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DNA BAR-CODING FOR IDENTIFICATION OF LOCAL AND EXOTIC INSECT SPECIES IN JINNAH GARDEN, LAHORE

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Abstract

DNA bar-coding is molecular identification of the world's insect fauna through the cytochrome c oxidase I (COI) gene. Pakistan has abundant biodiversity, many insect species had unknown classifications. In order to fill this gap, we used DNA bar-coding to survey the variety of insects, taxonomy identification and identify new insect species. The current investigation aims to differentiate among species at the order and family level. Almost 244499 insect specimens were collected through a Malaise trap in Jinnah Garden Lahore, Pakistan during 2021–2022. Collected samples were morphologically characterized in the Entomology Research Laboratory of LCWU, Pakistan for further molecular identification through the COI gene. Almost 1362 DNA barcodes were generated by submitting of COI gene of 8948 insect species on the BOLD and (96%) also had a visualization picture. Approximately 1362 BINs included 205 local insects (15.05% Unique insects) BINs and 1157 exotic insect (84.94% Non-unique insects) BINs. Local insect species contain a total of 9 orders and 17 orders of exotic insect species are identified. An overall total 4 classes and 17 orders of the local and exotic insect species were reported. Three orders represented 92.56% of the specimens: Diptera (66.80%), Hymenoptera (15.87%) and Hemiptera (9.92%). Two orders Coleoptera and Lepidoptera had 2.73% and 1.44% respectively. Most 88.26% specimens were place to a family (173). Therefore, current study is offered full and accurate key for identification different insect's fauna at family level of Pakistan. It is refilling the taxonomic and scientific gap in the species identification of Pakistan's insect fauna.

1. INTRODUCTION

DNA Bar-coding is a molecular technique for isolating different new species through the CO1 gene. This technique is based on DNA short sequences, standardized gene fragments called DNA barcodes. The mitochondrial DNA sequence is the most commonly used for DNA bar-coding in insects. With over a million distinct species documented, insects are the most varied category of creatures and may be found everywhere except

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in the ocean (Sanchez *et al*., 2008). In terrestrial ecosystems, insects make up more than half of the biodiversity and very essential to the health of nature (Siemann *et al*., 2004). The season availability, size, trophic level, life history, movement, strategy, and habitats of insects vary widely. Due to the richness of their species and other living forms, insect communities are a crucial component of terrestrial ecosystems. Due to their diversity, ecological functions, and effects on agriculture, natural resources, and human health, insects play a significant part in many food webs and lengths of food chains (Adjaloo *et al*., 2012).

Seven biomes parts of the Palearctic and Indo-Malayan biogeography domains are also present in Pakistan, which has area, 882,000 km^2 (Dinerstein *et al*., 2017). Its fauna is highly diverse because of this physiographic and climatic variance (Anwar *et al*., 2008). Past investigations on other animal ancestries have been limited to locations or particular taxa, despite the fact that its vertebrate wildlife is fine acknowledged (Inayat *et al*., 2010; Iftikhar *et al.,* 2016a; Manzoor, Khan and Shah, 2022). More than half of the world's reported biodiversity is made up of arthropods, which also outnumber all other known organisms and live in a great diversity of functional niche and microhabitats in terrestrial ecosystems (Stork*et al*., 2018).

Order level normally is a vital factor for identification and classification of insect biodiversity. Without knowledge of the orders level, it is difficult to distinguish the type of insect species. Scientific researchers have managed to the classification and identification of thirty-two insect orders in the natural ecosystem. Orders of insects before 2002 have been identified.Almost twenty-one different variables must be assessed, including body type, number of wings, and head shape and number of feet (Gullan*et al*., 2014).

Several earlier research investigated whether arthropod genera, most of which were found to belong to same genus but trophic niche, interact through various microbial groups. Mealy bugs (Lin *et al*., 2019), wasps and bees (Skrodenytė-Arbačiauskienė *et al*., 2019; Suenami *et al*., 2019), fruit flies (Ventura *et al*., 2018; Yong *et al*., 2017), reduviids (Rodríguez-Ruano *et al*., 2018), mosquitoes (Mancini *et al*., 2018), beetles (Hulcr *et al*., 2012; Kolasa *et al*., 2019), silkworms (Chen *et al*., 2018), aphids (Fakhour *et al*., 2018; Gallo-Franco *et al*., 2019; McLean *et al*., 2019; Zepeda-Paulo *et al*., 2018) and Lepidoptera (Liu *et al*., 2020; van Schooten *et al*., 2018), have all been discovered to different from other species of insects.

DNA barcodes are short and standardized molecular DNA sequences used for species identification and had been discovered in the last 20 years (Hebert*et al*. 2004). Modern biodiversity studies have quickly adopted DNA bar-coding due to its efficiency in identifying specimens and discovering new species (Hebert *et al.,* 2003; Huemer *et al*., 2014; Kress *et al*., 2015; Ashfaq and Hebert. 2016; DeSalle and Goldstein, 2019). In order to identify unidentified specimens, DNA barcodes compare a particular genetic marker to a reference sequence library. DNA barcodes should typically distinguish between low and high species. The vouchered sample may simply be utilized to remove short genetic

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sequences, which have been applied to successfully differentiate populations as well as species. By amplifying wildly varying sections, such as the DNA barcode region of mitochondrial genomes, chloroplast or nuclear, used in PCR (polymerase chain reaction), species can be identified. Standard molecular biology procedures are the foundation of the methods used in DNA-based identification systems. After sample selection and documentation, the laboratory approach entails DNA extraction, amplification by polymerase chain reaction (PCR) and identification through DNA sequencing. Sequence analysis and barcode identification are both involved in managing data while simultaneously time (Ahmed*et al*., 2022). The primary grouping (CBOL) controls the collection of DNA barcodes and for molecular identification creates a global reference library is the Bar-coding of Life Program (BOLD, http://www.bar-codinglife.org). The BOLD system now has over 400,000 insect DNA barcodes. Many of these are currently undergoing identification and/or confirmation. RBD (Reference Barcode Database) of BOLD struggles to minimize any preferences brought on by insufficient sampling or incorrect species identification (Ratnasingham*et al*., 2007).

Research is being conducted locally and globally to create DNA reference libraries and to use DNA barcodes to catalogue biodiversity. Worldwide DNA barcode reference libraries are now being built quickly for some key insect orders, such as Trichoptera (caddisflies), Odonata, Ephemeroptera (mayflies), Plecoptera (stoneflies), and others insects. These libraries are linked to voucher specimens identified by qualified taxonomists and kept in permanent repositories (dragonflies and damselflies). Surveying all insect species at specific areas is being done using a supplementary strategy known as bar-coding biotas. Such efforts enhance the worldwide taxonomy libraries of the target taxonomic groupings and advance our knowledge of biodiversity in important ecosystems. This technique is used to analyse biodiversity quickly (Zhou *et al*., 2010).

1.1 Aims and Objectives

The aim of this research was to identification, investigation and documentation of insect data in the Jinnah Garden Lahore. The keys were developed by reading a variety of literary works and looking through a field, museum collections and molecular identification through the cytochrome c oxidase I (COI) gene. Therefore, the current study was offered a full key for the identification of Pakistan's different insect fauna at family level.

The specific objectives of the study were:

- Specimen collection of insect biodiversity from Jinnah Garden Lahore.
- To preserve the insect species for morphological characterization.
- Molecular identification amplification by target 658bp barcode region using specific universal forward LCO1490 and reverseHCO2198 primers.
- Submission of sequences to BOLD system for assigning barcode index number (BIN).

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2. REVIEW OF LITERATURE

Ashfaq *et al*. (2017) determined that sequences derived from the mitochondrial COI gene's DNA barcode region are useful for identifying specimens and identifying new species. 4.5 million Animal data from the BOLD were now stored there. These records have been given more than 490,000 distinct BINs, which act as a species. BOLD has a significant capacity to both classify specimens in orders and to investigations of fauna connection because a quarter of these BINs are derived from Lepidoptera. 4503 moths representing 981 BINs from 52 families were collected from 329 locations across Pakistan and provided DNA barcode sequences. Except for 5 species that had a BIN split, all 379 species with Linnaean name assignments were represented by a single BIN. Of the 981 BINs, less than half (44 percent) had international analogues; the remaining BINs were exclusive to Pakistan. Prior to comparison with all 116,768 BINs for this order, 218 more Lepidoptera BINs from Pakistan were included with the 981 from this study. As was to be expected, the highest levels of faunal overlap were found in India (21 percent), Sri Lanka (21 percent), the United Arab Emirates (20 percent), and other Asian countries (2.1 percent), while it was extremely low on other continents like Africa (0.6 percent), Europe (1.3 percent), Australia (0.6 percent), Oceania (1.0 percent), North America (0.1 percent), and South America (0.1 percent). In spite of taxa not having been designated to a Linnaean species, this work demonstrates how DNA bar-coding makes it possible to estimate faunal overlap.

