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Molecular genetic identification of two *bracon* species based on RAPD-PCR and 16S rRNA genes

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Abstract

Random amplified polymorphic DNA (RAPD)-PCR genomic fingerprinting and partial sequencing of the 16S rRNA gene were evaluated on two insects collected from the Egyptian field which could belong to *B. hebetor* and *B. brevicornis* to investigate their genetic relatedness and to establish the value of techniques for their identification. Nearly identical RAPD-PCR profiles and identical 467 bp fragments of the 16S rRNA genes indicated many of genetic diversity between the two insects under study. The low levels of similarity (78.21% in the partial 16S rRNA genes and 86% in RAPD-PCR) appeared between the insects *B. hebetor egypt* and *B. brevicornis egypt.* However, 16S rRNA genes and RAPD-PCR provided an effective means of differentiating between members of the taxa. Moreover, a phylogenetic tree constructed from 16S rDNA sequences showed that *B. hebetor egypt* clustered with the *B. hebetor* with a degree of similarity 92%, but *B. brevicornis Egypt* clustered in a separated group. However, RAPD-PCR and partial sequencing of the 16S rDNA analysis raises questions about the taxonomic positioning of the two insects isolated from the Egyptian environment.

Keywords: RAPD-PCR and 16S rRNA genes - *bracon* species

INTRODUCTION

The identification and the use of correct parasitoids species is very important step in biological control programs to be released in the field. The identification of these parasitoids is difficult due to the most parasitoids are small, even minute, and evolving rapidly. Related species often have few or no known sufficiently invariant distinguishing morphological characters for reliable discrimination (Pungerl, 1986; Landry *et al*., 1993; Pinto *et al*., 1993; Demichelis & Manino, 1998; Kimani-Njogu *et al*., 1998; Stouthamer *et al*., 1999; Barnay *et al*., 2001; Chang *et al*., 2001) and their identification at species level depends mainly on male genitalia. Failure or delay the definition of parasite affect the outcome of the control of pest where the success of biological control programs often depends on correct identification of natural enemies (DeBach and Rosen, 1991). Today's technology allows us to identify any living thing by using a single cell.

The classification of species based on morphological features has problems because morphological attributes could change by environment (Shouche and Patole, 2000). Many molecular techniques allow ecologists and biologists to determine the genetics array

of a wide variety of closely related individuals (Wolf and Rijn, 1993). Among these techniques are DNA sequencing, restriction fragment length polymorphisms (RFLP), microsatellites analysis and random amplified polymorphic DNA (RAPD) (Mulcahy *et al*., 1993). *Bracon* spp is minute and indistinguishable morphologically, further, the environmental factors influence significantly its morphology and physiology. So, identification of the wasp is problematic and its systematic needs to be clarified (Pinto, 1998).

MATERIALS AND METHODS

Collection of samples:

Two spices of *Bracon* insects presented in the Egyptian fields. They may be *Bracon. hebetor* and *Bracon. brevicornis* and they individuals were kept in liquid nitrogen until use.

DNA extraction

Total genomic DNA was extracted from fifteen bulk of the two individual species by using GeneJET^{TM} Genomic DNA Purification kit (Fermentas). DNA samples were diluted by TE-buffer to final concentration of 50 ng/µl to be used in polymerase chain reaction (PCR) and stored at -20°C.

PCR conditions and purification of PCR products

For identification of two *Bracon* species, we used two categories, RAPD-PCR and sequencing a part of mt16S rRNA genes. In RAPD-PCR method, twenty primers (table 1), obtained from Pharmacia Biotech. (Amersham Pharmacia Biotech UK Limited, Ebgland HP79 NA), were used in this experiment to identify the insects. Moreover, A partial of mitochondrial 16S rRNA gene (467 bp) was amplified and sequenced by using primers 16sWb sequence: 5'-CACCTGTTTATCAAAAA-CAT-3'; 16s.Sh sequence: 5'-AGATTTTAAAAGTCGAA-CAG-3' which were published by Heimpel *et al*., 1997.

Table 1: Twenty primer sequences used in identification of two *Bracon* species.

