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Hierarchical Analysis of Variation in the Mitochondrial 16SrRNA Gene among Five Different Insect Orders

Chatterjee Susmita

Division of Genetics, Indian Agricultural Research Institute, New Delhi, India
Email: susmita.chatterjee1@gmail.com

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Abstract

Nucleotide sequences from a 500 bp region of the 16SrRNA gene were analyzed for ten insect pests of five different orders to examine the patterns of variation within the gene fragment and the taxonomic levels for which it showed maximum utility in phylogeny estimation. A hierarchical approach was adopted in the study through comparison of levels of sequence variation among taxa at different taxonomic levels. Among them, partial 16SrRNA gene was amplified in ten insects of five different orders. As previously reported for many holometabolous insects, the 16SrRNA gene data is reported here for 5 different orders were highly AT-rich and exhibited strong site-to-site variation in substitution rate. The partial 16SrRNA genes of five out of ten insects were reported first time. Primers were made from blasting 2 different genera of the order Diptera. These primers were proven to be universal as it amplified the partial 16SrRNA gene in ten different insects across five different orders, Diptera, Coleoptera, Heteroptera, Lepidoptera and Hymenoptera. Later, a phylogenetic tree was also constructed for understanding and analyzing the relation of above five orders. This study resulted in unusual findings which were as follows: All the species of Drosophila of order Diptera were evolutionary more closely related to *Dysdercus koenigii* of order Heteroptera than *Bactrocera cucurbitae* of Drosophilan order, Diptera in terms of partial 16SrRNA gene sequence. Similarly, *Spodoptera litura* and *Helicoverpa armigera* belonged to same family Noctuidae whereas *Pieris brassicae* belonged to family Pieridae. All belonged to order Lepidoptera. The results showed that *Spodoptera litura* in terms of partial 16SrRNA gene sequence was evolutionary more close to *Pieris brassicae than Helicoverpa armigera.*

Keywords

16SrRNA Gene, PCR, Amplification, Phylogenetic Tree

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1. Introduction

Drosophila is a model organism for research study for a longtime. However, other insects, especially pests which have a great significant value in agriculture were rarely studied. Selecting a gene for phylogenetic analysis required matching the level of sequence variation to the desired taxonomic level of study. The mitochondrial DNA of mammals had been used for molecular evolution studies [1] and similar techniques had been applied to insects as well [2]-[5]. Because of its high rate of evolution, mitochondrial DNA had been extremely useful molecule for high resolution analysis of evolutionary processes [6]. It had been used in the phylogenetic analysis of insects [7]. Factors other than the distribution of rate variation among sites could determine the shape of the sequence divergence accumulation curve. For instance, in many holometabolous insects, including Hymenoptera and Diptera, mitochondrial DNA exhibited a nucleotide composition which is strongly biased toward adenine and thymine (AT bias). For some groups, the mean percentage of AT could be higher than 80% [8]-[9]. When the base composition is biased to that degree, obviously, the ratio of transversions (tv) to transitions (ti) increased. These led to further reduce the ability to correctly estimate the number and proportion of hidden mutations and, hence, it also reduced the ability to correct sequence divergence for hidden changes. 16SrRNA of mitochondrial genome holds a crucial role in the mRNA translation and remained highly conserved throughout the evolutionary process. Here, 16SrRNA gene was selected for study across the insect orders. Primers were constructed by blasting two different genera of Diptera and were used for amplification in insect pests belonging to Diptera, Coleoptera, Heteroptera, Lepidoptera and Hymenoptera. Later, a phylogenetic tree was also constructed for understanding and analyzing the relation of five above orders.

