databanks (Pearson *et al.*, 1997)

Organisms	**Proteins**	**Identities (Position of Overlap)**
Tetrahymena thermophila	DYNEIN HEAVY CHAIN (412 aa)	96% in 141 aa overlap (425-3:136-276)
Anthocidaris crassispina	DYNEIN HEAVY CHAIN (4466 aa)	61% in 139 aa overlap (419-3:1989-2127)
Tripneustes gratilla	DYNEIN HEAVY CHAIN (4466 aa)	59% in 139 aa overlap (419-3:1989-2127)
Drosophila hydei	DYNEIN HEAVY CHAIN (4564 aa)	59% in 139 aa overlap (419-3:2082-2220)
Chlamydomonas reinhardtii	DYNEIN HEAVY CHAIN (2405 aa)	60% in 141 aa overlap (425-3:732-872)
Paramecium tetraurelia	LEFT-RIGHT DYNEIN (4488 aa)	55% in 139 aa overlap (419-3:2011-2149)
Paramecium tetraurelia	OUTER ARM DYNEIN BETA HEAVY CHAIN(4588 aa)	54% in 142 aa overlap (425-3:2026-2167)
Tetrahymena thermophila	CILIARY OUTER ARM DYNEIN BETA HEAVY CHAIN (4589 aa)	51% in 142 aa overlap (425-3:2031-2172)
Chlamydomonas reinhardtii	DYNEIN HEAVY CHAIN (4568 aa)	50% in 140 aa overlap (419-6:2056-2195)

Table 11. Possible identities of the amplicons through BLASTN and FASTXY search

Amplicons	Possible Identities
A1L	Dehydrogenase gene / dioygenase gene
A1Tm	Dehydrogenase gene / NADH-ubiquinone Oxidoreductase gene
BT1	Dehydrogenase gene
CT1	Dynein heavy chain gene

FIGURES

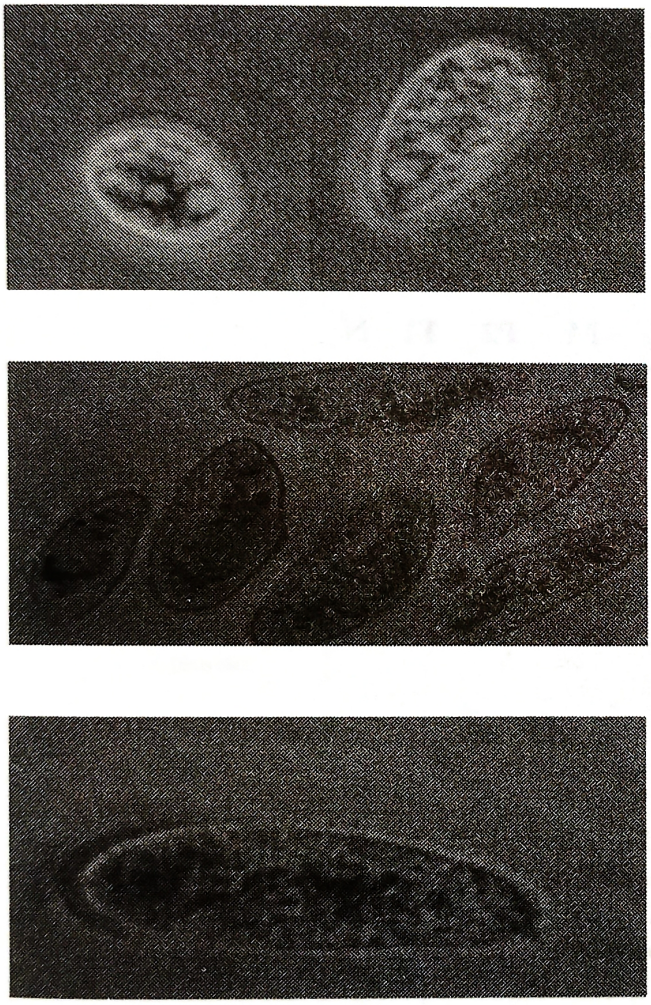

Figure 1. The reference ciliates *Tetrahymena thermophila* B2698 (T1), *Paramecium caudatum* (P1) and *P. tetarurelia* (P2) at 4000X magnification.

