ITS sequence as barcode for identifying closely related species of *Chelonus blackburni* Cameron, and *Apanteles angaleti* Mueseback, a biocontrol agent of cotton ecosystem

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Abstract:

The most important point for the success or failure of biological control is the correct identification of the pest or the natural enemy especially when either the pest or the natural enemy has closely related species that are often morphologically indistinguishable differing only in their behavior and physiology. In the current, the ITS sequences (Internal Transcribed Spacer) of two parasitoid species; *Chelonus blackburni* Cameron and *Apanteles angaleti* Mueseback were amplified using PCR. The ITS region of rDNA of both parasitoids *C. blackburni* and *A. angaleti* were sequenced. The ITS sequences were 818 nt and 848 nt in length for *C. blackburni* and A. angaleti, respectively. The complete sequence of ITS region in both species have been submitted to the GenBank (Bank kit assigned accession No. EU938531 *for C. blackburni* and EU938530 for *A. angaleti*). Comparison of the sequences with those of other genera of the same family from BLASTN showed that some part of the ITS sequence was conserved and distinct at species and genus levels. The aligned sequences of the ITS region of *C. blackburni* and *A. angaleti* showed maximum matching between two species but some gap between sequences are assumed to be due to diverged from evolution time . The results showed that this technique is a good tool to identify braconid parasitoid species, otherwise difficult to identify when using only morphological characters.

Introduction

Parasitoids belonging to the braconid genera, *viz*., *Apanteles* sp. and *Chelonus* sp have been found to be useful for controlling American bollworm complex on cotton. (Raodeo *et al*., 1978*;* Pawar *et al*., 1983*;* Hentz *et al*., 1997). However, the success or failure of biological control depends on the correct identification of the pest or natural enemy as the most successful natural enemy is highly host-specific. Therefore correct identification is especially important when either the pest or the natural enemy has closely related species that are often morphologically indistinguishable differing only in their behavior and physiology (Fernando and Walter, 1997).

In eukaryotes the genes encoding 18S and 28S rDNA are clustered as tandem repeats in the nuclear genome (Hoy, 1994). The internal non - coding transcribed spacer (ITS) region between 18S and 28S coding region usually has a higher degree of polymorphism than the coding region (Hoy, 1994). Hence, ITS has been used extensively in the examination of taxonomic status of species and for diagnostic purposes (Collin and Paskewitz, 1996).

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The ribosomal DNA (rDNA) has also been reported as an important tool since the ribosomal genes have been highly preserved throughout evolution (Orrego & Agudelo-Silva 1993, Hoy 1994, Pinto *et al*., 1997). Polymerase chain reaction based molecular techniques provide efficient tools for species-specific diagnostics and also for understanding intra specific genetic polymorphism. More recently, DNA sequencing of Internal Transcribed Spacer (ITS) of rDNA have been used as promising tools for taxonomic identification at different levels (Turbeville *et al*., 1991; Collins *et al*., 1996; Chang *et al*., 2001). So far, the differentiation of braconid parasitoids at species level by using ITS region is limited. In this study, therefore, we used amplification of ITS region in both parasitoids *C. blackburni* and *A. angaleti* with the purpose of providing ITS sequence of both species to database for future studies of relative closely species as well as population studies.

Materials and methods

Mass culturing of parasitoid *C. blackburni*

The mass culturing of egg-larval parasitoid, *C. blackburni* was started from the *C. blackburni* culture maintained continuously on the laboratory host *Corcyra cephalonica* in the Biological Control Laboratory, Division of Entomology, Indian Agricultural Research Institute, New Delhi - 110012. The adult parasitoids were kept in glass cages of 15x30x15 cm size and fed with 100% honey. Fresh unsterilized eggs of *C. cephalonica* were stuck on egg cards and offered to the adult parasitoids for parasitization. The egg cards were changed every day and the old cards were kept in a glass jar of 20x30x20 cm size with broken maize till emergence of adult parasitoids.

Mass culturing of *Apanteles angaleti*

The mass culturing of the larval parasitoid, *A. angaleti* was initiated from the *A. angaleti* culture maintained continuously on the laboratory host *Corcyra cephalonica* in the Biological Control Laboratory, Division of Entomology, Indian Agricultural Research Institute, New Delhi - 110012. Male and female adults were kept in glass cages of 20x20x20 cm size and provided with 1-2 day old larvae of *C. cephalonica* for parasitization for 24-36 hrs. The adult parasitoids were fed with opened resin. The parasitized larvae of *C. cephalonica* were transferred to glass jars filled with broken maize grains till the formation of parasitoid pupae in silk cocoons and emergence of parasitoid adults. Both cultures of *A. angaleti* and *C. blackburni* were maintained under laboratory conditions of 26 ± 3^0C and 65-70% RH.

