Chapter III

Molecular Characterization of Agriculturally Important Pests of Vadodara

3.1 Introduction

The roots of insect systematics go back to the sixteenth, seventeenth, and eighteenth centuries. Influential pioneers of entomology were the Italian naturalist Ulisse Aldrovandi (1522–1605), the Dutch doctor, and microscopist Jan Swammerdam (1637–1680), and the German naturalist August Johann Rösel von Rosenhof (1705–1759). The names of insects refer to the characteristics of the wings, e.g., Heteroptera (heterogeneous forewing), Hymenoptera (membranous wings), and Coleoptera (sheath-like forewing), etc. Insects are ancient (>450 million years ago) and taxonomically diverse group having a worldwide distribution and a complex evolutionary history (Speight, 2008; Sahney *et al.,* 2010) Many of them are considered agricultural pests, major disease vectors, pollinator of crops, parasites of other insects, and bio-indicator of environmental changes (Price *et al.,* 2013; Mandal, 2014). Identification of insects is crucial to managing endangered species, protected species, and invasive species. This management is essential for environmental quality indicators, basic research on evolutionary biology and ecology, agricultural pests/beneficial species and disease vectors/pathogens, and biodiversity study and conservation research.

The benefit of phylogenetic classification is that it reveals the underlying biological processes responsible for organism diversity. Via phylogenetics, scientists were able to trace the genetic history of different species and thus proved that the speciation process (Wiens *et al.,* 2003). Previously the phylogenetic classification was based on the morphological identification; due to the advancement of technology. There is a paradigm change in the classification where several research groups classify the DNA barcode method based on the insects. Moreover, the identification of organisms based on morphological characters often represents a challenging task requiring experienced taxonomists. These morphology-based procedures are usually time-consuming and may not always provide resolution to the species level (Cywinska *et al.,* 2007; Rindi *et al.*, 2008; Packer 2009) and there is substantial phenotypic plasticity of that has to lead to the misidentification of insects (Nekola *et al.,* 2000; Gutiérrez *et al.*, 2013). Different molecular studies have discovered the existence of cryptic insect species that have built up genetic divergence without accompanying morphological disparities and, thus, cannot be identified using the conventional morphological species concept. The recognition of such morphologically cryptic species poses a significant challenge for modern taxonomy (Heinrichs *et al.,* 2011).

Therefore to overcome this, scientists have developed nucleotide-based taxonomic classification, which was termed as DNA barcoding (Hebert *et al.,* 2003, 2004). This approach is much more efficient than the conventional morphological approach as it relies on a change in nucleotide sequence (mutations) that arouse due to environmental adaptability. So, DNA barcoding is a multidimensional system that identifies the insects in a robust approach. Insect species-level identification is critical in many applications of the economic and social importance of the nation. In such cases, rapid identification is a need of an hour. The insinuation of DNA barcoding has proven a wide range of success in several rapid biodiversity assessment studies (Valentini *et al.,* 2009), biomonitoring (Hajibabaei *et al.,* 2011; Sweeney *et al.,* 2011) including the monitoring of pathogen, spread and their associated vectors (Azpurua *et al.,* 2010) in forensics (Dawnay *et al.,* 2007), in the investigation of the illegal trade of endangered species and their products (Baker *et al.,* 2000; Muellner-Riehl *et al.,* 2011, Pečnikar and Buzan, 2014), in studies on feeding ecology (Rollo *et al.,* 2002), medicinal and poisonous plants (Phua *et al.,* 2008; Baker *et al.,* 2012; Fišer Pečnikar and Buzan, 2014), conservation initiatives (Smith *et al.,* 2005), developmental stages, castes polymorphism in social insects, sexually dimorphic, polyphenic and polymorphic individuals (Colgan *et al.,* 2011). DNA barcoding tackles many of the problems inherent to morphological taxonomy. With the number of taxonomists decreasing and the number of named species increasing, molecular tools have become a mainstay of modern taxonomic analysis. Only a small amount of tissue (one single cell at best) is required for species determination, as it gives the advantage to analyse the specimen without any prior knowledge and can be applied to all stages of development (Floyd *et al.,* 2002; Hebert *et al.,* 2003; Savolainen *et al.,* 2005).

Therefore, molecular methods provide a better in-depth understanding of insects' variations and similarities and even provide evolutionary explanations among different species. Molecular analyses and phylogenetic analysis using molecular markers can explain the relationships between different Molecular phylogenetics using molecules' structure and function and how they change over time to infer these evolutionary relationships. In recent years, a considerable amount of sequence information is available in publically accessible online databases that enabled molecular phylogenetics to grow and find new applications (Sanderson *et al.,* 2008). Different types of mitochondrial and nuclear DNA markers are available for phylogenetic analysis of insects. However, the choice of a molecular marker in a particular analysis is crucial since a sequence fragment, whose rate of substitution is inappropriate for the level of divergence understudy, can be a source of misleading data). The choice of markers lays its five main characteristics; for instance, firstly, in the study group, it needs to be suitably variable to discriminate among most species, but sufficiently conserved to be less variable within than between species. Second, priming sites need to be sufficiently conserved to permit a reliable amplification without the risk of false results when the study is done from the pooled samples, e.g., when the total of small insects from a sample is to be studied without separating individuals or of environmental DNA such as subfossil DNA remains from the soil (Rondon *et al.,* 2000; Willerslev *et al.,* 2004). Third, the gene should convey sufficient phylogenetic information to allocate species to major taxa using simple phenetic approaches. Fourth, its amplification and sequencing should be as robust as possible and under different lab conditions and protocols. Fifth, sequence alignment should be achievable also among distantly related taxa. The ideal barcoding gene should have a noticeable gap between intra- and interspecific levels of divergence and, most important, correctly identify species (Hebert *et al.,* 2004; Meyer and Paulay, 2005; Kohler *et al.,* 2008). Carefully planning is, therefore, necessary before any DNA barcoding experiments of insects. In a phylogenetic study, mitochondrial DNA has many advantages. They possess strict maternal transmission (Carelli *et al.,* 2011; Carelli, 2015) with a high mutation rate due to a limited repair system (5-10 times that of nuclear DNA) and a simple conserved structure.

These unique properties allow the development of universal primers and easy recovery from the small or degraded biological sample due to its high copy number in most cells with a different evolution rate in different regions of mitochondrial DNA. However, to overcome PCR biases and increase the detection of species, lots of metabarcoding studies employ multiple markers (Rossetto and Verbruggen, 2016). Therefore, more studies directly compare the utility of different markers for DNA barcoding and investigate its taxonomic implications in insects' species-rich groups.

COI as the Phylogenetic Marker

In 2003, a standardized 658 bp fragment of the mitochondrial cytochrome C oxidase subunit I gene (cox1 or COI) was proposed as a universal marker for species identification – to be used as a "DNA barcode" tagging any taxon in the animal kingdom (Hebert *et al.,* 2003). Following this idea, the number of partial COI gene sequences available in public data repositories has reached its apex. Till 2016, about 4.7 million COI barcodes are sequenced and stored in the Barcode of Life Data Systems database (BOLD, http://www.boldsystems.org/), and more than 3000 papers have been published on the application of COI barcodes to identification and discovery of animal species (Pentinsaari *et al.,* 2014). Due to sequencing advancements, COI is now the most extensively used in constructing the animal kingdom's phylogeny. Notably, most DNA barcoding studies published to date treat this gene region as a mere identification tag – in exact accordance with the concept of a conveniently readable "barcode." Therefore, the 5′ region of mitochondrial cytochrome c oxidase subunit I (COI) gene is recommended as the universal and standard barcoding marker for most animals (Hebert *et al.,* 2004 Ward *et al.,* 2005; Hajibabaei *et al.,* 2007; Ratnasingham and Hebert, 2007; Hebert *et al.,* 2016). In particular, for metazoans and insects, the popular "Faviolmer region," a 648 basepair fragment at the 5 ' end of the mitochondrial CO1 gene, was historically applied as a universal DNA barcoding region.

16s rRNA as a Phylogenetic Marker

Mitochondrial ribosomal RNA sequence has been widely used for phylogenetic studies. Mitochondrial rRNA gene sequences invertebrates appear to be as less conserved in evolution than their nuclear counterparts. Insect mitochondria contain two ribosomal RNA (rRNA) genes, 12S rDNA and 16S rDNA. 12s rDNA is highly conserved in insects and used to study genetic diversity in phyla, but the large subunit of ribosomal RNA (16S rDNA) is often used for low and intermediate studies families or genera (De Mandal *et al.,* 2014). Mitochondrial 16S rRNA has been used to reveal the evolutionary relationship of different castes of termite and has been well explored for their foraging behavior (Legendre *et al.,* 2008) and their genetic relationship (Singla *et al.,* 2013; Jadhav, 2019).

Further, a molecular phylogeny of cockroaches and related insects based on 16S rRNA is presented by Vaishampayan *et al.,* 2007. A study on 16S rRNA gene sequences from different taxa of Hymenoptera showed that the 16S rRNA gene is most informative for phylogenetic analysis among closely related species or populations, and among tribes, subfamilies, and families (Arévalo *et al.,* 2004; Wei *et al.,* 2010; Mao *et al.,* 2014). According to Chandish Ballal, Director ICAR-NBAIR (2016), 40 percent of insect species are likely to become extinct globally in the coming years. Indian entomologists agree that India is already witnessing a slump in insect numbers. More than 59,000 insect species have been described in India, of which only 4.6% barcodes have been generated from the known species, while the global record is about 16% of the described species (Jalali and Ojha, 2015).

After making an inventory on the diversity of Insects in the agriculture fields and observing the pest status of Vadodara and its surrounding (Chapter I and II), DNA barcoding will be employed for identification of insect pests belonging to four significant orders, i.e., Coleoptera, Orthoptera, Hemiptera, and Lepidoptera by using mitochondrial sequence information from COI and

16sRNA genes to shed light on molecular systematics of insect pests affecting the economically important vegetable crops. This will evaluate the determinants or the various sequence parameters of accuracy to study their divergences and phylogenetic relationships.

3.2 Materials and Methods

Procedure for DNA isolation

The collected pest species from all the study sites were preserved in 100% ethanol and stored at -80ºC in the laboratory and used for DNA isolation. Each specimen's femoral muscles were dissected out and used for Genomic DNA extraction using HiPurA® Insect DNA Purification Kit method (Cat.no. MB529).

1. The insect tissue (50 mg) was pulverized using sterilized mortar and pestle in 180 µl of Lysis Solution (AL) (DS0015) and 20 µl of the Proteinase K solution (20 mg/ml).
2. The minced tissue was then transferred to an autoclaved capped 2.0 ml microcentrifuge tube and was incubated at 55°C for 1- 3 hours.
3. As RNA-free genomic DNA was required, 20 µl of RNase (DS0003) was added and incubated for 2 minutes at room temperature (15-25°C).
4. Further, 200 µl of Lysis Solution (C1) (DS0010) was added to the sample. Was Vortex thoroughly for 15 seconds, and a homogeneous mixture was obtained, which was incubated at 70°C for 10 minutes.
5. The lysate was loaded onto HiShredder (DSCA02), placed in an uncapped 2.0 ml collection tube, and centrifuged for 2 minutes at 13,000 x g (≈14,000 rpm).
6. The flow-through fraction was transferred to a new 2.0 ml collection tube, taking care of not disturbing the cell debris pellet.
7. 200 µl of ethanol (96-100 %) was added to the lysate and was mixed thoroughly by vortexing for 5-10 seconds.
8. The lysate was loaded onto HiElute Miniprep Spin Column (Capped) [DBCA03] and centrifuged at ≥6,500 x g (≈10,000 rpm) for 1 minute. The

flows-through liquids were discarded and placed in the column of the same 2.0 ml collection tube.

9. 500 µl of diluted Wash Solution was added to the column and centrifuged at ≥6,500 x g (≈10,000 rpm) for 1 minute. The flow-through liquid was discarded.
10. 500 µl of diluted Wash Solution was added to the HiElute Miniprep Spin Column and centrifuge at 12,000-16,000 x g (≈10,000 rpm) for 3 minutes to dry the column. The flow-through was discarded. The column was centrifuged for 1 minute at the same speed for the complete removal of the ethanol.
11. 80 µl of the Elution Buffer (ET) (DS0040) was added directly into the column, taking care of not spilling to the sides, and was incubated for 5minutes at room temperature and centrifuged at 6,500 x g (≈10,000 rpm) for 1 minute to elute the DNA.
12. The eluate was transferred to an autoclaved capped 2ml collection tube for more extended DNA storage.

The integrity of DNA was checked by using 0.8% agarose gel electrophoresis. The isolated DNA samples were quantified to find out the amount of DNA using a Nanodrop Spectrophotometer. The quantification was done taking the A_{260}/A_{280} ratio, as it reveals contaminants' presence and gives evidence of possible degradation. An A_{260}/A_{280} ratio of 1.8 was considered acceptable for DNA.

The DNA product was then amplified for COI and 16s rRNA using PCR (prima-96, HiMedia, India) and primer, as shown in Table 3.1.

DNA marker	Primer sequence (5’ to 3’)	Reference	Amplicon Size
Cytochrome c oxidase subunit 1			
LCO-1490	**5’-GGTCAACAAATCATAAAGATATTGG -3’**	Folmer *et al.*, 1994	**720bp**
HCO-2198	**5’-TAAACTTCAGGGTGACCAAAAAATCA-3’**	Folmer *et al.*, 1994	
16s rRNA			
16Sar		Simon *et al.*, 1994	**620bp**
16Sbr	**5’- GCTCAGAACGAACGCTATC-3’**	Simon *et al.*, 1994	

Table 3. 1: PCR primers for COI and 16s rRNA

PCR Conditions

The DNA product was then amplified in PCR for COI at 94 °C denaturation for 1 min, 5 cycles of 94°C for 1:00 min, 45 °C annealing for 1:30 min, 72°Cfor 1:30 min, followed by 35 cycles of 94°C for 1:00 min, 50 °C for 1:30 min and 72 °C for 1:00 min, and extension was carried out 72 °C for 7 min. A total of 40 cycles were performed using primers given in Table 3.2. For amplification of 16s rRNA 94°C denaturation for 3 min, 98 °C annealing for 0:10 min and 50 °C for 1:30 min, and extension was carried out 72 °C for7 min. A total of 35 cycles were performed (Table 3.3) using primers 16Sar- 5’-CCGGTCTGAACTCAGATCACGT-3’, 16Sbr-5’GCTCAGAACGAACGCTATC-3’ (Simon *et al.*, 1994).

Stage 1 (1 cycle)	**Stage 1 (5 cycles)**	**Stage 2 (35 cycles)**	**Stage 3 (1 cycle)**
94° C 1:30 min	94°C 1:00 min	94°C 1:00 min	72°C 10:00 min
	45°C 1:30 min	50°C 1:30 min	4.0°C stop for ∞ time
	72°C 1:30 min	72°C 1:00 min	

Table 3. 2: PCR cycles for COI

Stage 1 (1 cycle)	Stage 2 (35 cycles)	Stage 3 (1 cycle)
94° C 3.00 min	98°C 0:10 min	72°C 08:00 min
	50°C 0:30 min	4.0°C stop for ∞ time
	72°C 1:30 min	

Table 3. 3: PCR cycles for 16s rRNA

Amplification of DNA was then checked by running the samples on 2% agarose gel using a 720bp DNA ladder and visualized in gel dock. The amplified products were then sent to commercial sequencing at Chromos Biotech Pvt. Ltd. and Eurofins Pvt Ltd, Bangalore, India, where the chain termination method was used for sequencing.

Sequencing

The DNA sample was divided into four separate sequencing reactions, containing all four standard deoxynucleotides (dATP, dGTP, dCTP, and dTTP) and the DNA polymerase. To each reaction, added only one of the four dideoxynucleotides (ddATP, ddGTP, ddCTP, or ddTTP), while the other added nucleotides were ordinary ones. The dideoxynucleotide was added to be approximately 100-fold lower in concentration than the corresponding deoxynucleotide, allowing for enough fragments to be produced while still transcribing the complete sequence. Following rounds of template DNA extension from the bound primer, the resulting DNA fragments were heat-denatured and separated by size using gel electrophoresis. This was frequently performed using a denaturing polyacrylamide-urea gel with each of the four reactions run in one of four individual lanes (lanes A, T, G, C). The DNA bands may then be visualized by autoradiography or UV light, and the DNA sequence can be directly read off the X-ray film or gel image. The sequences were trimmed, validated, and edited using Bio Edit v7.2.5 software. The assembled sequences were compared with Gene Bank data to confirm the morphometric identification, and the sequences were submitted to NCBI Gene Bank for acquiring Accession Number.

Bioinformatics Analysis

- %GC content analysis was done for each sequence.
- AT and GC skew analysis

  The sequenced data were subjected to strand asymmetric analysis, which was calculated using the formula(s) AT-skew = (A−T)/(A C T), GC skew = (G−C)/(G C C).

Rate of Synonymous and Non-synonymous Probability

The numbers of synonymous and non-synonymous differences between sequences were estimated using the Nei-Gojobori method. The significance for noted at $p<0.05$ for the synonymous and non-synonymous occurrence of nucleotides.

Phylogenetic Analysis

It was also subjected to tree construction using a statistical neighbourhood joining distance where the phylogeny test was performed with 500 replicates using the bootstrap method and was subjected to nucleotide type substitution. The maximum composite probability method and Gamma rate (G) were used to determine the diversity, and complete deletion was applied to obtain the full sequence. This was achieved by phyloT and presented in the interactive tree of life (iTOL) and re-confirmed in the program of MEGA X.

3.3 Result

A total of 163 insect pest species belonging to 45 families of 4 orders (Coleoptera, Hemiptera, Lepidoptera, and Diptera) were recorded from Vadodara's agricultural fields (Chapter II), from which 51 pest species were chosen based on its infestation economically important crops. The quality of the genomic DNA indicated an appreciable amplification process with an approximate 720 bp and 620 bp size for COI and 16s rRNA, respectively. The rate of amplification attainment was higher in pests belonging to Coleoptera and Orthoptera over Lepidoptera and Hemiptera. The sequences' similarity was checked using BLAST, and the identity ranged between 95 and 100%. A total of 102 samples were send for sequencing from which 97 sequences were obtained (51 COI and 46 16s rRNA) and out of it 91 sequences (47 COI and 44 16s rRNA) has obtained the Accession Number from NCBI out of which 5 (COI) sequences and 18 (16s rRNA) sequence of species were novel and first time recorded in the NCBI GenBank database (Table 5 and 4). The sequence with its Accession Number submitted to NCBI are presented in respective tables of four Orders.

Order	**Species**	**NCBI Accession No.**
		16s rRNA
Coleoptera	*Monolepta signata*	MT726206
	Aulacophora foveicollis	MT934448
	Oxycetonia versicolor	MT724791
	Myllocerus undecimpustulatus	MT154251
	Niphona picticornis	MT548060
	Chiloloba orientalis	MT725616
	Myllocerus dorsatus	MT934779
	Synhoria maxillosa	MT773627
	Mylabris pustulata	MT934445
	Lanelater fuscipes	MT603573
Orthoptera	*Acrida ungarica*	MT994522
	Choroedocus robustus	MT773626
	Hieroglyphus nigrorepletus	MT994558
	Acrida Conica	MT994552
Hemipera	*Nezara mendax*	MT994695
	Leptocentrus taurus	MT994692
	Coridius janus	MT774554
Lepidoptera	*Olene mendosa*	MT603575

Table 3. 4: Accession No. of first-time recorded species' nucleotide (16s rRNA) in NCBI

Order	**Species**	**NCBI Accession No.**
		COI
Coleoptera	*Chiloloba orientalis*	MT707357
	Myllocerus dorsatus	MT863617
	Synhoria maxillosa	MT765071
	Lanelater fuscipes	MT547190
Hemiptera	*Nezara mendax*	MT707352

Table 3. 5: Accession No. of first-time recorded species' nucleotide (COI) in NCBI

Bioinformatics analysis

GC Content and AT – GC Skew analysis of Order Coleoptera

A genomics analysis order wise GC% resulted that GC content of Coleoptera was recorded maximum in *Lanelater fuscipes* (42.7%) and minimum *Monolepta signata* (30%) (Table 3. 4)

Further, each order sequence was analyzed for GC and AT skews, where the GC skew of Coleoptera has resulted in maximum in *Myllocerus dorsatus* (0.13), whereas the minimum was found in *Lanelater fuscipes* (-0.26) among the selected species. AT skew of Coleoptera was computed the maximum in *Aulacophora indica* (0.16), whereas the minimum was found in *Aulacophora foveicollis* and *Mylabris pustulata* (− 0.18) among the selected species. (Table 3. 4 and Figure 3.1). We also did the AT and GC content of individual orders, where AT bias of 64.7 and GC of 35.3 for COI and 72.2 and 27.7 for 16 sRNA were demonstrated in Coleoptera. The estimated Transition/Transversion bias (*R*) for COI and 16srRNA was found to be 0.962 and 3.114 respectively. The nucleotide frequencies of COI were 30.74% (A), 33.49% (T/U), 18.73% (C), and 17.05% (G) and that of 16s RNA were 36.63% (A), 35.73% (T/U), 14.47% (C), and 13.17% (G). Table 3. 4 represents the Accession number for Order Coleoptera obtained after sequence submitted to NCBI.

Coleoptera	NCBI Accession No.		AT Skew	GC Skew	GC %	AT %
	COI	16s rRNA				
Monolepta signata	MT707359	MT726206	-0.10	0.06	30	70.05
Aulacophora foveicollis	MT863615	MT934448	-0.18	0.02	32.7	67.31
Oxycetonia versicolor	MT707356	MT724791	-0.15	-0.05	33.1	66.95
Altica cyanea	MT707358	MT726041	-0.07	-0.09	33.4	66.58
Aulacophora indica	MT863614	MT934447	0.16	0.06	33.6	66.36
Mylloceruss undecimpustulatus	MT547192	MT154251	-0.14	-0.07	34.3	65.67
Niphona picticornis	MT547191	MT548060	0.15	0.05	34.6	65.42
Chiloloba orientalis	MT707357	MT725616	-0.14	-0.02	34.7	65.27
Myllocerus dorsatus	MT863617	MT934779	0.08	0.13	34.8	65.24
Monochamus scutellatus	MT913389	--------	-0.13	-0.10	34.9	65.13
Protaetia aurichalcea	MT863616	MT934778	-0.09	-0.15	35.2	64.77
Sitophilus oryzae	MT731601	--------	-0.01	-0.16	36.1	63.86
Synhoria maxillosa	MT765071	MT773627	-0.07	-0.08	36.6	63.38
Mylabris pustulata	MT863613	MT934445	-0.18	-0.14	39.3	60.65
Myllocerus viridanus	MT863618	--------	-0.01	-0.10	40.7	59.32
Lanelater fuscipes	MT547190	MT603573	0.004	-0.26	42.7	57.25

Table 3. 6: Order Coleoptera with Accession no., AT-GC- Skew and AT- GC content

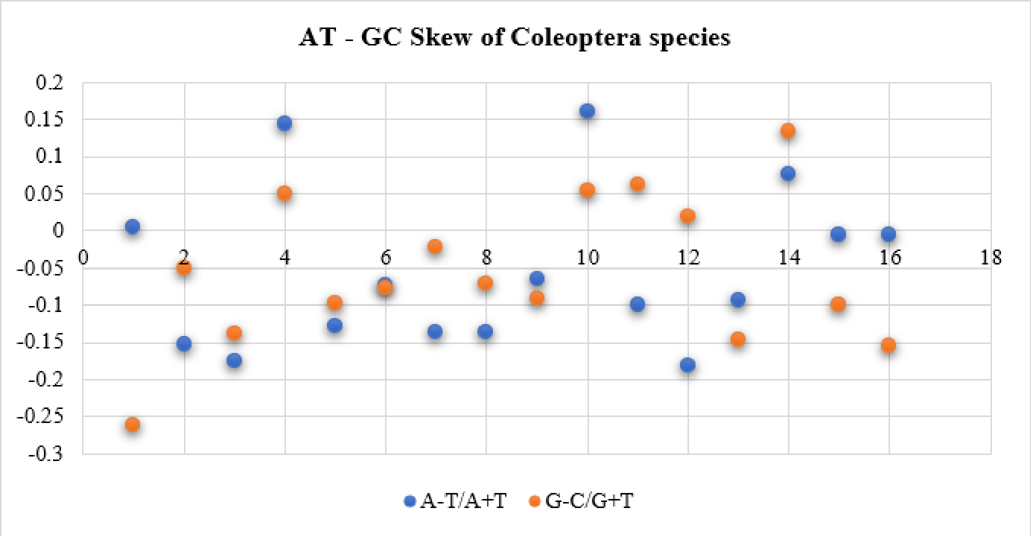

Figure 3. 1: AT - GC Skew Analysis of Coleoptera species

Codon-based analysis of neutrality between Coleoptera sequences was performed, and 16 nucleotide sequences were involved. It eliminated all unclear positions for each pair of sequences. The final dataset included a total of 136 places. A total of 20 significant ($p<0.05$) synonymous and non- synonymous rate was determined between the Coleopteran species for COI, shown in Table 3.7. 16s rRNA, 181 data position for 16 sequences were analyzed, and there was no significance noted in Table 3.8.