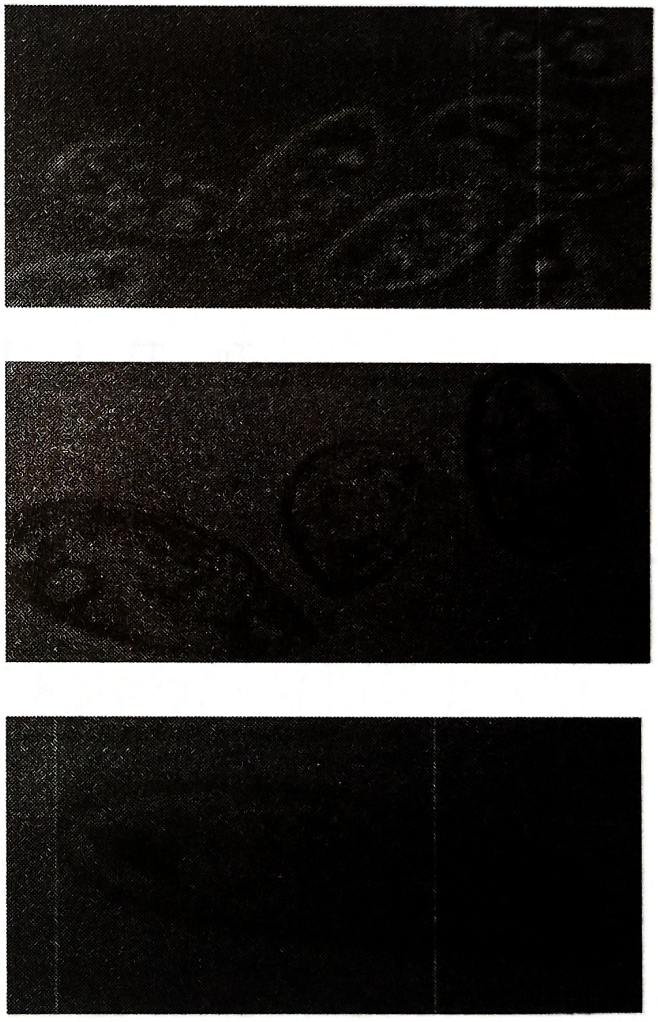

Figure 2. The Local ciliates *Tetrahymena* sp. (Tm) and *Tetrahymena* sp. (T3) at 4000X and *P. aurelia complex* (L) at 1000X

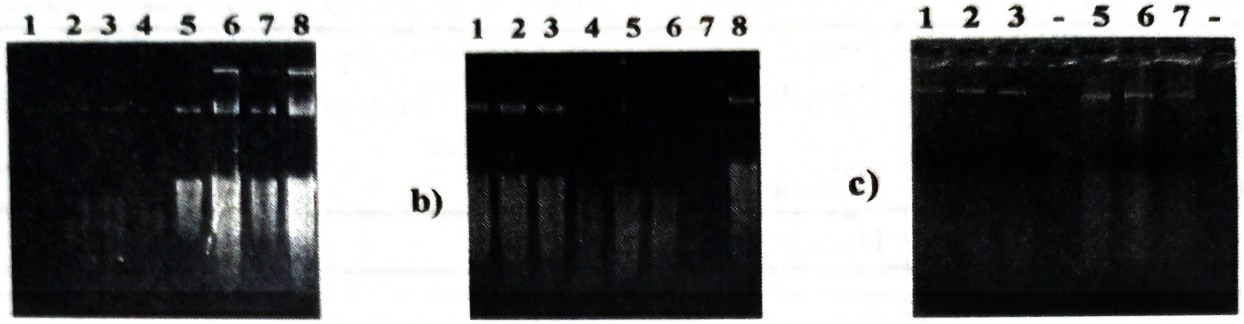

Figure 3 Agarose gel electrophoresis of genomic DNA from ciliates. a) lanes 1-4, T1 and 5-8, Tm ; b) lanes 1-4, T3 and 5-8, P1; and c) lanes 1-3, P2 and 5-7, L.