DNA extraction

Ten females (10 mg) of each species (*C. blackburni* and *A. angaleti*) were placed in 1.5 ml microcentrifuge tubes separately and ground with liquid N_2 with the help of a plastic grinder. Genomic DNA of each parasitoid species was isolated by using DNA extraction kit (QUIAGEN DNeasy blood and tissue kit Cat. 69504, Germany).

PCR, Cloning and Sequencing the ITS2 Region of the rDNA.

A PCR was performed in a total volume of 50 µl using a Biometra thermocycler. For one reaction, 2 µl of DNA template (100ng/µl) were used, with 48 µl of the PCR mix [5 µl of 10x PCR-buffer, 1 µl of dNTP's each at 10mM), 2µl of the ITS forward primer ITSa (5'- TCCGTAGGTGAACCTGGCGG-3') located in the 18s region of rDNA, 2 µl of the reverse primer ITS d (5'-TCCTCCGCTTATTGATATGC- 3'), located in the 28s region of the rDNA, 1 µl of TAQ polymerase 1units/µl and 24 µl of double distilled and autoclaved water]. The thermocycler program used for *C. blackburni* was as follows: 2 min at 94°C, followed by 30 cycles of 60 seconds at 94°C, 2min at 60°C and 3min at 72°C, with 10 min. at 72°C after the last cycle. The thermocycler program used for *A. angaleti* was as follows: 3min at 94°C, followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 1.30 min at 72°C, with 7 min. at 72°C after the last cycle.

Gel extraction of PCR products

PCR products were subjected to electrophoresis on 1% agarose gel. The desired fragments were excised from the gel and purified by using gel extraction kit (QIA Quick gel extraction kit, MBI, Germany) as per manufacturer's protocol. After purification, the PCR product was linked to a pGEM-T easy vector (Promega).The reaction essentially consisted of 50ng of vector DNA, 150ng insert DNA, and 1u T4 DNA ligase enzyme and the volume was made up to 20 µl. The reaction mix was incubated at 4^0C overnight.

Transformation of competent *E. coli* cells.

A maximum of 20µl of DNA ligation mix was added to 200 µl of competent cells (heat shock cell off DH5-□□*E. coli*). After that a heat shock was given at 42 ^{0}C for 2 min and again quickly incubated on ice for 5min. This was then transferred to a 1.5ml centrifuge tube with containing of 1000 µl of LB broth medium. This mixture was gently mixed and incubated with constant shaking at 200 rpm at 37^0C for 1hr. The cells were spread on agar plate containing a mixture of 200µl of X-gal (40 µg/ml), 20 of µl IPTG (100 µg/ml) and 100 µg/ml of ampicillin (100 µg/ml) in 100ml of LB agar). This plate then was incubated at 37^0C for at least 12-16 hrs and the white colonies were chosen for making the master plate. The white colonies were numbered for recombinant confirmation. We used PCR colony and Restriction confirmation. After positive results (Fig 1C, D and 2A,B)ITS fragments of both *C. blackburni* and *A. angaleti* were sequenced in the automatic sequencer facility at the South Campus, University of Delhi, New Delhi, India.