Hebert *et al*. (2018) studied that although HTS have essentially replaced their Sanger alternatives, their value for amplicon sequencing is limited by the short read sizes and great mistake rates of most stages. The current work uses the 658 bp amplicons of the mtCO1 gene as a framework to assess the ability of particular particle, real-time (SMRT) sequencing used on the SEQUEL technology to get over these restrictions. The effectiveness of SMRT sequencing was evaluated with amplicons displaying considerable diversity in Guanine-Cytocine components and diverse sequence properties by looking at templates from more than five thousand species and twenty thousand specimens. Sanger and SMRT sequences were highly comparable; however, SMRT sequencing offered more thorough coverage, particularly for amplicons with homopolymer tracts. The cost of analysis is minimal, far lower than that of first- or second generation sequencers.

DeSalle *et al*. (2019) determined that DNA bar-coding, which has been widely hailed as a revolutionary taxonomic discovery method, maybe the highest-quality approach now accessible for classifying samples and specimen dependent data for systematic study. Studies involving documentation, taxonomic classification, and finding of cryptic species have multiplied quickly and seem to have been an important factor in the overall rise in DNA barcode study publications. Biological conservation publications using DNA barcodes and forensic research have mostly followed the overall number of studies; however, they look too had increased significantly in 2017. The neighbor-joining and visual (tree-based) standards for species limitation continue dominant, despite the

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diversification of analytical theories, notably in response to the increased accessibility of tools in BOLD. We come to the conclusion that DNA bar-coding data practices and paradigms are likely to endure and continue to be an important instrument in taxonomic inquiry for taxa like Lepidoptera.

Ballare *et al*. (2019) evaluated that DNA sequencing innovations are advancing the biological sciences and opening up new prospects for fundamental and practical genomic research on non-model species. Despite these possibilities, a lot of next-generation sequencing techniques have been created based on the idea that a significant amount of great molecular weight DNA (less than 100 ng) is available, which might be challenging for many reading systems. Particularly, the potential for sequencing field-collected samples with varied degrees of DNA degradation is currently completely unexplored. In this work, we examine the effects of five common insect collection and preservation techniques on the success of ddRAD sequencing for three species of wild bees. We analyzed 105 samples altogether (between 7 and 13 samples per species and treatment). Additionally, we examined two DNA extraction techniques and looked at how several DNA quality metrics, such as pre-sequence concentration and damage, impacted the effectiveness of later sequencing. We report satisfactory library research for all specimens, with all managements and isolation techniques providing an adequate number of very dependable loci for population genetic studies. Although results varied between species, we discovered that specimens collected by net sampling straight into 100 percent ethanol or by passive trapping followed by 100 percent ethanol storage before pinning tended to create higher-quality ddRAD assemblages. This is probably because the specimens quickly dried out. We offer suggestions for every procedure, extraction technique, and evaluation of DNA quality. We also encourage researchers to think about using a wider range of samples for genomic studies.

Ahmad *et al*. (2019) examined that in Punjab, Pakistan, fruit flies (Diptera: Tephritidae), were molecularly identified and characterized using PCR analysis using primers based on the mt-COI gene. Additionally, phylogenetic examination was used to distinguish the detected fruit flies from those found in other parts of the world. The genomes of B. dorsalis, B. cucurbitae, and B. zonata were 99 to 100 percent comparable to fruit flies recorded from other nation state, according to sequencing results and phylogenetic analyses of collected specimens. This is the first account of fruit flies infesting fruits in Punjab, Pakistan. That had been molecularly identified and characterized.

DeWaard *et al*. (2019) evaluated that an organized library of verified reference sequences is necessary for the accurate taxonomic identification of species using DNA sequence data. But about 15% of the animal species that have been reported are thought to be available in public sequence collections.

To start addressing this gap, we offer DNA barcodes for fifteen lac animal specimens gathered from 23 global and aquatic ecozones at locations all over Canada, a country that makes up 7% of the planet's land area. In all, 64264 (BINs; a alternative for species) and total of 14 phyla, 43 classes, 163 orders, 1123 families, and 6186 genera are

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represented. 38% of the specimens had species-level taxonomy accessible, although larger percentages had genus (69.5%) and family (99.9%) assignments. At the Centre for Biodiversity Genomics, voucher specimens and DNA isolation were conserved and available for future study. The BOLD, Gen Bank, the Global Biodiversity Information Facility, and the Global Genome Biodiversity Network Data Entry all provide access to the associated sequence and taxonomy data.

3. RESEARCH METHODS

3.1. Sampling Site

Bagh-e-Jinnah (Punjabi, Urdu: باغ جناحor Jinnah's Garden) is an ancient botanical garden in the city of Lahore, Pakistan. It was in the past known as **Lawrence Gardens.** The study was conducted in Bagh-e-Jinnah (Latitude: 31° 33' 13.42" N Longitude: 74° 19' 52.69" E); District Lahore, Pakistan.

3.2. Sample Collection

The Global malaise trap (BT 1002) was used, which is appropriate for collecting flying insects all over their flight period in Jinnah garden. Malaise trap (BT1002) has been used to collect a significant portion of the regional arthropod fauna as shown in **Figure 3.2** below. A suitable place was explored in Jinnah garden for trap placement because it was appropriated place, when local variables like terrain, density, absence of vegetation, and atmospheric relationship, water, and other similar features typically favor insects in flight.

So, the trap was placed in nursery area of Jinnah garden where there was less vegetation and no interaction of most people. The trap was positioned at a right angle to insect flight line. Active collection (Aspirators, Vaccum Collector, White Pans, sweep nets, Beating trays, Insect nets and Sampling frames) and passive collection (bright traps, Malaise traps, pitfall traps, UV illuminated sheets, Burlese funnels) and hand collections were performed for the collection of insect Sample (Muhammad Ashfaq *et al*., 2021).

3.3. Insect Handling and Preparing Apparatus for Preservation

For the preservation of insects killing Jar, Vials, Pinning Insects, Mini Dissection Tray, Spreading Boards, Pinning Block, Forceps, Miuten pins, and many different cages (Sleeve Cage, Clip Cage, PVC Pipe Cage, Can and Wire Cage, Bucket Rearing Cage) were used for insect collection.

3.4. Collection of Weekly Samples

At the field site, the trap was assembled securely according to the malaise trap instruction sheet. Then the prepared collection bottle was affixed with 95% ethanol in it **(Figure 3.4.)**, to the trap head. One day was selected to start our collections weekly. It was necessary to consistently return for the duration of the sampling period. The trap was visited frequently to prevent collapsing and to avoid sample overflow. It was also checked regularly, particularly after strong winds and heavy precipitation.

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3.5. Replacement of Bottles

The catch was removed weekly and replaces the collection bottle with a new one; which also contain 250ml of 95% ethanol and labeled with collection details in pencil. Then collection details were recorded in the malaise log sheet. After this, all the different orders of insects were separated in separate Petri dishes and count the number of each insect as described below **(Figure 3.5)**.

Figure 3.5: Insects Collect in Petri Dish

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3.6. Insect Preservation

Huge samples were fixed as well as kept dry or put in ninety-five percent ethanol-filled Matrix tubes. In 96-well microplates, little samples were each inserted in well having 30ml of 95 percent EtOH (as shown in **Figure 3.6**).

Figure 3.6: Insect Preservation Matrix Tubes with 95% Ethanol

3.7. Storage of Sampled Insects

Collected samples were placed in a standard house hold freezer at -20^{o}C as shown in **Figure 3.7**. The uality and quantity of sample insects were checked regularly. All the insect order was identified at specific temperature and humidity.