Coleoptera species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Lanelater fuscipes																
Oxycetonia versicolor	0.61															
Mylabris pustulata	0.83	0.66														
Niphona picticornis	0.96	0.06	0.77													
Monochamus scutellatus	0.41	0.06	0.34	0.22												
Synhoria maxillosa	0.02	0.03	0.77	0.59	0.03											
Chiloloba orientalis	0.71	0.00	0.99	0.13	0.12	0.01										
Myllocerus undecimpustulatus	0.37	0.90	0.85	0.45	0.00	0.32	0.92									
Altica cyanea	0.52	0.00	0.18	0.04	0.17	0.02	0.00	0.23								
Aulacophora indica	0.18	0.16	0.01	0.61	0.08	0.01	0.29	0.04	0.53							
Monolepta signata	0.68	0.24	0.97	0.02	0.69	0.02	0.79	0.08	0.41	0.22						
Aulacophora foveicollis	0.31	0.07	0.74	0.25	0.92	0.42	0.01	0.34	0.48	0.17	0.01					
Protaetia aurichalcea	0.80	0.00	0.80	0.37	0.11	0.05	0.07	0.40	0.26	0.50	0.06	0.32				
Myllocerus_dorsatus	0.69	0.51	0.00	0.94	0.42	0.92	0.52	0.22	0.62	0.70	0.73	0.16	0.42			
Myllocerus viridanus	0.24	0.01	0.29	0.45	0.36	0.79	0.24	0.92	0.01	0.19	0.16	0.73	0.05	0.12		
Sitophilus oryzae	0.67	0.67	0.80	0.01	0.55	0.66	0.14	0.23	0.21	0.27	0.68	0.69	0.02	0.19	0.40	

Table 3. 7: Codon-based Test of Neutrality analysis between COI sequences of Coleoptera

Coleoptera species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Lanelater fuscipes														
Oxycetonia versicolor	1.00													
Mylabris pustulata	1.00	1.00												
Niphona picticornis	0.54	1.00	1.00											
Synhoria maxillosa	1.00	1.00	1.00	1.00										
Chiloloba orientalis	1.00	1.00	1.00	0.49	1.00									
Myllocerus undecimpustulatus	1.00	1.00	1.00	1.00	1.00	1.00								
Altica cyanea	1.00	1.00	1.00	1.00	1.00	1.00	1.00							
Aulacophora indica	1.00	1.00	1.00	0.54	1.00	1.00	1.00	1.00						
Monolepta signata	1.00	1.00	1.00	0.58	1.00	0.50	1.00	1.00	1.00					
Aulacophora foveicollis	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00				
Protaetia aurichalcea	1.00	1.00	1.00	0.19	1.00	1.00	1.00	1.00	1.00	0.55	1.00			
Myllocerus dorsatus	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.18	1.00	0.43		
Sitophilus oryzae	1.00	1.00	1.00	0.57	1.00	1.00	1.00	1.00	1.00	0.34	1.00	0.20	0.56	

Table 3. 8: Codon-based Test of Neutrality analysis between 16s rRNA sequences of Coleoptera

Similarly, the A+T contents of the 1st, 2nd, and 3rd codons were 56.68, 57.72, and 79.65%, respectively, and G+C contents of the 1st, 2nd, and 3rd codons were 43.32, 42.28, and 20.34% respectively percentage was calculated. The overall mean pairwise distance for Coleoptera was calculated to be 2.94 and 7.46 for COI and 16s rRNA, respectively. The pairwise distance between *Sitophilus_oryzae and Niphona picticornis* (6) was found to be maximum, and *Chiloloba orientalis* and *Oxycetonia versicolor* (0.14) was found to be least for COI (Table 3.9). The pairwise distance of 16s rRNA sequences were found maximum between *Protaetia aurichalcea* and *Myllocerus undecimpustulatus* (11.83) and was found a minimum between *Altica cyanea* and *Oxycetonia versicolor* (3.52) (Table 3.10).

Coleoptera species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Lanelater fuscipes																
Oxycetonia versicolor	2.94															
Mylabris pustulata	3.97	2.78														
Niphona picticornis	2.46	2.17	3.27													
Monochamus scutellatus	2.65	2.59	2.60	1.79												
Synhoria maxillosa	2.10	2.44	2.46	2.21	2.02											
Chiloloba orientalis	2.90	0.14	3.01	2.60	3.08	2.44										
Myllocerus undecimpustulatus	2.91	2.74	2.80	2.52	0.24	2.64	3.21									
Altica cyanea	2.66	0.23	2.75	2.37	3.76	2.50	0.29	3.09								
Aulacophora indica	2.99	1.91	3.04	2.35	2.99	2.91	2.10	3.08	1.88							
Monolepta signata	2.91	2.77	2.59	4.11	1.74	2.58	2.75	1.70	2.62	3.20						
Aulacophora foveicollis	4.08	2.86	2.27	3.03	3.20	3.02	2.18	3.08	4.37	2.76	2.51					
Protaetia aurichalcea	2.44	2.64	3.78	2.80	1.85	2.68	2.56	1.99	2.97	1.77	2.11	2.72				
Myllocerus_dorsatus	4.76	3.12	3.36	4.42	3.88	5.06	2.74	3.84	4.18	3.16	4.88	3.87	4.60			
Myllocerus viridanus	4.14	2.42	2.16	2.08	1.96	2.04	2.83	2.41	2.14	2.27	2.22	2.27	3.78	4.40		
Sitophilus oryzae	4.75	3.25	4.10	6.00	3.88	4.62	4.35	4.09	4.10	2.99	4.62	4.70	4.05	4.61	3.02	

Table 3. 9: Pairwise distance analysis COI sequences of Coleoptera

Coleoptera species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Lanelater fuscipes														
Oxycetonia versicolor	7.02													
Mylabris pustulata	5.14	6.82												
Niphona picticornis	9.66	9.84	8.59											
Synhoria maxillosa	5.39	7.83	5.21	7.41										
Chiloloba orientalis	6.75	5.78	10.06	9.51	7.82									
Myllocerus undecimpustulatus	9.14	9.67	6.36	6.53	9.77	6.76								
Altica cyanea	6.97	3.52	7.27	11.42	7.21	4.96	7.01							
Aulacophora indica	8.69	9.17	5.97	6.28	6.86	6.73	6.88	7.12						
Monolepta signata	7.53	6.92	5.51	7.67	6.16	7.54	9.56	6.08	8.89					
Aulacophora foveicollis	9.19	6.30	5.54	4.47	3.98	6.56	6.28	7.99	5.93	6.32				
Protaetia aurichalcea	7.85	9.56	9.42	4.98	9.64	9.04	6.25	8.59	7.26	6.51	8.25			
Myllocerus dorsatus	6.57	6.25	6.92	9.76	6.94	6.30	11.83	9.29	8.93	10.32	9.68	7.34		
Sitophilus oryzae	8.17	6.65	6.94	6.17	7.12	6.78	7.56	6.90	7.11	7.09	6.65	8.66	9.74	

Table 3. 10: Pairwise distance analysis 16s rRNA sequences of Coleoptera

Phylogenetic analysis of Order Coleoptera

Further, we tried to look into the phylogeny of individual species considering only barcoded species using the NJ method. Bootstrap analysis was used with 500 replicates to understand the phylogeny of different species of insect orders. The study showed that in order Coleoptera, a representative of six families were in the order of (Chrysomelidae + (Cerambycidae + (Curculionidae + Meloidae+ (Scarabaeidae + Elateridae)))). Thus, the result suggests that Chrysomelidae was closest to Cerambicidae, while Curculionidae was closest to Meloidae, and Scarabidae was with Elateridae. A total of 16 species phylogeny was resolved, and the results showed that in the family Chrysomelidae, *Aulacophora foveicollis* and *A. indica* were were found to be monophyletic while it was *paraphyletic* to *Monolepta signata.* Together they had a common ancestor and polyphyletic sister clade of *Alitica cyanea.* While in Cerambycidae, the species *Monochamus scutellate* and *Niphona picticornis* were found to be monophyletic. The Curculionidae family's phylogeny revealed the least distance was in *Millocerus dorsatus*, *M. viridanus*, and *M. undecimpustulatus*, and they were monophyletic to each other. While the closest sister taxa found was *Sitophilus oryzae*, and it was paraphyletic to it. In the family Meloidae, two species were found: *Mylabris pustulata* and *Synhoria maxillosa*, which had the closest distance. While the family Scarabaeidae, three pest species were found where, *Protaetia aurichalcea, Chiloloba orientalis,* and *Oxycetonia versicolor* was monophyletic to each other. In Elateridae, we only found one pest species: *Lanelater fuscipes*, which had its closest relative, *P. aurichalcea* (Figure 3.2).

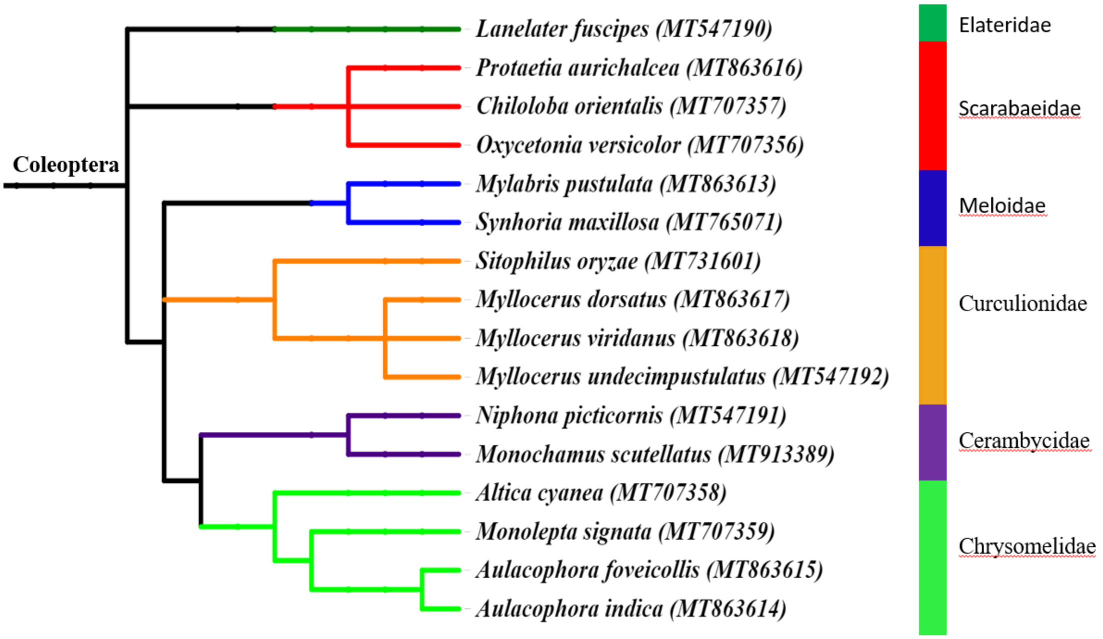

Figure 3. 2 : Phylogenetic tree of order Coleoptera with its species

The following are the sequences (COI and 16s rRNA) which are obtained through Sanger Sequencing

- ***Lanelater fuscipes*** **- MT547190 (*COI*)**

ATACTCGGAGCCCCAGACATAGCATTCCCTCGAATAAATAACATAAGATTCTGACTA
CTTCCCCCATCATTATCCCTTCTTCTGATAAGAAGAATTGTTGAAAACGGAGCCGGG
ACCGGATGAACAGTCTATCCACCCCTATCAGCAAACATCGCCCATAGAGGATCTTC
AGTTGACCTCGCAATTTTTAGCCTCCATTTGGCAGGTATTTCATCCATTCTAGGAGCC
GTAAATTTTATCTCTACTGTAATCAATATACGATCAACAGGAATTTCATTTGACCGT
ATACCCCTATTTGTATGAGCTGTAGCAATTACAGCCCTTCTCCTCCTCCTATCCCTGC
CGGTTCTAGCAGGAGCAATCACTATACTGTTAACAGATCGAAACCTAAAT

- ***Lanelater fuscipes*** **- MT603573 (16s rRNA)**

TAGTCTTGCGGCCAGCTCGCCTGTTTTTCAAAAACATGTCCTTTTGAAAATTATTTAA
GGTCTAATCTGCTCAATGAGGATTTAAATTGCCGCGGTATTCTGACCGTGCGAAGGT
AGCATAATAATTAGTTTCTTTATTGGAAGCTGGAATGAATGATTGGACGAGAAAAA
TTTTGTCTCTGCTTGATTTTTTTTTGAATTTAACTTTTAAGTTAAAAGGCTTAAATGTT
CTTTAAAGACGAGAAGACCCTATAGAGTTTAATATTCCTTCTTTGTTTAGAGTTTTTG
GTATTCGTTGTCTATATTTAGTGGATTATTTGGTTGGGGTGACTGGAAGATTGATAA
AACTCTTCTAAAATTTTGATTACTGATTTGTAAATTTATGATTCTAATTTTTTCAAGT
TTAAATTAAATTACCTTAGGGATAACAGCGTAATTTCTCTTTAGAGTTCTTATTGAA
GGGGAAGTTTGCGACCTCGATGTTGGATTAAAGTTAATGCTGGGCGCAGTTGCTTAG
TTATTAGGTCTGTTCGACTTTTGAAACTTTACATGATCTGAGTTTCAACCGGTAAAA
ACCTAAAGGGTCTAATCTG

- ***Oxycetonia versicolor - MT707356 (COI)***

ATAATTTTTTTCATAGTAATACCAATTATGATTGGTGGTTTTGGAAATTGGCTTGTAC
CCTTAATACTAGGGGCCCCTGATATAGCCTTTCCTCGAATAAATAACATAAGATTTT

GATTATTGCCTCCATCATTAACTTTACTTTTAATAAGAAGAATAGTAGAAAGAGGGG
CAGGAACTGGATGAACTGTCTATCCGCCTCTTTCAAGAAATATTGCTCATAGAGGAG
CATCTGTTGATTTAGCTATTTTTAGACTTCATCTTGCAGGTATTTCATCAATTTTAGG
TGCTGTTAATTTTATTACTACTGTAATTAACATACGATCTGCAGGAATAACTTTTGAT
CGAATACCTTTATTTGTTTGATCTGTTGCTCTTACTGCCTTATTACTTTTATTATCCTT
ACCTGTCTTAGCAGGAGCTATTACAATACTACTTACAGATCGAAATATTAATACTTC
CTTCTTTGACC

- ***Oxycetonia versicolor*** **- MT724791 (16s rRNA)**

TCAGATCATGTAAAATTTTAAAGGTCGAACAGACCTAACCTTTTAGCTTCTACACCA
AAAGTTAATTTTAATCCAACATCGAGGTCGCAAACTCTTTTTTCGATAAGAACTCTC
AAAAAAAATTACGCTGTTATCCCTAAGGTAATTTAATCTTTTAATCGTAAATAACGG
ATCAACTACTCATAAATCAATGTAAATATACAAAAAAAGTTTCACTAATTTTCCTGT
CACCCCAACAAAATAATTATTAATATATAAATTCAAAACATTCTAAATTATAAATAT
ACTAATAAATATAAAACTCTATAGGGTCTTCTCGTCTTTTAAAATCATTTAAGCTTTT
TTACTTAAAAATAAAGTTCTAATTAAAATTAAATTGAGACAGTCATTTTCTCGTCCG
ACCATTCATACCAGCTTTCAATTAAAAAACTAATGATTATGCTACCTTTGCACGGTC
AAAATACCGCGGCCATTCAAATACTCATTGGGCAGGCCAGACTTTAAATTATACTCA
AAAAGACATGTTTTTG

- ***Mylabris pustulata*** **- MT863613 (COI)**

TACAATGTTATTGTTACAGCCCATGCATTTATCATAATTTTCTTTATGGTAATACCTA
TCATGATTGGCGGCTTTGGGAATTGGCTTGTACCCTTAATGTTAGGGGCCCCTGATA
TAGCCTTTCCTCGTATAAACAATATAAGATTTTGATTACTTCCACCTTCATTAACACT
TCTAATCATAAGAAGAATTGTAGAAAATGGTGCAGGAACTGGATGAACGGTGTACC
CTCCACTCTCATCTAATATTGCCCATGATGGTTCTTCTGTAGATTTAGCCATCTTTAG
CCTCCACTTAGCCGGGGTTTCTTCTATCCTGGGAGCAGTCAATTTCATTTCTACTGTC
ATCAACATACGCCCAGCTGGAATAACATTCGATCGTATACCCTTATTTGTATGAGCA
GTTGTTATTACTGCTCTCCTCCTTCTATTATCATTACCTGTCCTTGCAGGTGCAATTA

- ***Mylabris pustulata*** **(16s rRNA)**

CCAGTCCCCTTAGGCCACGTCCCCGGTCCGGTTGAACTCAGATCATGTAAAGTTTCA
AAAGTCGAACAGACTCAATATCCTAGCTTCTGCACCAAGAATTAACTTTAATCCAAC
ATCGAGGTCGCAATCCCTTTCATCGATTAGAACTCTCTGAAAAGATAACGCTGTTAT
CCCTAAGGTAATTTTTTCTTTTAATCTTCAATAAAGGATCAATTAATCATTAATCAAT
GTATAAACATAAAGAAGTTCATTCAATTCTATTGTCACCCCAACCAAATAGAAATCA
ATACAAAAAACTTAAATCCAAAATCCTTATATATTTTAGTCTATTAAACTCTATAGG
GTCTTCTCGTCTTTATATAAAATTTAAGCTTTTTTACTTAAAAATAAAATTCAATTCA
AATTATTCATGAGACAGTTATTTCCTCATCCGACCATTCATACCAGCTTCCAATTAA
AAAACTAATGATTATGCTACCTTTGCACAGTCAAAATACTGCGGCTATTCAATTAAT
CATCATTGAGCAGGCCAGACCTTAAACAATTCACAAAAAGCCATGTTTTTGTAAAAC
AGGGGAAAGGGTTTGAAAACGGACT

- ***Niphona picticornis*** **- MT547191 (COI)**

ATAAAATTTACAGCGCCTAGAATAGAGGAGACTCCAGCTAAATGAAGACTAAAAAT
AGCTAAATCGACTGAAGCTCCATTATGAGCAATATTTGCTGCTAAAGGGGGATATA
CAGTTCATCCAGTTCCAGCCCCCTTTTCTACAATTCTTCTTATTAAAAGAAGGGTTAA
TGAAGGAGGTAAAAGTCAAAATCTTATATTATTTATTCGAGGGAAGGCTATGTCTGG
AGCTCCTAATATTAAAGGAACTAATCAATTACCAAACCCGCCAATTATAATTGGCAT

AACTATGAAAAAAATTATGATGAAGGCGTGAGCAGTAACGATAACATTATAAATTT
GGTCATT

- ***Niphona picticornis - MT548060 (16s rRNA)***

TCGCCTGTTTTTACAAAAACATGGCCTTTTGATTATAATTTAAGGTCTAGTCTGCCCA
CTGATTAGTTTTAAAGGGCCGCGGTATTTTGACCGTGCTAAGGTAGCATAATCATTA
GTTTTTTGATTGAAAGCTGGTATGAATGGCTGGATGAGAAATATACTGTCTCTGTTT
AATTTTAAATTAATTTTATTTTTAAGTAAAAAAGCTTAAATTTATTTAAAAGACGAG
AAGACCCTATAGAGTTTTATAAATGAAAAATTTTTAAGATTTAGAAATATCAATTAG
AAATTTTGAATTTATTTGATTGGGGTGATTGAAAAATTTAAGAAACTTTTTTTATATT
TGAACATTTATATATGAAAAATTGATCCATAATTAATGATTATAAGATTAAATTACC
TTAGGGATAACAGCGTAATTTCTTTTAAGAGTTCTAATCGATAAAGAAGATTGCGAC
CTCGATGTTGGATTAAAATAGAATATTTGGTGTAGAAGCTAAATTATTTGGTCTGTT
CGACCATTAAAATTTTACATGATCTGATTTC

- ***Monochamus scutellatus*** **- MT913389 (COI)**

ATAGTAGGAACATCATTAAGTCTACTCATCCGATCTGAGTTAGGAAACCCTGGAACT
CTAATTGGTAATGACCAAATTTATAATGTTATCGTTACTGCTCACGCCTTCATCATAA
TTTTTTTCATAGTTATGCCAATTATAATTGGCGGGTTTGGTAATTGATTAGTTCCTTT
AATATTAGGAGCTCCAGACATAGCCTTCCCTCGAATAAATAATATAAGATTTTGACT
TTTACCTCCTTCATTAACCCTTCTTTTAATAAGAAGAATTGTAGAAAAGGGGGCTGG
AACTGGATGAACTGTATATCCCCCTTTAGCAGCAAATATTGCTCATAATGGAGCTTC
AGTCGATTTAGCTATTTTTAGTCTTCATTTAGCTGGAGTCTCCTCTATTCTAGGCGCT
GTAAATTTTATTACAACTGTAATCAATATACGACCAAGAGGAATGTCCTTTGACCGT
TTACCTTTATTTGTTTGAGCAGTGAAAATTACAGCTATTCTTCTTCTTTTATCCCTTCC
AGTTCTTGCTGGAGCAATTACTATACTTTTAACTGATCGAAATTTAAATACTTCATTC
TTTGACCCA

- ***Synhoria maxillosa*** **- MT765071 (COI)**

GGAGCCTGATCAGGAATTCTAGGAACCTCACTAAGAGTTCTAATTCGACTAGAACTT
GGTAATCCTGGATCATTCATTGGAAACGATCAAATTTATAATGTAATTGTTACAGCC
CATGCTTTCGTAATAATTTTCTTTATAGTTATACCGATTGTTATTGGAGGATTTGGAA
ATTGACTTGTACCATTAATGCTAGGAGCCCCTGATATAGCATTCCCACGAATAAACA
ATATAAGATTTTGATTACTACCCCCCTCCCTTACTCTTCTCATTATAAGAAGAATTGT
AGAAAATGGAGCTGGTACTGGATGAACTGTTTATCCCCCACTTTCATCCAATATTGC
TCATAGAGGATCTTCTGTAGACCTAGCTATTTTTAGTCTTC

- ***Synhoria maxillosa*** **- MT773627 (16s rRNA)**

ATTTTCCCCTTTTATTCTCTTTCCGGGTGAACTCAGATCATGTACAGTTCTTAACGTC
GAACAGACTCAAATTATTAGCTTCTACCCCAATTTTTAACTTTAATCCAACATCGAG
GTCCCAATCTCTCTTATCGATTAGAACTCTCCAAGAAAATTACGCTGTTATCCCTAA
GGTAATTTATTCTTATAATCCATAAATTAGGATCATTTATTCATAAATCAATGATTTT
CAAAATAGAAGTTAAATCAATTCAAGTGTCACCCCAACAAAATAAACTAAAAATTA
AATAAAATAAAACCCTCAAAAATTTAACTTATAAATTTATTAAACTCTATAGGGTCT
TCTCGTCTTTTTAAAAAATTTAAGCTTTTTTACTTAACAATAAATTTCGATTTAATTA
AAAAGCAGCACAGTTTATTTCCTTATTCAGCCATTCATTCCAGCTTTCAATTAAAAA
ACTAATGATTATGCTACCTTTGTACAGTCAAGATACTGCAGCTATTTAAATAATCAT
GGAGCAGGCTAGACTTTAGATAATTCTCAAAAAGACATGTTTTTTT

- ***Chiloloba orientalis*** **- MT707357 (COI)**

ATAATTTTCTTCATAGTGATGCCAATTATAATTGGCGGATTTGGTAATTGGCTTGTTC
CTCTAATACTAGGGGCCCCAGATATAGCCTTTCCTCGAATAAATAACATAAGATTTT
GATTATTACCCCCGTCATTAACTTTACTTTTAATGAGTAGAATAGTTGAAAGAGGTG
CTGGAACAGGATGAACAGTATACCCCCCTCTTTCTAGAAATATCGCCCATAGAGGTG
CCTCTGTTGATTTAGCTATTTTCAGTTTACATTTAGCTGGAATCTCTTCTATTTTAGG
AGCTGTTAATTTTATTACTACTGTTATTAACATACGATCAACAGGAATAACATTTGA
TCGAATACCTTTATTTGTTTGATCTGTAGCTTTAACTGC

- ***Chiloloba orientalis*** **- MT725616 (16s rRNA)**

CTCAGATCATGTAAAATTTTAAAGGTCGAACAGACCTAACCTTCTAGCTTCTGCACC
AAAAGTTAATTTGTAATCCAACATCGAGGTCGCAAACTTTCTTTTCGATATGAACTC
TCAAAAAAAAGTACGCCTGTTATTCCCTAAGGTAATTTAATCTTATAATCAAAAATA
ACGGATCAAATAATCATAAACTAATGTTAAAATATAAAAAAAGCTGCACCAATTTT
TCTGTCACCCCAACAAAATAATTATATTAATATAAATTCCAAACATACTAAATTATT
TATATTTAATAATTATAAAACTCTATAGGGTCTTCTAGTCTTTTAAACTTATTTAAGC
TTTTTTACTTAAAAATAAAATTATAACTAAAATTAAATTGAGACAGTTATTTTCTCGT
CCAACCATTCATTCCACCTTTCAACTAAAAAACTAATGATTATGCTACCTTTGCACG
GTCAAAATACACGCGTGCGCATTCAAATCCTCATTGGGCAGGTCAGACTTTAAATTA
TTATCAAAAAGACATGTTTTTGAT

- ***Myllocerus undecimpustulatus*** **- MT547192 (COI)**

ATAGTAGGAACCTCCTTAAGAATTTTAATTCGAACAGAACTAGGAAACCCGGGTTCT
TTAATTGGTGACGATCAAATTTATAACGTAATTGTTACAGCCCATGCTTTCATTATA
ATTTTCTTTATAGTTATACCTATAATGATTGGAGGATTTGGGAATTGACTTGTTCCTT
TAATATTAGGAGCACCTGATATAGCTTTTCCTCGTCTTAACAATATAAGATTTTGACT
TTTACCCCCATCTCTCTCCCTTCTTCTTATAAGAAGAATCGTAGATAAGGGGGCAGG
TACTGGTTGAACAGTTTACCCACCTTTATCAGCTAATATTGCTCATGAAGGATCTTCT
GTTGACCTAGCAATTTTTAGACTCCATATAGCAGGAGTTTCTTCTATCCTAGGAGCC
GTAAATTTTATTTCTACAGTAATTAATATACGTCCATCAGGAATATCTTTTGATCGAC
TACCTCTATTTGTGTGGGCCGTTAAAATTACAGCTATTTTACTTCTTTTATCTTTACC
AGTTTTAGCTGGAGCTATTACTATACTATTGACAGATCGTAATATTAATACAT