Primer code	Sequences	Primer code	Sequences
OPA-01	CAGGCCCTTC	OP-B15	GGAGGGTGTT
OPA-02	TGCCGAGCTG	OP-B16	TTTGCCCGGA
OPA-04	AATCGGGCTG	OP-B18	CCACAGCAGT
OPA-05	AGGGGTCTTG	OP-B20	GGACCCTTAC
OP-A09	GGGTAACGCC	OP-C04	CCGCATCTAC
OPA-11	CAATCGCCGT	OP-C05	GATGACCGCC
OPA-16	AGCCAGCGAA	OP-C13	AAGCCTCGTC
OPA-18	AGGTGACCGT	OP-C16	CACACTCCAG
OPA-19	CAAACGTCGG	OP-C19	GTTGCCAGCC
OPA-20	GACCAATGCC	OP-Q19	CCCCCTATCA

PCR reactions were performed with GoTaq® Flexi DNA Polymerase kit (Promega), in a total volume of 25 µl volume reaction mixture containing: 50 ng of total cellular DNA (1µl DNA extraction), 100 µM of primer (1 µl), 5 µl of 5X green Taq DNA polymerase buffer, 5U/µl of GoTaq DNA polymerase (0.25µl) (Promega), 10 mM of each dNTP (0.5µl), 25mM $MgCl_2$ (4µL), up to 25µl by nuclease-free water. PCR was performed in a DNA thermocycler (Biometra, Germany). For RAPD-PCR program, samples were first heated at 94°C for 3 min and subjected to 35 cycles of the following cycle: 45 seconds at 94°C, 45 seconds at 37°C, 1.5 min at 72°C. A final step of 5 min at 72°C was always run. For a partial 16S rDNA fragment amplification, PCR reaction mixture heated at 94°C for 3 min and subjected to 18 cycles of the following cycle: 45 seconds at 94°C, 45 seconds at 44°C, 45 seconds at 72°C. A final step of 5 min at 72°C. PCR reaction was tested on 1.8% agarose (Genetics) gel, 100 bp

DNA Ladder H3 RTU (Genetics) and 1Kb DNA ladder (GeneRuler^{TM}) were used as the standard markers.

Sequencing

For sequencing of 16S rDNA fragment (~467bp) we used sequencing unit which is equipped with a Tecan robot installed on a platform Genesis Workstation 150 (capillary electrophoresis) and performs the reactions are then analyzed via the sequencer 3100 Genetic Analyser (Applied Biosystems). DNA fragments to be sequenced are prepared in a final reaction concentration 6µl content 30 ng of PCR products and 5 pmol of the 16sWb or 16s.Sh primers. Sequence analysis was done with the Sequencher 3.0 software.

Data analysis

Data of reducible RAPD markers were scored "1" or "0" for each sample "1" was assigned for the presence of a band and "0" for its absence. These data were used in counting the number of total amplified markers in two *Bracon* spp. Moreover, pairwise comparisons of the two species, based on the presence or absence of unique and shared polymorphic products, were used to determine similarity coefficients, according to Jaccard (1908).

Three of partial sequences of 16S rDNA were obtained from http://blast.ncbi.nlm.nih.gov/Blast.cgi, that in additional our two sequences were used for constructing the UPGMA phylogenetic tree. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007). Sequence alignments were carried out using the site http://www.ebi.ac.uk/Tools/msa/clustalw2/.

RESULTS

For estimation of genetic diversity of two species of insects presented in the Egyptian environment which could belong to *Bracon hebetor* and *Bracon brevicornis* we used RAPD-PCR and 16Ss rDNA sequences comparison.

RAPD-PCR is a powerful tool for *the analysis of genetic diversity* and was successfully used to compare genetic variation among the two species. RAPD produced by all 20 primers were used to evaluate the similarity between the studied insects as shown in Fig 1 and Table 2. The RAPD bands were ranged from 30 to 1500 bp. The twenty primers produced total 184 bands with average 9.2 per primer. The all primers generated a total of 137 monomorphic (~74 %) and 47 polymorphic (~26%) bands in two insect under study.

The produced bands polymorphisms varied from primer to another. Among these primers, only 5 did not reveal any polymorphic bands and they produced the highly monoymorphic percentage (100%), these are identified as B18, A05, Q16, C13 and A02. On the other hand, A16, A18, A19, B15, C04 and C05 showed highest percentage of polymorphism (46%, 40%, 50%, 44%, 50% and 56%), respectively. Generally, the polymorphic percentage mean was 23.05%. Moreover, we observed variation in number of bands presented by each primer. Whereas, the primer A09 amplified the highest number of bands (15 bands) with sizes ranged from 100 to 1330 bp. While the lowest number of bands (4 bands) were produced by the A02 primer.