3.8. Morphological Identification of Insects

Field collected insects transported for identification insects at species-level to the Entomology Research Laboratory, LCWU. Insects were well-known to the lowest taxonomic rank possible and counted. Identification of insects was ended with the help of key (Laboratory procedure and essentials of entomology by W. A. Foster, 2005), insect identification with casual behavior and with reference to Pakistan insect museum as descried below **Figure 3.8.**

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Figure 3.8: Identification of Insects

3.9. Molecular Identification of Insects

DNA isolation, polymerase chain reaction, extension, DNA sequencing and DNA bar-coding were passed out for molecular identification of insects. Following molecular techniques were performed to investigate the COI gene by extracting mitochondrial DNA of insects for the identification.

3.10. Genomic DNA Extraction of Insects

Body parts of insects that used as tissue sources for DNA extraction were legs and thorax region. DNA from each adult insect was extracted separately by using manual method of (Huang *et al*., 2004). Depending on size, a single or three legs were removed from the majority of specimens. DNA prepared and stored at -80^{o}C.

Insect sample placed into 1.5ml micro-centrifugation tube and then kept on ice. 400µl of TEN extraction buffers (10 Mm Tris-HCL (Cat# T3253; Lot# SLCD9663), 2mM EDTA, 0.4M NaCl (Cat# S270706; Lot# 70740) and pH 8.0) was added and insect sample was homogenized by Mortar and Pestle.10 µl of 10% SDS (Cat# 28312; Lot# 80300) and 8µl of 20mgml^{-1} of Proteinase K (Cat# PRK403; Lot# SP27100) to each tube were added.

The solution was mixed by vortex and then incubated at 55 ^{o}C for 1h and then Added 300 µl of 6M NaCl (Cat# S270706; Lot# 70740) to each tube. Centrifuge of each tube at 14,000rpm for 20mins and then added supernatants to new tube and remove pellet. Precipitation DNA by adding 500µl of 2-propanol (Cat# S010306; Lot#SZBCO23OV) and was freeze at -20^{o}C for 20min. After doing repeat centrifugation (20min, 14,000rpm) remove supernatant and wash pellet with chilled 70% ethanol (Cat# S240697; Lot# SHBL6735) then dried by evaporation at room temperature. The pellet was dissolved in 50µl of H_2O. Store the Sample at -20^{o}C for PCR and used 1µl for further PCR analysis.

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3.11. Gel Electrophoresis

To examine the gene expression, agarose gel electrophoresis was performed.

Afterward steps were performed for the gel electrophoresis.

3.11.1. Preparation of Reagents

3.11.1.1. Preparation of 50*X TAE (Tris Acetate EDTA) Buffer Stock Solution*

For the preparation of a 1.0 litre 50X standard solution of TAE, 242g of trihydroxy-methyl amino-methane (Tris) base (Cat# T838; Lot# SPSO838013B) was added to double-distilled H_2O. After 100ml of 0.5M Ethylene Di-ammine Tetra-Acetate (EDTA) solution, pH= 8.0 was added. There was also added 57.1m*l glacial acetic acid (Cat#A35*-500; Lot# B261651423). After adding the distilled H_2O, the resulting volume of the solution was 1000ml.

3.11.1.2. Preparation of 1*X TAE Buffer Working Solution*

In the measuring flask 20ml of 50*X TAE buffer was taken. Distilled H_2O 980ml was added, to get 1 liter. Solution was mixed carefully and put in storage in air fitted glass vessel to avoid impurities.*

3.11.2. Preparation of Gel

Agarose was measured into an Erlenmeyer flask to the appropriate quantity. Agrose gel was maked using weight to volume percent solution. Concentration of Agarose in a gel was depended on size of DNA segment to be separated. 0.8 Agarose powder (SIGMA-ALDRICH; Lot No SLBN601V) was added in 100ml of TAE buffer for making 0.8% gel. Agrose/buffer mixture was heated in an oven until the clear solution was formed that indicated the complete mixing of agarose powder in buffer solution. Ethidium bromide (EtBr) (Code# D0197; Lot#GE53632C) was added to a concentration of 9-12 µl and mixed it slightly. The gel casting tray was covered by tap on both sides for solidification of gel. Gel was poured in the casting tray and comb was placed in the gel and allowed to cool and solidified.

3.12. Amplification of Insect CO1 Gene by PCR Method

Mitochondrial CO1 gene fragment was used for amplification. A pair of universal forward LCO1490 and reverse HCO2198 primers was used for COI gene amplification (Vrijenhoek *et al*., 1994). For amplification, CO1 gene was used to amplify the various insect's fauna. Polymerase chain reaction (PCR) with specific primer situated in flanking regions of target fragments was performed to amplify the fragment of interest.

Table 3.12: Universal Forward and Reversed Primers Sequence for Amplification of Mitochondrial CO1 Gene

Accession NO	Primers sequence		Temp˚C
LCO1490	(5′-GGTCAACAAA TCATAAAGATATTGG-3′)	Universal forward primer	54 ˚C
HCO2198	(5′-TAAACTTCAGGGTGACCA AAAAATCA-3′)	Universal reverse primer	56 ˚C

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3.13. Composition of Master Mix for PCR Reaction:

For amplification of the mitochondrial DNA following method used and chemicals were added in PCR tubes to make 12.5ul of PCR solution.

Table 3.13: Master-Mix Recipes for PCR Amplification (in µl)

Reagents	Quality (µl)
Water	7.2µl
Forward primer	1µl
Reverse primer	1µl
DNA	2µl
Taq polymerase	0.8µl
Master mix	12µl

3.14. Polymerase Chain Reaction

Half volume reactions of the PCR were carried out and adjusted, including 12.5 l of MasterMix with loading dye, 10 litre of clean distilled H_2O, 1.5 l of extracted DNA (about 20 ng L1), and 0.5 l of forward and reverse primers (10 M).

For Polymerase chain reaction (PCR) of mtDNA conditions and steps were used in following order.

1. Initially, DNA was heated at 94˚C temperature for denaturing, which separated dsDNA to single-stranded DNA. The temperature at which fifty percent of the dsDNA was denatured is known as the melting-temperature (Tm) and was determined by G+C content, the length of sample, and the concentration of ions (primarily Mg^{2+}).
2. All through the annealing stage, sample cooled to 51˚C, allowing the primers to attach to the target DNA.
3. The ending polymerase chain reaction step was occurred at 72˚C, and known as extension. During this stage, DNA polymerase was extended the DNA from the primers; making new double stranded DNA with one from the past strand DNA and one new DNA strand.

Table 3.14.1: Showing PCR Temperature, Time and Phases of the Cycle.

Cycle	Temperature (˚C)	Time (min/sec)	Phase
35X	94 ˚C	5 min	Denaturation
	94 ˚C	45 sec	Denaturation
	51˚C	60 sec	Annealing
	72˚C	60 sec	Extension
	72˚C	10 min	Final Extension

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4. RESULTS

4.1. Sample Collection

Through the Malaise trap, 242,226 specimens collected from Jinnah Garden, Lahore during 2021-2022. It is botanical Garden of the Lahore.

4.2. Morphological Identification

Identification of a few insect species had reported through Entomology Research Laboratory, Lahore College for Women University, Pakistan. Identification of local and exotic insect species studied with reference key (Laboratory procedure and essential of entomology by W.A. Foster 2005).

4.3. Molecular Identification

Total 242,226 specimens were testified for DNA isolation through manual method. The DNA of 354 local insects and 8605 exotic insect species were examined.

4.4. Amplification of Cytochrome c Oxidase 1 (CO1) Gene

Amplified DNA of 242,226 specimens by using forward (LCO1490) and reversed (HCO2198) primers, targeted the CO1 gene.