- ***Myllocerus undecimpustulatus*** **- MT154251 (16s rRNA)**

TCCGGTTGAAACTCAGATCATGTAAAATTTTAAAGGTCGAACAGACCTAAAGTTTCA
GCTTCTACACCAAAATTTAATTTTAATCCAACATCGAGGTCGCAATCTTTTTTATCGA
TTTGAACTCTCCAAAAAAATTACGCTGTTATCCCTAAGGTAATTTTATCTTTTAATCT
CAAAAAAAGGATCATTTACTCATAAATTAATGTTTAAATAAAAAAAAAGTTTATTA
AATTTTTCAATCACCCCAATTAAATTAATTTTTTTAATAAAAATAATAAATACTAAA
AATTACATTAAAAGAATTAATAAAACTCTATAGGGTCTTCTCGTCTTCTATAAATAT
TTAAGCTTTTTAACTTAAAAATAAAATTCAATAAAATTTTTATAGAGACAGTCATTT
TCTCATCCAACCTTTCATTCCAGCTTTCAATTAAAAAACTAATGATTATGCTACCTTT
GCACGGTCAAAATACCGCGGCCATTTAACTTATCATTGGGCAGGCTAGACCTTAAAT
TATAATCAAAAGGGCCATGTTTTTGAAAAAAAGGCGGG

- ***Altica cyanea*** **- MT707358 (COI)**

ATAATTTTCTTTATAGTAATACCCATAATAATTGGAGGTTTTGGAAACTGATTAGTC
CCTTTAATAATTGGGGCCCCTGATATAGCTTTCCCTCGAATAAATAATATAAGATTC
TGGTTACTCCCGCCATCAATTTTTTTATTATTAATAAGAAGGTTAACAGAAAGAGGA

GCAGGAACTGGATGAACGGTTTATCCCCCCCTATCATCCAATCTTGCCCATAATGGG
CCATCTGTGGATTTAGCTATTTTTAGCCTTCATTTAGCAGGAATTTCATCAATTCTAG
GTGCTATTAATTTTATTACTACAATAATCAATATACGACCTAAAGGAATATCTATAG
ATCAAATACCTTTATTTGTATGAGCTGTCCTTATTACAGCAATTCT

- ***Altica cyanea* - MT726041 (16s rRNA)**

CTCAGATCCTGTAAAATTTTTAAAGTCGAACAGACTTAATCTTTTAGCTGCTACACC
AAAATTTAATTTTAATCCAACATCGAGGTCGCAATCTTTATTATCGATTTGAACTCTC
CAATAAAATTACGCTGTTATCCCTAAGGTAATTTAATCTTATAATCATTTATATGGAT
CAAAAATATATAAATTTATAAATAAAAATAAAAAAAGTTTATTAAATTTTTCAATCA
CCCCAATTAAATTTTTATAAAAAATATAATTTTAAATTCTAAACATTATATAAAATA
AAAAAAATCAAACTCTATAGGGTCTTCTCGTCTTTTAAAAAAATTTAAGCTTTTTAA
CTTAATAATAAAATTTTAATTTAATTATCAAAAGACAGTTTTTTTTCATCAAACCCT
TCATTCTAGCTTTCAATTAAAAAACTAATGATTATGCTACCTTAGCACAGTCAAAAT
ACTGCGGCCATTTAAATTCTCAGTGGGCAGGTAAGACCTAAAATTATCTTTCAATAG
GCCATGTTTTTGAAAAACAGGCGA

- ***Aulacophora indica* - MT863614 (COI)**

TCTGTTAATAATATTGTAATGGCTCCAGCTAAAACTGGTAGAGATAATAATAATAAT
ACAGCTGTAATAACAACAGCTCATACAAATAGTGGTATTCGGTCTAGGGTTATTCCT
TTAGGACGCATATTAATTACGGTTGTGATAAAATTAATTGCTCCTAAAATTGAAGAA
ATTCCGGCTAAATGTAAACTGAAAATTGCTAAATCAACAGAAGAACCTCCATGGGC
AATATTTGAAGAAAGAGGAGGGTACACAGTTCAACCAGTTCCAGCCCCTCTTTCAA
CAACTCTACTTATAATTAATAAAAATAGAGAAGGAGGAAGTAAT

- ***Monolepta signata* - MT707359 (*COI*)**

ATAATTGGTGGATTCGGAAATTGGTTAGTTCCATTAATAATTGGAGCTCCTGATATG
GCTTTTCCTCGAATAAATAATATAAGATTTTGATTGTTGCCCCCATCATTATTTTTAT
TAATTATAAGAAGAATTGTTGAAAGAGGAGCAGGGACAGGATGAACTGTTTATCCT
CCATTATCATCTAATATTGCTCATGGAGGAGCTTCTGTAGATTTAGCAATTTTTAGAC
TTCATTTAGCAGGAATTTCTTCAATTTTAGGAGCTATTAATTTTATTACAACTATTAT
TAATATACGTCCAAGAGGAATATCTATAGATCGTATACCTTTATTTGTTTGAGCTGTT
ATAATTACAGCTATTTTACTTCTATTATCATTACCAGTATTAGCTGGAGCAATTACAA
TATTATTAACAGATCGAAACTTAAATACTTC

- ***Monolepta signata* - MT726206 (16s rRNA)**

CAAAAACATGTCTTTTTGTAAAAATATAAAGTCTAACCTGCCCTATGAAAATTTAAA
TGGCCGCGGTATATTAACCGTGCTAAGGTAGCATAATCATTAGTTTTTTAATTGAAA
GCTGGAATGAAGGGTTGGATAAAAAAATAACTGTCTCTATAAAAATGATTGAATTT
TATTTTTAAGTTAAAAAGCTTAAATTTTTTTAAAAGACGAGAAGACCCTATAGAGTT
TAATATAAAATCTAATTATTTTTTTTAGTATTTATTTAAATAATTAGATTTTATATTTA
GTTGGGGTGATTGAAAAATTAAATGAACTTTTTTTGTATAAAAATATATTTTTATAA
ATTATTGATCCAAAAATTTTGATTATAAGATAAAATTACCTTAGGGATAACAGCGTA
ATTTTATTAGAGAGTTCTTATTGATAATAAAGATTGCGACCTCGATGTTGGATTAAA
ATTTATAATTGGTGCAGAAGCTATATTATTAAGTCTGTTCGACTTTTAAAATTTTACA
TGATCTGAG

- ***Aulacophora foveicollis* - MT863615 (COI)**

GGAAATGATCAAATTTATAATGTAATTGTCACTGCCCATGCATTCATTATAATTTTTT
TTATAGTTATACCAATTATAATCGGAGGGTTTGGAAACTGATTAGTACCCTTAATAA

TTGGGGCTCCTGATATAGCTTTCCCTCGTATAAATAATATAAGATTTTGATTACTTCC
TCCTTCTCTATTTTTATTAATTATAAGTAGAGTTGTTGAAAGAGGGGCTGGAACTGG
CTGAACTGTGTACCCTCCTCTTTCTTCAAATATTGCCCATGGAGGTTCTTCTGTTGAT
TTAGCAATTTTCAGTTTACATTTA

- ***Aulacophora foveicollis*** **(16s rRNA)**

GCTAGACTTTTAAATTAAAAATTCCCGGTTGAAATCAGATCATGTAAAATTTTAAAA
GTCGAACAGACTTAATTATTTAGCCACTACACCAAATAGAAATTTTAATCCAACATC
GAGGTCGCAATCTTTATTATCAATATGAACTTTCTAATAAAATTACGCTGTTATCCCT
AAAGTAATTTTATCTTATAATCAATAATATTGGATCATAAATTTATAAATTTATATA
AAAATATAAAAAAAGTTAATTAAATTTTTTAATCACCCCAACCAAATAAACATTTCA
AAATAAAAAATTAATTCTAAAAAAATATTTTAAAATATTTATAAAACTTTATAGGGT
CTTCTCGTCTTTTAATTACATTTAAACTTTTTTACTTAAAAATAAAATTTTAATAAAA
TTAAACTAAAAAAGTTATTTTTTCATCCAACCCTTCATTCCAGCTTTCAATTAAAAAA
CTAATGATTATGCTACCTTAGCACGATCAAAATATCGCGGCCATTTAAACTTCATTG
GGCAGGCTAGACTTTAAATTTTAAACAAAAAGACATGTTTTTGAAAAACAGGCGAA
ATTTGAACAGACTTAATT

- ***Protaetia aurichalcea*** **- MT863616 (COI)**

ATCCGAGCCGAACTAGGAAACCCTGGATCTTTAATTGGCGACGACCAAATTTATAAT
GTAATTGTTACAGCTCACGCTTTCATCATAATTTTTTTCATAGTAATACCAATCATAA
TTGGTGGATTTGGAAATTGACTTGTCCCTCTAATACTTGGGGCACCAGATATAGCTT
TCCCACGAATAAACAACATAAGTTTTTGACTTCTCCCCCCTTCACTAACTTTACTTCT
AATAAGAAGAATAGTTGAAAGAGGAGCAGGAACTGGATGAACTGTTTATCCCCCCT
TATCTAGAAATATTGCTCATAGTGGAGCTTCAGTAGACCTAGCTATTTTCAGATTAC
ATCTAGCTGGAATTTCCTCAATTTTAGGTGCTGTTAATTTTATTACTACTGTAATTAA
TATACGATCAACAGGAATAACATTCGATCGAATACCTTTATTTGTTTGATCTGTTGCT
TTAACTGCTTTACTTCTTCTACTTTCACTTCCTGTCTTAGCTGGAGCAATTACTATACT
TTTAACTGATCGAAATATTAATACCTC

- ***Protaetia aurichalcea*** **(16s rRNA)**

TTCGCCCTGTTTTTCAAAAACATGTCTTTTCTGATTATAATTTAAAGTCTGACCTGCC
CAATGTAGGAGTTGAATGGCCGCGGTATTTTGACCGTGCAAAGGTAGCATAATCATT
AGTTTTTTAATTGAAAGCTGGAATGAACGGTTGGACGAGAAAATAACTGTCTCAAT
ATAATTTAAATTAGAATTTTATTTTTAAGTAAGAAAGCTTAAATATATTTAAAAGAC
GAGAAGACCCTATAGAGTTTTATATTTATTATTATATTTATTATTAAGTATGGTATAA
GTTTTTATAATAATAAATATTTTGTTGGGGTGACAGGAAAATTGAATGAACTTTTTTT
ATATTATTAACATTGATTTATGAGTGTTTGATCCATTATTAATGATTAAAAGATTAA
ATTACCTTAGGGATAACAGCGTAATTTTTTTTGAGAGTTCTTATCGAAAAAAGAGTT
TGCGACCTCGATGTTGGATTAAAATAAACTTTTGGTGTAGGAGCGAGAAAGTTGGG
TCCTGTTCGACCTAATTATAAATTTTACTTGATCTAGTTTCGGGGGGGAGG

- ***Myllocerus dorsatus*** **- MT863617 (COI)**

TGATGTATTAATATTACGATCAGTTAAAAGTATGGTGATTGCTCCTGCTAATACTGG
GAGGGAAAGAAGAAGAAGAATGGCAGTAATTTTTACAGCTCATACAAATAAAGGA
AGACGGTCAAAAGATATTCCTGAAGGACGTATATTAATAACTGTAGAGATAAAATT
AACTGCTCCTAGGATTGAAGATACCCCAGCTATATTAAGACTAAAAATTGCTAGATC
TACAGAAGATCCTTCATGGGCGATGTTAGCTGATAAAGGTGGGTAAACCGTTCATCC
TGTTCCTGCTCCTTTATCAACAATTCTTCTTATTAATAGAAGAGATAATGAAGGGGG

TAAAAGTCAGAATCTTATATTATTAAGACGTGGAAAAGCTATATCTGGTGCCCCTAA
TATTAAAGGTACTAGTCAGTTTCCGAATCCTCCGATTATTATGGGTATAACTATAAA
AAAAATTATAATAAAAGCATGTGCTGTTACAATAACGTTGTAAATTTGGTCGTCTCC
AATTAGGGATCCTGGGTTTCCTAATTCA

- ***Myllocerus dorsatus* (16s rRNA)**

CCTTCTCCCCCCTGGTTTTTTCACAAAACATGCGCCTTTTGAGAATAATTTAAGGTCT
GGGCCTGCCCAATGCATAAGTTTAATGGCCGCGGTATTTTGACCGTGCAAAGGTAGC
ATAATCATTAGTTTTTTAATTGAAAGCTGGTATGAAAGGTTAGATGAAAAAATTTCT
GTCTCTGTTTAATTTAAATTGAATTTTATTTTTAAGTTAAAAAGCTTAAATAATTATA
GAAGACGAGAAGACCCTATAGAGTTTAATTAAAAGTTTTATTTAATTTTTAAGGATT
TATTGTTTTTAGTAAAGAATTTAATTTAATTGGGGTGATTGAAAAATTTAAAAAACT
TTTTTTAAAGATAAACATTGATTTATGAAAATTTGATCCTTTTTTTAAGATAAAAAG
ATAAAAATACCTTAGGGAGAACAGCGTAATTTTTTTGGAGAGTTCAAATCGATAAA
AAAAATTGCGACCTCGATGGTGGATAAAAACTGAATTTTGGTGTAGTAAGCGAGAT
AGGTTAAGTCTGTCCTACATTTAAAAAGGTATGTGTTTTTAATAGCAAGCCCGAGGA

- ***Myllocerus viridanus* - MT863618 (COI)**

GATAAAGGAGCAGGAACGGGGTGAACAGTCTACCCGCCCCTTTCAGCTAATATCGC
CCATGAGGGCTCCTCAGTAGATTTAGCAATTTTCAGCCTACATATAGCAGGGGTATC
ATCAATTCTAGGTGCAGTAAATTTTATTTCCACAGTAATTAATATACGCCCAATAGG
AATATCTTTTGATCGACTACCTCTATTTGTATGAGCGGTAAAAATTACAGCAATTCT
CCTACTCCTTTCATTACCAGTTCTTGCAGGAGCAATTACTATACTTCTCACAGATCGA
AATATTAATACATCTTTCTTTGACCCAGCAGGGGGAG

- ***Sitophilus oryzae* - MT731601 (COI)**

TACCAATTATAATTGGAGGATTTGGAAACTGATTAATCCCATTAATATTAGGAGCCC
CAGATATAGCATTCCCCCGTTTAAATAATATAAGATTTTGATTACTTCCACCCTCCTT
AACTCTTTTACTAATAAGAAGATTTATTGAAAAGGGAGCAGGAACAGGATGAACCG
TCTACCCCCCGCTCTCATCCAATATTGCCCATGAAGGAGCTTCTGTTGATCTGGCCAT
TTTCAGTTTACATATAGCAGGAATTTCATCTATTCTAGGAGCTATTAATTTTATTACA
ACAGCCTATAATATACGACCCTCAGGAATATTAT

- ***Sitophilus oryzae* (16s rRNA)**

CTTTTTGATTATAATTTAAAGTCTAACCTGCCCAATGATAAAATTTAAATGGCTGCG
GTATTTTGACCGTACAAAGGTAGCATAATCATTAGTTTTTTAATTGAAAGCTGGAAT
GAAAGGTTGCATGAAAAAATGACTGTCTCTATTTAAATTTAATTGAATTTTATTTTTA
AGTAAAAAACGCTTAAATTTTTCTAAAAGACGAGAAGACCCTATAGAGTTTTATAA
ATTTATTATAAGGAATTTTTAGGATTTAAAATAATTTATATTTAATTTATTTAATTGG
GGTGATTGAAAAATTTGTTAAACTTTTTTATTTATATTACATTAATTTATGAGTTTTT
GATCCTTACTTTAAGATTAAAAGATAAAATTACCTTAGGGATAACAGCGTTATGCGC
ATTGAGAGTTCAAATCGAAAAAAAAGATTGCGACCTCGATGTTGGATTAAAATAAA
TTTTTGGTGTAGAAGCTAAATTTTTGATTATAATTTAAAGTCTAACCTGCCCAATGAT
AAAATTTAAATGGCTGCGGTATTTTGACCGTACAAAGGTAGCATAATCATTAGTTTT
TTAATTGAAAGCTGGAATGAAAGGTTGCATGAATGCATGACTGTCTCTATTTAAATT
TAATTGAATTTTATTTTTAAGTAAAAA

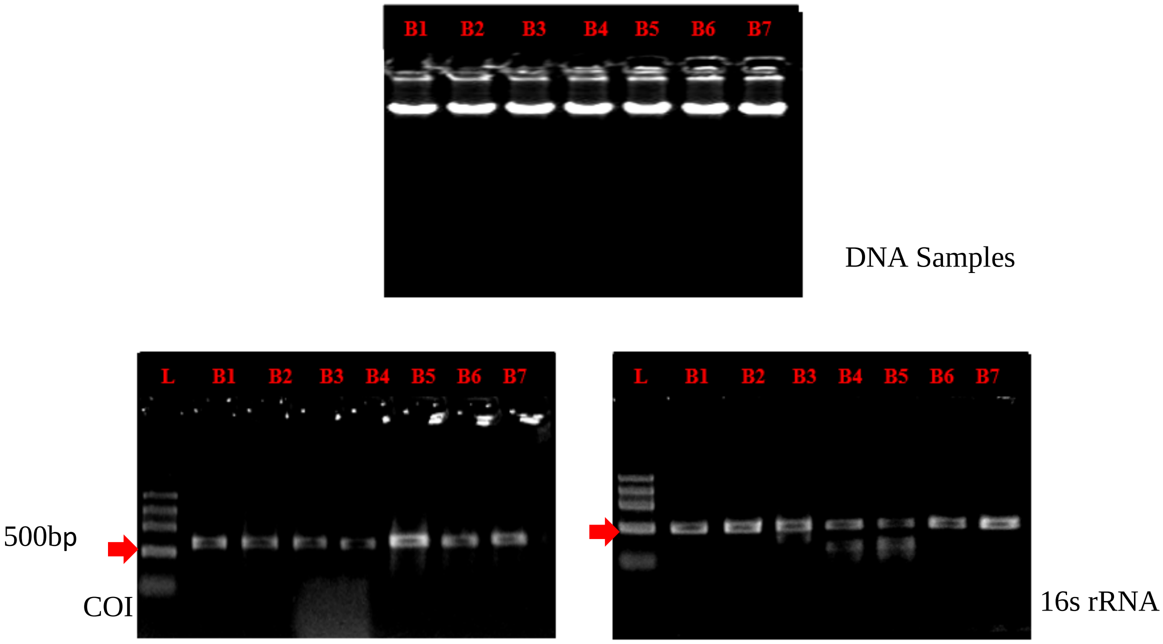

Figure 3. 3: Electrophoresis gel image of DNA, PCR product of COI and 16s rRNA of Coleoptera species representatives

GC Content and AT – GC Skew analysis of Order Orthoptera

The order wise genomic analysis of GC% of Orthoptera was shown highest with *Choroedocus robustus* (37.6%) and lowest with *Aiolopus thalassinus* (31.3%) and AT content was maximum with *Acrida exaltata* (68.7%) and minimum with *Oxya hyla hyla* (62.4%). Table 3. 11 represents the Accession number for Orthoptera obtained after sequence submitted to NCBI. Further, each order's sequence was analyzed for GC and AT skews, where the GC skew of Orthoptera was calculated the maximum in *Aiolopus thalassinus tamulus* (0.21), whereas the minimum was seen in *Hieroglyphus nigrorepletus* (-0.14) among the selected species. Computed AT skew was maximum in *Atractomorpha sinensis* (0.06), whereas the minimum was found in *Choroedocus robustus* (-0.11) among the selected species. (Table 3.11 and Figure 3. 4).

Orthoptera	NCBI Accession No.		AT Skew	GC Skew	GC %	AT %
	COI	16s rRNA				
Acrida ungarica	MT859408	MT994522	-0.06	0.15	36.6	63.3
Choroedocus robustus	MT707353	MT773626	-0.11	-0.07	37.6	62.4
Aiolopus thalassinus	MT731611	MT994517	0.05	-0.10	31.3	68.7
Aiolopus thalassinus tamulus	MT859409	MT994525	-0.01	0.21	35.8	64.1
Trilophidia annulata	MT859410	MT994526	-0.10	-0.11	35.7	64.3
Acrida willemsei	MT547193	MT102740	0.01	-0.11	33.3	66.6
Acheta domesticus	MT859331	MT994502	-0.02	-0.05	33.1	66.9
Hieroglyphus nigrorepletus	MT859411	MT994558	-0.04	-0.14	36.5	63.5
Chorthippus curtipennis	MT709100	--------	-0.04	-0.02	33.1	66.8
Acrida Conica	--------	MT994552	-0.04	-0.05	34.9	65.0
Locusta migratoria	--------	MT994524	-0.01	-0.07	33	67.0
Hieroglyphus banian	--------	MT994520	-0.07	-0.08	35.7	64.3
Acrida exaltata	--------	--------	-0.03	0.07	32.7	67.3
Oxya hyla hyla	MT994515	--------	-0.05	-0.04	31.5	68.4
Atractomorpha sinensis	MT994515	MT994587	0.6	-0.11	32.7	67.2

Table 3. 11: Order Orthoptera with Accession no., AT-GC- Skew and AT- GC content

The AT and GC content of individual orders, where in Orthoptera, demonstrated AT bias of 66.2 and GC of 33.8 for COI and 68.6 and 31.4 for 16s rRNA. The estimated Transition / Transversion bias (*R*) for COI and 16s rRNA was found to be 0.841 and 0.473, respectively. The nucleotide frequencies of COI were 31.76% (A), 33.74% (T/U), 17.62% (C), and 16.88% (G) and that of 16s rRNA were 38.50% (A), 38.78% (T/U), 12.53% (C), and 10.19% (G).

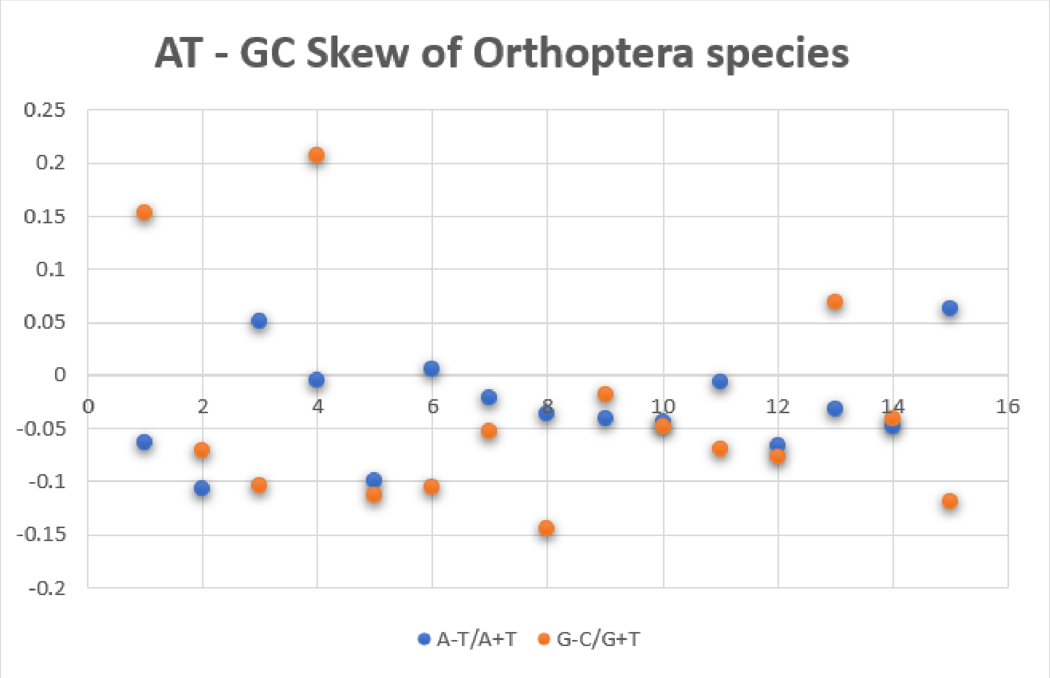

Figure 3. 4: AT - GC Skew Analysis of Orthoptera species

Nei-Gojobori analysis of the Orthopteran sequence involved 15 nucleotide sequences. All ambiguous positions were removed for each sequence pair for considering the probability of synonymous and non- synonymous. There was a total of 287 positions in the final dataset. A total of 20 significant mutations ($p<0.05$, $p<0.01$) rate was found for COI (Table.3.12). And for 16s rRNA, 78 positions were validated where the significance ($p<0.05$, $p<0.01$) was reported among the seven species Table 3.13.

