DNA BARCODE FOR IDENTIFICATION OF *Chelonus blackburni* (Hymenoptera: Braconidae), A BIOCONTROL AGENT IN COTTON CROP

Tran Thi Kieu Trang[1], V.G Malathi[2] and Debjani Dey[2]

[1] Cuu Long Delta Rice Research Institute, Thoi Lai, Can Tho, Vietnam; [2] Indian Agricultural Research Institute, NewDelhi, 110012. For correspondence, email: trangvl62@yahoo.com

ABSTRACT

The family Braconidae well–known attacked on many parasitoid species of key pest of agriculture. It plays a major role as important biocontrol agents in cotton crop for many Lepidopteran pests. Proper identification of some species of parasitoids is further complicated by physiological and morphological differences that are induced by the host in which they develop. With the advance of molecular techniques, DNA based approaches have been proposed to generate genetic fingerprinting of numerous organism. In this study we used polymerase chain reaction based method to characterize C. blackburni at molecular level. Recent work suggests that sequence of 898bp region of the mitochondrial gene, cytochrome c *oxidase I (COI), may serve as a DNA barcode for the identification of parasitoid species.*

Keywords: *DNA barcode, Chilonus blackburni, molecular technique, PCR*

INTRODUCTION

In the last decade, mitochondrial genes for cytochrome b (Cyt-b) and cytochrome oxidase 1 (Co-1) have been most frequently used for taxonomic and phylogenetic analysis at the species - family level. These genes proved to be advantageous over other genes for estimating divergence in taxa up to the family level in many animal groups. This technique has been investigated for their utility in identification of some species of *Peristenus* (Tilmon *et al*., 2000; Erlandson *et al*., 2003; Zhu *et al*., 2004). These molecular marker systems include PCR-based methods that potentially preclude the need for tedious and time-consuming dissection and rearing procedures. This will be advantageous as dissection provides no information as to which parasitoid species is present (Carignan *et al.,* 1995). Tilmon *et al*., (2000) developed molecular markers based on the cytochrome oxidase 1 (CO1) gene for *P. pallipes*, *P. digoneutis*, and *P. conradi* to assess parasitism rates in *L. lineolaris* in the field. Vink *et al*., (2003) worked on *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae) which has been introduced into North America for biological control of weevils of the genera *Sitona* and *Hypera* (Coleoptera: Curculionidae) and into Australia and New Zealand for control of *Sitona discoideus* Gyllenhal. They found that various geographic and host-associated populations of *M. aethiopoides* exhibited differences in host preference, host range, and adult morphology. These differences have generally been interpreted as indicative of genetically differentiated biotypes of *M. aethiopoides*. Further they generated nucleotide sequence data from the gene regions COI, 16S, 28S, and β-tubulin to assess genetic variation among *M. aethiopoides* reared from various host species collected in Australia, Iran, New Zealand, the United States, and 10 other European countries. The results shows that parsimony and maximum likelihood analyses of the COI, 16S, and β-tubulin sequences provided strong support for the presence of at least two *M. aethiopoides* biotype, one associated with *Hypera* species and the other with *Sitona* species. There are also evidence for genetic divergence among parasitoids associated with different *Sitona* species.

Morphological variation are also closely correlated with host species, but the occurrence of morphological variation in the absence of genetic variation suggests morphological characters should be used cautiously with *M. aethiopoides* biotypes. Hebert *et al.*, (2003) cited that the mitochondrial gene cytochrome *c* oxidase I (COI) can serve as the core of a global bioidentification system for animals as the cytochrome *c* oxidase I gene (COI) has two important advantages. First, the universal primers for this gene are very robust, enabling recovery of its 5' end from representatives of most, if not all, animal phyla (Folmer *et al*., 1994; Zhang and Hewitt, 1997). Second, COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene In fact, the evolution of this gene is rapid enough to allow the discrimination of not only closely allied species, but also phylogeographic groups within a single species (Cox and Hebert, 2001; Wares and Cunningham, 2001). Although COI may be matched by other mitochondrial genes in resolving such cases of recent divergence, this gene is more likely to provide deeper phylogenetic insights than alternatives such as cytochrome *b* (Simmons and Weller, 2001) because changes in its amino-acid sequence occur more slowly than those in this, or any other, mitochondrial gene (Lynch and Jarrell,1993). Mardulyn and Whitfield (1999) explored the phylogenetic signal by using three genes, viz., mitochondrial COI and 16S, and nuclear 28S fragments for an assessment of their utility in resolving generic relationships within this species-rich insect group. These genes are chosen because their level of sequence divergence is thought to be appropriate for this study and because they have resolved relationships among other braconid wasps at similar taxonomic levels. They suggest that the lack of phylogenetic signal observed is an indication of the presence of many short internal branches on the phylogeny being estimated, which in turn might be the result of a rapid diversification of the taxa examined. Relative specialization of diet, which is typically associated with parasitic behaviour, is believed to result in high radiation rates, which may have been especially high in microgastrine wasps because of the great diversity of their lepidopteran hosts. This hypothesis of a rapid diversification caused by an abundance of host species remains speculative and suggested that more data will be needed to test it further. In this studies we want to prove that the CO1 gene can be used as DNA barcode for identification of relative species of parasitoids that can not be differentiated by morphology.