Table 4.4.1: Target gene sequence for amplification of mitochondrial CO1 gene

Region	Gene Location	Accession NO	Primers sequence		Reference
Mitochondria	CO1	LCO1490	(5′-GGTCAACAAA TCATAAAGATATTGG-3′)	Universal forward primer	Hebert *et al*. 1003a,b
		HCO2198	(5′-TAAACTTCAGGGTGACCA AAAAATCA-3′)	Universal reverse primer	

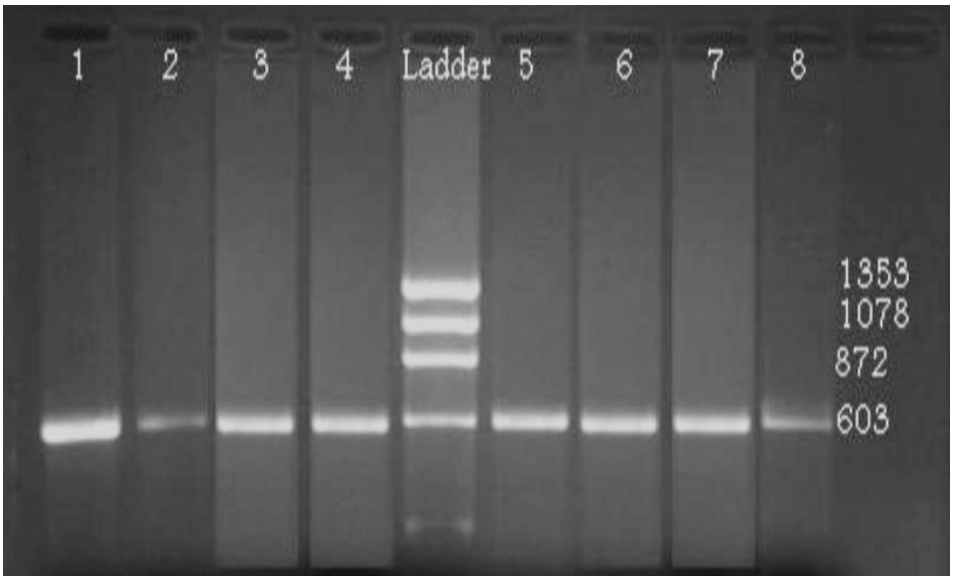

Figure 4.4.1: DNA Ladder of Cytochrome Oxidase (CO1 gene)

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4.5. Gene Sequencing

Gene sequence of 9007 insect species was submitted on the BOLD system. Identified the gene sequence of local and exotic insect species at family level was submitted on the BOLD system, which assigned 1362 BIN numbers that is available on the website (www.boldsystems.org). Report is indicated the local insect species 354 and exotic insect species8605.

4.6. DNA Bar-coding

After it BOLD system was assigned BIN (Barcode Index Number) against each insect species. On the BOLD system, data is accessible in BIN detail with insect representative images and insect taxonomical records.

4.7. Diversity of Insects in Jinnah Garden, Lahore

DNA barcodes were recovered from 242,226 of the 244499 specimens analyzed. A total number of 1362 BINs was recovered. One thousand, three hundred sixty-two BINs were divided into 205 BINs of Local (Unique) insect species, 1157 BINs of exotic (Non-unique) insect species and 59 split BINs of exotic insect species.

4.7.1. Diversity of Local Insect Species

Three classes (Insecta, Collembola and Arachnida) and eight orders (Diptera, Coleoptera, Lepidoptera, Hemiptera, Hymenoptera, Thysanoptera,Entomobryomorpha and Araneae) of local insect specieswere reported. Largest number of insect species recorded belonging to the class insecta. Maximum richness (96.78%) of insect species was belonging to class insecta and least richness (1.46%) of class arachnida. Four major orders of local insects indicated considerable variation in diversity (Hymenoptera: 44.90%, Diptera: 29.73%, Hemiptera: 10.50%, Coleoptera: 8.16%), and the least count of the order Araneae (0.29%). as described in **Table 4.7.1.**

Table 4.7.1: Relative Abundance (%), Average and Orders of Local Insect Species, Jinnah Garden Lahore, Pakistan

Class	Local insect order	Average	% Relative Abundance
Insecta	Diptera	102	29.73%
	Coleoptera	28	8.16%
	Lepidoptera	5	1.46%
	Hemiptera	36	10.50%
	Hymenoptera	154	44.90%
	Thysanoptera	7	2.04%
Collembola	Entomobryomorpha	6	1.74%
Arachnida	Araneae	1	0.29%
		4	1.17%
Total		343	99.98%

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4.7.2. Diversity of Exotic Insect Species

Four classes (Insecta, Collembola, Arachnida and Malacostraca) and seventeen orders (Diptera, Coleoptera, Lepidoptera, Hemiptera, Hymenoptera, Thysanoptera, Odonata, Orthoptera, Psocodea, Neuroptera, Entomobryomorpha, Symphypleona, Araneae, Sarcoptiformes,Mesostigmata, Trombidiformes and Isopoda) of exotic insect specieswere reported. Largest number of insect species recorded belonging to the class insecta. Highest richness (96.09%) of insect species was belonging to class Insecta and least richness (0.03%) of class Malacostraca. Three major orders of exotic insects indicated considerable variation in diversity (Diptera: 68.20%, Hymenoptera: 14.71% and Hemiptera: 9.90%) and the least count of the order Neuroptera (0.01%). as described in **Table 4.7.2.**

Table 4.7.2: Percent Relative Abundance (%), Average and Orders of Exotic Insect Species, Jinnah Garden Lahore, Pakistan

Class	Exotic insect order	Average	% Relative Abundance
Insecta	Hymenoptera	1266	14.71%
	Hemiptera	852	9.90%
	Lepidoptera	124	1.44%
	Diptera	5876	68.20%
	Thysanoptera	75	0.87%
	Coleoptera	217	2.52%
	Odonata	5	0.05%
	Orthoptera	5	0.05%
	Psocodea	30	0.34%
	Neuroptera	1	0.01%
Collembola	Symphypleona	6	0.06%
	Entomobryomorpha	76	0.88%
Arachnida	Araneae	27	0.31%
	Sarcoptiformes	3	0.03%
	Mesostigmata	24	0.27%
	Trombidiformes	15	0.17%
Malacostraca	Isopoda	3	0.03%

4.7.3. Overall Local and Exotic Insect Species Diversity

An overall total 4 classes (Insecta, Collembola, Arachinda, and Malacostraca) and 17 orders (Diptera, Coleoptera, Lepidoptera, Hemiptera, Hymenoptera, Thysanoptera, Odonata, Orthoptera, Psocodea, Neuroptera, Entomobryomorpha, Symphypleona, Araneae, Sarcoptiformes,Mesostigmata, Trombidiformes, and Isopoda)of the local and exotic insect species were recognized and reported.

Overall 17 orders to all 242,226 insects with barcode index numbers were allocated while 88.26% of insects had 173 families. Three orders represented 92.56% of the specimens: Diptera (66.80%), Hymenoptera (15.87%) and Hemiptera (9.92%). Two orders Coleoptera and Lepidoptera had 2.73% and 1.44% respectively.

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While remaining 12 orders (Thysanoptera, Odonata, Orthoptera, Psocodea, Neuroptera, Isopoda, Trombidiformes, Mesostigmata, Sarcoptiformes, Araneae, Symphypleona, and Entomobryomorpha) possessed fewer representatives. Class Insecta included 10 orders. This showed 98.14% of overall specimens. Class Collembola included two orders (Entomobryomorpha: 0.92% and Symphypleona: 0.07%).

Class Arachnida had 4 orders (Araneae, Sarcoptiformes, Mesostigmata, and Trombidiformes) which represented 0.82%. Four members of class Arachnida were identified at the order level. Class Malacostraca had only one order (Isopoda: 0.03%) as shown in **Table 4.7.3.**

Table 4.7.3: Overall Relative Abundance (%), Member Average and Orders of Local and Exotic Insect Species, Jinnah Garden Lahore, Pakistan

Class	Overall Order	Average	% relative Abundance
Insecta	Diptera	5978	66.80%
	Coleoptera	245	2.73%
	Lepidoptera	129	1.44%
	Hemiptera	888	9.92%
	Hymenoptera	1420	15.87%
	Thysanoptera	82	0.92%
	Odonata	5	0.06%
	Orthoptera	5	0.06%
	Psocodea	30	0.34%
	Neuroptera	1	0.01%
Collembola	Entomobryomorpha	82	0.92%
	Symphypleona	6	0.07%
Arachnida	Araneae	28	0.31%
	Sarcoptiformes	3	0.03%
		4	0.04%
	Mesostigmata	24	0.27%
	Trombidiformes	15	0.17%
Malacostraca	Isopoda	3	0.03%
Total		8948	99.97%

4.8 Data Analysis

The final dataset (N=244,499) included 242,226 new barcode recorded and 9007 public records on BOLD from specimens collected in Jinnah garden Lahore, Pakistan.