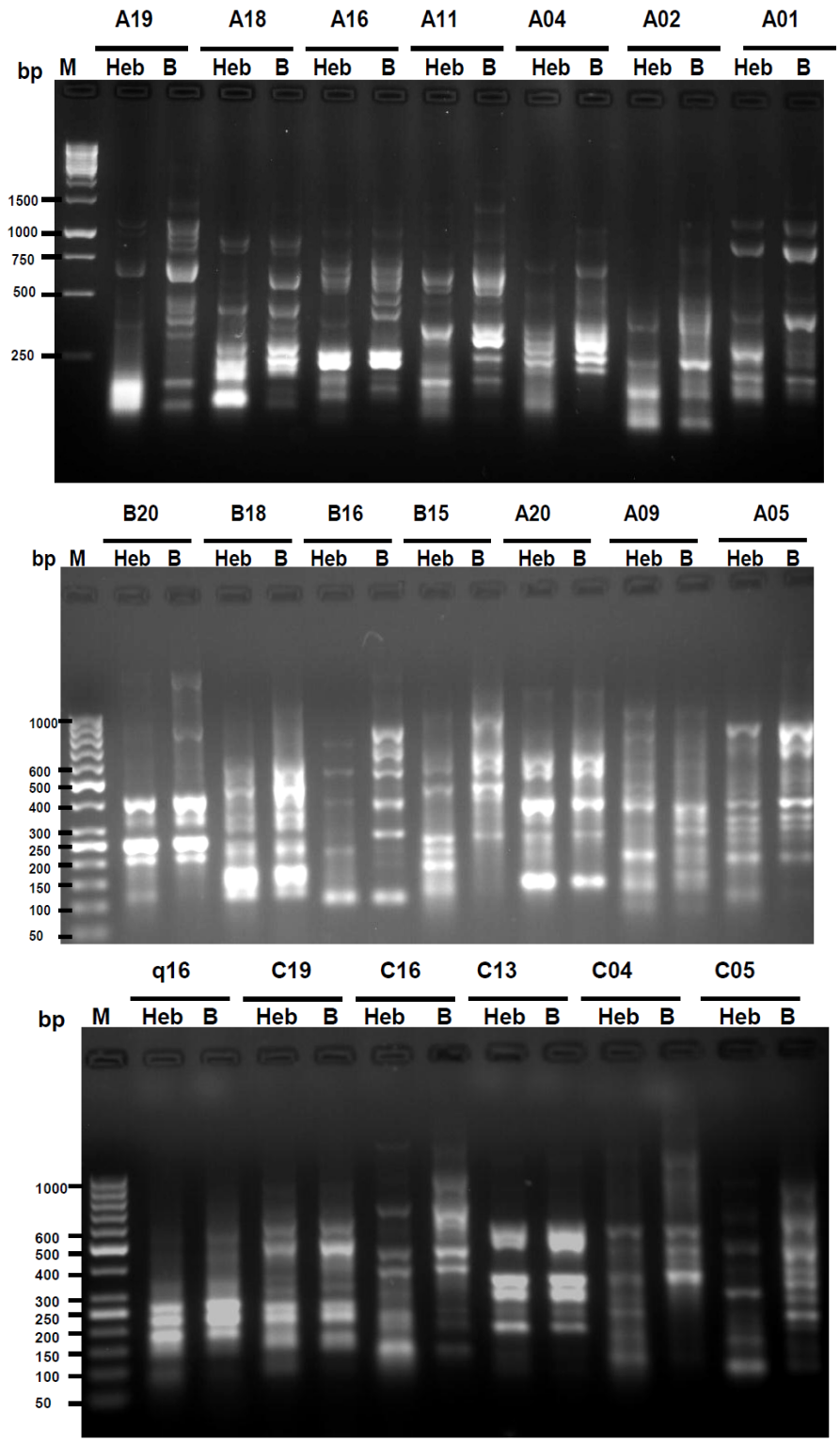

Fig. 1: RAPD amplified fragment produced by all 20 primers were used to evaluate the similarity and to compare genetic variation among the two insects. Heb: *Bracon hebetor*, B: *Bracon brevicornis*, M: Standard marker.