MATERIALS AND METHODS

Chelonus blackburni culture

Cultures of *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 adults of parasitoid were kept in glass cages of 15x30x15 cm size and fed with 100% honey. Fresh unsterilized eggs of *C. cephalonica* was stuck on egg cards and offered to the adult parasitoids for parasitization. The egg card was changed every day and the old card was kept in a glass jar of 20x30x20 cm size with broken maize till emergence to adult parasitoids. The culture was maintained under laboratory conditions of 26±3^{0}C and 65-70% RH.

DNA extraction

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

Polymerase chain reaction (PCR), Cloning and Sequencing the CO1 gene of *C. blackburni*

The primer sequences used to amplify the Cytochrome oxidase subunit 1 (CO1) gene for *C. blackburni* were:

COF (forward primer) 5’TCCAATGCACTAATCTG CCATATT 3’

COR (Reverse primer) 5’TTCATTTTTTGGTCATCCAGAAG T 3’

PCR program for amplification of CO1 gene of *C. blackburni* was followed:

1. Denaturation of DNA at 94^0 C for 3 min in the first cycle

2. 40 cycles which had 3 steps was

a. Denaturation of DNA at 940^C for 30 seconds

b. Primer annealing temperature at 48^0C for 45 seconds

c. Primer extension at 72 ^{0}C for 1min.

3. One cycle for final extension at 72 ^{0}C for 20 min.

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

Cloning of PCR products

The pGEM-T easy vector (Promega) was used to clone the PCR amplified fragment of DNA

Bacterial transformation

Preparation of *Escherichia coli* competent cell by $CaCl_2$ method (Mendel and Higa, 1970) with bacterial culture medium Luria –Bertani Broth (LB)

The *E. coli* strain DH5α was used for transformation of cloned DNA fragment. The strain has the following features: F^-, end A_1, hsd R17 (rk^-, mk^+), sup E44, thi, Rec A1, Gyr a96, reA1, ΔLac, and ZM 15

Transformation of competent *E. coli* cells

A maximum of 20µl of DNA ligation mix was added to 200 µl of competent cells and incubated on ice for 1hr. 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

Recombinant confirmation of cloned DNA fragment

Selection of transformations

The recombinant clones were screened by growing the transformed competent cells on appropriate selection media. For cloning in vector *pGEM-T* easy with *LacZ* α gene which facilitate the blue/white screening among the recombinants, the cells transformed with the ligation mix was plated on LB agar medium supplemented with X-gal (40 µg/ml) IPTG (100 µg/ml) and appropriate antibiotics ampiciline (100 µg/ml). The recombinant colonies showing white colour were selected and recombinant plasmids were confirmed by colony PCR and their specific restriction digestion pattern with appropriate enzyme.

Colony PCR confirmation

The presence of recombinant plasmid in transformed bacterial colonies was confirmed by colony PCR

Restriction confirmation

Restriction reaction: 5 µl of the isolated DNA plasmid from miniprep (Brirnboin and Dolly, 1979) along with 1 µl of *ECo*R1 restriction enzyme (Geini company), 2 µl of restriction buffer (10x)and 1 µl RNase was mixed and put into 0.5ml Eppendoff tube and the volume was made up to 20 µl with distilled water and a short spin was given. Thereafter it was incubated overnight at 37^0C. The digested product was added with 3 µl of 6x loading dye (15% Ficoll l400; 0.06% Bromo phenol blue; 0.06% Xylene cyanol FF and 30mM EDTA) and analyzed by gel electrophoresis. The band was visible and photographed under UV to

confirm the size of inserted DNA fragment from the recombinants.