4.8.1. Class Insecta

Total ten orders of class insect e.g. Diptera, Coleptera, Hemiptera, Lepidoptera, Hymenoptera, Thysanoptera, Odonata, Orthoptera, Psocodea and Neuroptera were covered as shown in **Table 4.8.1.**

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Table 4.8.1: Overall Relative Abundance (%), Member Average and Orders of Local and Exotic Insect Species in Class Insecta, Jinnah Garden Lahore, Pakistan

Class	Order	Average	% Relative Abundance
Insecta	**Diptera**	5978	66.80%
	Coleoptera	245	2.73%
	Lepidoptera	129	1.44%
	Hemiptera	888	9.92%
	Hymenoptera	1420	15.87%
	Thysanoptera	82	0.92%
	Odonata	5	0.06%
	Orthoptera	5	0.06%
	Psocodea	30	0.34%
	Neuroptera	1	0.01%
Total		8783	98.14%

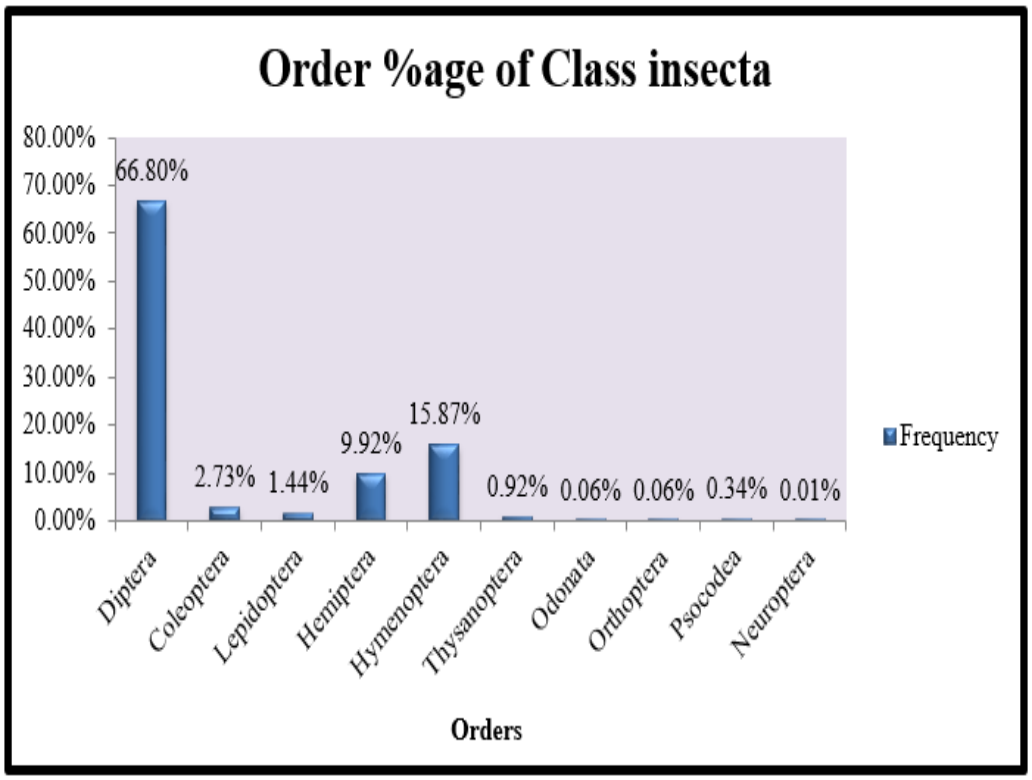

Figure 4.8.1: Overall Percent Relative Abundance of Class Insect at the Order Level

4.8.1.1. Order Diptera

Overall 98.14% relative abundance of order Diptera was calculated. Forty families of order diptera were reported. It had 5978 specimens in overall families as shown in **Figure 4.8.1.1.**

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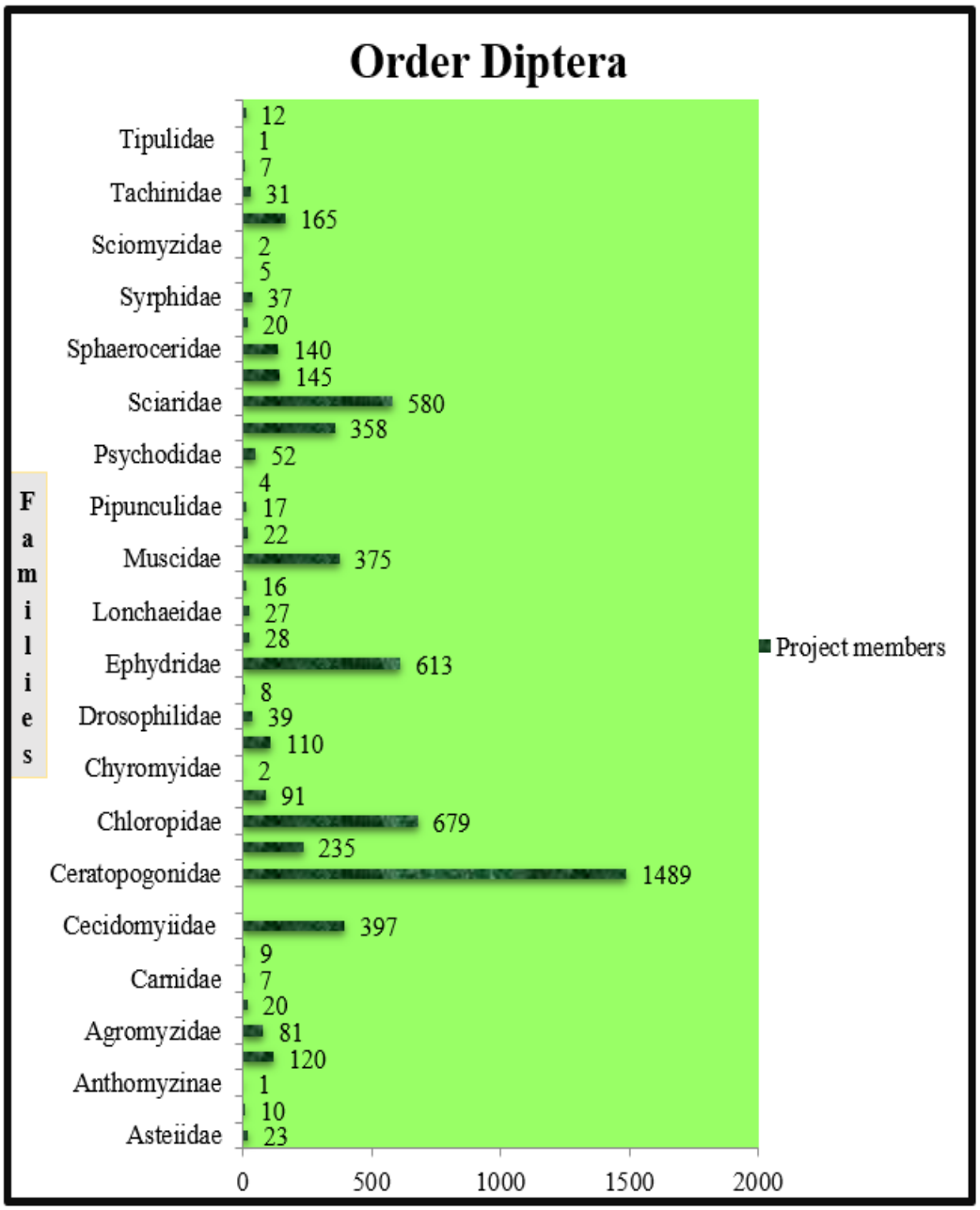

Figure 4.8.1.1: Order Diptera Showing Families and Members in Projects

4.8.1.2. Order Coleoptera

Overall 2.73% relative abundance of order Coleptera was calculated. Twenty four families of order Coleoptera were reported. It had 245 specimens in overall families as depicted in **Figure 4.8.1.2.**