Table 2: Polymorphisms and F value were revealed by the twenty primers that used for the identification of two *Bracon* species.

Primer	Total Amplification products	Total bands		Monomorphic bands	Polymorphic bands		Polymorphic bands Size with bp	Polymorphism percentage	F Value %
		brevicornis	*hebetor*		*brevicornis*	*hebetor*			
A01	7	6	7	6	0	1	220	14%	92%
A02	4	4	4	4	0	0	-	0%	100%
A04	7	7	6	6	1	0	80	14%	92%
A05	10	10	10	10	0	0		0%	100%
A09	15	11	15	11	0	4	180-200-260-350	27%	85%
A11	10	8	10	8	0	2	40-250	20%	89%
A16	11	8	9	6	2	3	110-140-370-400-460	46%	71%
A18	10	7	9	6	1	3	170-240-350-540	40%	75%
A19	12	7	11	6	1	5	280-330-360-380-410-460	50%	67%
A20	8	8	7	7	1	0	220	13%	93%
B15	9	8	6	5	3	1	130-200-240-420	44%	71%
B16	9	7	8	6	1	2	690-760-870	33%	80%
B18	10	10	10	10	0	0	-	0%	100%
B20	7	7	6	6	1	0	120	14%	92%
C04	10	9	6	5	4	1	120-180-230-260-390	50%	67%
C05	11	7	9	5	2	4	210-260-290-320-410-440	56%	63%
C13	6	6	6	6	0	0	-	0%	100%
C16	10	9	9	8	1	1	300-370	20%	89%
C19	10	9	9	8	1	1	360-390	20%	89%
Q16	8	8	8	8	0	0	-	0%	100%
Total	184	156	165	137	19	28			
Mean								23.05%	86%

In order to study the similarity index between insects under study, we calculated the F value of each primer. The primers B18, A05, Q16, C13 and A02 show 100% identical similarity between the two species, otherwise lowest similarities 63%, 67% and 67% were detected by using primers C05, A19 and C04, respectively. In general, mean of the similarity detected by all primers was 86%. RAPD results showed successfully the variation between the two insects.

For more clearly identification of the insects under study, we performed sequencing of mitochondrial 16S rDNA. 467 bp fragment of the mitochondrial 16S rRNA gene was successfully sequenced for the two insects (Fig. 2).

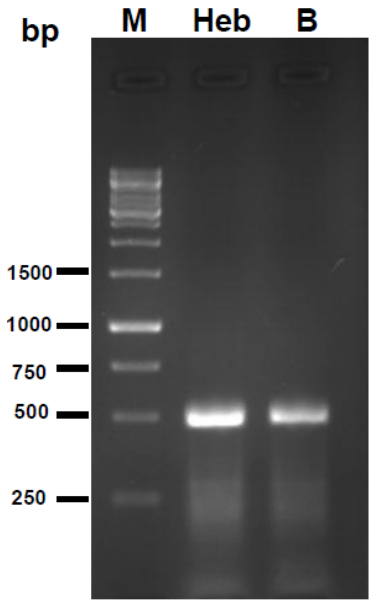

Fig. 2: Polymerase chain reaction (PCR) amplified 467bp of 16S rDNA fragments from to species *B. hebetor egypt* and *B. brevicornis egypt.*

And the alignment of the two sequences considered in this study show several insertions or deletions and revealed many variable positions on 467 bp analyzed (Fig. 3). Moreover, the similarity between the two 16S rRNA gene sequences was 78.21%.