Orthoptera species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Acrida ungarica															
Choroedocus robustus	0.112														
Aiolopus thalassinus	1.000	1.000													
Aiolopus thalassinus tamulus	0.010	0.254	0.304												
Trilophidia annulata	0.001	0.157	0.555	0.088											
Acrida_willemsei	0.389	1.000	1.000	0.311	0.204										
Acheta domesticus	0.208	0.372	1.000	0.012	0.214	0.045									
Hieroglyphus nigrorepletus	0.304	0.002	1.000	0.251	0.075	0.139	0.001								
Chorthippus curtipennis	0.171	1.000	1.000	0.123	0.523	1.000	0.064	1.000							
Acrida Conica	0.178	1.000	1.000	0.406	1.000	0.221	0.000	0.180	1.000						
Locusta migratoria	0.204	1.000	1.000	0.148	0.563	1.000	0.122	1.000	0.000	1.000					
Hieroglyphus_banian	0.191	1.000	1.000	0.092	1.000	1.000	0.034	1.000	0.000	1.000	0.000				
Acrida_exaltata	0.085	1.000	0.335	0.278	0.298	0.412	0.370	1.000	1.000	0.483	1.000	1.000			
Oxya_hyla hyla	0.089	0.059	0.330	0.092	0.021	0.001	0.006	0.023	1.000	0.003	1.000	1.000	1.000		
Atractomorpha sinensis	0.374	0.082	1.000	0.129	1.000	0.007	0.002	0.001	1.000	0.006	1.000	1.000	0.315	0.070	

Table 3. 12: Codon-based Test of Neutrality analysis between COI sequences of Orthoptera

	Orthoptera species	1	2	3	4	5	6	7	8	9	10	11	12
1	*Acheta domesticus*												
2	*Hieroglyphus nigrorepletus*	1.000											
3	*Aiolopus_thalassinus_tamulus*	1.000	0.484										
4	*Trilophidia annulata*	0.092	1.000	0.110									
5	*Acrida ungarica*	1.000	0.282	1.000	1.000								
6	*Atractomorpha sinensis*	0.549	0.003	0.025	0.136	0.383							
7	*Aiolopus thalassinus*	0.502	0.209	0.298	0.444	1.000	0.272						
8	*Locusta migratoria*	1.000	0.412	0.161	0.547	0.532	0.021	0.088					
9	*Acrida conica*	0.414	0.011	1.000	1.000	0.293	1.000	0.081	1.000				
10	*Hieroglyphus Banian*	0.253	0.076	0.015	1.000	0.004	0.139	0.078	0.480	1.000			
11	*Acrida_willemsei*	1.000	0.359	0.513	0.316	0.285	0.186	0.560	0.275	0.472	0.173		
12	*Choroedocus robustus*	1.000	0.534	0.243	1.000	0.354	0.522	0.039	0.305	1.000	0.293	0.576	

Table 3. 13: Codon-based Test of Neutrality analysis between 16s rRNA sequences of Orthoptera

Similarly, the A+T contents of the 1st, 2nd, and 3rd codons were 56.68, 57.72and 79.65%, respectively, and G+C contents of the 1st, 2nd, and 3rd codons were 43.32, 42.28, and 20.34% respectively percentage was calculated. The overall mean pairwise distance for Orthoptera was calculated to be 3.82, and 2.8 for COI and 16s rRNA, respectively. The pairwise distance for *Hieroglyphus banian* and *Trilophidia annulata* (6.5) was found to be maximum, and *Hieroglyphus banian* and *Hieroglyphus nigrorepletus* (0.16) was found to be least for COI (Table.3.14). The pairwise distance of 16s rRNA sequences were found maximum between *Acrida willemsei* and *Acheta_domesticus* (4.32) and was found a minimum between *Aiolopus thalassinus* and *Acrida ungarica* (3.52) (Table.3.15).

	Orthoptera species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*Acrida ungarica*															
2	*Choroedocus robustus*	3.13														
3	*Aiolopus thalassinus*	2.85	4.48													
4	*Aiolopus thalassinus tamulus*	2.50	2.33	4.41												
5	*Trilophidia annulata*	4.92	2.87	5.57	3.35											
6	*_Acrida willemsei*	4.89	2.48	4.58	3.21	2.42										
7	*Acheta domesticus*	2.25	2.12	5.08	4.34	3.00	1.94									
8	*Hieroglyphus nigrorepletus*	3.84	2.77	5.21	2.25	2.11	1.98	2.95								
9	*Chorthippus curtipennis*	3.82	5.26	1.91	4.04	5.95	6.43	5.77	6.17							
10	*Acrida Conica*	3.20	3.32	5.08	2.96	2.14	1.98	2.96	2.01	5.68						
11	*Locusta migratoria*	4.06	6.34	1.96	4.05	5.91	6.33	4.46	5.94	0.21	5.53					
12	*Hieroglyphus_banian*	4.36	5.07	1.93	4.30	5.74	6.50	5.12	5.97	0.16	5.59	0.20				
13	*Acrida_exaltata*	5.86	4.19	3.36	5.93	5.52	5.34	4.24	3.20	2.98	3.48	3.38	3.35			
14	*Oxya_hyla hyla*	5.71	1.85	5.77	2.71	2.29	2.16	1.72	2.09	4.48	1.45	4.90	5.00	5.43		
15	*Atractomorpha sinensis*	3.59	3.14	6.04	3.79	1.70	2.07	2.18	2.39	6.10	2.03	5.96	6.14	5.06	2.16	

Table 3. 14: Pairwise distance analysis COI sequences of Orthoptera

	Orthoptera species	1	2	3	4	5	6	7	8	9	10	11	12
1	*Acheta domesticus*												
2	*Hieroglyphus nigrorepletus*	2.25											
3	*Aiolopus_thalassinus_tamulus*	2.95	2.98										
4	*Trilophidia annulata*	3.28	2.53	3.26									
5	*Acrida ungarica*	2.73	2.65	2.67	2.43								
6	*Atractomorpha sinensis*	3.08	3.38	2.45	2.20	2.45							
7	*Aiolopus thalassinus*	2.65	2.97	2.12	2.95	0.66	3.37						
8	*Locusta migratoria*	2.88	2.66	3.10	1.98	2.25	2.65	2.82					
9	*Acrida conica*	3.27	2.88	2.06	2.41	3.12	2.39	2.98	3.87				
10	*Hieroglyphus Banian*	3.94	3.19	1.95	2.44	2.64	2.29	1.93	2.56	1.18			
11	*Acrida_willemsei*	4.32	2.91	2.75	3.12	3.77	2.80	3.57	2.88	3.71	3.88		
12	*Choroedocus robustus*	2.24	2.41	2.54	3.01	3.33	3.08	2.40	3.12	4.07	2.53	2.66	

Table 3. 15: Pairwise distance analysis 16s rRNA sequences of Orthoptera

Phylogenetic analysis of Order Orthoptera

For Orthoptera, three representative families, namely Acrididae, Pyrgomorphidea, and Gryllidae sequences, were obtained, which were representing 15 species. Among all the genus, Acrida species dominated five members and had minimum pairwise distance compared to others (Figure 3. 5). In comparison, the other representative members were showing closeness among themselves. In order to Orthoptera, the sequencing of three families representing the pest species was obtained, namely, Acrididae, Pyrgomorphidea, and Gryllidae. Acrididae was representing five sub-families, which was having 14 species. Acrida genus was dominating among all, and the representatives *A. ungarica*, *A. exaltata*, *A. willensei*, *A. conica* were found to be monophyletic to each other. While species H. nigrorepletus and H. banian were having the closest distance and *O. hyla hyla* was found to be paraphyletic to it. Further, the *A. thalassinus* had its closest relative, *A. thalassinus tamulus*, and both were paraphyletic to *T. annulate* and *L. migratoria*. The closest distance-related taxa of *L migratoria* was found to be *C. curtipennis* followed by *C. robustus*. The Pyrgomorphidea and Gryllidae families had a single representative of each, *A. sinensis* and *A. domesticus,* respectively, polyphyletic to the Acrididae family, and the closest distant relative was found to be *C. robustus*.

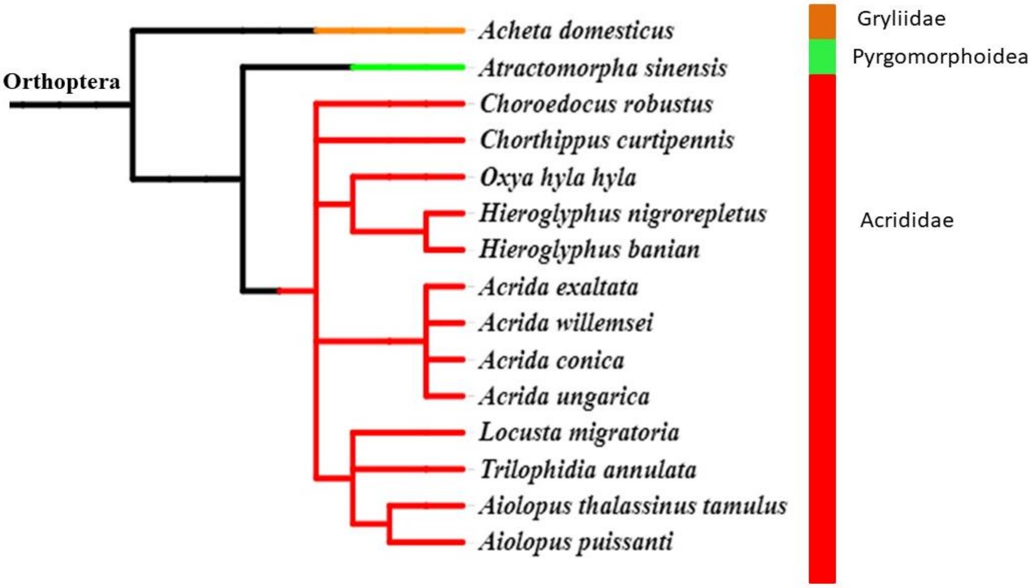

Figure 3. 5: Phylogenetic tree of Orthoptera species

The following are the sequences (COI and 16s rRNA) which are obtained through Sanger Sequencing

- ***Acrida ungarica*** **- MT859408 (COI)**

AGGGCAGTGATTGATACTGATCAAACAAATAATGGTGTTTGATCTAATGTTATTCTT
TCTGATCGTATATTAATTGCTGTGGTAATGAAGTTTACTGCACCTAAGATTGATGAA
ATACCTGCTAAATGTAATGAAAAGATTGCTAGGTCTACTGATGCTCCTCCATGAGCA
ATAGCTCCTGCTAGTGGGGGGTACACTGTTCATCCTGTACCTACTCCTCTATCTACTA
TTGATGATGAAATGAGAAGGGTTAATGATGGTGGTAATAATCAAAATCTTATGTTAT
TTATTCGAGGAAATGCC

- ***Acrida ungarica*** **- MT994522 (16s rRNA)**

CTCAATAATTAGGTCCTTTTCAGAATCGCCTGTTTTTCAAAAACATGTCTTCTTGATA
TATATTTTGAAGTCTGACCTGCTCACTGATAATTTTTAAAGAGCCGCGGTAATTTAG
ACCGTGCAAAGGTAGCATAATCATTAGTCTTTTAATTGGAGGCTGGAATGAATGGTT
TGACGAAAAATCAACTGTCTCTTATTAATTTTTTGAATTTAACTTTTGAGTTAAAAGG
CTTAAATTTACCTTTAGGACGAGAAGACCCTATAGAGCTTAACAGATTAAAATATTA
TTTATTTTAGTATATTTTTTAAATGATTTTTTATTTTGTTTAGTTGGGGTGACGTGAAG
AATAAATAAACTCTTCATTATTAAATCATTGATTTATGTTTATATGATCCATAAATTA
TGATCATAAGATTAAGTTACCTTAGGGATAACAGCGTAATTGTTTTTAAGAGCTCTT
ATCGACAAAACAGATTGCGACCTCGATGTTGGATTAAGATTATTTTCGGGTGCAGTA
GCTCAATAATTAGGTCTGTTCGACCTTTAATTTCTTACATGATCTGAGTTCAACCGGG
TTTTTTTTTTAAAAGGA

- ***Choroedocus robustus*** **- MT707353 (COI)**

TTAGGTCAACCAGGATCGTTAATTGGAGATGATCAAATTTATAATGTTATTGTTACA
GCTCACGCATTTGTAATAATTTTCTTTATAGTTATACCAATCATAATTGGTGGGTTTG
GTAATTGATTAGTCCCATTAATAATTGGAGCACCTGATATAGCATTCCCTCGAATAA
ATAATATGAGTTTTTGACTCTTACCTCCTTCCCTGACCCTACTCCTTTCTTCCTCAATG
GTTGATAACGGAGCTGGCACAGGATGAACAGTGTACCCTCCACTGGCAAGAGCTAT
CGCTCATGGGGGTGCTTCAGTAGACTTAGCAATTTTCTCACTTCATCTGGCTGGTGTT
TCATCAATTTTAGGAGCAGTTAATTTTATTACAACAGCAATTAATATACGATCAGAA
AGAATGACTCTAGATCAAACCCCCTTATTTGTGTGATCAGTTGCTATTACAGCACTA
TTATTACTCTTATCATTACCTGTCCTAGCAGGA

- ***Choroedocus robustus*** **- MT773626 (16s rRNA)**

CGCCTGTTTTTACAAAAACATGTCCTCTTGATGATATTTTGAGGTCCGGCCTGCTCAC
TGACAAGTTTTAAAGAGCCGCGGTATTTTGACCGTGCAAAGGTAGCATAATCATTAG
TCTCTTAATTAGGGGCTGGAATGAATGGTTTGACGAGAAATCAACTGTCTCTTAATA
ATTTTTAAAATTTAACTTTTGAGTTAAAAGGCTTAAATTATTCTTTAGGACGAGAAG
ACCCTATAGAGCTTAACATTTTATTTTTATATAGTTTTTGGATAATCTTTTTATATTTA
ATAATAATGTTTTGTTGGGGTGACATGAAGAATAATTAAACTCTTCATTATAAAATC
ATTGATTTATGTTTATAATGATCCATAATTTATGATCATAAGATTAAGTTACCTTAGG
GATAACAGCGTAATTGTTTTTGAGAGCTCATATCGACAAAGCAGATTGCGACCTCGA
TGTTGGATTAAGAAAAATGTTGGGTGCAGTAACTCAATGATTAGGTCTGTTCGACCT
TTAAATTCTTACATGATCTGAG

- ***Aiolopus thalassinus* - MT731611 (COI)**

TGATAATGGAGTTGGTACTGGTTGAACAGTATACCCCCCACTTGCAGGAGCAATTGC
TCATAGAGGAGTATCCGTTGATCTAGCAATTTTTTCATTACACCTAGCAGGTATTTC
ATCTATTCTAGGAGCAATTAACTTCATTACCACAACAATCAACATACGATCTGAAAG
AATAACTATAGACCAAACACCCCTATTTGTTTGATCAGTAGCAATTACAGCACTGCT
ATTATTATTATCATTACCAGTACTAGCAGGAGCTATTACAATATTATTAACAGACCG
AAACTTAAATACATCATTCTTTGACCCTGCTGGTGGAGGTGATCCAATTTTATATCA
ACACTTATTTTGATTTTTTGGACACCCAGAAGTATATATTTTAATTTTACCAGGATTT
GGTATTATCTCTCATATTGTATGTCAAGAAAGAGGAAAACTTGAATCATTTGGAACA
TTAGGTATAATTTATGCAATACTTTCAATTGGATTAATAGGATTTATTGTATGAGCA
CACCATATATTCACAGTAGGAATAGACGTTGACACACGAGCATACTTTACATCAGC
AACCATAATTATTGCAGTACCAACAGGAATTAAAGTATTTAGATGAATAGCAACAC
TATATGGAACCAAATTCAAATTCAACCCACCATTATTATGAGCATTAGGATTCATTT
TCCTATTTACTATAGGAGGATTAACAGGATTAATTTTAGCTAATTCATCACTTGATAT
TGTATTACACGATACATATTATGTAGTAGCACACTTCCACTATGTATTATCCATAGG
AGCAGTATTTGCAATTATAGGAGGAATTATTCAATGATACCCTTTATTCACAGGATT
AACAATAAATAATAAATGATTAAAAATTCAATTTTCTATTATATTCATTGGAGTAAA
TATAACATTCTTCCCTCAACACTTCCTTGGATTAGCTGGAATACCACGACGATATTCT
GATTATCCAGATGCATATACATCATGAAACGTAATTTCTAGAATTGGATCTACCATT
TCAATCATTGGAATCATTATATTCATTATAATTATATGAGAAAGAATAATTAAAAAC
CGAACAATTATATTCAGAACAAATATAAGAAGATCAACAGAATGATTACAAAATAA
CCCACCA

- ***Aiolopus thalassinus - MT994517 (16s rRNA)***

CCCAATTATTAGTTCCTGTTACGACCTATCGCCTGTTTTTCAAAAACATGTCCTCTTG
ATAATATTTTGAGGTCTGGTCTGCTCACTGAAGAATTTTAAATAGCCGCGGTATTTT
GACCGTGCAAAGGTAGCATAATCATTAGTCTTTTAATTAAAGGCTGGAATGAATGG
CTTGACGAGAAATTAACTGTCTCTTAATAATTTTTAAAATTTAACTTTTGAGTCAAA
AGGCTTAAATTTTTTTTAAGGACGAAAAGACCCTATAAAGCTTAACATTAATATTAT
ATTTGGTTTTTAGTTAATCTTTCTAAATTTAATGATAATGTTTTGTTGGGGTGACATG
AAAAATAATTAAACTTTTCATTATTAAATCATTGATAAATGTTTTATTGATCCATAAT
TTATGATCATAAGATTAAGTTACCTTAGGGATAACAGCGTAATTGTTTTTGAAAGCT
CATATCAACAAAATAAATTGCGACCTCCATGTTGGATTAAAATAAATCTTGGGTGCA
GTAGCACACGTTCCCATTTTAACGTCCTTTT

- ***Aiolopus thalassinus tamulus* - MT859409 (COI)**

GTAATAGCTCCTGCTAGTACTGGTAATGATAATAATAATAGCAGTGCTGTAATTGCT
ACTGATCAAACAAATAGGGGTGTTTGGTCTATAGTTATTCTTTCAGATCGTATGTTG
ATTGTTGTGGTAATGAAGTTAATTGCTCCTAGAATAGATGAAATACCTGCTAGGTGT
AATGAAAAAAATTGCTAGATCAACGGATACTCCTCTATGAGCAATTGCTCCTGCAAGT
GGGGGGTATACTGTTCAACCAGTACCAACTCCATTATCAGTTATTGATGATGAAATA
AGTAAAATTAATGCTGGTGGTAAAA

- ***Aiolopus thalassinus tamulus* – MT994525 (16s rRNA)**

AAAAATTAGGTCCTTTTCCAACTTAATTCTTCGCCTGTTTTTACAAAAACATGTCTTC
TTGATAATATTTTGAAGTCTAACCTGCTCACTGATATTATATTAAATAGCCGCGGTA

TTTTGACCGTGCAAAGGTAGCATAATCATTAGTCTTTTAATTGTGGACTGGAATGAA
TGGTTTAACGAGAAATTAACTGTCTCTTAATAATTTTTAGAATTTAACTTTTGAGTTA
AAAGGCTTAAATTTTTCTTTAGGACGAGAAGACCCTATAGATCTTTACATTTAAATT
TTTATATTTTTTTTAGTTAATCTTTTTATATTAAATTTTAATGTTTTGTTGGGGTGACA
TGAAGAATAGTTAAACTCTTCATTATTAAATCATTTATTTATGATTATTTGATCCATA
ATTTATGATCATAAGATTAAGTTACCTTAGGGATAACAGCGTAATTATTATTGAGAG
TTCATATCGACATAATAGTTTGCGACCTCGATGTTGGATTAAGATTAATTTTAGGTG
TAGTAGCTTAAAAATTAGGTCTGTTCGACCTTTAGATTCTTACATGATCTGAGTTCA
ACCGGAAGAAAAATTTTGAAAGTCTAACCTGC

- ***Trilophidia annulata*** **- MT859410 (COI)**

TTATTACCACCATCATTAATCCTCCTCCTATCATCCTCAATAGTTGATAGGGGAGCC
GGTACAGGATGAACAGTTTACCCCCCATTAGCAGGAGCTATTGCTCATGGAGGAGC
ATCAGTAGATTTAGCTATTTTTTCCTTGCATTTAGCAGGTGTTTCTTCCATTCTAGGA
GCAGTTAATTTTATTACAACAGCAATTAATATACGATCAGAAAGAATAACTTTAGAT
CAAACACCATTATTTGTTTGATCTGTTGCAATTACAGCAATTTTATTACTGTTATCTT
TACCTGTATTA

- ***Trilophidia annulata*** **- MT994526 (16s r RNA)**

AAAAATTAGTTCTTTTCGAAAATACGCCTGTTTTTCAAAAACATGTCTTCTAGAATA
TATTTTGAAGTCTGACCTGCTCACTGATGAATTTAAAGAGCCGCGGTATTTTGACCG
TGCAAAGGTAGCATAATCATTAGTTTCTTAATTAGGGGCTGGAATGAATGGTTTGAC
GAAAAATTAACTGTCTCTTAATAATTAATTGAATTTAACTTTTGAGTTAAAAGGCTT
AAATTTATCTTTAGGACGAGAAGACCCTATAGAGCTTGACATCTAGTTTTATATATA
TTTTTAGTTTATTTATTTATATAATTTAAAATGTTTTGTTGGGGTGACATGAAGAATA
AATAAACTCTTCATTATTAAATCATTAATTTATGTTTAAATGATCCATAATTTATGAT
CATAAGATTAAGTTACCTTAGGGATAACAGCGTAATTGTTTTTGAGAGCTCATATTA
ACAAAGCAGATTGCGACCTCGATGTTGGATTAAGACAAATTTTAGGTGAAGTAGCT
TAAAAATTAGGTCTGTTCGACCTTTAAATTCTTACATGATCTGAGTTTCAACCGGAG
GGGTTATATTTTTGAAGTCTGACCTG

- ***Acrida willemsei*** **- MT547193 (COI)**

ATAATTGGAGATGATCAAATTTATAATGTTATTATCACAGCTCACGCATTTATTATA
ATCTTCTTTATAGTAATACCAATTATAATTGGAGGATTTGGTAATTGATTAGTACCTT
TAATAATTGGTGCACCAGATATGGCATTTCCTCGAATAAATAACATAAGATTTTGAT
TATTACCACCATCATTAACCCTTCTCATTTCATCATCAATAGTAGATAGAGGAGTAG
GTACAGGATGAACAGTGTACCCCCCACTAGCAGGAGCTATTGCTCATGGAGGAGCA
TCAGTAGACCTAGCAATCTTTTCATTACATTTAGCAGGTATTTCATCAATCTTAGGTG
CAGTAAACTTCATTACCACAGCAATTAATATACGATCAGAAAGAATAACATTAGAT
CAAACACCATTATTTGTTTGATCAGTATCAATCACTGCCCTTCTACTATTATTATCAT
TACCAGTTCTAGCAGGAGCTATTACA

- ***Acrida willemsei*** **- MT102740 (16s rRNA)**

AAAATAATCTTAATCCAACATCGAGGTCGCAATCTGTTTTGTTGATAAGAGCTCTTA
AAAACAATTACGCTGTTATCCCTAAGGTAACTTAATCTTATGATCATAATTTATGGA
TCATATAAACATAAATCAATGATTTAATAATGAAGAGTTTATTTATTCTTCATGTCA

CCCCAACCAAACAAAATAAAAAAATCATTTAACAATATACTAAAACTGTTAACCTC
AATTGGGTCTTCTCGTCCGGGTCATAACTTCCAACCTTTAAATTTAAACCTTTTATTC
AAAAATTTAATTTCAAAAACTTAATTTTACCTCGTTGCTTTCTCTCCAACCTCTTATT
TCAAGCCTCCAGTTAAAGCACCCTTTGTACGGTCACCTTTGCGCGGCCCTTTAACAT
GGGTCCTTGAACATTTCTCACTTAGCACGCCATACCTAAAAAACATGATTAAGATAA
ACTGTTGATGAAAAAGAGGCGT

- ***Acheta domesticus* - MT859331 (COI)**

TTAATTCGAACTGAACTTGGACAACCAGGATATTTAATTGGAGATGATCAAACCTAT
AATGTAATTGTAACTGCACACGCATTTATTATGATTTTTTTTATAGTTATGCCAATTA
TAATTGGAGGATTTGGAAATTGACTTGTACCTTTAATATTAGGAGCTCCTGATATAG
CATTTCCACGAATAAATAACATAAGTTTTTGATTATTACCACCATCATTAATTCTCCT
ACTAACCAGAAGAATAGTCGAAAATGGAGCAGGTACTGGATGAACAGTCTACCCAC
CTTTATCTACAGGAATTGCACATGCTGGAGCATCAGTAGATTTAGCAATTTTTTCAC
TCCATCTTGCAGGAATTTCATCAATTTTAGGAGCCGTAAACTTTATTACTACAATAA
TTAATATACGAACCCCAGGAATATCTTTAGATCAAACACCCTTATTTGTATGAGCAG
TAGGAATTACTGCTTTATTATTATTATTTTCATTACCCGTATTAGCAGGTGCAATTAC
AATATTATTAACCGATCGAAATTTAAATACATCATTCTTTGATCCGGCTGGAGGAGG
TGATCCTA

- ***Acheta domesticus* - MT994502 (16s rRNA)**

TCTCGCCCTGTTTTTCAAAAACATGTCTTCTTGGATTGATATGAAGTCGTGCCTGCCC
ATTGAGTATTTTAACGGCCGCGGTATATCTGACCGTGCAAAGGTAGCATAATCATTA
GTCTTTTAATTGGAGGCTGGAATGAATGGTTTGACGAGACAATAACTGTCTCAATTT
AATTATATTGAATTTTATTTTTAAGTTAAAAAGCTTAAATAATAATAGGGGACGAGA
AGACCCTATAGATCTTGAAATGATATTTTAATAATTCGCGTTTAGATCTTAAAGATG
ATTTTTGGATTATTATTTTTGGTGGGCGTGACCAGGGAATATAAAAACTTCTTTAAT
ATTAACTACGATATTAGGTTAGATGATCCTATAATATGGAATTAAGATAAAGATACC
TTAGGGATAACCAGACGCAATATATATGTGCTAGAGTTCATATTGATAACAAAGATT
GTGACCTAGATGTCGTACTTAATGAATAATAGTTAGGAGCAGGACCTTAATTCCATT
CCGGTAAATCGCCCTAAA