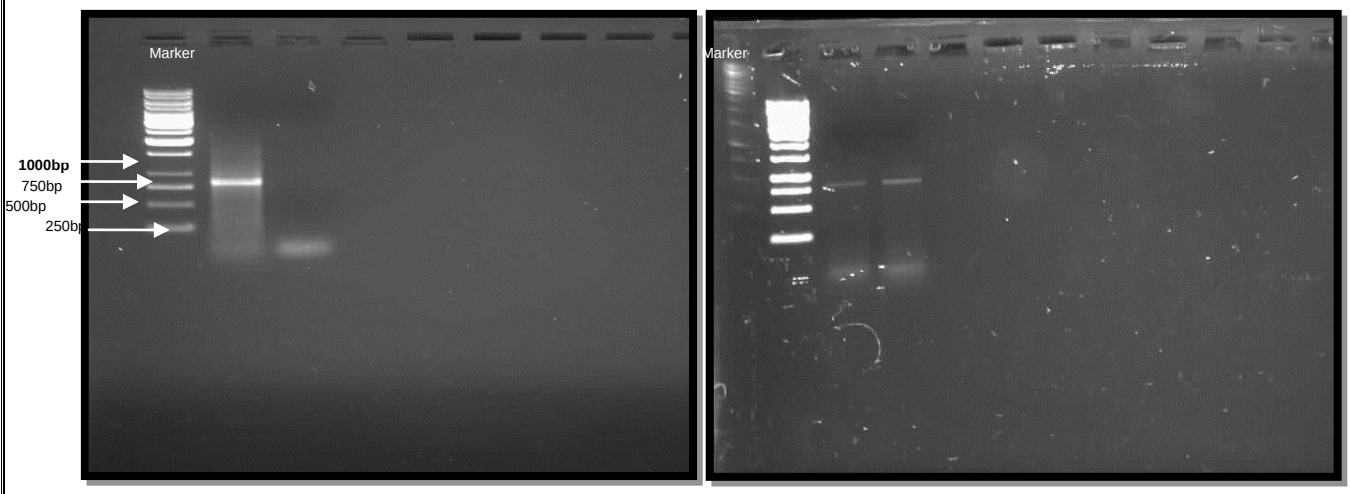
Alignment of Sequences

Nucleotide sequences of the two parasitoids, *viz*., *C. blackburni* and *A. angaleti* were aligned and analyzed by using Clustal W alignment programme and deposited in the GenBank database.

Results and Discussions

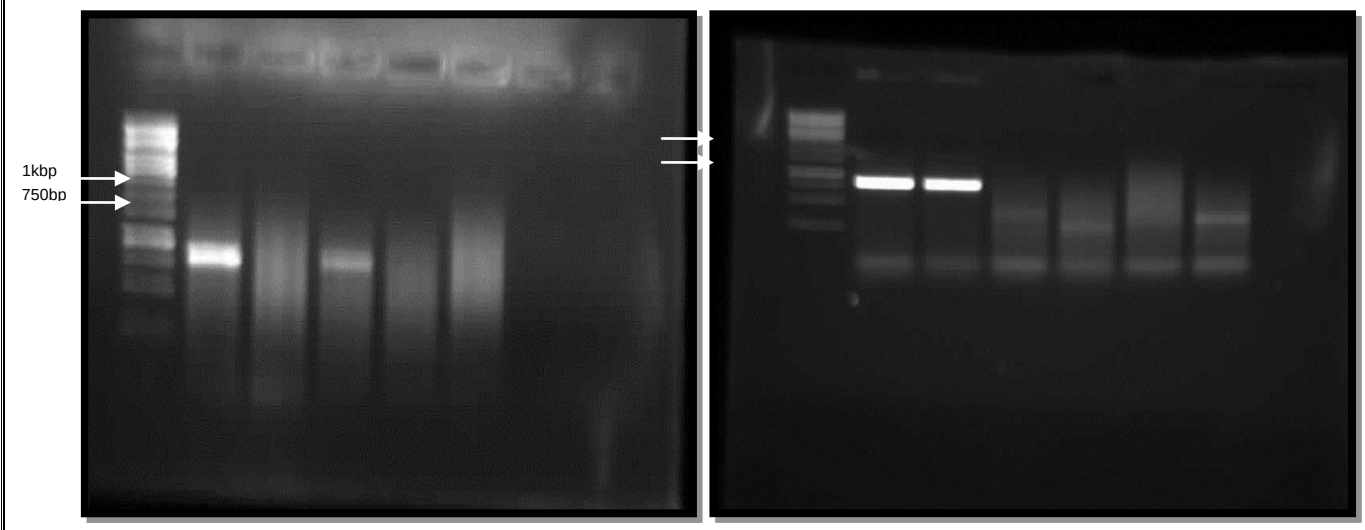
The Polymerase Chain Reaction (PCR) amplification of DNA of two parasitoids, *viz*., *C. blackburni* and *A. angaleti* was carried out with the same Internal Transcribed Spacer (ITS) primers, *viz*., ITSa and ITSd. A total of two reactions were performed with DNA extracted from pooled sample of *C. blackburni* out of which two were positive and showed a PCR amplicon of about 800 bp in gel electrophoresis (Fig 1). Likewise, among the PCR reactions performed for *A. angaleti*, only one ITS-PCR reaction was positive. PCR products clearly revealed that ribosomal DNA (rDNA) primer ITSa (5'-TCCGTAGGTGAACCTGCGG-3') and ITSd (5'-TCCTCCGCTTATTGATATGC-3') amplified the entire Internal Transcribed Spacer region of about 800 bp (Fig 1B)in both cases of *C. blackburni* and *A. angaleti* (Fig 1,2).