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B_brevicornis_Egypt      TCAAAAAAACCCAAACAATTTTTTTTTTTACATCTGATCC-TACATAAACAATTCATGGC 59
B_hebetor_egypt          -----AGGAATTAAA-----TTTTAATTCAGATTTGCCCAATGAATAAATAA---AAGGG 47
                              *  *   ***     ****  ** * ** **  *  *  ***** **   * **

B_brevicornis_Egypt      TGC-AGCCCTTTACTGAA--AAAAGTCGCAAATAATTTGGCTGTT--ATCCAAAACGAAA 114
B_hebetor_egypt          TGCCATTTTTTTAACGGTCCAAAGGTAGCATATTAATTGTTTTTTTAATTAAAAATTAGA 107
                         *** *    ****  *    *** ** *** ** * ***  * **  **  ****  * *

B_brevicornis_Egypt      ATGAAAGATTTAATGAAAAAAAATCTGTTTCAAATAAATTAATTAA-TTTATTATTTAAG 173
B_hebetor_egypt          AGGAAAGTTTTAATGAGATAAAATCGGTTTCAAATAAATTATTTAAATTTATAATTTAAG 167
                         * ***** ******** * ****** ************** **** ***** *******

B_brevicornis_Egypt      TAAAAAAACTTAAATTTTTTATAAAGACGATAAGACCCTACAGAATTTTATTTATTTAAT 233
B_hebetor_egypt          TAAAAAAACTTAAATTTTTTATAAAGAGGTAAAGCCCCTATAGAATTTTTTTTATTTAGT 227
                         ************************** *  *** ***** ******** ******** *

B_brevicornis_Egypt      AAAAAAATTTTTAAAAAAA--TTAATCTGGGGGACAAAACAACTAAATAAATTTTTT--A 289
B_hebetor_egypt          AAAAAAATTTTTTAAATAAATTTAATTGGGGTAATAAAAAAATTAAATAAATTTTTTTTA 287
                         ************ *** **  *****  ***  * **** ** **************  *

B_brevicornis_Egypt      AAATTTTACCTTAATTATTGAATTAA--TAATTAAAGA--GGCCTAATTTTTAA--TAAA 343
B_hebetor_egypt          AATTTTTACATAAATTAATGAATTTAATTAATTAAAATATGTCTTAATTTTTAAATTAAA 347
                         ** ****** * ***** ****** *  ********    * * **********  ****

B_brevicornis_Egypt      -----CAATCATCCCTTGGATA-CA-CACAATTTTTTT-ACAAGTC--TATCA-TAAAAA 392
B_hebetor_egypt          AAAATTAATTACCTTAGGGATAACAGCATAATTTTTTTTAAGAGTTCTTATCAATAAAAA 407
                               *** * *    ***** ** ** ********* *  ***   ***** ******

B_brevicornis_Egypt      ----CAC-ACCCC-ATGTGAATCATATAAA-TTCAATGCAAAA--TTAAAATT------- 436
B_hebetor_egypt          AGATTATGACCTCGATGTGAATAAGATAAAATTAAATGCAAAAATTTAAAAATTTTTTGG 467
                              *  *** * ******** * ***** ** *********  ****** *
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Fig. 3: Sequences alignment of a partial mitochondrial 16S rRNA gene of the two insects by using ClustalW2 alignment.

BLAST analysis of 467 bp from the two insects which could belong to *Bracon hebetor Egypt* and *Bracon brevicornis Egypt* (presented in Egypt, figure) showed significant homology with *Bracon hebetor*, *Callibracon limbatus* and *Bracon phylacteophagus.* However, we did not find sequences published to *Bracon brevicornis* in the NCBI-Gen Bank database. The phylogenetic UPGMA tree was carried out using MEGA 4 software (Fig. 4). The UPGMA tree was constructed based on the multiply aligned sequence data for five types of insects. The tree separates the genomes into two distinct groups, whereas the insects *Bracon hebetor*, *Callibracon limbatus*, *Bracon phylacte-ophagus* and *Bracon hebetor Egypt* were presented in one group but only the insect Bracon brevicornis Egypt was found in another group. 16S rRNA gene sequence informatics is one of the most attractive potential tools to provide genus and species identification and reclassification for the two insects under study.