DNA sequence

CO1 fragment of *C. blackburni* were sequenced in the automatic sequencer facility at the South Campus, University of Delhi, New Delhi, India.

Statistical analysis

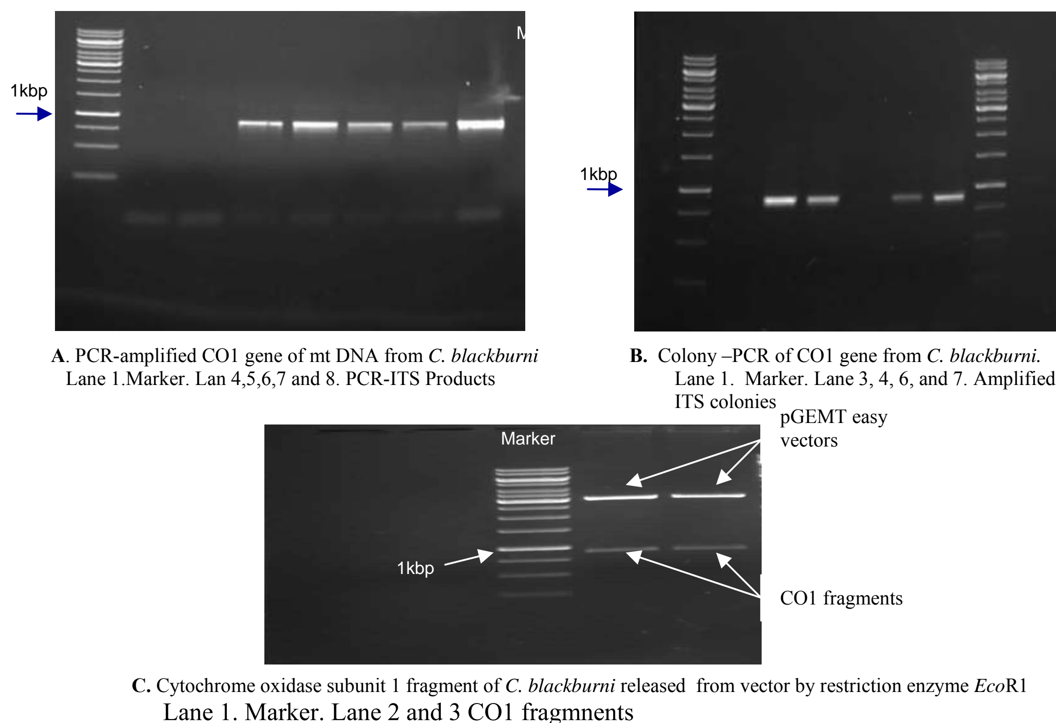
Nucleotide sequences of the parasitoids, *C. blackburni* was aligned and analyzed by using Clustal W alignment programme and deposited in the Gene Bank database

RESULTS AND DISCUSSION

Sequence of CO1 of *C. blackburni*

Mitochondrial DNA (mtDNA) is a valuable marker to indicate maternal gene flow and mtDNA is more numerous than nuclear DNA. Partial CO1 sequence of *C. blackburni* was 898 bp (Fig. 1 A,B,C). It was deposited in the Gen bank (assigned accession No. FJ441075) and the data were then analyzed by BLAST. Nucleotide sequence of other braconid parasitoids taken from Gene Bank database for comparison, the details of which have been given in Table 1 and 2.

From BLAST, contrastingly, the sequence of CO1 of mtDNA of *C. blackburni* showed 83-89 % sequence identity with different species of *Chelonus.* The region which completely aligns with that of *Chelonus* sp. has nucleotide coordinate from 90 to 898 with 89% sequence identity compared with another genus in the same family, *viz.*, *Cotesia* in which the coverage region was from nucleotide 225 to 898, with 84% sequence identity. The analysis of CO1 gene indicated how it could be used as an efficient taxonomic tool for fixing the identity of closely related species, mimic species as well as different geographic populations (Table 3).