The ribosomal DNA (rDNA) present in all the organisms is composed of several regions including genes and spacers that evolve at different rates and can be used to distinguish closely related species. The ITS region of rDNA of both parasitoids *C. blackburni* and *A. angaleti* were sequenced. The length of ITS region of *C. blackburni* was found to be 818 bp and 849 bp for *A. angaleti*. The complete sequence of ITS region in both species have been submitted to the GenBank (Bank kit assigned accession No. EU938531 for *C. blackburni* and EU938530 for *A. angaleti*). The data was analyzed in BLASTN (http:// www. ncbi.nlm.nih.gov. blast), Biodit Sequence Aligmemt Editor Version 7.0.5 and Gene Runner version 3.05 programme and compared with the nucleotide sequence of other braconid species present in the GenBank database.



A. PCR-amplified of ITS region from *A. angaleti* with ITSa and ITSd primers. Lane 1. Marker. Lane 2. Amplified ITS fragment

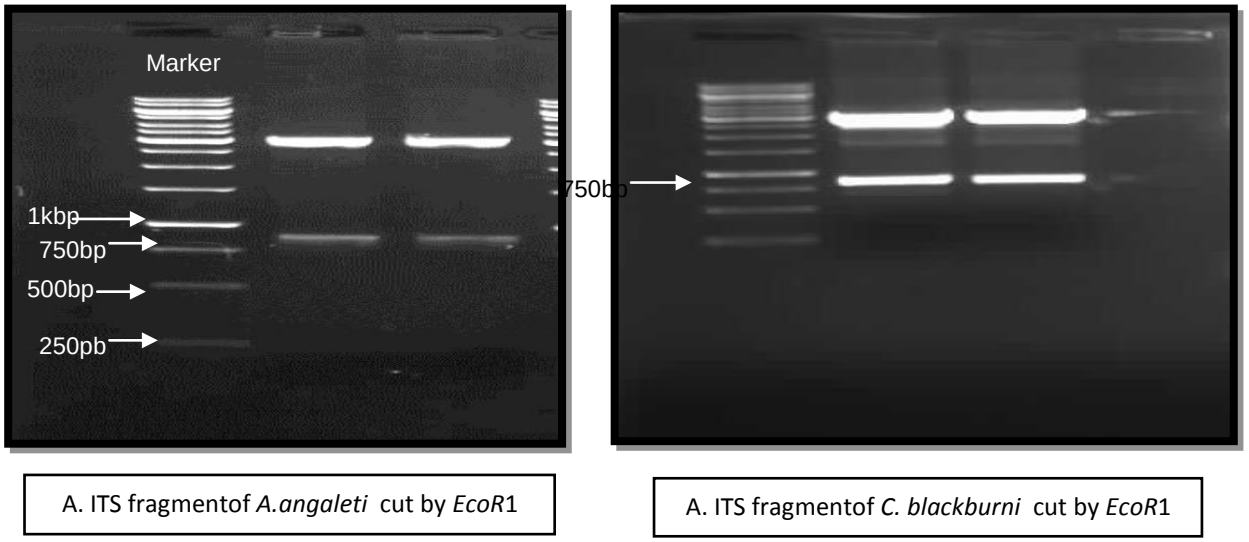
Lane 1. Marker. Lane 2. Amplified ITS fragment



C. Colony- PCR of region ITS amplified from *A. angaleti*. Lane 1. Marker. Lane 2 and 4. Amplification of ITS colony.

D. Colony- PCR of region ITS amplified from *C. blackburni* Lane 1. Marker. Lane 2 and 3. Amplification of ITS colony.

Fig1. Gel electrophoresis of PCR- ITS products and ITS-colony PCR of *A. angaleti* and *C. blackburni*



A. ITS fragmentof *A.angaleti* cut by *EcoR*1

A. ITS fragmentof *C. blackburni* cut by *EcoR*1

Fig 2. Restriction analysis of ITS region of *A.angaleti* and *C.blackburni* with restriction enzyme *EcoR*1

Internal transcribed Spacer region of rDNA has been used extensively for identification of various species of genus *Trichogramma,* as due to its small size it is extremely difficult to differentiate the species by morphology (Chang *et al.*,2001; Ciociolar *et al*., 2001; Pinto *et al*., 2002; Xi *et al.*,2002). Similarly, molecular makers have been used for studies of the ITS regions for other insects also, viz., *P. digoneutis, P. stygicus* and *P. pallipes* (Erlandson *et al.*, 2003).