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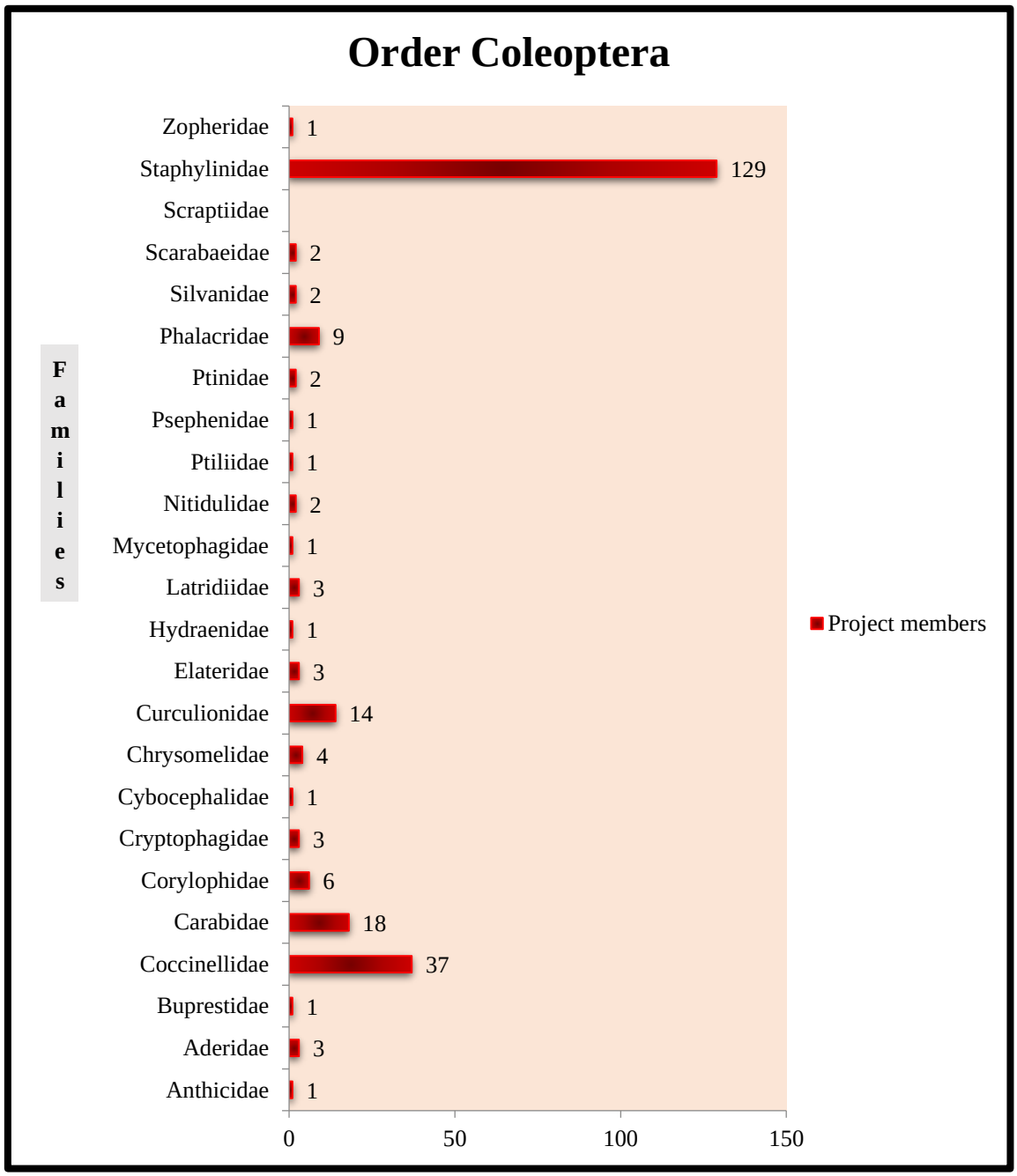

Figure 4.8.1.2: Order Coleoptera Showing Families and Members in Projects.

4.8.1.3. Order Lepidoptera

Overall relative abundance of order Lepidoptera 1.44% was calculated. Fifteen families of order Lepidoptera were reported. It had 126 specimens in overall families. Out of 129,

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three specimens were reported at the order level and not identified at their family level as shown in **Figure 4.8.1.3.**

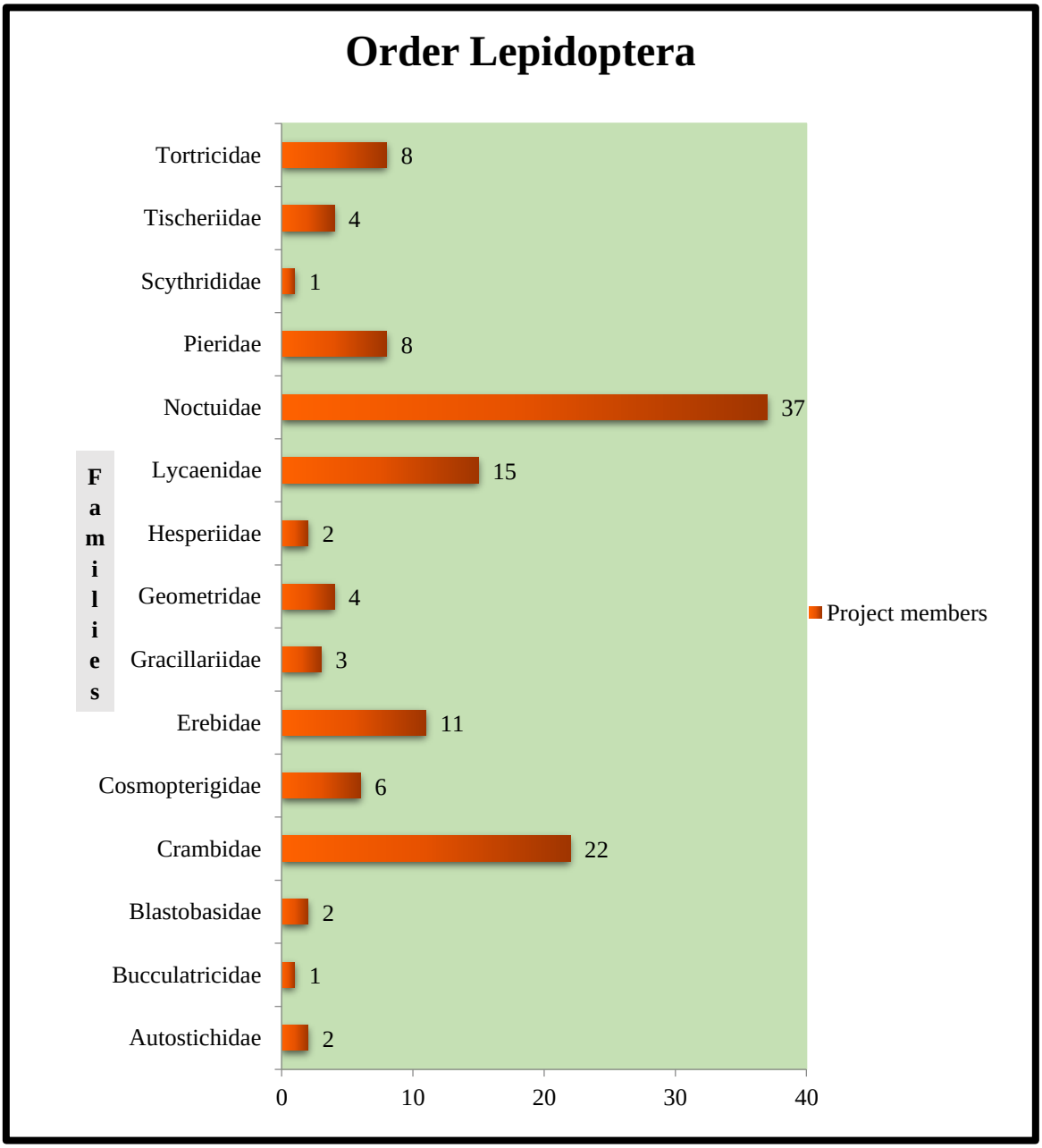

Figure 4.8.1.3: Order Lepidoptera Showing Families and Members in Projects

4.8.1.4. Order Hemiptera

Overall 9.92% relative abundance of order Hemiptera was calculated. Twenty-four families of order hemiptera were reported. It had 887 specimens in overall families. Out

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of 888, one specimen was reported at the order level and not identified at the family level as described in **Figure 4.8.1.4.**