2. Materials and Methods

2.1. Sources of Sequence Data

For 16S ribosomal partial gene sequences, I blasted *Drosophila melanogaster* partial 16S ribosomal RNA gene sequence (GenBank: X53506.1) and *Bacterocera cucerbitae* partial 16S ribosomal RNA gene sequence (GenBank: FJ168025.1). These were blasted in NCBI blast tool in Fasta format to obtain the conserved regions of 16S ribosomal RNA partial gene. Gene-specific primer used for amplification of the target partial gene was designed in-house manually. The sequence specificities of the primer sequence so designed was verified using the BLAST program available at the NCBI website (www.ncbi.nlm.nih.gov/BLAST/). The primer sequence was also evaluated for various other characteristics like melting point, presence of secondary structure formations like hairpins and propensity for dimer formation using software available in internet, Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html).

2.2. Isolation of DNA

DNA was extracted from ten different insect species of five different orders (**Table 1**) by using Qiagen tissue kit and was verified by running in 1% agarose gel and then quantified by Spectramax M5, a dual-monochroma- tor and further processed for partial 16SrRNA gene amplification.

The PCR was carried out using Taq DNA polymerase (Fermentas) with the following general conditions: 15 - 20 Ng of genomic DNA was used in a 20 µl reaction with 5 U/µl of Taq DNA polymerase, 2.5 mm each dNTP mix and 10 pM/µl of each primer (**Table 2**) with the following condition.

Table 1. Following species were considered for study.

Order	Name of Species
Diptera	*Drosophila ananesse, Drosophila jambulina, Drosophila melanogaster, Bacterocera cucerbitae*
Coleoptera	*Coccinella septempunctata*
Heteroptera	*Dysdercus koenigii*
Lepidoptera	*Spodoptera litura, Helicoverpa armigera, Pieris brassicae*
Hymenoptera	*Apis mellifera*

Table 2. Primers used for gene study.

Gene Name	Primer Name	Primer Sequence (5' - 3')	Tm (˚C)	Targeted Region (bp)
16SrRNA	Forward	TAAAGTCTGACCTGCCCACTGAAT	56	500
	Reverse	CTTAATCCAACATCGAGGTCGCAA	56	

Following an initial denaturation at 94˚C for 5 min, 37 cycles at 94˚C were performed, each annealing at 59˚C for 45 s and extension at 72˚C for 30 s. A final extension was run at 72˚C for 5 min. The PCR products (5 µl) were resolved in 1% agarose gel which was run at 80 V for 45 minutes. Negative PCR control was also run with double distilled water instead of DNA to eliminate doubts. The amplified products were eluted from the gel and purified by gel purification kit before sending for sequencing. The sequencing both through forward and reverse primers were done by Sanger method through outsourcing.

The gene sequences obtained from the samples were aligned through clustal W software in www.genome.jp/tools/clustalw/ and similarities and dissimilarities were assessed.

2.3. Construction of Phylogenetic Tree

Based on homology and genetic dissimilarities, a phylogenetic tree was constructed using rooted and branched clustal W software, www.genome.jp/tools/clustalw/.

Finally the sequences were submitted to NCBI database in www.ncbi.nlm.nih.gov/.

3. Results and Discussion

Bands amplified (**Figure 1**) have been verified to be ribosomal DNA by direct sequencing of the PCR products and blasting the obtained sequences with NR sequences of the NCBI database. The sequences were obtained both by forward and reverse primers and then verified and finally submitted to the NCBI database. In *Coccinella septempunctata*, *Dysdercus koenigii*, *Spodoptera litura*, *Helicoverpa armigera* and *Pieris brassicae*, this partial gene was reported first time. The sequences were published in NCBI database and have gene bank accession numbers (**Table 3**). The versatility of these ribosomal primers in insects was clearly demonstrated. Experimental studies confirmed the theoretical expectations based on sequence conservation about the relative performance of the forward and reverse primers. Since these primers amplified different orders, The identity score of all the sequences with each other were as follows: 1. *Drosophila ananesse*; 2. *Drosophila melanogaster*; 3. *Drosophila jambulina*; 4. *Bactrocera cucurbitae*; 5. *Coccinella septempunctata*; 6. *Dysdercus koenigii*; 7. *Spodoptera litura*; 8. *Helicoverpa armigera*; 9. *Pieris brassicae*; 10. *Apis mellifera*.