Our results of sequencing of the Internal Transcribed Spacer of ribosomal DNA of *C. blackburni* indicated the presence of 818 bp and 849 bp for *A. angaleti*. Comparison of the sequences with those of other genera of the same family from BLASTN showed that some part of the ITS sequence was conserved and distinct at species and genus levels. The aligned sequences of the ITS region of *C. blackburni* and *A. angaleti* showed maximum matching between two species but some gap between sequences are assumed to be due to diverged from evolution time (Fig. 3). The sequence variation in between two different genus was not more and they can use for designing of specific primers of ITS region at species level. The use of PCR for the parasitoid can enable entomologists to determine levels of parasitism under field conditions without delay (Amornsak *et al*., 1998). When any sequences of the species present in one country or other population in different geographic is already available, it is possible to determine if a new species is present or not by simply comparing the sequences of the populations under study. The use of this molecular tool may solve many problems among closely related species of parasitoids as identification improves the success of biological control using these minute wasps.

Fig 3. Internal Transcribed Spacer sequences of *C. blackburni* and *A. angaleti* was analyzed by Clustal W version 7.0.5.3. The asterisk presenting the conserved nucleotides.

Chelonus blacburni. ITS sequen 1 TCCGTAGGTG AACCTGCGGA AGGATCATTA ACGTATAACA

Apanteles angaleti-ITS sequenc 1 TCCGTAGGTG AACCTGCGGA AGGATCATTA ACGTATAAAA

Clustal Consensus 1 ********** ********** ********** ******** *

Chelonus blacburni. ITS sequen 41 AATT-TCTTA AAATTTGTAA AATACAATAA CTTTTAAAAG

Apanteles angaleti-ITS sequenc 41 TACAATAACA AGTTTTGTTA ATGTCATTAT TATATATCAC

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Clustal Consensus          39  *  *  * * ******* *  ** **   * ** *
Chelonus blacburni. ITS sequen  80  TGTTAACGCA CGAACAAATA TAAATCAACA CAAA---ATT
Apanteles angaleti-ITS sequenc  81  AACAAATATG CTAACGCATA TACATTATGA TATATTTGTT
Clustal Consensus          58    **    * ***  *** ** ** * *  * * *   **
Chelonus blacburni. ITS sequen  117 AAATGAGCCT TCAATATATA TCATGGTAAC CTAGT--AAA
Apanteles angaleti-ITS sequenc  121 GAAGGAGACT TTGCTGTCGA TTATAAATTT ATAATTCATG
Clustal Consensus          77  ** *** ** *  * * * * **      ** * *
Chelonus blacburni. ITS sequen  155 GTAATGTTTC TTTCTTCAGA AAGAGATG-A TTGCTAAGGT
Apanteles angaleti-ITS sequenc  161 GTAACCTATT TTAGACTTAT ACCTTGTGTA TGGTTGAGGT
Clustal Consensus          96  **** * * **       *    ** * * * * ****
Chelonus blacburni. ITS sequen  194 TTTTAAATGA ATATTCACCA TGATA-ATTG AAGCTCATGT
Apanteles angaleti-ITS sequenc  201 TTTTAAATGA ATGTTCGCCA TGATTTGTTT GATTATATTA
Clustal Consensus          115 ********** ** *** *** ****  **  *   **
Chelonus blacburni. ITS sequen  233 GTGTTGATTT TTTTCTCTAT GCCATAGCAT -TATGATGAT
Apanteles angaleti-ITS sequenc  241 AAAAAGCTCT CTGTTGCCAT ATC-CATCAT GCATGTATAG
Clustal Consensus          141    * * * * * * ***  * * ***  ***  *
Chelonus blacburni. ITS sequen  272 ATGGCCGATG CACATTTGTT ACATC----- ---TGT-AAT
Apanteles angaleti-ITS sequenc  280 ATGGCATTTT TACATTGATT ATATCATTAT ATTTGTTAAT
Clustal Consensus          159 *****  *  ***** ** * ***       *** ***
Chelonus blacburni. ITS sequen  303 TTATGTGTAT ATCGTTTTGA TTATGAAATA TGTACAGATC
Apanteles angaleti-ITS sequenc  320 CAATGTAAAA GACAATTTTG TCTTTAAGAA ATTATGTAAT
Clustal Consensus          181  **** *  * ***  * * ** *  **  *
Chelonus blacburni. ITS sequen  343 ACATTGAC-- TTAAAATGAT TGACAAAAAT --AACAAATG
Apanteles angaleti-ITS sequenc  360 ATGTACACAA TAAAGAAAAT TTAAAATTTT CTAAAGATTG
Clustal Consensus          199 * * **  * *** * ** * * **  *  ** * **
Chelonus blacburni. ITS sequen  379 ATTATTCTAA ACGGTGGATC ACTTGGCTCG TAAATCGATG
Apanteles angaleti-ITS sequenc  400 ATTATCCTGA ACGGTGGATC ACTTGGCTCG TAAATCGATG
Clustal Consensus          219 ***** ** * ********** ********** **********
Chelonus blacburni. ITS sequen  419 AAGAACGCAG CTAATTGCGC GTCAACTTGT GAACTGCAGG
Apanteles angaleti-ITS sequenc  440 AAGAACGCAG CTAATTGCGC GTCAACTTGT GAACTGCAGA
Clustal Consensus          257 ********** ********** ********** **********
Chelonus blacburni. ITS sequen  459 ACACATGAAC ATCGACATTT CGAACGCATA TTGCGGTCTA
Apanteles angaleti-ITS sequenc  480 ACACATGAAC ATCGACATTT CGAACGCACA TTGCGGTCCA
Clustal Consensus          296 ********** ********** ******** * ******** *
Chelonus blacburni. ITS sequen  499 CGGATCCAAT TCCCAGACCA CACTTAGCTG AGGGTCGTAT
Apanteles angaleti-ITS sequenc  520 CGGATCCAAT TCCCGGACCA CACTTGGCTG AGGGTCGTTT
Clustal Consensus          334 ********** **** ***** ***** **** ******** *
Chelonus blacburni. ITS sequen  538 -TTATCTATA AAACTGCATA TTATT---TT TGTA-TTATG
Apanteles angaleti-ITS sequenc  560 ATTATATA-A AAACTGCATA CAATTACACT TGTAATTGTA
Clustal Consensus          370  **** ** * **********   ***    * **** ** *
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Chelonus blacburni. ITS sequen 574 CGAATG-TAT TTAAATGAGT ATTCA-TCAT TTATATTTAT