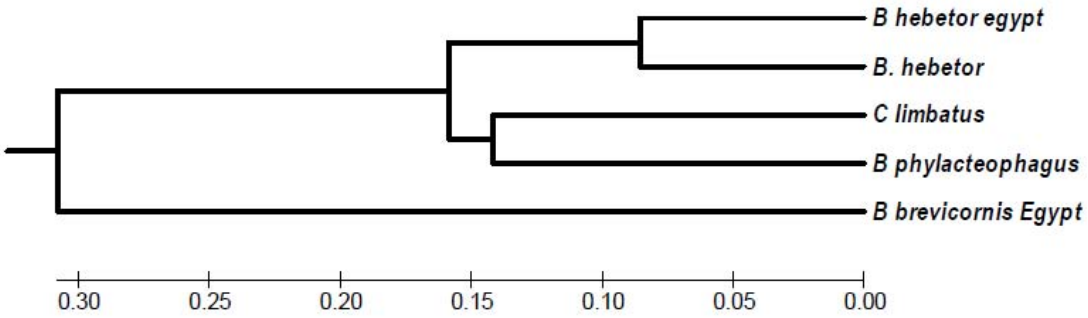

Fig. 4: The UPGMA tree was constructed based on the multiply aligned sequence data for two insects collected from Egyptian environment (*Bracon hebetor Egypt* and *Bracon brevicornis Egypt*) and other three insects sequences (*Bracon hebetor*, *Callibracon limbatus* and *Bracon phylacteophagus* published in the NCBI-GenBank database.

DISCUSSION

The molecular studies of *Bracon* species have produced interesting outcomes about the hidden relationships among species which could not be observed well by the phenotypic or behavioural studies (Aruggoda, *et al*., 2010). RAPD-PCR and 16S rDNA gene partial region were performed in order to infer the relationship of two insects.

The RAPD-PCR results were summarized in Table 2 and they revealed genetic variations between *B. hebetor Egypt* and *B. brevicornis Egypt*. As a matter of fact, the polymorphic percentage mean and the similarity were ~23% and 86%, respectively. These results suggested that *B. hebetor Egypt* and *B. brevicornis Egypt* have a common ancestor but this percentage of polymorphism could make us to think more in their taxonomy. This difference between the two insects could due to the natural environment and it is in agreement of Jain *et al*. 2010 who reviewed the importance to study the insects' ecology to understand their evolution and diversification, and their influence on the functional and trophic links between different components of associated habits. Moreover, The RAPD markers technique has been reported to be an efficient tool to discriminate genetically isolated species and to verify the existence of spices that presented as a result of genetic drift or natural selection (Fuchs *et al*., 1998). So the RAPD marker is useful in taxonomic and classification studies (Gala, 2009).

The partial 16S rDNA sequences uses in taxonomic studies (Hebert *et al*., 2003), has been applied mostly to known species. This region presents near the 3' end of the mitochondrial 16S rRNA gene and these sequences are adequate for species discrimination in various arthropod taxa (reviewed by Heimpel *et al*., 1997). Our 16S rDNA sequencing alignment data showed variability that was up to 78.21% between the two insects. The alignment of 16S rRNA genes revealed differences including substitutions at a higher level. Of course 16S rDNA sequencing has played a pivotal role in the accurate identification and discriminate the genus or species level (Aruggoda, *et al*., 2010). Although, the percentage of similarity that outcomes from either RAPD-PCR or from 16S rDNA sequencing infer phylogenetic relationships between the two insects, but this percentage could be less than the rate needed to be in the same genus. Whereas, Fry *et al*., 1991 revealed that the homology of the 16S rDNA sequences is less than 98%, then a variety can be considered different and if the homology is less than 93-95%, then a genus can be considered to be different.

In conclusion, 16S rRNA gene sequencing is more powerful than RAPD-PCR for identification of the two insects under study. The similarity revealed differences between the insects arrived to 78.21% and 86% by using 16S rRNA gene sequencing and RAPD-PCR. The phylogenetic studies provided the evolutionary relationships among different species that reflects their sharing a common ancestor. Moreover, the average genetic differences are high and that make us to reconsider their taxonomy.

REFERENCES

Aruggoda A. G. B., Shunxiang R. and Baoli Q. (2010). Molecular Phylogeny of Ladybird Beetles (*Coccinellidae*: *Coleoptera*) Inferred from Mitochondrial 16S rDNA Sequences. Tropical Agricult. Res., 21(2): 209-217.

Barnay, O., Hommay, G., Gertz, C., Kienlen, J.C., Schubert, G., Marro, J.P., Pizzol, J. and Chavigny, P. (2001). Survey of natural populations of Trichogramma (Hym., Trichogrammatidae) in the vineyards of Alsace (France). Journal of Applied Entomology 125: 469–477.