Sequences (1:2) Aligned. Score: 90.7834
Sequences (1:3) Aligned. Score: 95.0521
Sequences (1:4) Aligned. Score: 76.0925
Sequences (1:5) Aligned. Score: 68.0782
Sequences (1:6) Aligned. Score: 88.4106
Sequences (1:7) Aligned. Score: 67.8378
Sequences (1:8) Aligned. Score: 68.2266
Sequences (1:9) Aligned. Score: 69.4511
Sequences (1:10) Aligned. Score: 60.8466
Sequences (2:3) Aligned. Score: 86.9792
Sequences (2:4) Aligned. Score: 74.2931
Sequences (2:5) Aligned. Score: 67.7524
Sequences (2:6) Aligned. Score: 96.0265
Sequences (2:7) Aligned. Score: 64.3243
Sequences (2:8) Aligned. Score: 65.5172
Sequences (2:9) Aligned. Score: 68.9737
Sequences (2:10) Aligned. Score: 60.0529
Sequences (3:4) Aligned. Score: 70.0521
Sequences (3:5) Aligned. Score: 52.1173

Sequences (3:6) Aligned. Score: 86.4238
Sequences (3:7) Aligned. Score: 57.2973
Sequences (3:8) Aligned. Score: 66.6667
Sequences (3: 9) Aligned. Score: 60.9375
Sequences (3:10) Aligned. Score: 56.8783
Sequences (4:5) Aligned. Score: 58.9577
Sequences (4:6) Aligned. Score: 75.4967
Sequences (4:7) Aligned. Score: 58.9189
Sequences (4:8) Aligned. Score: 56.0411
Sequences (4:9) Aligned. Score: 60.1542
Sequences (4:10) Aligned. Score: 47.8836
Sequences (5:6) Aligned. Score: 55.6291
Sequences (5:7) Aligned. Score: 65.798
Sequences (5:8) Aligned. Score: 56.6775
Sequences (5:9) Aligned. Score: 71.6612
Sequences (5:10) Aligned. Score: 50.8143
Sequences (6:7) Aligned. Score: 60.596
Sequences (6:8) Aligned. Score: 65.894
Sequences (6:9) Aligned. Score: 68.2119
Sequences (6:10) Aligned. Score: 54.3046
Sequences (7:8) Aligned. Score: 70.2703
Sequences (7:9) Aligned. Score: 77.5676
Sequences (7:10) Aligned. Score: 52.7027
Sequences (8:9) Aligned. Score: 65.5172
Sequences (8:10) Aligned. Score: 61.9048
Sequences (9:10) Aligned. Score: 60.582

As per the above score and the phylogenetic tree (**Figure 2**), it was clear that though, *Drosophila ananesse*, *Drosophila melanogaster*, *Drosophila jambulina* and *Bactrocera cucurbitae* belonged to same order Diptera, all the species of Drosophila in terms of partial 16SrRNA gene sequence were evolutionary more close to *Dysdercus*