Apanteles angaleti-ITS sequenc 599 CATGCGATAA ATGTATGAAC TTGGAATGAA TGATTGTCGT

Clustal Consensus 399 * * ** * **** * * * * * ** * *

Chelonus blacburni. ITS sequen 612 ATGCAAATGA TGTTACTTGA -AATTATATA AACGATGATT

Apanteles angaleti-ITS sequenc 639 TTTGAGTTGA TTATACATAA TAACGATATC ATTTTAAATT

Clustal Consensus 416 * * *** * *** * * ** **** * ***

Chelonus blacburni. ITS sequen 651 AGACAACACA ATTGAAGTTA ATCGTCCGGT CCATGAGTAA

Apanteles angaleti-ITS sequenc 679 AAAAAACATA CTCGAT-TTA GTGAAATTGT C-----ATTA

Clustal Consensus 438 * * **** * * ** *** * ** * * *

Chelonus blacburni. ITS sequen 691 TAATTGTTTA TTG--TAAAT GGGGA-CGGA TTGATTTATA

Apanteles angaleti-ITS sequenc 713 TAATCGTTTA TCGAGTCTAT GAAAAATATA TAAATTTGTA

Clustal Consensus 457 **** ***** * * * ** * * * * **** **

Chelonus blacburni. ITS sequen 728 ACTTT----- -TAATTGTAT GTCATCATTT TTCTTATTAG

Apanteles angaleti-ITS sequenc 753 AGACCGATAA ATAATTTTAC CAAATCATAA ATTCACTTGA

Clustal Consensus 481 * ***** ** ***** * **

Chelonus blacburni. ITS sequen 762 CGACCTCAGA TTAAGTGAGA CTACCCGCTG AATTTAAGCA

Apanteles angaleti-ITS sequenc 793 CGACCTCAGA TTAAGTGAGA TTACCCGCTG AATTTAAGCA

Clustal Consensus 497 ********** ********** ********* **********

Chelonus blacburni. ITS sequen 802 TATCAATAAG CGGAGGA

Apanteles angaleti-ITS sequenc 833 TATCAATAAG CGGAGGA

Clustal Consensus 536 ********** *******

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