A. PCR-amplified CO1 gene of mt DNA from *C. blackburni* Lane 1.Marker. Lan 4,5,6,7 and 8. PCR-ITS Products

B. Colony –PCR of CO1 gene from *C. blackburni.* Lane 1. Marker. Lane 3, 4, 6, and 7. Amplified ITS colonies

C. Cytochrome oxidase subunit 1 fragment of *C. blackburni* released from vector by restriction enzyme *Eco*R1 Lane 1. Marker. Lane 2 and 3 CO1 fragmnents

Figure 1. Gel electrophoresis of Cytochrome oxydase subunit 1(CO1) gene from *C. blackburni*. **A.** PCR-amplified CO1 gene of mt DNA from *C. blackburni* **B**. Colony –PCR of CO1 gene from *C. blackburni.* **C**. Cytochrome oxidase subunit 1 fragment of *C. blackburni* released from vector by restriction enzyme *Eco*R1

Table1. BLAST sequences which were used for comparison

No	Name of Insects	Name of gene	Source	Gene bank ID
1	*Chelonus sp*	(CO1)		AF102723
2	*Cotesia koebelei*	(CO1)	USA	AY333888
3	*C. acuminate*	(CO1)	Sweden	AY333869
4	*C. acuminate*	(CO1)	China	AY333871
5	*C. biquellii*	(CO1)	Finland	AY333890
6	*C. melitaearum*	(CO1)	Spain	AY333881
7	*C. acuminate*	(CO1)	France	AY333872
8	*C . melitaearum*	(CO1)	Hungary	AY333885
9	*C. acuminate*	(CO1)	Spain	AY333887
10	*C. acuminate*	(CO1)	Russia	AY3338873
11	*C. melitaearum*	(CO1)	Sweden	AY333877
12	*C. marquiniventris*	(CO1)	France	AY333880
13	*C. marquiniventris*	(CO1)	Russia	AY333879
14	*C. marginiventris*	(CO1)		AF102705

Table 2. Comparison of nucleotide sequence of cytochrome oxidase subunit 1 (CO1) of *C. blackburni*

No	Name of Insects	Query coverage (%)	Max. Identity (%)	Source/voucher
1	*Chelonus* sp	90	89	
2	*Cotesia koebelei*	74	85	USA
3	*C. acuminate*	76	84	Sweden
4	*C. acuminate*	75	84	China
5	*C. biquellii*	84	82	Finland
6	*C. melitaearum*	78	83	Spain
7	*C. acuminate*	79	83	France
8	*C. melitaearum*	76	84	Hungary
9	*C. acuminate*	75	83	Spain
10	*C. acuminate*	79	83	Russia
11	*C. melitaearum*	75	83	Sweden
12	*C. marquiniventris*	75	83	France
13	*C. marquiniventris*	75	83	Russia
14	*C. marginiventris*	79	83	

The mitochondrial cytochrome oxidase subunit 1 (*CO1*) gene is among the most popular markers in molecular systematics - partitions of this gene are frequently employed. CO1 is currently in the focus of special interest: its 5′ partition is used for the 'Barcoding of Life' initiative (Hebert *et al*., 2003; Stoeckle, 2003). The nucleotide sequence of this particular 640 nucleotide region (Folmer *et al*., 1994) shall be the unique identification code for all species to facilitate the correct determination of specimens and the discovery of new species (Moritz and Cicero 2004). Mitochondrial DNA has been used widely in taxonomic, population studies, phylogenetic relationship and evolutionary studies (Avise *et al.,* 1987; Simon *et al*., 1994; Lang *et al.,* 1999; Saw *et al*., 2006; Vahtera and Muona, 2006). Some studies for the use of mtDNA include taxonomy and population genetic studies on biotypes of cereal aphids, fruit flies and hymenoptera parasitoids (Gasparich *et al*., 1997; Taylor *et al.,* 1997), genetic variation in

bees (Crozier and Crozier, 1993) and Vink *et al*., (2003) in studies on genetic variation in *Microtonus aethipoides.*

CONCLUSION

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. Correct identification is often very difficult, therefore an attempt is made to use polymerase chain reaction based molecular techniques. The PCR amplified cytochrome oxidase subunit 1 (CO1) gene from *C. blackburni* shows a sequence of 898 bp and the sequence are analyzed by Blast (GenBank Accession No. FJ441075).