- ***Hieroglyphus nigrorepletus* - MT859411 (COI)**

GGAGACGATCAAATCTATAATGTTATCATTACAGCACATGCATTTGTAATAATTTTC
TTTATAGTAATACCAATTATAATTGGTGGATTTGGTAATTGACTCGTACCTTTAATAA
TTGGAGCACCAGATATGGCATTCCCTCGAATAAATAATATAAGTTTCTGGCTTTTAC
CACCATCACTAACACTATTACTTGCATCCTCTATAGTAGATAACGGAGCTGGTACTG
GTTGAACAGTTTACCCTCCACTAGCAGGAGCCATTGCTCATGGAGGAGGGTCCGTTG
ATCTTGCAATCTTTTCATTACACCTTGCAGGTGTATCATCAATTCTTGGAGCAGTAAA
TTTCATTACTACTGCAATTAATATACGATCAGAAAGAATAACACTAGACCAAACACC
ACTATTTGTTTGATCAGTAGCTATTACTGCTCTGCTCCTCCTCCTATCACTACCAGTA
CTAGCAGGAGCAATTACTATATTATTAACTGATCGTAATTTAAATACCTCATTC

- ***Hieroglyphus nigrorepletus* - MT994558 (16s rRNA)**

TTCCTCGCCTTGTTTTTCAAAAACATGTCCTTTTGATTAATATTTTAAGGTCTGGCCT
GCTCACTGACAAGGTTTTAAAGAGCCGCGGTATTTTGACCGTGCAAAGGTAGCATA
ATCATTAGTCTCTTAATTAGAGGCTGGAATGAATGGCTTGACGAGAAATCATCTGTC

TTTTAATAATTTATATAATTTAACTTTTGAGTTAAAAGGCTTAAATTTTTTTTTAGGA
CGAGAAGACCCTATAGAGCTTAACATTTTATTTATATATAGTTTTTTGTTGGTTTTTC
TATAAATATTGTTGATGTTTTGTTGGGGTGACATGAAGAATAAATAAACTCTTCATT
ATTAAATCATTAGTTTATGTTATTATGATCCATAATTTATGATCATAAGATTAAGTTA
CCTTAGGGATAACAGCGTAATTGTTTTTGAGAGCTCACATTCGCGCTAGTCAAGTCT
AAAACACCTACGCACCATTGAACGAGATACATTTAGGCGTATTAAGTCTTCAAAATT
CCAATACACAAGCCTATT

- ***Chorthippus curtipennis*** **- MT709100 (COI)**

AACTTTATATTTTTTATTTGGAGCATGAGCAGGAATAGTAGGAACATCAATAAGCAT
AATTATTCGTGCAGAACTTGGACAACCAGGATCTCTTATTGGAGATGATCAAATTTA
TAATGTTATCATTACAGCTCATGCATTTGTAATGATTTTCTTTATAGTAATACCTATT
ATAATTGGAGGATTTGGTAATTGACTTGTACCATTAATAATTGGAGCACCAGATATA
GCTTTTCCACGAATAAATAACATAAGTTTCTGATTACTACCACCATCATTAACCCTTT
TAATTGCATCATCTATAGTGGATAATGGTGTAGGAACAGGATGAACAGTTTACCCTC
CACTTGCCGGAGCTATTGCCCATGGTGGAAGATCAGTAGATCTAGCTATTTTTTCAT
TACATCTAGCCGGTGTTTCATCCATTTTAGGAGCAGTAAACTTTATTACAACAGCAA
TTAATATACGTTCAGAAAGAATAACACTGGATCAAACACCATTGTTTGTTTGATCAG
TAGCAATTACGGCACTTTTATTATTACTATCATTACCAGTTTTAGCAGGAGCAATTA
CTATATTATTAACTGATCGAAATTTAAATACATCATTTTTTGATCCAGCTGGAGGTG
GAGACCCAATTCTTTACCAACATTTATTT

- ***Acrida conica*** **(COI)**

TCTGATTTCATCATCAAYAGTAGATAGAGGAGTAGGTACAGGATGAACAGTATACC
CTCCACTAGCAGGAGCTATTGCCCACGGGGGGGCATCAGTAGACTTAGCAATTTTTT
CATTACATTTAGCAGGTATTTCATCAATTCTAGGTGCAGTAAATTTTATTACCACAG
CAATTAACATACGATCAGAAAGAATAACATTAGATCAAACACCATTATTTGTTTGAT
CAGTATCAATTACTGCCCTCTTATTATTACTATCATTACCAGTTTTAGCAGGAGCTAT
TACAATATTATTAACTGATCGAAACCTAAATACATCTTTTTTTGACCCAGCAGGTGG
TGGTGATCCAATCCTATATCAACATTTATTTTGATTCTTTGGACATCCAGAAGTTTAC
ATTCTAATTTTACCAGGATTTGGTATTATTTCACATATTGTTTGTCAAGAAAGAGGTA
AAATTGAATCATTTGGAACATTAGGAATAATTTATGCAATATTATCAATT

- ***Acrida conica-*** **MT994552 (16s rRNA)**

GTTTTATAGGTTTCCTTTTTCCCCAACTTCGCCCTGGTTTTTCAAAAACATGTCTTTTT
GTGATTAATTTAAAGTCGGTCCTGCCCACTGAATATATTTGAATGGCCGCGGTATCT
TGACCGTGCAAAGGTAGCATAATCATTAGTCTTTTAATTGAAGGCTGGAATGAATGG
TTTGACGAAGTATAAGCTGTCTCTGTTTAATTAATGGAATTTGTCTTTTTAGTTAAAA
GACTTAAATAATAATAAGGGACGAGAAGACCCTATAGAGCTTTATATTAATATATA
ATTTATGTTTTTTCAGATGTTTTATCAATTTATAATATATTTAATATTGTGTTGGGGT
GACATGAAGATAAAATTAACTCTTTATTAGATAAACATAGATTTATGGATAATTGAT
CCAATATTATTGATTAAAAGATAAAGTTACCTTAGGGATAACAGCGTAATCCTTCTT
GAGAGTTCATATCGAAAGAGGGGGTTGCGACCTCGATGTTGGACTAAGAAAATGAC
TAAATGCAGCAGTTTTAGTTTATAGGTCTGTTCGACCTTTAAATTCTTACATGATCTG
AGTTTCAACCGGGGGGGCCGGCG

- ***Locusta migratoria*** **(COI)**

TACATTGTATTTTATATTCGGGGCATGAGCTGGAATAGTAGGAACATCAATAAGAAT
AATTATTCGAGCTGAATTAGGTCAACCAGGAACAATAATTAATGATGATCAAGTAT
ATAATGTAATTATTACAGCACACGCATTTGTTATAATTTTCTTCATGGTTATGCCTAT
TATAATTGGAGGATTCGGAAATTGATTAGTACCATTAATAATTGGAGCTCCAGATAT
AGCTTTCCCACGAATAAATAATATAAGATTTTGATTATTACCACCATCATTAACACT
CCTACTAATGTCTTCTTTAGTAGATAATGGAGCTGGTACAGGATGAACAGTATATCC
CCCACTAGCTAGAGTCATTGCTCATAGAGGAGCTTCTGTAGATTTAGCAATTTTCTC
ATTACATCTAGCAGGTGTTTCCTCAATTTTAGGAGCCATTAATTTCATTACGACAGC
AATCAACATACGATCAAATAATATAACCCTTGATCAAACACCATTATTTGTTTGATC
AGTAGCAATTACAGCCTTATTACTTTTATTATCATTACCAGTATTAGCTGGAGCAATT
ACTATATTATTAACCGATCGAAACCTTAATACATCATTCTTTGACCCAGCAGGAGGA
GGTGATCCAATTCTATATCAACACTTATTT

- ***Locusta migratoria*** **- MT994524 (16s rRNA)**

GAAAAAATTCTGCTGGGGCATCGCCTGTTTTATCAAAAACATGTCCTTTTGATTAAT
ATTTTAAGGTCTGGCCTGCTCACTGACAAGGTTTTAAAGAGCCGCGGTATTTTGACC
GTGCAAAGGTAGCATAATCATTAGTCTCTTAATTAGAGGCTGGAATGAATGGCTTGA
CGAGAAATCATCTGTCTTTTAATAATTTATATAATTTAACTTTTGAGTTAAAAGGCTT
AAATTTTTTTTTAGGACGAGAAGACCCTATAGAGCTTAACATTTTATTTATATATAGT
TTTTTGTTGGTTTTTCTATAAATATTGTTGATGTTTTGTTGGGGTGACATGAAGAATA
AATAAACTCTTCATTATTAAATCATTAGTTTATGTTATTATGATCCATAATTTATGAT
CATAAGATTAAGTTACCTTAGGGATAACAGCGTAATTGTTTTTGAGAGCTCATATCC
ACAAAGCAGATAGCGACCTCCGATCTTGCAATTAAGAAAAATTTTAGGTGTAGGAG
TCTAAAAATTAGGTCTGTTCGACCTTTAAATTCTTACATGATCTGAGTTCAACCGGA
GGTAAAATTTTTAAGGGTCTGGG

- ***Hieroglyphus banian*** **(COI)**

CACTTTATATTTTTTATTTGGAGCATGAGCAGGTATAGTTGGAACATCAATAAGAAT
ATTAATCCGAGCAGAATTAGGTCAACCAGGATCTTTAATTGGAGATGATCAAATNT
ATAATGTTATTATTACAGCACACGCATTTGTAATAATTTTCTTTATAGTTATACCAAT
TATAATCGGTGGATTTGGAAATTGACTTGTACCACTAATAATTGGAGCACCAGATAT
AGCATTCCCACGAATAAATAATATAAGTTTCTGACTTTTACCACCCTCACTGACACT
ATTACTCACGTCCTCTATAGTAGATAACGGAGCTGGCACTGGTTGAACAGTTTACCC
TCCGCTGGCAGGNGCCATTGCTCACGGTGGTGGATCCGTTGACCTTGCCATTTTTTC
ATTACACCTTGCTGGTGTTTCATCTATTCTTGGAGCAGTTAATTTCATTACTACTGCA
ATTAATATACGATCAGAAAGTATAACATTAGATCAAACACCATTATTTGTTTGATCA
GTAGCAATTACTGCCCTACTTTTACTACTATCATTACCAGTTCTGGCAGGAGCTATTA
CTATATTATTAACTGATCGAAATCTAAATACATCATTCTTTGATCCAGCTGGAGGGG
GTGATCCAATTCTATATCAACATCTATTT

- ***Hieroglyphus Banian*** **- MT994520 (16s rRNA)**

CTAATAATTAGGTCTTGTTCGACACTTCGCCCTGTTTTATCAAAAACATGTCCTTTTG
ATTAATATTTTAAGGTCCGGCCTGCTCACTGACAAGGTTTTAAAGAGCCGCGGAATT

TTGCCCGTGCAAAGGTAGCATAATCATTACTCTCTTAATTAGAGGCTGGAATGAATG
GCTTGACGAAAAATCATCTGTCTTTTAATAATTTATATAATTTAACTTTGGAGTTAAA
AGGGCTTAAATTTTTTTTTAGGACGAGAAGCACCCTATAGAGCTTAACATTTTATTT
ATATATAATTTTTTGTTGGTTTTTCTATAAATATTGTTGATGTTTGGTGGGGGGGACA
GGAAAAAAAAATAAACTCTTCATTATTAAATCATTAGTTTATGTTATTATGACCCAA
AATTTAGGATCATAAAATTAAGTTACCTTAGGGAAAACAGCGTAATTGTTTTTGAAA
GCTCATATCAACAAACCAAATTGCAACCTCAATGTTGGATTAAAAAAAATGTTAGG
GGTAGGAGTCTAAAAATTAGGCCTGTTCAACCTTTAAATTCTTACATGATCTGAGTC
CAACCGGATTTTACAGACTGGCCT

- ***Acrida exaltata* (COI)**

TGGTCAAATAGGATCACCACCTCCTGCTGGGTCAAAGAATGATGTATAATAAGGAT
TTAAATTTCGATCTGTTAATAGTATTGTAATTGCTCCTGCTAATACAGGTAATGATA
GTAATAATAATAATGCAGTAATTGCTACAGATCAAACAAATAAAGGTGTGGCATCT
AATGATATATTTTTTGATCGTATATTGATTCTAGTTGTAATGAAGTTAATTGCTTCTA
AAATTGAAGATACACCTGCTAAATGAAGTGAAAAAAATTGCAAGATCTACTGATGAT
CCTCTATGTGCAATAATTGGCCCGCATCATGCTCCTTAATAAGGATAATGGTGGGTA
AACTGTTCATCCTGTACCTACTCCTGTATTTACTAATGATGATCAATATTAATAAGGT
TAATGAGGGTGGTAAAAGTCAGAATCTTATATTATTTATTCGAGGGCTATATCTGGC
ATTGAAGTTCCTATTATTCCTGGGCTCCAATTATTAAAGGTACTAATCAATTTCCAA
ACCCTCCAATTATAATTGGTATTACTATAAAGAAAATTATGATAAATGCGTGGGATG
TGATGATTACATATATAATGGCCTAATTTGGTCAAATAGGCCATCAACACCTCCTGC
TGGCCCTAATTCAGAATCGGATGTATTTAAATTCTCGATCTGAAGTTAATAGTATTC
CTAATCATGCTCCTGCTAATACAGGTAATGATAGTAATAATTTTAATGCAGTAATTG
CTACAGATCAAACAAATAAAGGTGTGGGCCTAATGATATATTTTTTGATCGTATATT
GATTCTAGTTGTAATGAAGTTAATTGCTTGAAGATACACCTGCTAAATGAAGTGATT
TTATTGCAAGATCTACTCTAATTTGTTCATCGGCCATTAAATTTCCTGGTTGCCCTAA
TTCTGCTCGGATTATTATTCT

- ***Oxya hyla hyla* – MT994515 (COI)**

GTAGGAACATCAATAAGTATAATTATTCGAGCTGAACTTGGTCAACCAGGATCATTA
ATTGGAGATGATCAAATTTATAATGTAATTATTACAGCACATGCATTTGTTATAATT
TTTTTTATAGTTATACCAATTATAATTGGTGGATTTGGTAATTGATTAGTTCCATTAA
TAATTGGAGCACCAGATATAGCATTCCCACGAATAAATAATATAAGATTTTGATTAC
TACCACCATCTTTAACACTTCTTATTATATCCTCTATAGTTGATAATGGAGCTGGGAC
AGGATGAACAGTTTACCCTCCACTAGCAGGAGCTATTGCACACGGAGGATCTTCAG
TAGATCTAGCCATTTTCTCACTTCATCTTGCTGGTGTTTCATCAATTCTTGGAGCAGT
AAATTTTATTACAACAGCAATTAATATACGATCAGAAAGAATAACACTTGATCAAA
CACCATTATTTGTTTGATCAGTTGCTATTACAGCTCTTTTATTATTATTATCATTACCA
GTTTTAGCTGGAGCTATTACAATATTATTAACA

- ***Atractomorpha sinensis* - MT994515 (COI)**

ATGAGAATGTTAATCCGAGCAGAACTTGGTCAACCAGGAAATTTAATTAATGATGA
ACAAATCTATAATGTAATCATTACATCTCATGCATTTATCATAATTTTCTTCATAGTA
ATACCAATTATAATTGGAGGATTTGGAAACTGATTAGTACCTTTAATAATCGGAGCT
CCAGATATAGCCTTTCCTCGAATAAATAACATAAGATTCTGATTACTTCCGCCTTCA
TTAACCCTATTAATTAGATCCTCAATAGTAAGTACAGGAGTAGGAACAGGATGAAC

GGTATACCCCCCATTAGCAAGATCTATTGCACATAGAGGTACATCAGTAGATCTTGC
AATTTTTTCTCTTCATCTAGCAGGAGTATCTTCAATTCTAGGAGCAGTAAATTTTATT
ACAACTAGAATCAATATACGATCAAAAAACATATACCTAGATCAAACACCTTTATTT
GTATGATCAGTAACAATCACTGCATTACTATTATTATTATCACTACCTGTATTAGCA
GGAGCAATTACAATACTATTAACAGACCGAAATTTAAATACATCATTCTTTGATCCA
GCAGGAGGAGGTGATCCAAT

- ***Atractomorpha sinensis* – (MT994587)16s rRNA**

TTTAGAAACTTAGGTTCTTTTCCACGGTATTATGACCGTGCAAAGGTAGCATAATCA
TTAGTCTTTTAATTTGAGGCTGGAATGAATGGTTTGACGAGGGAGGGACTGTCTCAA
TTTTAATTAATAAAATTTAACTTTTAAGTTAAAAGGCTTAAATTTGATTTTAGGACG
AGAAGACCCTATAGAACTTTATATATTATTTATAACTGATTTGCTGATATTTTGCTTC
ATTTTATTAAGATTATATTTTGTTGGGGTGACATGAAGAATAATTAAACTCTTTATTT
TTAATATCATTTATTTATGATTGTTTGATCCATAATTTGTGATTATAAGATTAAGTTA
CCTTAGGGATAACAGCGTAATTGTCTTTTAGAGTTCATATCAACAAGACTGATTGCG
ACCTCGATGTTGGATTAAGGATTTAGTACTTAGGTGTATCAGTTTAGTAACTAGGTC
TGTTCGACCTTTAAATTCTTACATGATCTGAGTTCAACCGGGAATTGTGTAGAAGAG
CCG

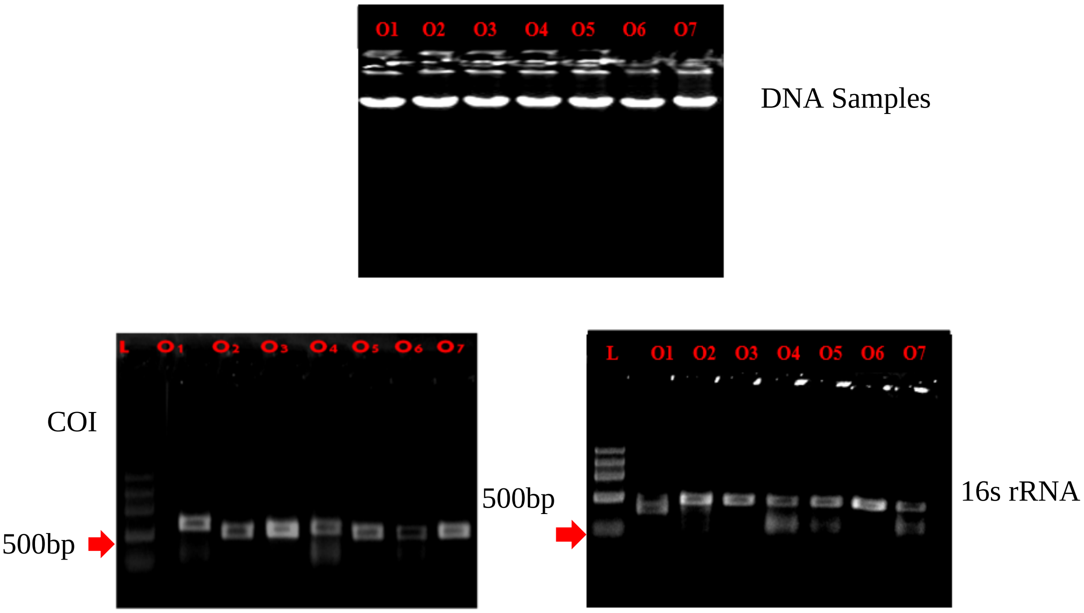

Figure 3. 6: Electrophoresis gel image of DNA, PCR product of COI and 16s rRNA of Orthoptera species representatives

GC Content and AT – GC Skew analysis of Order Hemiptera

A genomics analysis order wise GC% resulted that the GC content of Hemiptera resulted in maximum with *Cletus punctiger* (36.4%) and minimum with *Melanaphis sacchari* (21.5%) and AT content was maximum with *Nezara antennata* (78.5%) and minimum with *Leptocentrus Taurus* (63.6%) (Table 3.16).

	Hemiptera	**NCBI Accession No.**		**AT Skew**	**GC Skew**	**GC%**	**AT%**
		COI	**16s rRNA**				
1	*Melanaphis sacchari*	MT707350	MT994694	-0.06	-0.17	21.5	78.5
2	*Dysdercus cingulatus*	MT765073	MT994689	-0.07	-0.08	31.4	68.63
3	*Carbula insocia*	MT707346	-------	-0.03	-0.04	32.9	67.05
4	*Halyomorpha halys*	MT707347	-------	-0.003	-0.14	33.5	66.49
5	*Nezara mendax*	MT707352	MT994695	-0.02	-0.07	33.8	66.2
6	*Leptocentrus taurus*	MT707350	MT994692	-0.04	-0.13	33.9	66.14
7	*Nezara viridula*	MT707348	MT994699	-0.02	-0.04	34.2	65.78
8	*Nezara antennata*	MT707349	MT994693	0	-0.04	34.4	65.52
9	*Coridius janus*	MT765072	MT774554	-0.01	-0.09	35	65.03
10	*Cletus punctiger*	MT568728	MT102349	-0.07	-0.10	36.4	63.56

Table 3. 16: Order Hemiptera with Accession no., AT-GC- Skew and AT- GC content

The AT and GC content of individual orders, where in Hemiptera, demonstrated AT bias of 67.4 and GC of 32.6 for COI and 72.3 and 27.7 for 16 sRNA. The estimated Transition/Transversion bias (*R*) for COI and 16srRNA was found to be 0.577 and 0.566 respectively. The nucleotide frequencies of COI were 32.40% (A), 34.66% (T/U), 17.62% (C), and 15.32% (G) and that of 16s RNA were 32.97% (A), 39.63% (T/U), 12.38% (C), and 15.02% (G). Table 3. 16 represents the Accession number obtained after sequence submitted to NCBI.

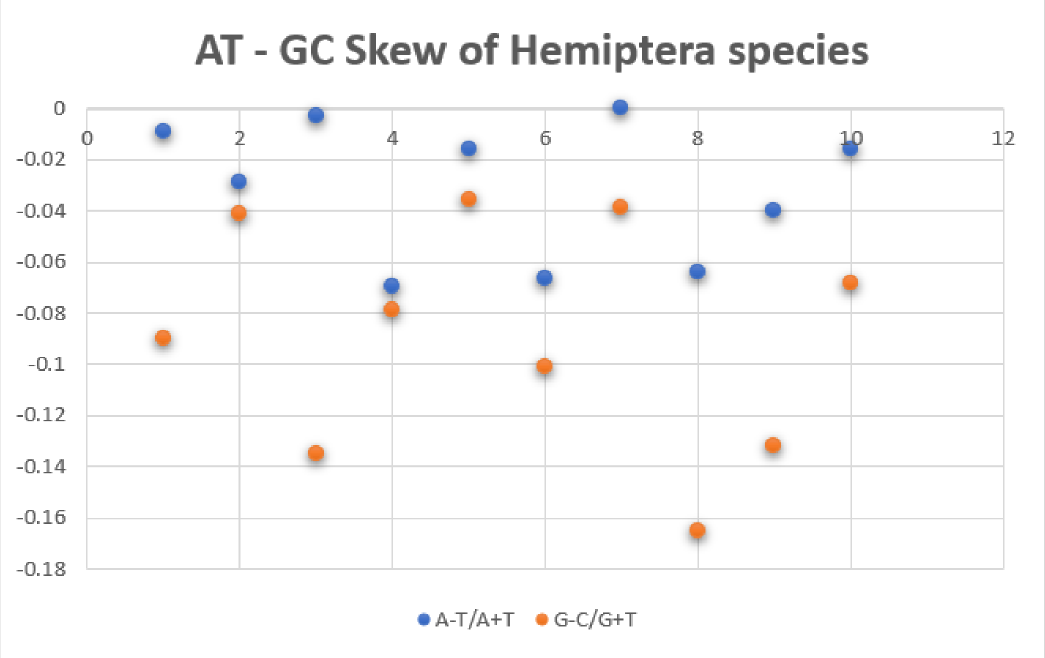

Figure 3. 7 : AT - GC Skew Analysis of Hemiptera species

Ten nucleotide sequences involved the possibility of synonymous or non-synonymous analysis. All uncertain positions were removed for each pair of sequences, and the final dataset contained 135 positions. The probability rate was found to be between 18 species ($p<0.05$, $p<0.01$) for COI (Table.3.17); on the other hand, for 16s rRNA, the analysis involved 9 nucleotide sequences (Table.3.18) and a total of 102 positions were analyzed in the final dataset. The probability rate was significant ($p<0.05$, $p<0.01$) between the six species.

Similarly, the A+T contents of the 1st, 2nd, and 3rd codons were 60.45, 60.47, and 81.18 %, respectively, and G+C contents of the 1st, 2nd, and 3rd codons were 39.55, 39.54, and 18.82% respectively percentage was calculated. The overall mean pairwise distance for Hemiptera was calculated to be 2.77, and 1.77 for COI and 16s rRNA respectively, the pairwise distance for *Nezara antennata* and *Cletus punctiger* (4.67) was found to be maximum, and *Nezara mendax* and *Nezara viridula* (0) was found to be least (Table 3.19). The pairwise distance of 16s rRNA sequences were found maximum between *Melanaphis sacchari* and *Carbula insocia* (2.09) and was found minimum between *Melanaphis sacchari* and *Dysdercus cingulatus* (1.41) (Table 3.20).