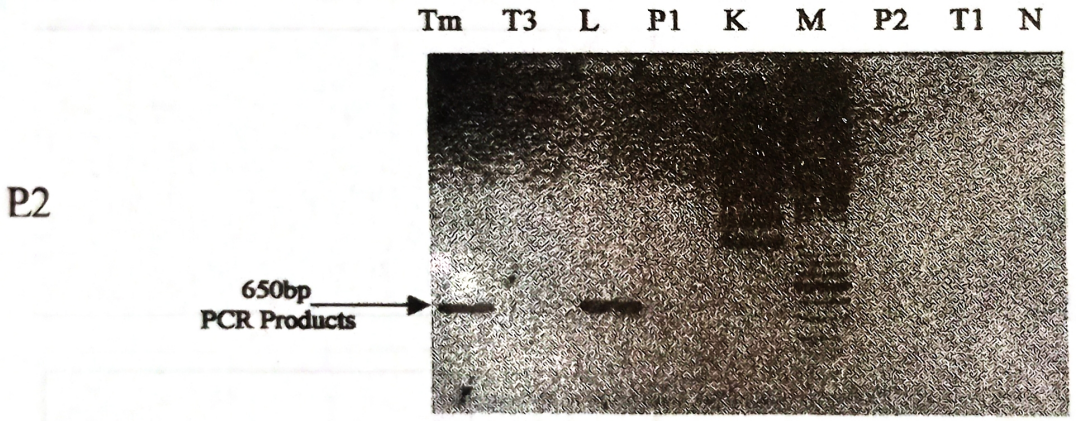

Figure 4a Agarose gel electrophoresis of PCR products of bphA1 primers Lanes Tm, T3, and L , local ciliates; P1, P2 and T1, reference ciliates; K, *Klebsiella pneumoniae* (the bacterial food of *Paramecium*); M, 100bp ladder; and N, negative control. Local isolates Tm and L yielded approximately 650bp amplified products.