Chang, S.-C., Hu, N.-T., Hsin, C.-Y. and Sun, C.-N. (2001). Characterization of differences between two Trichogramma wasps by molecular markers. Biological Control 21: 75–78.

DeBach, P.; Rosen, D. (1991). Biological control by natural enemies. Cambridge, UK; Cambridge University Press, 440 pp.

Demichelis, S. and Manino, A. (1998). Electrophoretic detection of parasitism by Dryindae in Typhlobcybinae leafhoppers (Homoptera: Aucheno-rrhyncha). Canadian Entomologist 130: 407-414.

Fry N. K., Warwick S., Saunders N. A. and Embley T. M. (1991). The use of 16S ribosomal RNA analyses to investigate the phylogeny of the family *Legionellaceae*. J. Gen. Microbiol. 137:1215-1222.

Fuchs H., Gross R., Stein H. and Rottamann O., (1998). Application of molecular markers for the differentiation of bream (Abramis bramaL.) populations from the rivers Main and Danube, J Appl Ichthyol, 14(1-2): 49-55.

Galal F. H. (2009). Comparison of RAPD and PCR-RFLP markers for classification and taxonomic studies of insects Egypt. Acad. J. biolog. Sci., 2 (2): 187-195.

Hebert P. D. N., Cywinska A., Ball S. L., and DeWaard J. R. (2003). Biological identifications through DNA barcodes. Proc. R. Soc. B 270:313-321.

Heimpel G. E., Antolin M. F., Franqui R. A. and Strand M. R. (1997). Reproductive isolation and genetic variation between two "strains" of *Bracon hebetor* (*Hymenoptera*: *Braconidae*). Biol. Control. 9: 149-156.

Jaccard, P. (1908). Nouvelles recherches sur la distribution florale. Bull. Soc. Vaud. Sci. Nat. 44:223–270.

Jain S. K., Neekhra B., Pandey D. and Jain K. (2010). RAPD marker system in insect study: A review. Indian Journal of Biotechnology 9: 7-12.

Kimani-Njogu, S.K., Overholt, W.A., Woolley, J.B. and Omwega, C.O. (1998). Electrophoretic and phylogenetic analyses of selected allopatric populations of the Cotesia flavipes complex (Hymenoptera: Braconidae), parasitoids of cereal stem borers. Biochemical Systematics and Ecology 26:285–296.

Landry, B.S., Dextraze, L. and Boivin, G. (1993).Random amplified polymorphic DNA markers for DNA fingerprinting and genetic variability of minute parasitic wasp species (Hymenoptera: Mymaridae and Trichogrammatidae) used in biological control programs of phytophagous insects. Genome. Jun; 36(3):580- 587.

Mulcahy, D.L., M. Cresti, S. Sansavini, G.C. Douglas, H.F. Linskens, G.B. Mulcahy, R. Vighani, and M. Pancaldi. (1993). The use of random amplified polymorphic DNAs to fingerprint apple genotypes. Scientia Hort. 54: 89–96.

Pinto, J.D. 1998. Systematics of the North American species of Trichogramma (Hymenoptera: Trichogrammatidae). Mem. Entomol. Soc. Wash. Washington, Allen Press Inc., 22-287p.

Pinto, J.D., Platner, G.R. and Sassaman, C.A. (1993). Electrophoretic study of two closely related species of North American Trichogramma: T. pretiosum and T. deion (Hymenoptera: Trichogrammatidae). Annals of the Entomological Society of America 86: 702–709.

Pungerl, N.B. (1986). Morphometric and electrophoretic study of Aphidius species (Hymenoptera: Aphidiidae) reared from a variety of aphid hosts. Systematic Entomology 11:327–354.

Shouche Y. S. and Patole M. S. (2000). Sequence analysis of mitochondrial 16S ribosomal RNA gene fragment from seven mosquito species. J Biosci. 25(4):361-366.

Stouthamer, R., Hu, J., van Kan, F.J.P.M., Platner, G.R. and Pinto, J.D. (1999). The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of Trichogramma. BioControl 43: 421-440.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599.

Wolf FC and Rijn JPV (1993). Rapid detection of genetic variability in Chrysanthemum (Dendrantema grandiflora Tzvelev) using random primers. Heredity 71:335-341.