	Hemiptera species	1	2	3	4	5	6	7	8	9	10
1	*Coridius janus*										
2	*Carbula insocia*	0.000									
3	*Halyomorpha_halys*	0.542	0.225								
4	*Dysdercus cingulatus*	0.004	0.011	1.000							
5	*Nezara viridula*	0.005	0.002	1.000	0.007						
6	*Cletus punctiger*	0.007	0.081	1.000	0.270	0.200					
7	*Nezara antennata*	0.108	1.000	0.021	1.000	1.000	0.293				
8	*Melanaphis sacchari*	0.055	0.004	1.000	0.016	1.000	0.244	0.037			
9	*Leptocentrus_taurus*	0.064	0.233	1.000	0.035	0.016	0.103	0.412	0.004		
10	*Nezara mendax*	0.004	0.002	1.000	0.001	1.000	0.200	1.000	1.000	0.016	

Table 3. 17: Codon-based Test of Neutrality analysis between COI sequences of Hemiptera

	Hemiptera species	1	2	3	4	5	6	7	8	9
1	*Coridius janus*									
2	*Carbula insocia*	0.333								
3	*Dysdercus_cingulatus*	0.094	1.000							
4	*Nezara viridula*	1.000	1.000	0.066						
5	*Cletus punctiger*	0.467	0.048	0.198	0.044					
6	*Nezara antennata*	0.462	0.283	0.079	0.141	1.000				
7	*Melanaphis sacchari*	1.000	1.000	1.000	0.032	0.462	0.548			
8	*Leptocentrus_taurus*	1.000	0.327	1.000	0.118	0.010	0.248	0.024		
9	*Nezara mendax*	1.000	0.353	0.529	0.001	1.000	0.434	0.466	0.424	

Table 3. 18: Codon-based Test of Neutrality analysis between 16s rRNA sequences of Hemiptera

	Hemiptera species	1	2	3	4	5	6	7	8	9
1	*Coridius janus*									
2	*Carbula insocia*	3.29								
3	*Halyomorpha_halys*	4.21	3.80							
4	*Dysdercus cingulatus*	2.06	1.73	3.98						
5	*Nezara viridula*	2.36	1.87	2.71	2.00					
6	*Cletus punctiger*	1.94	2.75	3.40	2.76	2.39				
7	*Nezara antennata*	4.45	4.61	3.32	3.71	4.54	4.67			
8	*Melanaphis sacchari*	2.80	1.68	1.91	1.71	0.40	2.12	4.52		
9	*Leptocentrus_taurus*	2.48	2.62	4.18	2.04	2.83	2.52	4.41	2.57	
10	*Nezara mendax*	2.45	1.93	2.72	2.08	0.00	2.39	4.55	0.40	2.83

Table 3. 19: Pairwise distance analysis COI sequences of Hemiptera

	Hemiptera species	1	2	3	4	5	6	7	8	9
1	*Coridius janus*									
2	*Carbula insocia*	1.96								
3	*Dysdercus_cingulatus*	1.76	1.81							
4	*Nezara viridula*	1.67	1.79	1.35						
5	*Cletus punctiger*	2.00	1.98	1.88	1.84					
6	*Nezara antennata*	1.88	1.84	1.44	1.62	1.79				
7	*Melanaphis sacchari*	1.89	2.09	1.41	1.53	1.64	1.70			
8	*Leptocentrus_taurus*	1.95	1.83	1.65	1.75	1.99	1.52	1.59		
9	*Nezara mendax*	1.89	1.97	1.73	1.93	1.70	1.75	1.56	1.94	

Table 3. 20: Pairwise distance analysis 16s rRNA sequences of Hemiptera

Phylogenetic analysis of Order Hemiptera

Hemiptera, five representative family sequences were obtained- Pentatomidae, Coreidae, Pyrrhocoridae, Aphididae, and Membracidae. The phylogenetic pair wise closes distance was found in the manner of (Pentatomidae + (Coreidae + (Pyrrhocoridae + (Aphididae + Membracidae)))) representing nine species. The highest no of species representation was observed in Pentatomidae. *N. antennata*, *N. mendax* and *N. viridula* were monophyletic to each other, having a paraphyletic sister clade of *C. insocia* and *C. janus*. Family Coreidae, Pyrrhocoridae, Aphididae and Membracidae were having *C. punctiger*, *D. cingulatus*, *M. sacchari*, and *L. taurus* respectively as the represented species. The Coreidae and Pyrrhocoridae were having the least distance and were paraphyletic to each other; on the other hand, they were found to be polyphyletic to Aphididae and Membracidae (Figure 3.8).

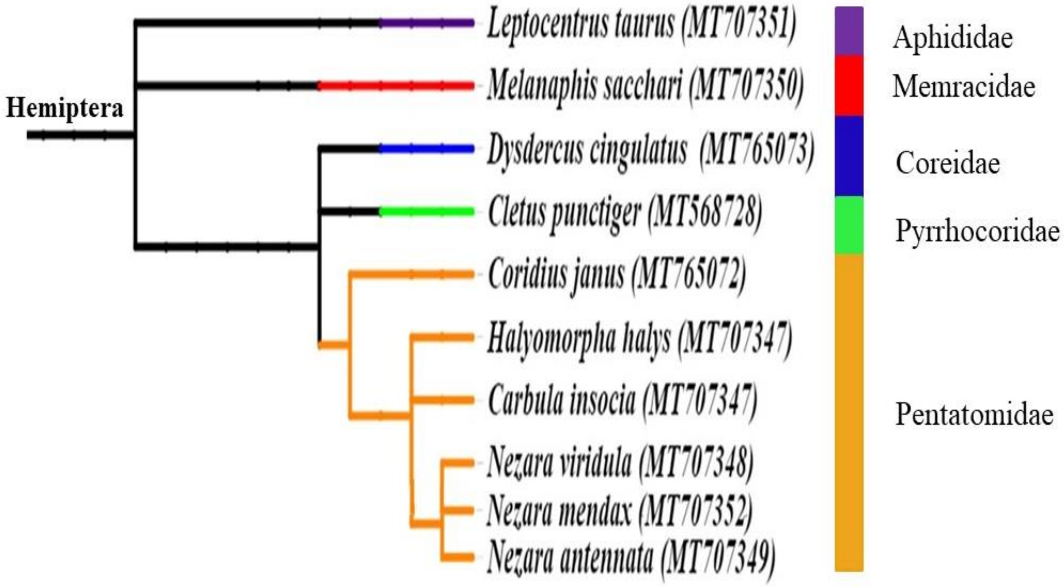

Figure 3. 8: Phylogenetic tree of Hemiptera species

The following are the sequences (COI and 16s rRNA) which are obtained through Sanger Sequencing

- ***Coridius janus*** **- MT765072 (COI)**

ATCGGTGATGATCAAATTTATAATGTTATTGTAACAGCACATGCATTTGTAATAATTT
TCTTTATAGTTATACCTATTATAATTGGAGGATTTGGTAACTGATTAGTACCACTAATA
ATTGGAGCACCAGATATAGCATTCCCCCGAATAAATAATATAAGATTTTGACTTCTAC

CCCCCTCTTTAACACTTCTATTAGTTAGAAGAACTGTAGAAATAGGAGCAGGGACAG
GATGAACAGTTTACCCTCCTTTATCAAGTAACCTATCCCATGCAGGACCAGCAGTAGA
TCTAGCAATTTTTTCCCTTCACTTAGCAGGAGCTTCTAGAATTATAGGAGCTGTAAATT
TTATTTCAACAATCATCAATATGCGACCGGTTGGAATAACTCCAGAACGAACACCCTT
ATTTGTATGATCGGTTGGAATTACAGCTCTACTTTTACTATTATCATTACCCGTGCTAG
CAGGAGCTATTACTATATTATTAACAGATCGAAACTTTAATAC

- ***Coridius janus - MT774554*** **(16s rRNA)**

TCCCGGTTGAAAATCAGATCATGTAAATAATTAAAGGTCGAACAGACCTAGAAAATT
GAGCTTCTACACCCAAATCTTAATTTAATCCAACATCGAGGTCGCAAACTTTTATATC
AATAAGAACTCTCCATAAAAATTACGCTGTTATCCCTAAGGTAGTTTAATCTTTTAAT
CCTAAATAAGGGATCCTTAAAATATAAATAAATATATCATATAAATAGAGAGTTAAA
TAAATCTCCATGTCGCCCCAACAAAACTATCATCACATTATTTAACATTATAAATAAC
CAAAAAAGTAATAAGTCCATAATAGTAAAACTCTATAGGGTCTTCTCGTCCTCCATAA
ACATTTAAGCTTTTTAACTCAAATATTAAATTTATCATATTTATATAAAGAAAGTAAA
CATTTCATCAAACCATTCATACAAGCCTACAATTAATAGACAAATGATTATGCTACCT
TCGCACAGTCAAATTACTGCGGCCCTTCAATAATACTATCAGTGGGCAGGTTTGACCT
AATATAAATTCATCAATAGGACATGTTTTTGA

- ***Carbula insocia*** **- MT707346 (COI)**

GAATTAGGACAACCAGGAAGATTCATTGGTGATGATCAAATTTATAATGTAGTAGTA
ACAGCTCATGCCTTTATTATAATTTTCTTTATAGTTATACCAATTATAATTGGAGGATT
TGGAAATTGACTAGTACCTTTAATAATTGGAGCCCCTGATATAGCATTCCCTCGAATA
AATAACATAAGATTTTGATTATTACCCCCTTCATTAACTCTACTAATAATTAGAGGGC
TAGCAGAATCAGGAGCAGGAACTGGATGAACAGTATATCCCCCTCTCTCTAGAAATA
TTTCACATAGAGGAGCATCAGTAGATTTAGCTATTTTTTCATTACATTTAGCTGGTGTA
TCATCAATTTTAGGAGCAGTAAATTTCATTTCAACTATTATTAATATACGACCAGAAG
GTATAACTCCTGAACGCATTCCATTATTTGTATGATCTGTAGGAATCACAGCCTTATT
ATTATTATTATCCCTACCAGTACTAGCTGGAGCTATTACCATGTTATTAACTGAT

- ***Carbula insocia*** **(16s rRNA)**

TTCTAGGTCGTCACCCAACCCAGTTCGCCTGTTTTTCAAAAACATAGCCTTCAGCAAA
CCAACAGACAAGTATTGAAGGTGATGCCTGCCTCGCCTCTTTTTCAAAAACATGTCTT
GATGAGTGTTTATATTAAGTCTGGCCTGCTCAATGATGCTATTACTTAACATAACCGC
ACTATATTGACTGTGCCAAAGGTACCATAATCATTTGTCCTTTAATTGAAGGCTTGAA
TGAATGGTTTGATGAAATAAAGACTTTCCTTATATTAATTATTGAACTTAATTTTTTAA
TTAAAAACCTTAAATTTATAACTGGGACCAGAAGAACCTATAAAATTTTATTTTTATT
TATAATTCTTTTTTTTGCATTTTAATTTACTACTTATACGTTAAGAATTTTGTTGGGGTG
ACAGAGAGGAATTTTTTAACTCTCATATTTGATTTTTTATTGATTGATATTTTTATGAT
CCTAATAAATGGATATTTAAATTAAATTACCCTACGGATAACAGCGTAATTTCTTTCC
AGAGATCCTATCAATAAAGAACTTTGCGACCCTACCATGTTGGATTAAATTCACAATT
AAAAGTACAAAGCTTGAAATTAAAGTTAAATTCAAGGTCTGGGCCTGCTC

- ***Halyomorpha halys*** **- MT707347 (COI)**

ACTAATTATCCGAATTGAATTAGGACAACCAGGAAGATTTATTGGAGATGATCAAAT
TTATAATGTAGTAGTTACAGCCCACGCATTCATTATAATTTTTTTTATAGTTATACCTA

TTATAATTGGGGGATTTGGTAATTGACTTGTACCTTTAATAATTGGAGCCCCTGATAT
AGCTTTCCCTCGAATAAATAATATAAGATTTTGACTATTACCCCCATCACTAACATTA
CTAATAATAAGAAGACTAACAGAATCTGGAGCAGGAACTGGATGAACAGTTTACCCC
CCTTTATCAAGTAATATTTCACACAGAGGATCATCAGTAGATTTAGCAATCTTTAGTC
TTCACTTAGCAGGAATCTCTTCTATCTTAGGTGCAGTAAATTTCATTTCAACAATCATT
AATATACGCCCAGCAGGAATAATTCCTGAACGAATTCCATTATTTGTATGATCAGTAG
GAATTACTGCCTTACTTCTACTTCTATCCCTTCCTGTATTAGCAGGAGCCATTACAATA
CTATTAACAGACCGAAACTTTAATACATC

- ***Dysdercus cingulatus*** **- MT765073 (COI)**

GATCAAATTTATAATGTTATTGTTACTGCACATGCATTCATTATAATTTTCTTCATAGT
AATACCTATTATAATTGGAGGTTTTGGTAATTGATTAGTACCTTTAATAATTGGAGCA
CCCGATATAGCATTCCCTCGAATAAATAATATAAGATTCTGACTTTTACCCCCTTCTTT
AACACTATTAGTATCAAGAAGATTAGTAGAAATAGGAGCAGGAACTGGATGAACAGT
TTATCCCCCATTATCAAGAAATATTTCCCATAGAGGACCTTCTGTAGATTTAGCTATTT
TTTCCCTACACCTAGCAGGTGTATCATCTATTTTAGGAGCTGTAAATTTTATTTCAACT
ATTATTAATATACGACCAATAGGAATAACATTAGAACGAACTCCTTTATTTGTATGAT
CGGTAGGAATTACAGCACTTTTACTTTTATTATCATTACCTGTATTAGCAGGAGCTATT
ACAATACTTCTTACAGATCGAAATTTTAATACATCTTTCTTTGATCCAACAGGTGGTG

- ***Dysdercus cingulatus*** **- MT994689 (16s rRNA)**

AATTTCTAGGTCTGTCACCCTTTCGCCTGTTTTTTCAAAAACATGTCTTATAGAGTTTA
ATTTTAAGTCTGACCTGCTCAATGATTTATTAAATTGCTATAGTATTTTAACTATACAA
AGGTAGCATAATAATTTGTCTTTTAATTGGAGGCTTGTATGAATGGTTGGACGAGAAG
TAATCTTTCTTAATTTAAATTAAATTGAATTTAATATTTTTGTAAAAAAGCTTAAATAT
AGTTGTGGGACGAGAAGACCCTTTAGAATTTTAATTAAATTAATAATTTTTTATTTTG
GGTAAAATTTTATAATATTTTATTAATTTAATTTTTGTTGGGGTGACAGTAAAAATTTT
TTAACTTTTATTATTAAAATAATCATAAATTAATGTATTATTGATCCTCTATATAGAGA
TTATAAGATTAAATTACCTTAGGGATAACAGCGTAATTACTTTGGAGAGTTCTTATCG
ATAAAGTAGTTTGCGACCTCGATGTTGGATTAAAGTTAGATTTATAGTGCAGCTGCTT
AATTTCTAGGTCTGTTCGACCTTTAAACCTTTACATGATCTGAGTTCAACCGGACGTTA
TTTAAAGTCTGACCTG

- ***Nezara viridula*** **- MT707348 (COI)**

CTAATTATTCGAATTGAACTAGGACAACCCGGAAGATTTATTGGAGATGATCAAATTT
ATAATGTAGTAGTAACAGCTCACGCATTTGTAATAATTTTCTTTATAGTAATGCCAAT
CATAATTGGAGGATTTGGAAATTGATTAGTACCTTTAATGATTGGTGCCCCAGACATA
GCATTCCCTCGAATAAACAATATAAGATTCTGACTATTACCCCCATCATTAACCCTTTT
AATAGTAAGAAGATTAGCAGAATCTGGAGCAGGGACAGGATGAACTGTTTATCCTCC
TTTATCTAGTAACTTATCCCATAGAGGAGCTTCAGTGGATTTAGCTATTTTTTCATTAC
ATCTAGCAGGAGTATCATCAATTTTAGGTGCAGTAAATTTCATTTCAACTATTATTAA
TATACGACCAACAGGTATAACTCCAGAACGAGTGCCACTATTTGTTTGATCAGTTGGA
ATCACAGCACTATTATTACTACTTTC

- ***Nezara viridula - MT994699*** **(16s rRNA)**

AGTTCTAGGTCCTTTGGAACCCTCGCCTGTTTTTCAAAAACATGTCTTGATGATTTTTA
TTTATTAAGTCTATCCTGCTCAATGAGTTATTATTAAATAGCCGCAGTATCTTGACTGT
GCGAAGGTAGCATAATCATTTGTCTTTTAATTAAAGGCTTGTATGAATGGTTGGATGA
GGGAATTACTTTCTTTATATTAAAAATTGAATTTAATTTTTAAGTTAAAAAGCTTAAAT
TTTTAAGTGGGACGAGAAGACCCTATAGAATTTTATTATAAGTTTTATATTTAATTTAT
GGTTATTTTTATTATTTATATGTCTTATAATTTTATTGGGCTGATATTGAGATTAAAAA
AACTCTCATAACTTAATATTTAATTATTTTTATTTTGTTAGGATCCTATTTTATGGATAT
TTAGATTAAATTACCTTAGGGATAACAGCGTAATTTTTCTGGAGAGTTCCTATCGATG
GAAAAGTTTGCGACCTCGATGTTGGATTAAAATTAGATATAAGAGCAGAAGCTCGTA
AAGTTCTAGGTCTGTTCGACCTTTAAATTTTTACATGATCTGAGTTCAACCGGAGACA
CAATGTACAAAGTCTATCCTG

- ***Cletus punctiger - MT568728*** **(COI)**

ATAATTTTTTTTATAGTAATACCCATTATAATTGGCGGATTTGGAAATTGACTTGTCCC
ATTAATAATTGGGGCACCTGATATAGCATTCCCACGTATAAATAACATAAGATTTTGA
TTACTACCCCCTTCACTGACTCTCTTATTAACTAGTAGAATAGTAGAGAAGGGTGCTG
GAACTGGTTGAACAGTCTACCCTCCTCTATCAAGTAATTTATCACACAGAGGTGCATC
AGTAGATTTAGCAATTTTTTCATTACATTTAGCAGGTGTATCCTCAATTTTAGGAGCTG
TTAACTTCATTTCTACTATTATCAATATACGCCCAGTAGGTATGACCCCCGAGCGTAC
TCCA

- ***Cletus punctiger - MT102349*** **(16s rRNA)**

TCGCCCTGTTTAACAAAAACATGTCTTATAGATTTTTAATTTTAAGTCTAATCTGCCCG
GTGATTAATTAAACGGCCGCAGTATTCTGACTGTGCGAAGGTAGCATAATCGTTTGTC
TCTTAATTGGGGGCTTGTATGAATGATTGGACGAGGGACTGACTTTTTTTATTTAATTT
TTATGAATTTGATTTTTGAGTTAAAAAGCTTAAATTTTTTTGCAAGACGAGAAGACCC
TATAGAATTTTATTTTTAATTAATTACTTACTATTTGGTTTATTAACATTAGGTAGTTTT
TTAAGAATTTTATGGGGGCGATATTGAAATTAAAATAACTTTCATTTATAATTTTTCAT
TATTTAATGTATTTTTTTGATCCTTTATTATGGATTATTAGATTAAATTACCTTAGGGAT
AACAGCGTAATTTTTTCGGAGAGTTCTTATCGATGAATTAAGTTTGCGACCTCGATGT
TGGATTAAAGTAAATTTTTGGTGCAGAAGCTAAAAAATTGAGTCTTTCGACTCTTAAA
TCTTTACATGATCTGAGTTCAACCGGA

- ***Nezara antennata - MT707349*** **(COI)**

TTGAACTAGGACAACCCGGAAGATTTATTGGAGATGATCAAATTTATAATGTAGTAGT
AACAGCTCACGCATTTGTAATAATTTTCTTTATAGTAATGCCAATCATAATTGGAGGA
TTTGGAAATTGATTAGTACCTTTAATGATCGGTGCCCCAGACATAGCATTCCCTCGAA
TAAACAATATAAGATTCTGACTATTACCCCCATCATTAACCCTTTTAATAGTAAGAAG
ATTAGCAGAATCTGGAGCAGGGACAGGATGAACTGTTTATCCTCCTTTATCTAGTAAC
TTATCCCATAGAGGAGCTTCAGTGGATTTAGCTATTTTTTCATTACATCTAGCAGGAG
TATCATCAATTTTAGGTGCAGTAAATTTCATTTCAACTATTATTAATATACGACCAAC
AGGTATAACTCCAGAACGAGTGCCACTATTTGTTTGATCAGTTGGAATCACAGCACTA
TTATTACTACTTTCATTACCTGTACTAGCAGGTGCAATTACAATATTATTAACAGATCG
AA

- ***Nezara antennata* - MT994693 (16s rRNA)**

AAAACTAGACTTAAGTGACAGTCGCCTGTTTTTCAAAAACATGTCTTGATGATTTTTATTTATTAAGTCTATCCTGCTCAATGATTTATTATTAAATAGCCGCAGTATCTTGACTGTGCGAAGGTAGCATAATCATTTGTCTTTTAATTAAAGGCTTGTATGAATGGTTGGATGAGGGAATTACTTTCTTTATATTAAAAATTGAATTTAATTTTTAAGTTAAAAAGCTTAAATTTTTAAGTGGGACGAGAAGACCCTATAGAATTTTATTATAAGTTTTATATTTAATTTATGGTTATTTTTATTATTTATATGTCTTGTAATTTTATTGGGGTGATATTGAGATTAAAAAAACTCTCATAACTTAATATTTAATTATTTTTATTTTGTTAGGATCCTATTTTATGGATATTTAGATTAAATTACCTTAGGGATAACAGCGTAATTTTTCTGGAGAGTTCTTATCGATGGAAAAGTTTGCGACCTCGATGTTGGATTAAAATTAGATTTAAGTGCAGAAGCTTAAAGTTCTAGGTCTGTTCGACCTTTAAATTTTTACATGATCTGAGTTCAACCGGAACGCCTTATTTACCCACCCTCTATACCTGC

- ***Melanaphis sacchari* - MT707350 (COI)**

ATTTTAATTCGATTAGAATTAAGTCAAATTAATTCAATTATTAATAATAATCAATTATATAATGTTATTGTTACAATTCATGCATTTAATATAATTTTTTTTATAACTATACCAATTGTAATTGGGGGATTTGGAAATTGATTAATTCCTATAATAATAGGTTGTCCAGATATATCTTTTCCGCGATTAAATAATATTAGATTTTGACTTTTACCTCCATCATTAATAATAATAATTTGTAGATTTATAATTAATAATGGAACAGGTACAGGTTGAACTATTTATCCACCATTATCTAATAACATTGCACATAATAATATTTCAGTAGATTTAACTATTTTTTCACTTCATTTAACAGGAATTTCATCTATTTTAGGTGCAATTAATTTTATTTGTACAATTTTAAATATAATACCTAATAATTTAAAATTAAATCAAATTCCTCTATTCCCATGATCAATCTTAATTACAGCTACACT

- ***Melanaphis sacchari* - MT994694 (16s rRNA)**

AAAATTTTTAAGTTTTAGTTCTTTTTCCGCTTCGCCTGTTTTTCAAAAACATTGTATTAAGTTTTATTTAATATAGGATCTGCTCAATGATTTATTAAATAGCTGCAGTATTTTGACTGTGCAAAGGTAGCATAATAATTTAGCCTTTTAATTGAAGACTTGTATGAAAGATTTGATGAGAAATAAACTTTATTATTTATATAATTAAAATTTTATTTTTAAGTAAAAAAGCTTAAATGATTTAAAGGGACCATAAAACCCTATAAAACTTTATAAATTATAATTTTAATTTTTTTGGATTAATAAATATTTTATTTTTATAATTTATTTTATTGGGGTGATAAAAAAAATTGATTAACTTTTTTTAAAATTTAACATTATTTAATGATTTATTGAATTAAAATTTTTAATTAAAGGAAAAAGTTACTTTAGGGATAACAGCGTAATTAATTTTTTAAGTTCAAATTTAAAAAATTAGTTTGCGACCTCGATGTTGAATTAAGATTAATCTTAGGTGCAAAATTTTAGTGTTATATAGGACTGCTCATGATTATTAATAGCTGCGTATTTTGACTGTGCAAAGGTAGCATAATTATTTAGCCTTTTTTTG

- ***Leptocentrus Taurus* - MT707350 (COI)**

CCTGACATAGCATTCCCCCGACTAAATAATATAAGATTTTGACTTTTACCACCTTCATTAATAATACTGATATTAAGATCAAGAATTGAGTCTGGAGTAGGCACTGGGTGAACTATATACCCTCCACTTTCATCAAATGTGGCTCACTCATCACCTAGAGTGGATATAGCAATTTTCTCCCTCCATTTAGCTGGTATTTCATCAATTTTAGGAGCCATTAATTTTATTACAACAGTTATTAATATACGAGCAAATGGGATAAAGATAGATCAAACACCTTTATTTGTGTGATCAGTATTGATTACAGCTTTTCTATTACTTCTGTCACTACCAGTATTAGCAGGTGCTATTACTATGTTATTAACAGATCGAAATATTAAC

- ***Leptocentrus Taurus*** **- MT994692 (16s rRNA)**

CGCTCTAATCTGAGACCCTTCGCCTGTTTTATCAAAAACATTGTATTAAGTTTTATTTA
ATATAGGATCTGCTCAATGATTGGTGATTAAATAGCTGCAGGATTCTGACTGTGCAAA
GGTAGGCATAATAATTAGTCTTTTAATTGAGGACTTGCATGAAAGATTTGATGACAAA
TAAACTATATTATTTATATAATTAAAATTTTATTTTTAAGTAAAAAAGCTTAAATTATT
TAAAGGGACGATAAAACCCTATACAAACTTTATAAATTATAATTTTAATTTTTTTGGA
TTAATAAATATTTTATTTTTATAATTTATTTTATTGGGGTGATAAAAAAAAATTGATTAA
CTTTTTTTAAAATTTAACATTATTTAATGATTTATTGAATTAAAATTTTCAATTAAAGG
AAAAAGTTACTTTAGGCATAACAGCGTAATTAATTTTTTAAGTTCAAATCTAAAAATT
AGTTTGCCACCTCGATGTTGAATTAAGATTAATCTAAGGTGCAAAATGTTTAAGTTTT
AGGTCTGTTCGACCTTTAAATTCTTACATGATCTGAGTTCAACCGGAGGTCTTTACAG
GCGTCCCCTCA