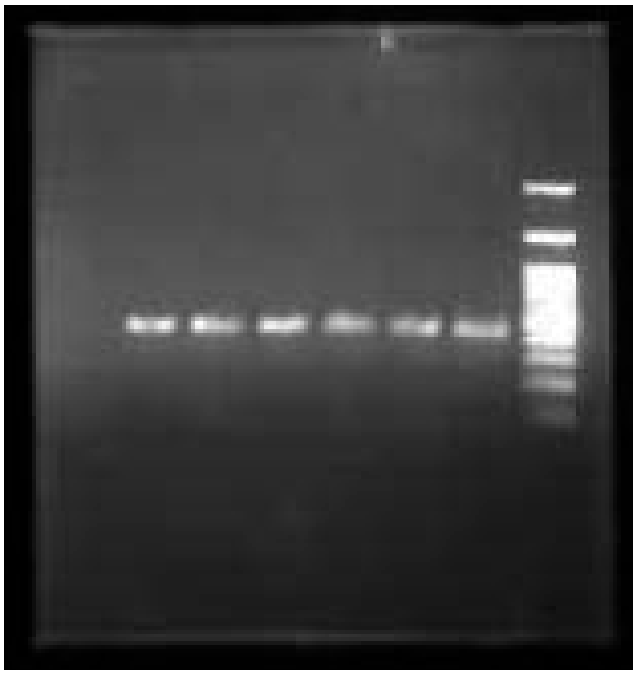

Figure 1. Distinct amplification of samples approximately corresponds to the 500 bp of the DNA marker (extreme right).

koenigii of order Heteroptera than *Bactrocera cucurbitae*. *Bactrocera cucurbitae* was placed in different branch and *Dysdercus koenigii* was placed in same branch of phylogenetic tree with respect to all species of Drosophila. Similarly, *Spodoptera litura*, *Helicoverpa armigera* and *Pieris brassicae* belonged to same order Lepidoptera. *Spodoptera litura* and *Helicoverpa armigera* belonged to same family Noctuidae whereas *Pieris brassicae* belonged to family Pieridae. The results showed that *Spodoptera litura* in terms of partial 16SrRNA gene sequence was evolutionary more close to *Pieris brassicae*. *Spodoptera litura* and *Pieris brassicae* were placed in same branch whereas *Helicoverpa armigera* was placed in different branch of the phylogenetic tree. Although we could not judge the sequence similarities by studying a partial sequence of 16SrRNA, this finding was unexpected as it was expected that genera of same orders and families were evolutionary close to each other. Similar results were showed by Shouche *et al.* [10], where mitochondrial 16SrRNA gene fragment was analyzed in mosquito. A phylogenetic tree was displayed and it showed that all studied species of Aedes, Culex and Anopheles belonging to same order Diptera and same family Culicidae were not in the same branch and thus might have had different evolutionary origins.

4. Conclusion

Insects having same taxonomic group might have different evolutionary origin in respect of a partial conserved gene, 16SrRNA.

Table 3. Name of the organism with accession numbers.

S.no.	Name of the Organism	Accession Numbers
1	*Drosophila ananesse*	JX896435
2	*Drosophila melanogaster*	JX896436
3	*Drosophila jambulina*	JX912717
4	*Bactrocera cucurbitae*	JX912718
5	*Coccinella septempunctata*	JX896437
6	*Dysdercus koenigii*	JX974556
7	*Spodoptera litura*	KC352618
8	*Helicoverpa armigera*	KC352619
9	*Pieris brassicae*	KC978889
10	*Apis mellifera*	KC422452

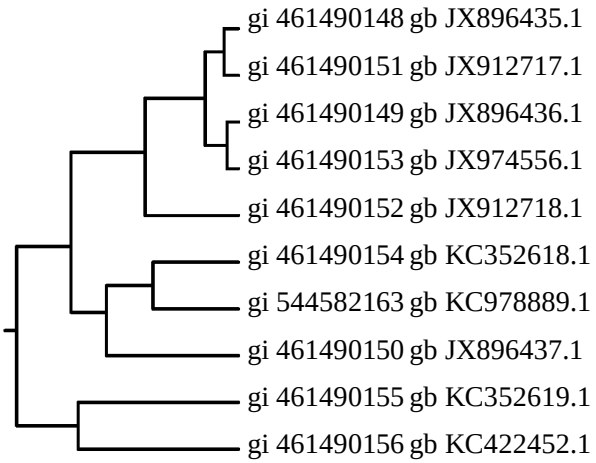

Figure 2. Phylogenetic tree of the above sequences based on rooted and branched clustal W software.

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