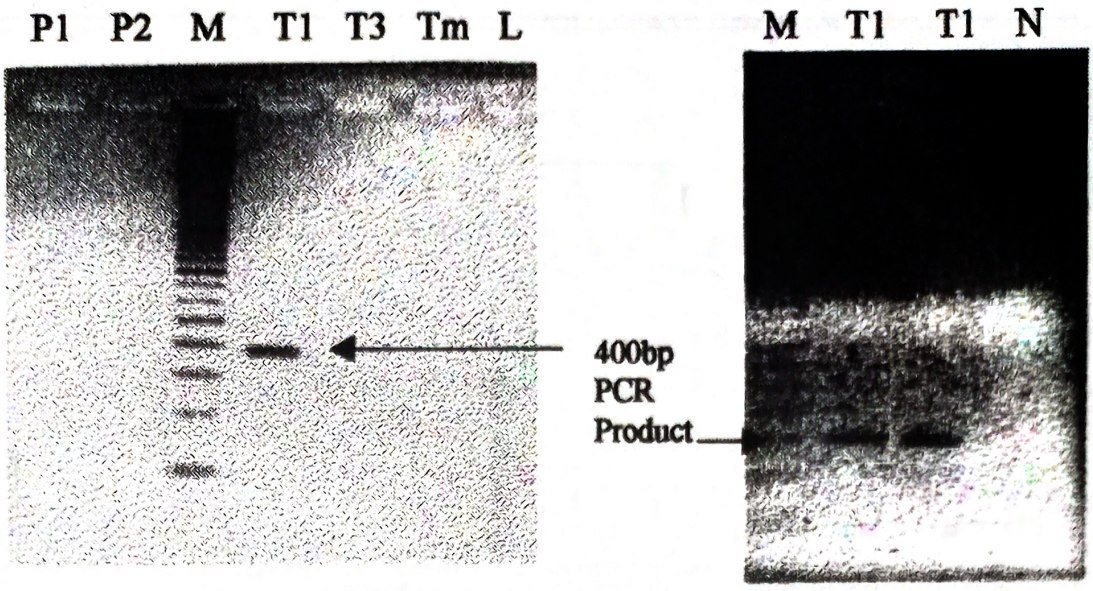

Figure 4b Agarose gel electrophoresis of PCR products of bphB primers. Lanes P1, P2, and T1, reference ciliates; M, 100bp ladder; T3, Tm, and L, local ciliates; and N, negative control. Reference ciliate T1 yielded approximately a 400bp product.

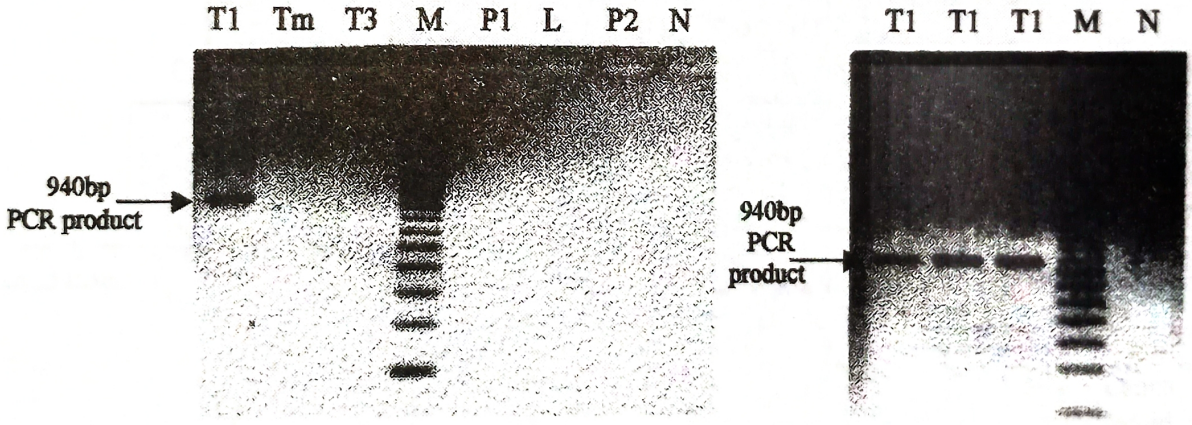

Figure 4c Agarose gel electrophoresis of PCR products of bphC primers. Lanes T1, P1, and P2, reference ciliates; M, 100bp ladder; Tm, T3 and L, local ciliates; and N, negative control. Reference ciliate T1 yielded approximately a 940bp amplified product.