- ***Nezara mendax*** **- MT707352 (COI)**

CTAATTATTCGAATTGAACTAGGACAACCCGGAAGATTTATTGGAGATGATCAAATTT
ATAATGTAGTAGTAACAGCTCACGCATTTGTAATAATTTTCTTTATAGTAATGCCAAT
CATAATTGGAGGATTTGGAAATTGATTAGTACCTTTAATGATTGGTGCCCCAGACATA
GCATTCCCTCGAATAAACAATATAAGATTCTGACTATTACCCCCATCATTAACCCTTTT
AATAGTAAGAAGATTAGCAGAATCTGGAGCAGGGACAGGATGAACTGTTTATCCTCC
TTTATCTAGTAACTTATCCCATAGAGGAGCTTCAGTGGATTTAGCTATTTTTTCATTAC
ATCTAGCAGGAGTATCATCAATTTTAGGTGCAGTAAATTTCATTTCAACTATTATTAA
TATACGACCAACAGGTATAACTCCAGAACGAGTGCCACTATTTGTTTGATCAGTTGGA
ATCACAGCACTATTATTACTACTTTCATTACCTGTACTAGCAGGTGCAATTACAATATT
ATTAACAGATCGAAACTTTAATACATCATTCTTTGACCCTT

- ***Nezara mendax*** **- MT994695 (16s rRNA)**

TTACACTTCTAGGCCCCTTTCCCCCCCGCCTGTTTTTTCAAAAACATGTCTTGATGATT
TTTATTTATTAAGTCTATCCTGCTCAATGATTTATTATTAAATAGCCGCAGTATCTTGA
CTGTGCGAAGGTAGCATAATCATTTGTCTTTTAATTAAAGGCTTGTATGAATGGTTGG
ATGAGGGAATTACTTTCTTTATATTAAAAATTGAATTTAATTTTTAAGTTAAAAAGCTT
AAATTTTTAAGTGGGACGAGAACACCCTATAGAATTTTATTATAAGTTTTATATTTAA
TTTATGGTTATTTTTATTATTTATATGTCTTATAATTTTATTGGGGTGATATTGAGATTA
AAAAAACTCTCATAACTTAATATTTAATTATTTTTATTTTGTTAGGATCCTATTTTATG
GATATTTAGATTAAATTACCTTAGGGATAACAGCGTAATTTTTCTGGAGAGTTCTTAT
CGATGGAAAAGTTTGCGACCTCGATGTTGGATTAAAACTAGATTTAAGTGCAGACAG
CTTAAAGTTCTAGGTCTGTTCGACCTTTAAATTTTTACATGATCTGAGTTTCAACCGGG
ATAAAATATTTAATTAAAGTCTAT

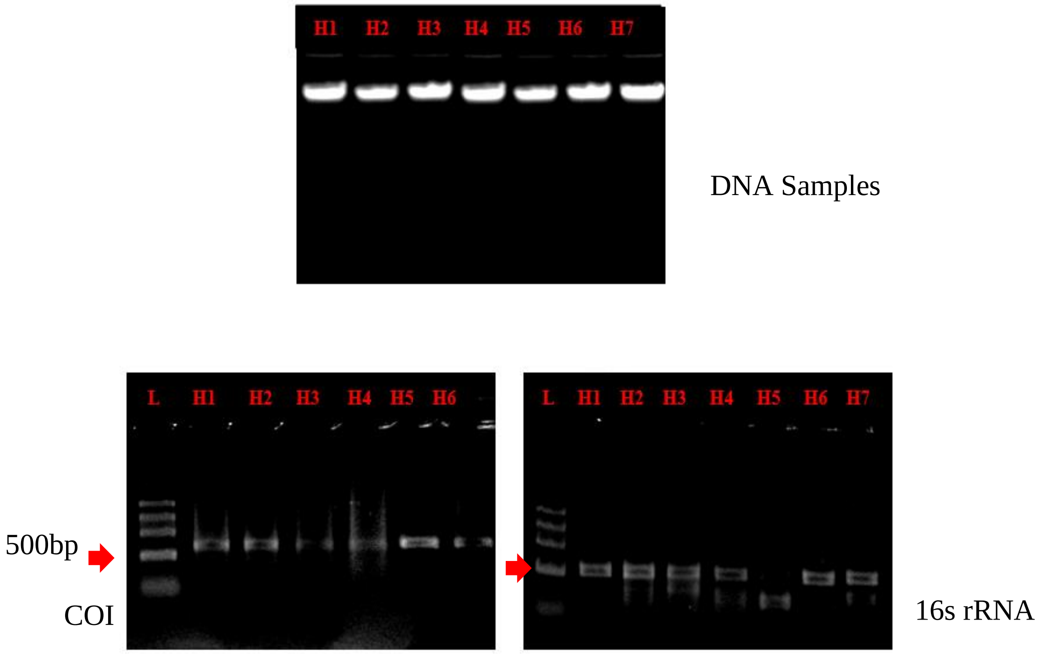

Figure 3. 9: Electrophoresis gel image of DNA, PCR product of COI and 16s rRNA of Hemiptera species representatives

GC Content and AT – GC Skew analysis of Order Lepidoptera

A genomics analysis order wise GC% resulted that GC content of Lepidoptera was recorded highest with *Olene mendosa* (32.6%) and lowest with *Spodoptera litura* (27.1%) and AT content was maximum with *Spodoptera frugiperda* (72.9%) and minimum with *Spoladea recurvalis* (67.4 %) (Table 3.19). Further, each order's sequence was analyzed for GC and AT skews, where GC skew of Lepidoptera was found the maximum in *Spoladea recurvalis* (0.01%), whereas the minimum was found in *Ariadne merione* and *Helicoverpa armigera* (-0.10%) among the selected species. AT skew of Hemiptera was found the maximum in *Spodoptera litura* (0.23%), whereas the minimum was found in *Ariadne merione* (-0.18%) among the selected species. (Table 3.19 and Figure 3. 10). Table 3. 19 also represents the Accession number obtained after sequence submitted to NCBI.

Lepidoptera	**NCBI Accession No.**		**AT Skew**	**GC Skew**	**GC%**	**AT%**
	COI	**16s rRNA**				
Spodoptera litura	MT568729	MT570012	0.23	-0.02	27.1	72.89
Spodoptera frugiperda	MT765070	MT774213	-0.17	-0.09	28.3	71.66
Ariadne merione	MT568733	MT603574	-0.18	-0.10	28.8	71.22
Helicoverpa zea	MT707354	MT774552	-0.12	-0.06	29.1	70.92
Helicoverpa armigera	MT568731	MT774154	-0.11	-0.10	29.3	70.72
Spodoptera sunia	MT707355	MT774553	-0.17	-0.08	29.4	70.6
Spoladea recurvalis	MT568730	MT102741	-0.07	0.01	30	70
Plutella xylostella	MT568735	MT570013	-0.16	-0.03	30.2	69.76
Spilarctia obliqua	MT568732	MT937230	-0.13	-0.07	30.4	69.63
Olene mendosa	MT568734	MT603575	-0.17	-0.06	32.6	67.36

Table 3. 21: Order Lepidoptera with Accession no., AT-GC- Skew and AT- GC content

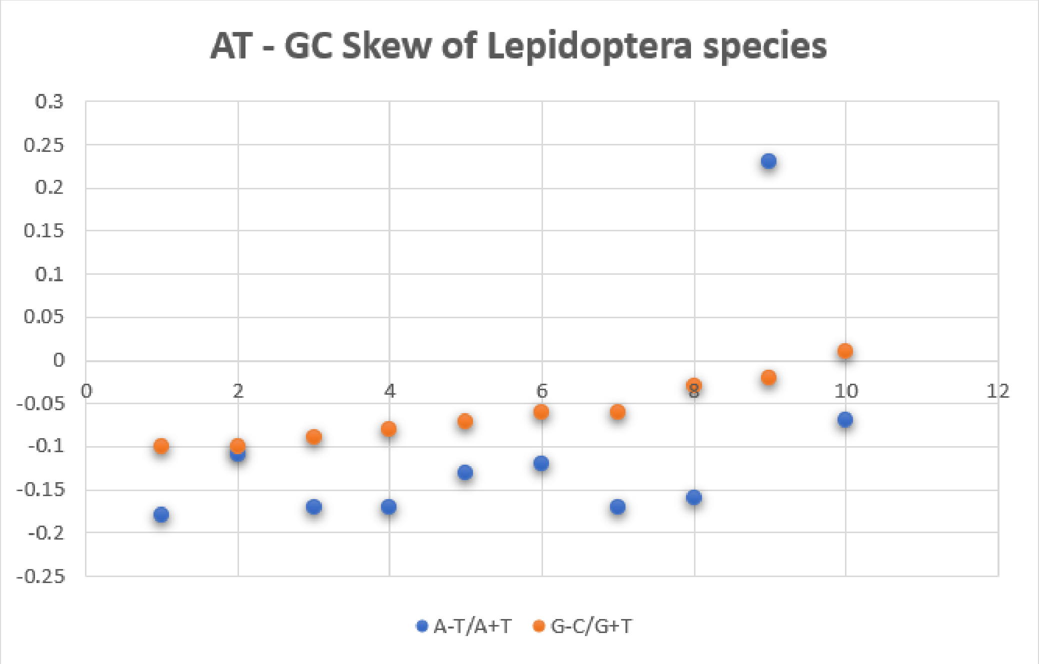

Figure 3. 10: AT - GC Skew Analysis of Lepidoptera species

We also did the AT and GC content of individual orders, where Lepidoptera demonstrated AT bias of 70.4 and GC of 29.6 for COI and 77 and 23.02 for 16s rRNA. The estimated Transition/Transversion bias (*R*) for COI and 16s rRNA was found to be 5.195 and 0.549, respectively. The nucleotide frequencies of COI were 31.79% (A), 38.42% (T/U), 15.65% (C), and 14.14% (G) and G = 16.57% and that of 16s rRNA was 38.50% (A), 38.78% (T/U), 12.53% (C), and 10.19% (G). The probability rate for synonymous to-nonsynonymous mutation was tested and the analysis involved 10 nucleotide sequences. A total of 153 positions in the final dataset was compared and 13 interspecies significance ($p<0.05$, $p<0.001$) was obtained for COI (Table.3.20) and that for 16s rRNA it was eight interspecies significance ($p<0.05$, $p<0.001$) for the mutation rate was obtained for 108 positions in the final dataset (Table 3.21).

	Lepidoptera species	1	2	3	4	5	6	7	8	9	10
1	*Spodoptera frugiperda*										
2	*Spodoptera litura*	0.082									
3	*Spoladea recurvalis*	0.130	0.008								
4	*Helicoverpa zea*	0.000	0.098	0.470							
5	*Spilarctia obliqua*	0.012	0.065	0.117	1.000						
6	*Ariadne merione*	0.008	0.007	0.030	1.000	1.000					
7	*Olene mendosa*	0.044	0.196	1.000	0.159	0.079	0.062				
8	*Helicoverpa armigera*	0.000	0.098	0.470	1.000	1.000	1.000	0.217			
9	*Plutella xylostella*	0.004	0.060	0.033	1.000	1.000	1.000	0.001	1.000		
10	*Spodoptera sunia*	0.004	0.112	1.000	1.000	1.000	1.000	0.035	1.000	1.000	

Table 3. 22: Codon-based Test of Neutrality analysis between COI sequences of Lepidoptera

	Lepidoptera species	1	2	3	4	5	6	7	8	9	10
1	*Spodoptera_frugiperda*										
2	*Spodoptera_litura*	0.353									
3	*Spoladea_recurvalis*	0.082	0.138								
4	*Helicoverpa zea*	1.000	0.259	0.112							
5	*Spilarctia obliqua*	0.073	0.013	0.251	0.010						
6	*Ariadne merione*	1.000	0.032	0.260	0.326	0.003					
7	*Olene mendosa*	1.000	1.000	0.040	0.064	0.553	0.207				
8	*Helicoverpa armigera*	1.000	0.263	0.127	1.000	0.010	0.378	0.037			
9	*Plutella_xylostella*	0.160	0.296	0.125	0.112	0.210	0.243	0.295	0.194		
10	*Spodoptera sunia*	1.000	0.549	0.112	0.218	0.023	0.528	0.196	0.212	0.116	

Table 3. 23: Codon-based Test of Neutrality analysis between 16s rRNA sequences of Lepidoptera

	Lepidoptera species	1	2	3	4	5	6	7	8	9	10
1	*Spodoptera frugiperda*										
2	*Spodoptera litura*	13.95									
3	*Spoladea recurvalis*	9.05	8.38								
4	*Helicoverpa zea*	7.10	6.53	8.45							
5	*Spilarctia obliqua*	8.22	6.68	7.77	0.19						
6	*Ariadne merione*	7.22	6.28	7.56	0.18	0.16					
7	*Olene mendosa*	6.53	9.67	0.17	7.09	7.55	6.29				
8	*Helicoverpa armigera*	7.42	6.53	8.45	0.00	0.20	0.18	7.21			
9	*Plutella xylostella*	7.68	7.74	8.38	0.28	0.32	0.26	6.76	0.29		
10	*Spodoptera sunia*	7.91	7.48	8.94	0.14	0.13	0.17	6.77	0.14	0.27	

Table 3. 24: Pairwise distance analysis COI sequences of Lepidoptera

	Lepidoptera species	1	2	3	4	5	6	7	8	9	10
1	*Spodoptera_frugiperda*										
2	*Spodoptera_litura*	3.44									
3	*Spoladea_recurvalis*	3.17	2.07								
4	*Helicoverpa zea*	0.48	3.39	4.40							
5	*Spilarctia obliqua*	2.21	3.49	2.55	3.04						
6	*Ariadne merione*	3.32	3.16	2.74	3.19	3.36					
7	*Olene mendosa*	4.52	2.76	3.36	3.48	3.31	4.25				
8	*Helicoverpa armigera*	0.46	3.24	3.66	0.01	3.15	3.05	4.26			
9	*Plutella_xylostella*	3.24	2.41	2.30	2.74	2.94	3.09	2.77	2.80		
10	*Spodoptera sunia*	1.66	2.83	3.30	0.96	3.42	4.21	4.17	0.99	2.33	

Table 3. 25: Pairwise distance analysis 16s rRNA sequences of Lepidoptera

Phylogenetic analysis of Order Lepidoptera

Lepidoptera was represented by five families with 10 species barcoded sequences. The most dominant was found to be Noctuidae with three species of barcode data. The pairwise distance was minimal in Noctuidae and Erabidae, while Crambidae, Nymphalidae, and Plutellinidae showed related closeness (Figure 3. 11). Therefore, the resolved phylogeny for order Lepidoptera was found to be (Noctuidae + (Erabidae + (Crambidae + (Nymphalidae + Plutellinidae)))). In Lepidoptera, five families were found: Noctuidae, Erabidae, Crambidae, Nymphalidae, and Plutellinidae. The prominent sequenced representatives belonged to Noctuidae, and the three species of Spodoptera *S. sunia*, *S. frugiperda*, *S. littura* were monophyletic to each other. In comparison, it was paraphyletic to *H. armigera* and *H. zea*. This family was found to be paraphyletic to Erabidae, having two representatives- *S. obliqua* and *O. mendosa*. This was having the closest distance and was found to be sister clades of each other. Similarly, Crambidae had a single representative, *S. recurvalis*, having the closest *A. merione* of family Nymphalidae. The family Plutellinidae also had a single representation of *P. xylostella*, which was also closely related to *A. merione*.

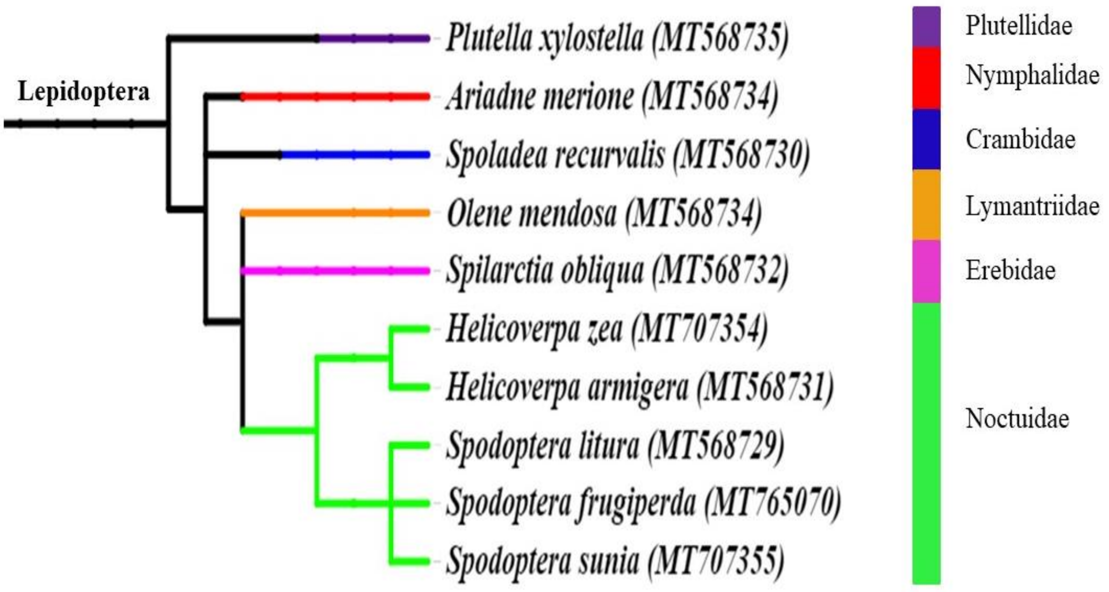

Figure 3. 11: Phylogenetic tree of Lepidoptera species

The following are the sequences (COI and 16s rRNA) which are obtained through Sanger Sequencing

- ***Spodoptera frugiperda* - MT765070 (COI)**

ATTTATAATACTATTGTAACAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCTATT
ATAATTGGAGGATTTGGAAATTGACTTGTACCTTTAATATTAGGAGCTCCTGATATAGCTT

TCCCACGTTTAAATAATATAAGTTTTTGACTTTTACCACCTTCTTTAACCTTACTAATTTCAAGTAGAATTGTAGAAAATGGAGCAGGAACTGGATGAACAGTTTACCCCCCCCTCTCCTCTAATATTGCTCATGGGGGAAGATCAGTAGATTTAGCTATTTTTTCCCTTCACTTAGCTGGAATTTCATCTATTTTAGGAGCTATTAACTTTATTACTACTATTATTAATATACGATTAAATAATTTATCATTTGATCAAATACCTTTATTTGTTTGAGCTGTAGGAATTACTGCATTTTTATTATTATTATCTTTACCTGTTTTAGCTGGAGCTATTACTATATTATTAACTGATCGAAATTTAAATACATCATTT

- ***Spodoptera frugiperda* - MT774213 (16s rRNA)**

TCCGGTTGAACTCAGATCATGTAAGATTTTAATGATCGAACAGATCAAAAATTTTAAACTTCTACATTTAAATTTTATCTTAATCCAACATCGAGGTCGCAAACTCTTTTTTTTATTCGAACTAAAAAAAAAAATTACGCTGTTATCCCTAAGGTAATTTTTTCTTATAATCATTAAAAATGGATCATAAAATCAATTATATATGTTAAAATTTAAAAAAAGTTATTTTTATTTTTTTGTCACCCCAACAAAATAATTAAAAATAATTTCAATTAAAAATTTTATAAATAATCTTAATTAATTTAAATATAAAACTCTATAGGGTCTTCTCGTCTTTTTAAATTATTTTAACTTTTTAATTAAAAAATTAAATTCTATAAATAATAAAGAGACAGTTTATATTTCATCCAATCTTTCATACAAGTCATCAATTAAATGACTAATGATTATGCTGACCTTTGTACAGTCAATATACTGCAGCCCCATAATATAAATTATATCAGTGGGCAGATTAAACTTTATATTAAATTCAAAAAGACATGTTTTTGTAAAACAGGCGA

- ***Spodoptera litura* - MT568729 (COI)**

ATAGTAATAGCTCCAGCTAAAACAGGTAAAGATAATAATAATAAAAATGCAGTAATTCCTACAGCTCAAACAAATAAAGGTATTTGATCAAATGATAAATTATTTAATCGTATATTAATAATAGTAGTAATAAAGTTAATAGCTCCTCCTAAAATAGATGAAATTCCAGCTAGATGAAGGGAAAAAATAGCTAAATCTACTGATCTTCCACCATGAGCAATATTA

- ***Spodoptera litura* - MT570012 (16s rRNA)**

TTCGGCCTTTTTTATCAAAAACATGTCTTTTTGAATTTAATATAAAGTCTAATCTGCCCACTGATATAATTTATATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTAGCATAATCATTAGTCATTTAATTGATGACTTGTATGAAAGATTGGATGAAATATAAACTGTCTCTTTATTATTTATAGAATTTAATTTTTTAATTAAAAAGTTAAAATAATTTAAAAAGACGAGAAGACCCTATAGAGTTTTATATTTAAATTAATTAAGATTATTTATAAAATTTTTAATTGAAATTATTTTTAATTATTTTGTTGGGGTGACAAAAAAATAAAAATAACTTTTTTTAAATTTTAACATATATAATTGATTTTATGATCCATTTTTAATGATTATAAGAAAAAATTACCTTAGGGATAACAGCGTAATTTTTTTTTTTAGTTCGAATAAAAAAAAAGAGTTTGCGACCTCGATGTTGGATTAAGATAAAATTTAAATGTAGAAGTTTAAAATTTTTGATCTGTTCGATCATTAAAATCTTACATGATCTGAGTTTCAACCGGA

- ***Spoladea recurvalis* - MT568730 (COI)**

ATAGTTATACCAATTATAATTGGAGGATTCGGAAATTGATTAGTACCTTTAATATTAGGAGCTCCAGATATAGCTTTCCCACGAATAAATAATATAAGATTTTGATTATTACCTCCTTCTTTAACTTTATTAATTTCTAGAAGTATTGTTGAAAACGGAGCTGGAACAGGATGAACAGTTTACCCCCCACTTTCATCTAATATTGCTCATGGAGGAAGATCAGTAGACTTAGCTATTTTTTCTTTACATTTAGCAGGAATTTCTTCAATTTTAGGAGCAATTAATTTTATTACAACTATTATTAACATACGAATTAATGGTTTACAATTTGATCAAATACCTTTATTTGTCTGAGCTGTTGGAATTACAG

- ***Spoladea recurvalis* - MT102741 (16s rRNA)**

TCTAATCTGCCCACTGATGAAATATTAAAGGGCTGCAGTATATTGACTGTACAAAGGTAGCATAATCAATAGTCTCTTAATTGGTGACTTGTATGAAAGATTGGATGAGATATAAACTGTC

TTAATAATAATTAAAGAAATTAATTTTTTATTTAAAAAGTTAAAATAAATTTAAAAGACGA
GAAGACCCTATAGAGTTTTATAAAATTTTTTATTAAAATTATATATAAAAATAATAATTAA
AATAAAAATTTTTATTTTGTTGGGGTGATAAAAAAATATAAATAACTTTTTTTTAATAAAA
ACATAAATAAATGAATAATTGATCCAAAATTATTTTGATTAAAAGAAAAAATTACCTTAG
GGATAACAGCGTAATTTTTTTTTTTAGTTCAAATAAAAAAAAAAGTTTGCGACCTCGATGT
TGGATTAAGATAAAATTTATATGCAAAAGTTTAAAATTTTGATCTGTTCGATCATTAAAAT
CTTACATGATCTGAGTTCAACCGGA

- ***Helicoverpa zea* - MT707354 (COI)**

ATAATTTTTTTTATAGTTATACCAATTATAATTGGTGGATTTGGTAATTGACTTGTACCTTT
AATATTAGGAGCCCCTGATATAGCTTTCCCCCGAATAAATAATATAAGTTTTTGATTACTT
CCCCCTTCTTTAACTTTACTTATTTCAAGAAGAATTGTAGAAAATGGAGCAGGAACAGGAT
GAACAGTTTACCCCCCACTTTCATCTAATATTGCACATGGAGGAAGATCAGTAGACCTAGC
TATTTTTTCTTTACATTTAGCTGGAATCTCATCTATTTTAGGAGCAATTAATTTTATTACTA
CTATTATTAATATAAAATTAAATAGCTTATCTTTTGATCAAATGCCATTATTTATTTGAGCT
GTAGGAATTACTGCATTTTTATTATTATTATCATTACCAGTTTTAGCAGGTGCTATTACTAT
ACTTTTAACAGATCGA

- ***Helicoverpa zea* - MT774552 (16s rRNA)**

CCGGTTGAAACTCAGATCATGTAAGATTTTAATGATCGAACAGATCAAAAATTTTAAACTT
CTGCATTTAAATTTTATCTTAATCCAACATCGAGGTCGCAAACTCTTTTTTTTATATGAACT
AAAAAAAAAAATTACGCTGTTATCCCTAAGGTAATTTTTTCTTATAATCAATAATATTGGA
TCAATTATTCACTTATTTATGTTTTATAAAAAAAAAGTTATTTTTATTTTTTTGTCACCCCA
ACAAATTAAATTAAATTATTTTAATTATTAATTTTATAAATAATCTTAATAATTATAATTTA
TAAAACTCTATAGGGTCTTCTCGTCTTTTTAATTAATTTTAGCTTTTTAACTAAAAAAATTAA
ATTTTATATTATAAATAAGAGACAGTATATATTTCATCCAATCTTTCATACAAGTCATCAA
TTAAATGACTAATGATTATGCTACCTTTGTACAGTCAAAATACTGCAGCCCTTTAATAATT
AAATCAGTGGGCAGATTAAACTTTATATTATTTACAAAAAGACATGTTTTTGTAAAACAGG
C