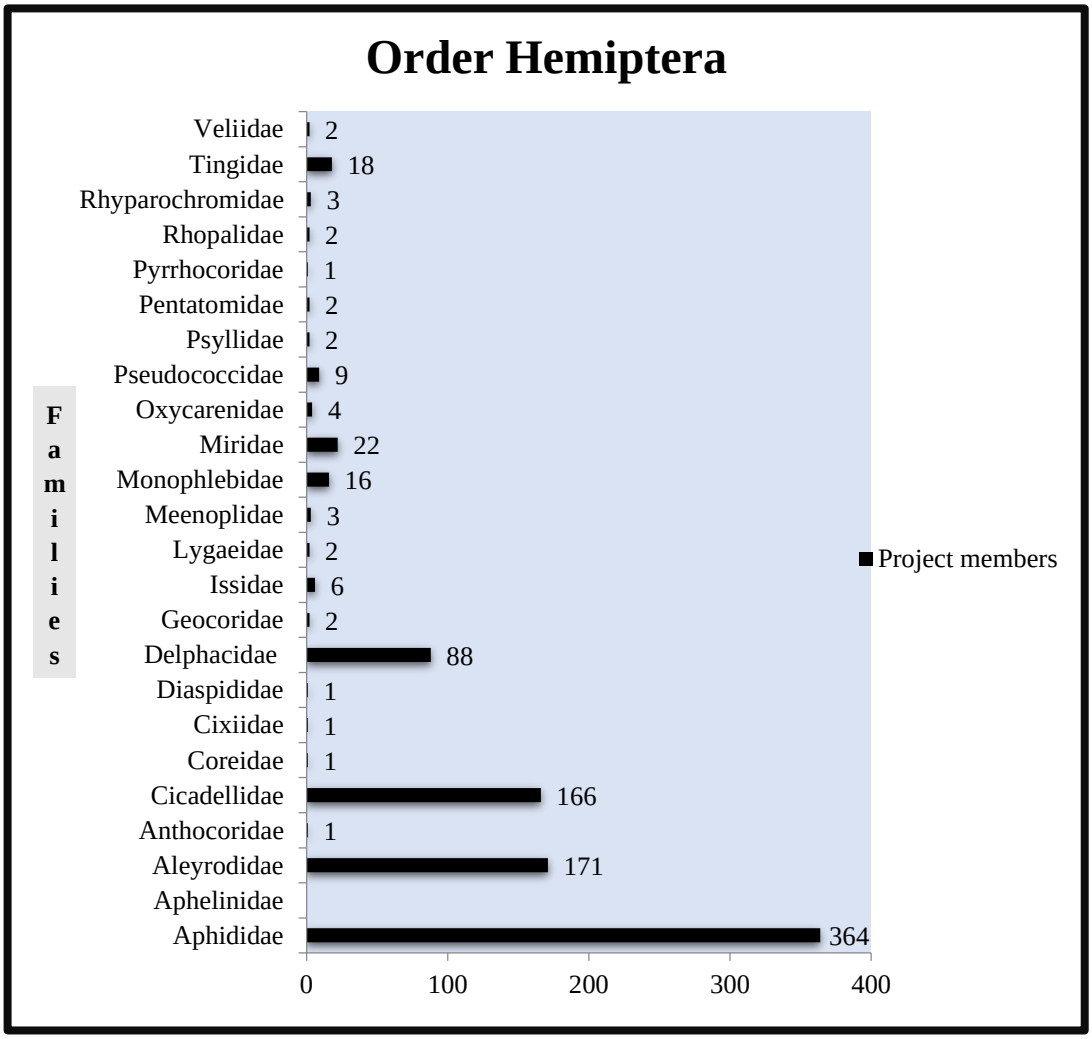

Figure 4.8.1.4: Order Hemiptera Showing Families and Members in Projects

4.8.1.5. Order Hymenoptera

Overall 15.87% relative abundance of Hemiptera was calculated. Forty families of order Hymenoptera were reported. It had 1411 specimens in overall families. Out of 1420, nine specimens were reported at the order level and not identified at their family level as presented in **Figure 4.8.1.5.**

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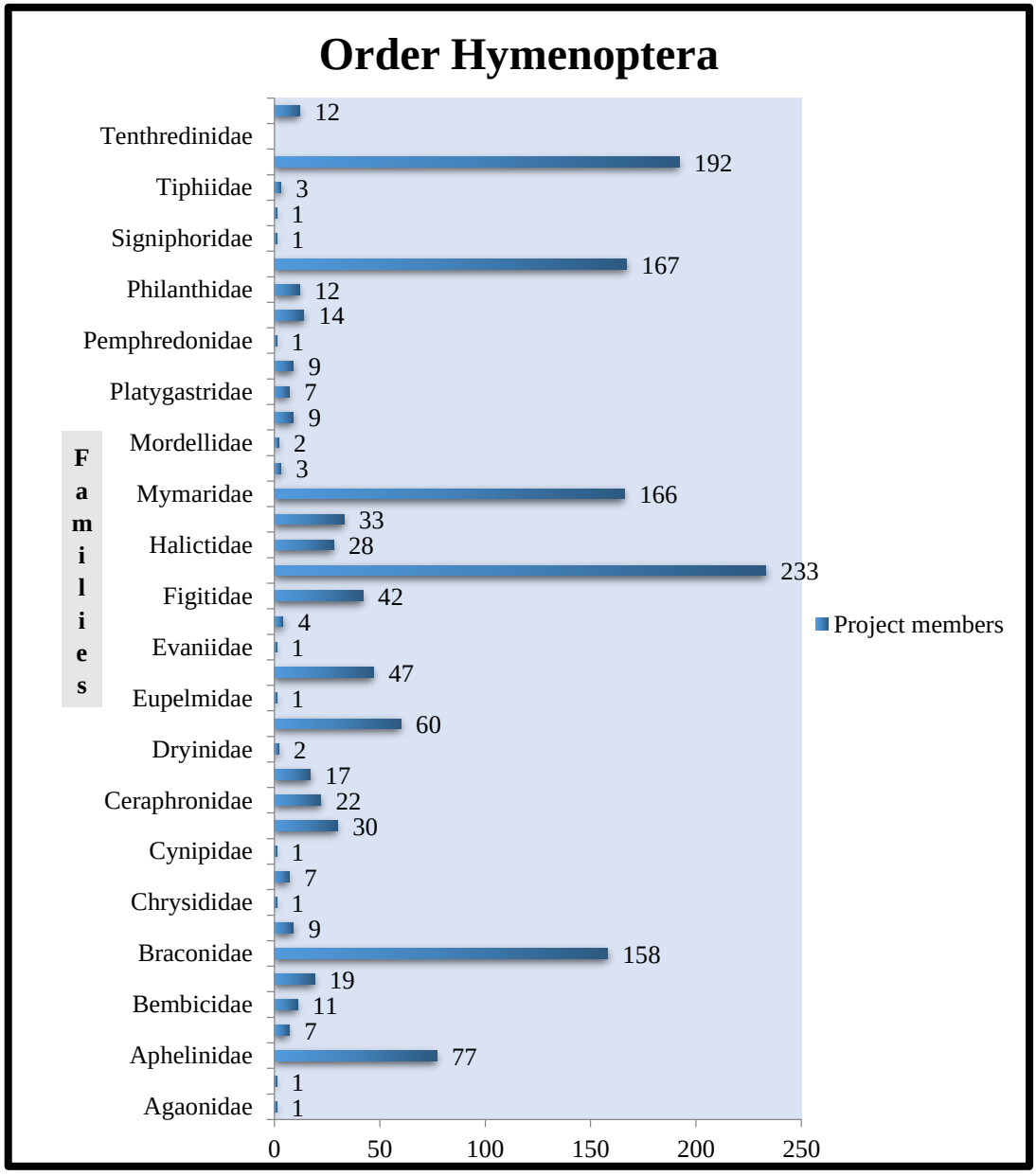

Figure 4.8.1.5: Order Hymenoptera Showing Families and Members in Projects

4.8.1.6. Order Thysanoptera

Overall 0.92% relative abundance of order Thysanoptera was calculated. Two families of Order Thysanoptera were reported. It had 82 specimens in overall families as described in **Figure 4.8.1.6.**