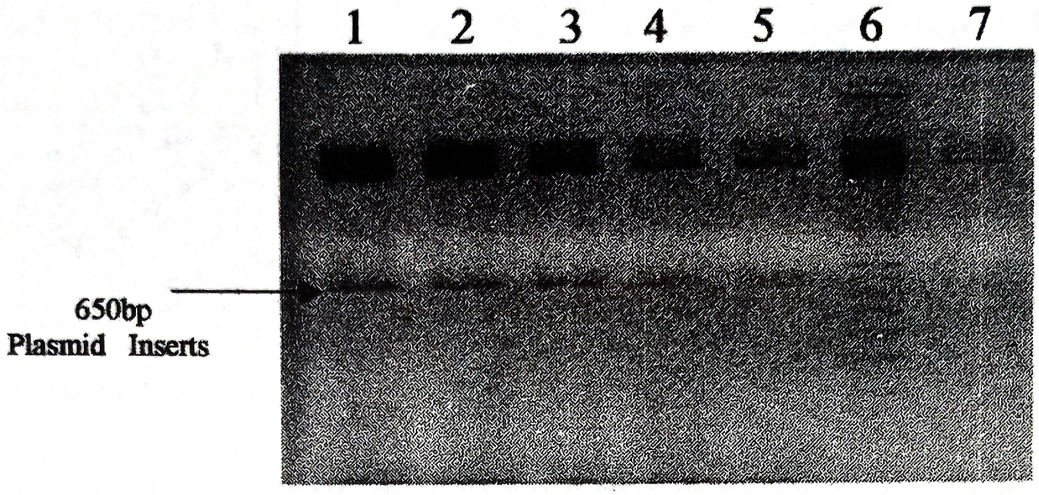

Figure 5a Agarose gel electrophoresis of EcoRI digests of A1Tm plasmids. Lanes 1-5, digested pA1Tm; 6, 100bp ladder; and 7, blue colony plasmid as negative control. All digested pA1Tm samples contain PCR inserts (650bp).

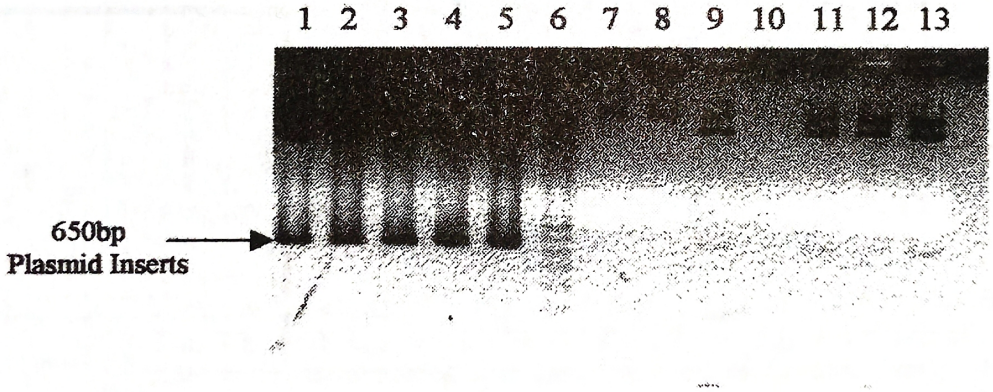

Figure 5b Agarose gel electrophoresis of EcoRI digests of A1L plasmids. Lanes 1-5, digested pA1L by Midi-prep Concert Kit preparation; 6, 100bp ladder; 7-8, digested blue colony plasmids as negative control; and 7-13, digested pA1L by LiCl preparation. PCR inserts (650bp) are present in lanes 1-5, 9, and 11-13.

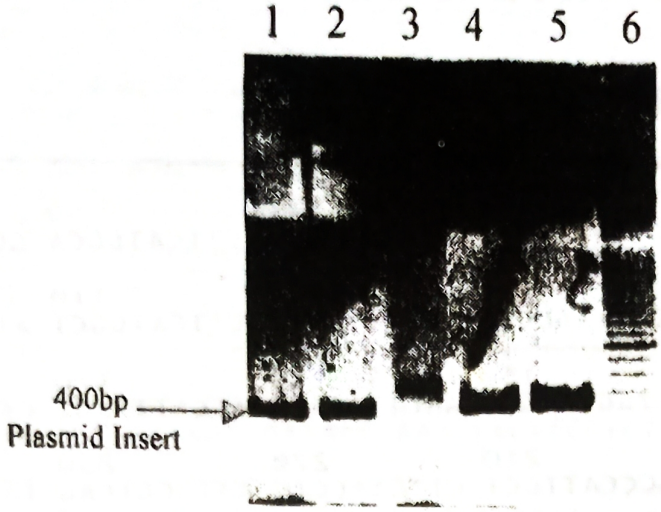

Figure 5c Agarose gel electrophoresis of EcoRI digests of BT1 plasmids. Lanes 1-5, digested pBT1 and 6, 100bp ladder. All digested pBT1 samples contain the PCR insert (400bp) except for lane 3.