- ***Spilarctia obliqua* - MT568732 (COI)**

ATAATTTTTTTTATAGTAATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTACCTTT
AATGTTAGGGGCCCCTGATATGGCTTTTCCACGTATAAATAATATAAGTTTTTGACTTTTA
CCCCCATCATTAACCTTATTAATTTCAAGAAGAATTGTAGAAAATGGTGCAGGAACAGGA
TGAACAGTTTACCCCCCACTCTCATCTAATATTGCCCATGGAGGAAGTTCAGTTGATTTAG
CTATTTTTTCCCTTCATTTAGCTGGAATTTCATCAATTTTAGGAGCTATTAATTTTATTACTA
CAATTATTAATATACGATTAAATAATCTATCATTTGATCAAATACCTTTATTTGTTTGAGCA
GTAGGAATTACAGCTTTTTTACTTCTTTTATCACTTCCTGTTTTAGCAGGGGCTATTACCAT
ACTTTTAACCGATCGAAATTTAAATACATC

- ***Spilarctia obliqua* -**

ACCCTATAGAGTTTTATTTATTTTTATATTATAACTATTTATAAATTTAAAATTTTTAATTTA
ATAATAAATTTTATTGGGGTGATGAAAAAATATGTTTAACTTTTTTTTTATGTATAATATAT
ATATATATATATATTAAACATTAATAAGTGATTAATTGATCCAATTTTATTGATTAAAAGA
AAAAATTACCTTAGGGATAACAGCGTAATTTATTTTTTTAGTTCATATAAAAAAAATGAGTT
TGCGACCTCGATGTTGGATTAAGATAAAAATTAAATGCAAAAGTTTAAATTTTTGATCTGT
TCGATCATTAAAATCTTACATGATCTGAGTTCAGACCGG

- ***Ariadne merione* - MT568733 (COI)**

ATAATTTTTTTCATAGTTATACCTATTATAATTGGTGGATTTGGTAATTGATTAGTACCTTT
AATATTAGGAGCTCCTGATATAGCTTTTCCTCGAATAAATAATATAAGTTTTTGACTCCTTC
CCCCATCATTAACTTTATTAATTTCTAGAAGAATTGTCGAAAATGGAGCAGGAACAGGAT
GAACAGTTTACCCCCCTTTATCTTCTAATATTGCACACGGAGGATCCTCAGTTGATTTAGC
TATTTTTTCCCTTCATCTAGCTGGAATTTCTTCCATTTTAGGAGCTATTAATTTTATTACTAC
AATTATTAATATACGTATTAATAATATATCTTTTGATCAAATACCATTATTTATTTGAGCTG
TAGGTATTACAGCTTTATTATTATTATTATCTTTACCTGT

- ***Ariadne merione* - MT603574 (16s rRNA)**

AGTGGGCAGACTAGACTCTGACCCAAACTCCGGGTGAACTCAGATCACGTAAGATTTTAA
TGATCGAACAGATCAAAATTTTAAACTTTTGCATTTAAATTTTATCTTAATCCAACATCGA
GGTCGCAAACTTTTTTTTTTATTTGTTCTAAAAAAAAATATTACGCTGTTATCCCCAAGGTA
ATTTTTTCTTTTAATCATTAATAATGGATCAATTTTTTACTTATTTATATTTATTAAAAAAA
AAAGTTTATTTAATTTTTTTATCACCCCAACAAAATATTTTTTATTAATTAAATAAATTTAT
ATATATATATATATATATATATATAATAAAAAAAAAATATAAAACTCTATAGGGTCTTCTC
GTCTTTTAATTAAATTCTAGCTTTTTAACTAAAAAATTAAATTCTATAAATAAATTAAAAA
CAGTTTATATTTCATCAAATCTTTCATACAAGTCTTCAATTAAAAGACTAATGATTATGCT
ACCTTTGTACAGTCAATATACTGCACCCCTTTAATTAATAAATGCAGGGGGCAGATTAAAC
TTTAAATTATTAAACAAAAAGACATGTTTTTGATAAACAGGGGACATTCGAAAGGATCAA
ATTTTA

- ***Olene mendosa* - MT568734 (COI)**

ATAGTTATACCAATTATAATTGGAGGATTTGGTAATTGATTAGTTCCTTTAATATTAGGAG
CCCCTGATATAGCTTTCCCCCGAATAAATAATATAAGATTTTGACTTTTACCTCCTTCTTTA
ACTCTTCTTATCTCAAGAAGTATTGTAGAAAATGGAGCTGGAACTGGATGAACAGTTTACC
CACCTCTTTCTTCTAATATCGCCCACGGAGGAAGTTCAGTTGATTTAGCTATTTTTTCTCTT
CACTTAGCTGGAATTTCTTCTATTCTTGGAGCAATTAATTTTATTACTACTATTATTAATAT
ACGACTTAATAATCTTTCATTTGATCAGATACCTTTATTTGTTTGAGCTGTTGGAATTACAG
CATTCCTTTTATTATTATCTTTACCAGTATTGGCAGGAGCTATTACTATACTTTTAACAGAT
CGAAATCTTAATACTTCATTTTTTGACCCTGCGGGAGGAGGAGATC

- ***Olene mendosa* - MT603575 (16s rRNA)**

CAGACAGCCCTCCTCCACGCCGGTTGAACTCAGATCATGTAAGATTTTAATGATCGAACA
GATCAAAAACTTTAAACTTCTGCATTTAAATTTTATCTTAATCCAACATCGAGGTCGCAAA
CTCTTTTTTTTATATGAACTAAAAAAAAAAAATTACGCTGTTATCCCTAAGGTAATTTTTTCT
TATAATCATTAAAAAATGGATCAAACATTCACTTATCTATGTTAAAATTAAAAAAAAAGTTT
ATTAAATTTTTTTATCACCCCAATAAAATTTTTTAATAAATTTAAATATTTCTTTTATAAAT
AATTATAATTTATTAAAAAATAAAACTCTATAGGGTCTTCTCGTCTTTTTTTTTTATTTTAA
CTTTTTAATTAAAAAATTAATTTCTATTTTATATTTTAAAGACAGTTTATATTTCATCCAAT
CTTTCATACAAGTCATCAATTAAATGACTAATGATTATGCTACCTTTGTACAGTCAAATTA
CTGCAGCCCTTTAATTTTCAGCAGTGGGGCAAATTAAACCTGATCCCGAAACGGAATCAA
AAAA

- ***Helicoverpa armigera* - MT568731 (COI)**

ATAATTTTTTTTATAGTTATACCAATTATAATTGGTGGATTTGGTAATTGACTTGTACCTTT
AATATTAGGAGCCCCTGATATAGCTTTCCCCCGAATAAATAATATAAGTTTTTGATTACTT
CCCCCTTCTTTAACTTTACTTATTTCAAGAAGAATTGTAGAAAACGGAGCAGGAACAGGAT
GAACAGTTTACCCCCCACTTTCATCTAATATTGCACATGGAGGAAGATCAGTAGACCTAGC

TATTTTTTCTTTACATTTAGCTGGAATCTCATCTATTTTAGGAGCAATTAATTTTATTACTA
CTATTATTAATATAAAATTAAATAGCTTATCTTTTGATCAAATGCCATTATTTATTTGAGCT
GTAGGAATTACTGCATTTTTATTATTATTATCATTACCAGTTTTAGCAGGTGCTATTACTAT
ACTTTTAACAGATCGAAACCTTAATACATC

- ***Helicoverpa armigera - MT774154***

TCCGGTTGAACTCAGATCATGTAAGATTTTAATGATCGAACAGATCAAAAATTTTAAACTT
CTGCATTTAAATTTTATCTTAATCCAACATCGAGGTCGCAAACTCTTTTTTTATATGAACT
AAAAAAAAAAAATTACGCTGTTATCCCTAAGGTAATTTTTTCTTATAATCAATAATATTGGA
TCAATTATTCACTTATTTATGTTTTATAAAAAAAAAGTTATTTTTATTTTTTTGTCACCCCA
ACAAATTAAATTAAATTATTTTAATTATTAATTTTATAAATAATCTTAATAATTATAATTTA
TAAAACTCTATAGGGTCTTCTCGTCTTTTTAATTAATTTTAGCTTTTTAACTAAAAAATTAA
ATTTTATATTATAAATAAGAGACAGTATATATTTCATCCAATCTTTCATACAAGTCATCAA
TTAAATGACTAATGATTATGCTACCTTTGTACAGTCAAAATACTGCAGCCCTTTAATAATT
AAATCAGTGGGCAGATTAAACTTTATATTATTTACAAAAAGACATGTTTTTGA

- ***Plutella xylostella* - MT568735 (COI)**

ATAATTTTTTTTATAGTTATACCTATTGTTATTGGAGGATTCGGAAATTGACTTATCCCATT
AATATTAGGAGCTCCTGATATAGCTTTTCCTCGAATAAATAATATAAGATTTTGACTACTT
CCCCCCTCATTAACCTTATTAATTTCCAGAAGAATTGTTGAAAATGGAGCAGGTACTGGAT
GAACTGTTTATCCTCCTTTATCTTCAAATATCGCTCATAGAGGAAGATCTGTCGATTTAGCT
ATTTTTTCTCTTCATTTAGCCGGTATTTCTTCAATTTTAGGAGCAATTAATTTTATTACGACT
ATTATTAATATAAAAAGAAATGGAATGTCATTTGATCGTATACCTTTATTTGTTTGAGCTG
TAGGAATTACAGCTATTTTATTATTGCTATCTCTACCAGT

- ***Plutella xylostella* - MT570013 (16s rRNA)**

GGGGGCGCGCACTTTTTTTTTTATATGATATAAAAAAAAAAAAAAACGCTGTTATCCCCGGG
GTGATATTCTCATTTAATCATATAATGTGGAGCAAATATTCATATATTTATGGGTATATAT
CATAAATATATATATATATATATATATATATATTTATAATATAAATAAAAAAAAAGTTAAT
TTAATTTTTTTATCACCCCAACAAAATAAAAAATTAAATTTTAATTTAAATTATATAAAAA
ATTTTAATTTAATTTTTTATAAAACTCTATAGGGTCTTCTCGTCTTTTTTTTTTATTTTAACT
TTTTAATAAAAAAATTAAATTCTATTATATTATAAAGAGACAGTTTATATCTTATCCAATC
ATTCATACAAGTCACCAATTAAGAGACTATTGATTATGCTACCTTTGTACAGTCAAAATAC
TGCAGCCCTTTAATTTTCATCAG

- ***Spodoptera sunia - MT707355* (COI)**

ATAATTTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGACTTGTACCTTT
AATATTAGGAGCTCCTGATATAGCTTTCCCACGTTTAAATAATATAAGTTTTTGACTTTTAC
CACCTTCTTTAACCTTACTAATTTCAAGTAGAATTGTAGAAAATGGAGCAGGAACTGGATG
AACAGTTTACCCCCCCCTCTCCTCTAATATTGCTCATGGTGGAAGATCAGTAGATTTAGCT
ATTTTTTCCCTTCACCTAGCTGGAATTTCATCTATTTTAGGAGCTATTAACTTTATTACTAC
TATTATTAATATACGATTAAATAATTTATCATTTGATCAAATACCTTTATTTGTTTGAGCTG
TAGGAATTACTGCATTTTTATTATTATTATCTTTACCTGTTTTAGCTGGAGCTATTACTATA
TTATTAACTGATCGAAA

- ***Spodoptera sunia* - MT774553 (16s rRNA)**

CCGGTTGAACTCAGATCATGTAAGATTTTAATGATCGAACAGATCAAAAATTTTAAACTTC
TACATTTAAATTTTATCTTAATCCAACATCGAGGTCGCAAACTCTTTTTTTTATTCGAACTA
AAAAAAAAAATTACGCTGTTATCCCTAAGGTAATTTTTTCTTATAATCATTAAAAATGGAT
CATAAAATCAATTATATATGTTAAAATTTAAAAAAAGTTATTTTTATTTTTTTGTCACCCCA

ACAAAATAATTAAAAATAATTTCAATTAAAAATTTTATAAATAATCTTAATTAATTTAAAT
ATAAAACTCTATAGGGTCTTCTCGTCTTTTTAAATTATTTTAACTTTTTAATTAAAAAATTA
AATTCTATAAATAATAAAGAGACAGTTTATATTTCATCCAATCTTTCATACAAGTCATCAA
TTAAATGACTAATGATTATGCTACCTTTGTACAGTCCAATATACTGCAGCCCTTTAATATA
AATTATATCAGTGGGCAGATTAAACTTTATATTAAATTCAAAAAGACATGTTTTTGATAAA
CAGGCGAGA

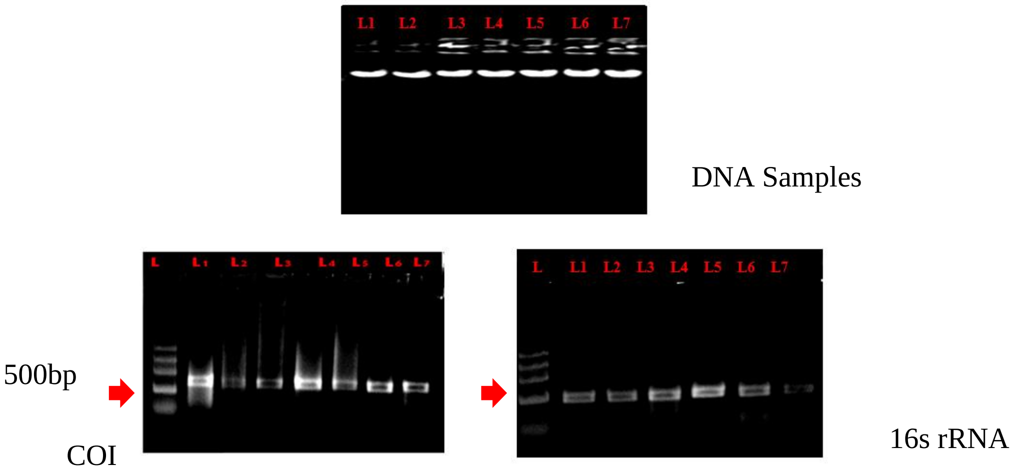

Figure 3. 12: Electrophoresis gel image of DNA, PCR product of COI and 16s rRNA of Lepidoptera species representatives

The overall Phylogeny of four Orders where green clades represents Coleoptera, orange one represents Lepidoptera, blue represents Orthoptera and red represents Hemiptera (Figure 3. 13).

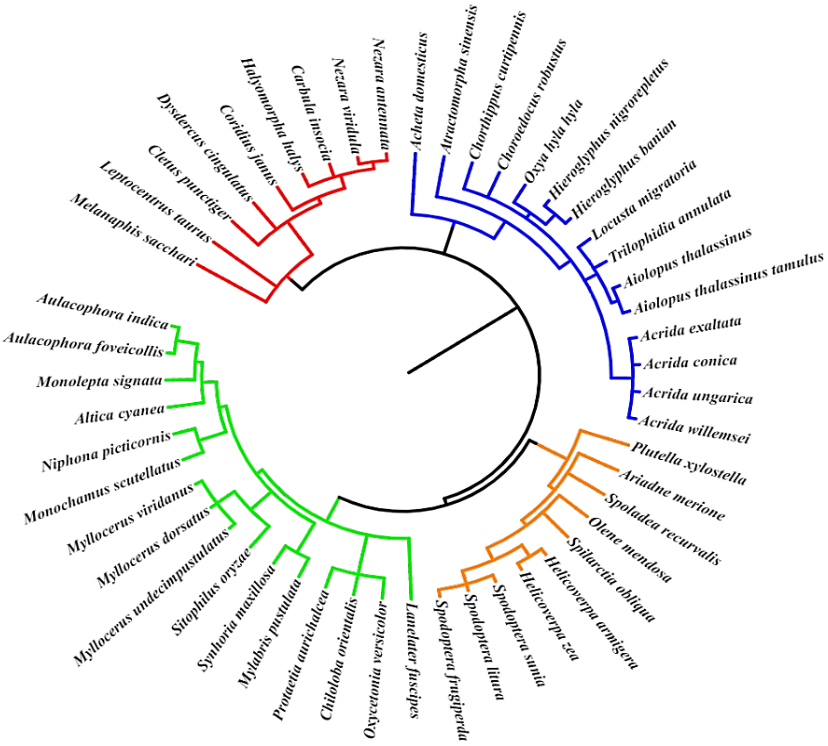

Figure 3. 13: Overall phylogeney Tree of four Orders

3.4 Discussion

In the present study, an attempt has been made to correlate the identification and assess the diversity of Coleoptera, Orthoptera, Hemiptera, and Lepidoptera of Vadodara genetically. The research on molecular ecology on pest insects of agricultural importance may lead to identifying new species (Armstrong and Ball, 2005) biotypes and cryptic species (Toda and Murai, 2007; Karthika *et al.,* 2016). The vast number of insect species is often challenging to identify using only a morphological approach (Witt, *et al.,* 2006) and thus presents an insurmountable obstacle for cataloging total biodiversity only through conventional taxonomy (Blaxter, 2004; Pentinsaari *et al.,* 2014), for which morphological recognition has fallen short and the barcoding of DNA has filled the void (Bourke *et al.,* 2013). Different scientists are now using DNA-sequencing to understand and resolve insects' phylogeny (Hebert *et al.,* 2003; Hajibabaei *et al.,* 2007). DNA sequences are characterized by length and base composition. To compare the nucleotide sequences in phylogenetic analysis, several additional parameters such as the overall nucleotide substitution rate, the ratio of two unique instantaneous substitution rate rates at which transitions and transversions occur, and the site rate variance play an essential role. They are essential for accurate phylogeny reconstruction, (DeWoody and Avise 2000; Karthika *et al.,* 2016; Singhal *et al.,* 2018). Thus, the current inventory was designed to unravel the phylogeny of different pest species of Coleoptera, Orthoptera, Hemiptera, and Lepidoptera using COI and 16s rRNA sequencing regime. The selection of this marker is based on its low rate of mutation caused by adaptive habitat-specific radiation (Raupach *et al.,* 2010) and for the construction of a concrete phylogenetic tree of the selected species using standard models.(Lanfear *et al.,* 2014).

Results indicated that the COI and 16s rRNA based insect pests identification was exceptionally sufficient because all the species were accurately and successfully identified based on the marker profile. Most of the phylogenetic information has been derived from mitochondrial DNA variations (DeWoody and Avise, 2000), and recently, DNA barcoding data have been used successfully to elucidate the relationships of many groups of insect species at the generic level (Wang *et al.,* 2009). The composition of the COI and 16s rRNA gene sequence in the present study was expectedly AT biased for all the orders and is generally observed in different previous studies reported (Zhang *et al.,* 2012; Karthika *et al.,* 2016) Overall, the frequency of transitional substitutions is known to be higher than transversional substitutions in the genome (Karthika, *et al.*, 2016) saturation possibly occurs when the plot shows no further increase in the observed number of transitions despite increasing genetic distances.

Thus, it has been found that saturation of transitions at high levels of sequence divergence indicates saturation in the data. Conversely, in the present study, the transversions rate was higher than that of transitions for COI and 16s rRNA for all the orders analysed. These substitutions perhaps lead to substantial saturation of the sequences. Analysing the transitions and transversions showed that transversions contain stouter phylogenetic signals compared to transitions and are capable of masking the distorted signals coming from the saturated transitions sites that may be misleading, which is reported by Karthika *et al.,* (2016), where they have studied pest insects in Southern India. Moreover, too divergent datasets are still usable, as the phylogenetic information is not entirely lost and maybe retained using sites that have experienced more rate of transversions mutations (Dwivedi and Gadagkar, 2009).

In the present study, the probability change is synonymous to non-synonymous was calculated for all the four orders. The available evidence suggests that non-synonymous point mutations have more significant fitness impacts than synonymous or noncoding mutations (Cuevas *et al.,* 2012). Significant fitness effects from synonymous substitutions are more often observed with massive scale changes than discrete mutations, such as a complete change in a gene's codon usage (Lyons and Lauring, 2017). Estimation of non-synonymous (Ka) and synonymous (Ks) substitution rates is of great significance in reconstructing phylogeny and understanding evolutionary dynamics of protein-coding sequences across closely related and yet diverged species(Fay and Wu, 2003). Previous studies reported that the usage of statistical methods in evolutionary genetics could evaluate the strength of selection operating on individual codons over particular branches or regions of a phylogenetic tree (Delport *et al.,* 2010; Motyka *et al.,* 2017; Bocak *et al.,* 2018). Thus, we found the significant change in the rate of synonymous to non-synonymous mutation rate for COI sequence for all the orders, while for 16s rRNA, the significance was noted for Orthoptera, Hemiptera, and Lepidoptera. Hence, the null hypothesis of neutral selection was rejected since the p-values <0.05 were considered significant.

The average genetic distance among insects' different orders reported in this study showed higher values at the 1st codon position revealing the evolutionary nearness among the related group. (Baldwin *et al.,* 2001; Jørgensen *et al.,* 2007; Song *et al.,* 2019). Maximum-likelihood and Baysian analyses produced broadly similar topologies to the MP analyses without 3rd codon positions. Concordant results between the different analytical approaches has provide some assurance in the sub-ordinal structure obtained for coleoptera: (Chrysomelidae + (Cerambycidae+ (Curculionidae+ Meloidae+ (Scarabaeidae + Elateridae)))). Thus, the result suggests that Chrysomelidae was closest to Cerambicidae, while Curculionidae was closest to Meloidae, and Scarabidae with Elateridae. The monophyly of Coleoptera is well supported by nucleotide sequences of protein-coding genes, which shows that mitogenome data are very effective in resolving relationships within this group (Singhal *et al.,* 2018).

In order Orthoptera, sequencing of three families representing the pest species were obtained namely, Acrididae, Pyrgomorphidea and Gryllidae. Concordant results between the different analytical approaches provide some confidence in the subordinal structure for Orthoptera: (Acrididae + (Pyrgomorphidae+ Gryllidae)). Overall, our data confirm the phylogenetic affinity of Orthoptera, and it shows the clear phylogenetic pattern of basal relationships between the Orthoptera clade and its potential sister group. Our these observations are in the accordance with the previous studies (Chintauan-Marquier *et al.,* 2016; Song *et al.,* 2018), where they have reported strong support for monophyletic suborders (Ensifera and Caelifera) as well as the families and their results corroborate most of the higher-level relationships .

For Hemiptera, the COI and 16sRNA sequence of five families were obtained. The highest number of species representation was observed in Pentatomidae. The concordant findings between the various analytical approaches affords confidence in the subordinate framework for Hemiptera: (Pentatomidae + (Pyrrhocoridae+ (Coreidae +(Memracidae + Aphididae)))), and endows with strong confirmation for understanding the phylogenetic relationships among all major lineages of ecologically diversified Hemipterans The present finding is in accordance with the previous studies (Foottit *et al.,* 2009; Song and Liang, 2013; Misof *et al.,* 2014; Havemann *et al.,* 2018; Johnson *et al.,* 2018) where they have confirmed the influence of phylogenomic approaches in deciphering difficult-to-resolve phylogenetic relationships regarding the evolutionary history of the aphids, planthoppers, bugs and water bugs.

In Lepidoptera, five families were found which were: (Noctuidae+ (Erebidae+ Lymantriidae + (Crambidae + (Nymphalidae +Plutellidae)))). The Bayesian phylogenetic analysis resulted in a topological closely resembling that of the ML analysis and short internodes linking the basal splits are which are a common feature of deep divergences in insect phylogenies (Whitfield and Kjer, 2008); their relative consistency can be seen as a support per se for their consequence even in the absence of significant bootstrap values, especially as there is much congruence with a published morphology-based phylogeny (Kaila, 2004; Van Nieukerken *et al.*, 2011; Kaila *et al.*, 2011; Sohn *et al.*, 2016).

Earlier molecular studies on species have explored the distinction whether belonging to a single genus, family, or species level in the neighbouring of individuals (Koch, 2010; da Silva *et al.*, 2013; Singhal *et al.*, 2018). On the contrary, for the present molecular identification of individuals was based on the rate of infestation of vegetable crops which stands out among the studies that have been performed so far. Moreover, it is important for the groups to accurately identify the species with maximum uniqueness for elucidating the DNA sequences' that can be precisely studied and sub essentially utilized for inferring the evolutionary data and the genetic divergence for properly congruency (Caeiro-Dias *et al.*, 2018; Liu *et al.*, 2019). Therefore, the present study had potentially provided more in-depth insights into pests' knowledge of Vadodara's major vegetable crops based on nucleotide sequences of COI and 16s rRNA. However, further research based on employing different species-specific primers is needed to yield qualitative amplified products upon which the success of sequencing is dependent. The studies should be more focused on the metagenome analysis of different insect pest species' mitochondria to better insight into the phylogeny and evolutionary ambiguity among the species.

3.5 Conclusion

Regardless of complimentary views, the taxonomic impairment remains the chief concern and thus stresses an urgent need for comprehensive biodiversity assessments due to biodiversity catastrophes: the risk of human activities causing mass extinction. Thus, sequencing can accelerate the process of taxonomic inventory. In conclusion, the present study incorporates the advanced analyzed genetic data that determines the nucleotide content and specificity in two candidate markers COI and 16s rRNA. It also resolves the taxonomy and the phylogeny and the status of unknown species of Coleoptera, Orthoptera, Hemiptera, and Lepidoptera:

- **Coleoptera-**
  (Chrysomelidae+(Cerambycidae+(Curculionidae+Meloidae+(Scarabaeidae+ Elateridae)))).
- **Orthoptera**-
  (Acrididae + (Pyrgomorphidae + Gryllidae))
- **Hemiptera-**
  (Pentatomidae+(Coreidae+(Pyrrhocoridae+(Aphididae+Membracidae))))
- **Lepidoptera-**
  (Noctuidae+(Erabidae+(Crambidae+(Nymphalidae+Plutellinidae)))).

Overall, this study has added basic knowledge to molecular phylogeny of important insect pests in the agricultural fields of Vadodara and this investigation could potentially be applied in agricultural researches to rapid identification of pests. However, further studies are necessitated to comprehend ecophysiological role of the cryptic pest species.