TTGATTTTTTGGTCATCCAGAAGTTTATATTTTAATTTTACCAGG
ATTTGGAATAATTTCTCATATTGTATTTTCTGAAAGAGGTAAAAA
AGAAGTTTTTGGATATTTAGGTATAGTTTATGCTATAATAACAAT
TGGATTTTTAGGATTTATTGTTTGAGCACATCATATATTTACAGT
TGGGATAGATGTAGATACTCGAGCTTATTTTACTTCTGCAACAA
TAATTATTGCAGTTCCGACAGGAATTAAAGTTTTTAGATGAATTA
GATCATTAATAGGAATAAAAATAAATTATTCATTAGGAATGATTT
GATCATTAGGATTTATTTTTTTATTTACAATGGGGGGTTAACTG
GAATTATTTTATCTAATTCTTCTTTAGATTTAGTTTTACATGATAC
ATATTATGTTGTAGCTCATTTTCATTATGTTTTATCTATAGGTGCT
GTATTTGCAATTATAGCAGGATTTATTTATTGATTTCCTTTATTTT
TTGGATATTTATTGGATTTAATTTTATTAAAAATTCAATTCTTTTT
AATATTTATTGGGGTTAATTTAACTTTTTTTCCTCAACATTATTTA
GGATTAAGAGGTATACCTCGTCGTTATTCTGATTATTCTGATATA
TTTATATATTGAAACATAATTTCTTCTATTGGTTCAATTATTACTT
TAGTATCAATATTTTTATTTTTTTATATTTTAATTGAAGGGTTTTA
TTCAAAACGTTTAATTTTATTAATACTTCTTTAGAATACTTCTTTA
GAGTGGATACAAAGGTTACCACCAAAAAATCATTGTTTCGCTGA
AAATCCTAAAATAATTATATAATAATTATAATAATAGTTGAATAA
AATTATATTTTCTAATATGGCAGATTAGTGCATTGGA

Figure 2. Nucleotide sequence of CO1 gene of mt DNA from *C. blackburni*

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SỬ DỤNG CHỈ THỊ DI TRUYỀN TẠO MÃ VẠCH DNA TRÊN LOÀI *Chelonus blackburni* (Hymenoptera: Braconidae) ĐỂ NGHIÊN CỨU CÁC LOÀI THUỘC HỌ Braconidae TRONG PHÒNG TRỪ SINH HỌC TRÊN CÂY BÔNG

Mã vạch DNA được sử dụng như chỉ thị di truyền của DNA cá thể để xác định sự liên quan của những loài đặc biệt mà không thể phân biệt dựa trên hình thái học. Mục tiêu của việc sử dụng mã vạch DNA là phân loại, xác định những loài chưa được biết đến, chưa được phân loại. Hầu hết các tế bào nhân chuẩn chứa ty thể (mitochondria) và đoạn mitochondrial DNA (mtDNA) giữa các loài hầu hết tương tự hoặc khác nhau rất nhỏ trong cùng một loài. Folmer và ctv (1994) tìm thấy với đoạn gen khoảng 658 bp (gọi là đoạn Folmer) của mitochondrial cytochrome c oxidase (COI) được xem là tiềm năng như một “mã vạch” sử dụng trong nghiên cứu hệ thống phát sinh hoài cũng như sự phân định giữa các loài có phức hợp tính trạng chứa nhiều bí ẩn. Tiềm năng sử dụng các loài ong ký sinh (parasitoids) trong phòng trừ sinh học đối với một số ấu trùng của Lepidoptera là rất phong phú. Tuy nhiên, trong tự nhiên việc xác định các loài ong ký sinh trong cùng một họ, một chi hay loài phụ bằng hình thái học có thể dẫn đến sự nhầm lẫn. Điều này có thể dẫn đến sự thất bại của biện pháp phòng trừ. Vì vậy, sự thành công của biện pháp sử dụng parasitoids trong phòng trừ sâu hại là phải xác định đúng đối tượng sử dụng. Đối với phạm vi nghiên cứu này chúng tôi đã xác định được trình tự của đoạn gen ***mtCO1*** của loài ong ký sinh trứng *Chelonus blackburni* với 898 bp có thể được sử dụng như một mã vạch DNA (mẫu ngân hàng gen số FJ441075) trong GenBank nhằm cung cấp dữ liệu trong nghiên cứu xác định loài trong hệ thống đa dạng loài.