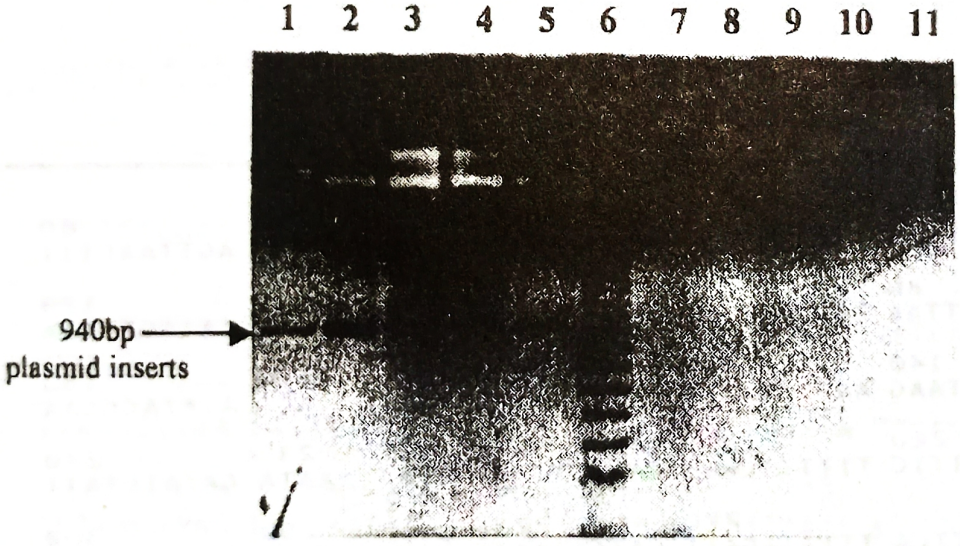

Figure 5d Agarose gel electrophoresis of EcoRI digests of CT1 plasmids. Lanes1-5, digested pCT1; 6, 100bp ladder; and 7-11, undigested pCT1. All digested pCT1 samples contain PCR inserts.

APPENDICES

Appendix 1. Consensus sequence of A1L

```
        10         20         30         40         50         60
ATATTCGGCA CCTAAAGCCA ACGTATCTTT TCATGGATCA CTTCATGGCA GGCTGACGCA

        70         80         90        100        110        120
TAATCGCCGG CAAGATTCAT CGCTAGCCAA TATTCTTGCC CTTCATGGCT ATCCAGGTCC

       130        140        150        160        170        180
AGATAGGCCA GATTTTCCGC TTGGTAAGGC AACTTACACA AGGATTTAGC CAACTTGGTG

       190        200        210        220        230        240
TAATGTGCCG CAATGGTAGC GCCCATTCCT CTTGAACCTG AATGGGTCAG TAAGCCTAAA

       250        260        270        280        290        300
TAGGCTCCGC TATCAATGTT CAAAACCTCA TCTCTTTCTT CAAATTCGAC GATACCAAAC

       310        320        330        340        350        360
TCCACAAAAT GATTCCCCCC ACCCGAAGAT CCCAATTGCG TCCAGGCCTT ATCCTTCAAT

       370        380        390        400        410        420
GAAGAAAGCA AACTGTTTTC CCTAAACAAC TTGTTATCTA GTACCTCATG ATTCAACGTC

       430        440        450        460        470        480
TCCGC..... .......... .......... .......... .......... ..........
```

Appendix 2. Consensus sequence of A1Tm

```
        10         20         30         40         50         60
CTGTTCTCCT CTATTAAGTA AAGCTTTTTC AGTTTCTAAA ATAGTATTCT AGTTAACTTT

        70         80         90        100        110        120
AAAACAAAAA TAATTATTAA TGAAAGCCTT TTTTCAGAAA AACATTGATT GAAAGTTAAA

       130        140        150        160        170        180
CTAATTTCTT AAATTTTAAG AAATTTTTTG TCACTTTTTG TTTTCATAAA ATATAGGGAA

       190        200        210        220        230        240
AAGTAAATAT TTTTGTTTTC TTTTTACATT CAAGCTCTCT TTATAAAATA GATATCTATT

       250        260        270        280        290        300
TTTGAACAGC TCTTGTTTCA TTTTTAGATT CATGAATTTC TATATCCTTC TCGTGAATCT

       310        320        330        340        350        360
ACTTTTAGTA TTCTGAATGG ATTTACTTCA GCAACTCTTC ATAATCTCTA ATTCTTATTT

       370        380        390        400        410        420
CGTATGCTTC TTTTTCTTCC TTAAATATTT TTTCTTGTCT CTCAACTGAT CTTAGTTAGG

       430        440        450        460        470        480
AGTTACACTT AATTTAAATT TACTAAAATT CTTCAATAGC CAGTTTTTAA CCAAAATTTT

       490        500        510        520        530        540
CTTCTTTCAA TTAATTAATT TCATAAATTA AATTCTGTTT TTCATTTTCG TATGAACGAA

       550        560        570        580        590        600
TAACTTCTGA ACTTGAGTTT ATATTAGTTT CATTCAACTT GAGCTC.... ..........
```

Appendix 3. Consensus sequence of BT1

```
        10          20          30          40          50          60
TGGAAAAATG AAACTGAAAG GTGTTTTAGG GAAATCTAAT TGCTGATTTA CTCCCATCTG

        70          80          90         100         110         120
CTAGTTATTT TTTTTCTTTT TACCTTCAGA TGAAAAGATG AACTCCATTG CTCTACCAAT

       130         140         150         160         170         180
AAACATTCTC TATAGAAGAT ATTAAGCAAT TTCTTCATCT ATTTATATTA GCCTAAATAT

       190         200         210         220         230         240
AAGTTCAAAG TATTCTTAAT AGTTTTCAGC AATATTCAAA CAATAAATAA GAGCATTTAT

       250         260         270         280         290         300
GAATTATCCA AGTAAAGTTT TAGGCAAATC TGTCTCTTCT TCAACATCAT GAAGATGAGC

       310         320         330         340         350         360
CTTTTCTAAG CTAAACAAAA CCTTACAAGA TTACTCTAAG ATTATAATAA CTATTTCCTA

       370         380         390         400         410         420
AGCATCGACG AAGGAGACAG .......... .......... .......... ..........
```

Appendix 4. Consensus sequence of CT1

```
        10          20          30          40          50          60
CAGTGACGAA TAGCAAGTAA TTCACTAAGC TAAACAACCT TCAATACAAA TTCTGGATCA

        70          80          90         100         110         120
GGGTCAAGCT TATTTTCAAT GCAAACATCT GTAATAATTT TTTCAAAATC AAGATCTTTC

       130         140         150         160         170         180
TTAGGCTTAA TATTGATACC AGGGAAGAGA TCACCTAAAA GGCCGTGGAA GACATACAAA

       190         200         210         220         230         240
TCTTAGAAAG CAATTTTAGG AATATTGAAA TCTCTTAAAG CTCTCATAAG CAAGGCTTAT

       250         260         270         280         290         300
TCAGCAATTT CAGGTTCAGA ACGTTTGAAA CCACCAGCGA CGACCAAGAC AGACTTAATG

       310         320         330         340         350         360
GCACGGAGAC CCCAATCATA GTGAAGTTAT TTAGAGAGTA AATCACGGCA AAGCATATAA

       370         380         390         400         410         420
AGGGTAACGA ATTTTTTGGC AAGAATTTTA GCTTCAATAA AACCTTCAGC CATAAGCATA

       430         440         450         460         470         480
TTTTGC.... .......... .......... .......... .......... ..........
```