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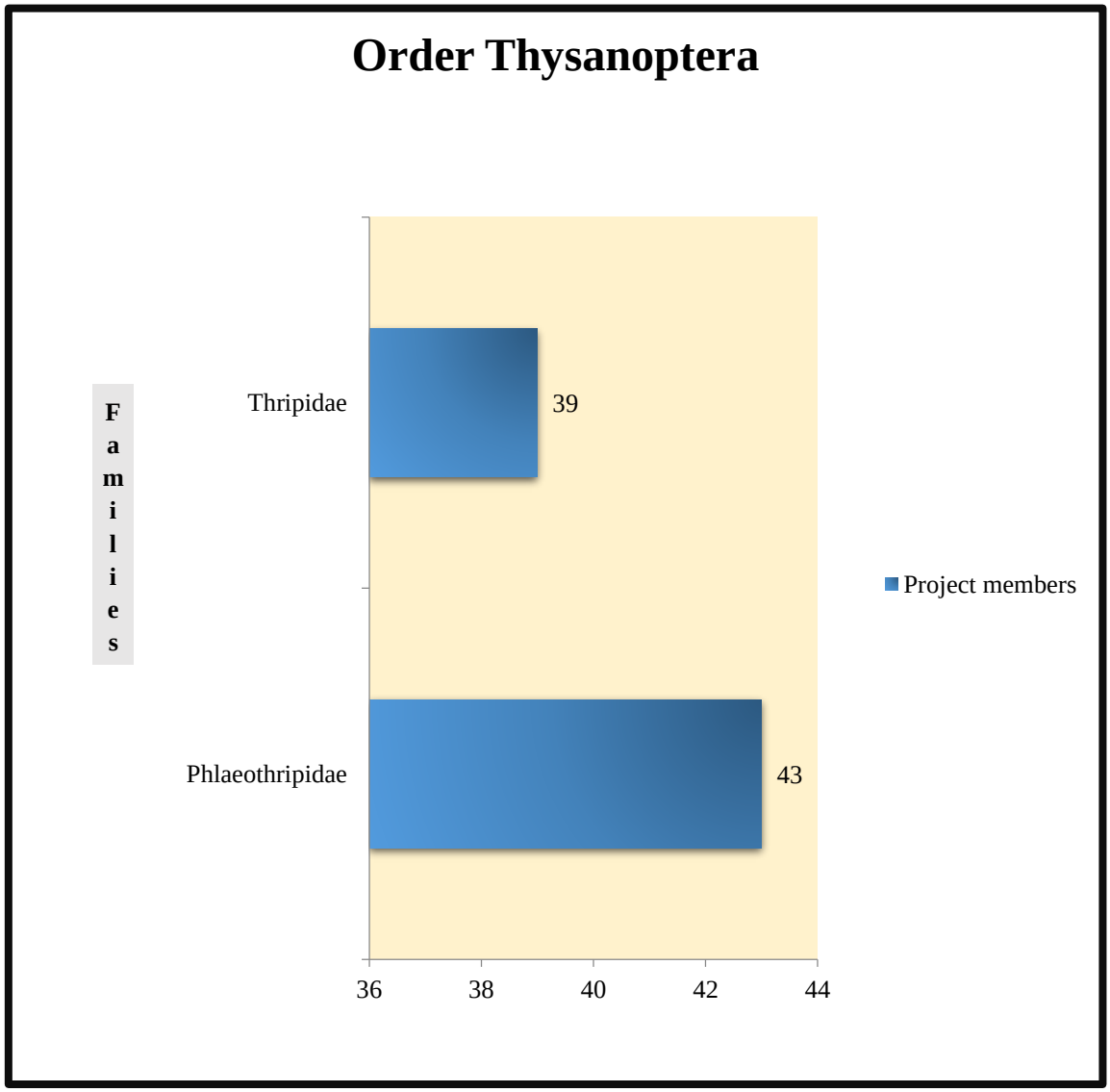

Figure 4.8.1.6: Order Thysanoptera Showing Families and Members in Projects

4.8.1.7. Order Thysanoptera,Odonata, Orthoptera and Neuroptera

Overall 0.92% relative abundance of Thysanoptera was calculated. Two families of order Thysanoptera were reported. It had 82 specimens in overall two families. One family of order Odonata and order Orthoptera was reported and its overall 0.06% relative abundance was calculated. Both orders had 5 specimens in one family. Order Neuroptera had only one family (No. of specimen=1) and its overall 0.01% relative abundance was calculated as depicted in **Figure 4.8.1.7**

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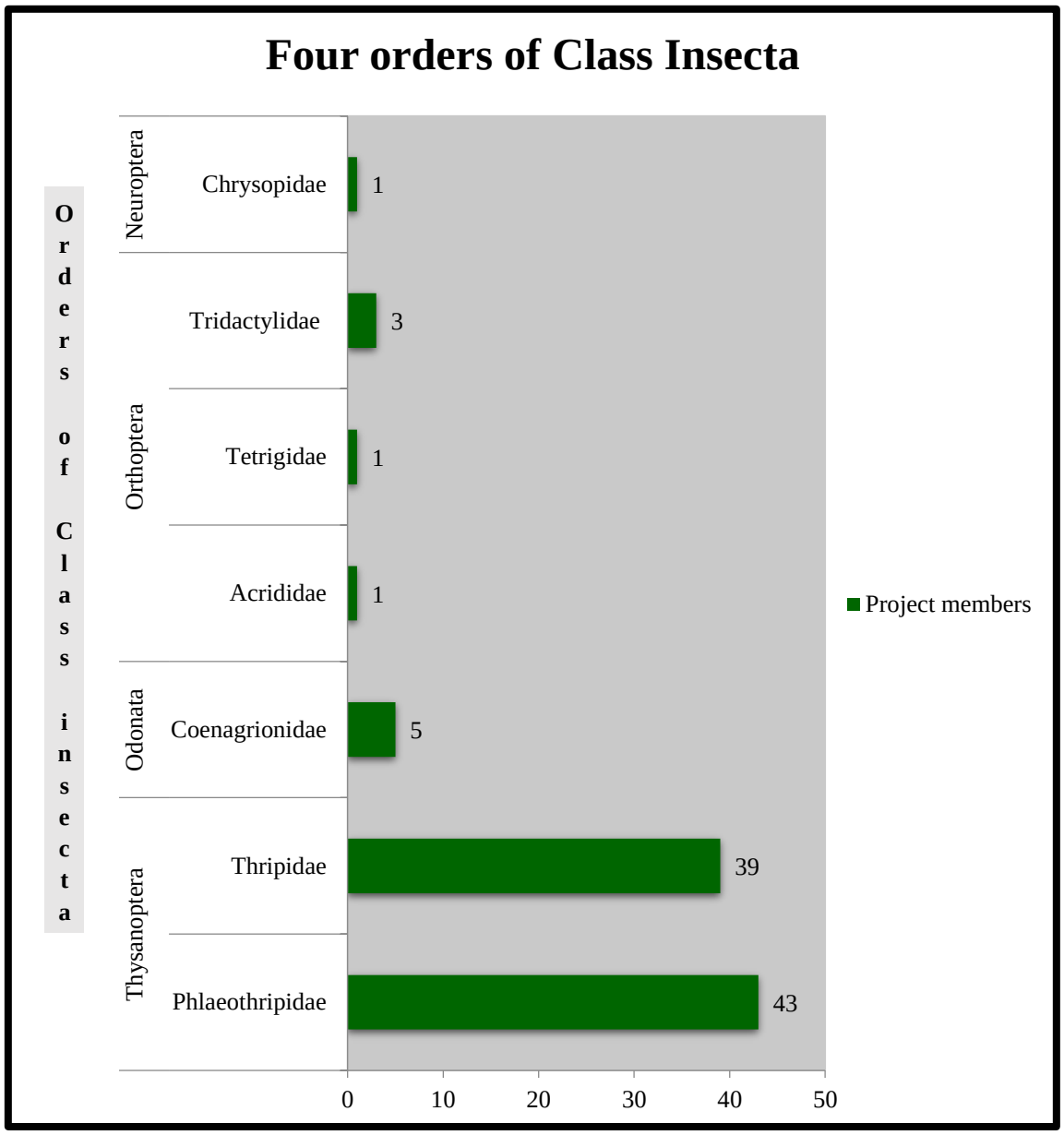

Figure 4.8.1.7: Orders Thysanoptera, Odonata, Orthoptera and Neuroptera Showing Families and Members in Projects

4.8.1.8. Order Psocodea

Overall 0.34% relative abundance of order Psocodea was calculated. Four families of order Psocodea were reported. It had 27 specimens in overall families as presented in **Figure4.8.1.8.** Out of 30, three specimens were reported at the order level and not identified at their family level.

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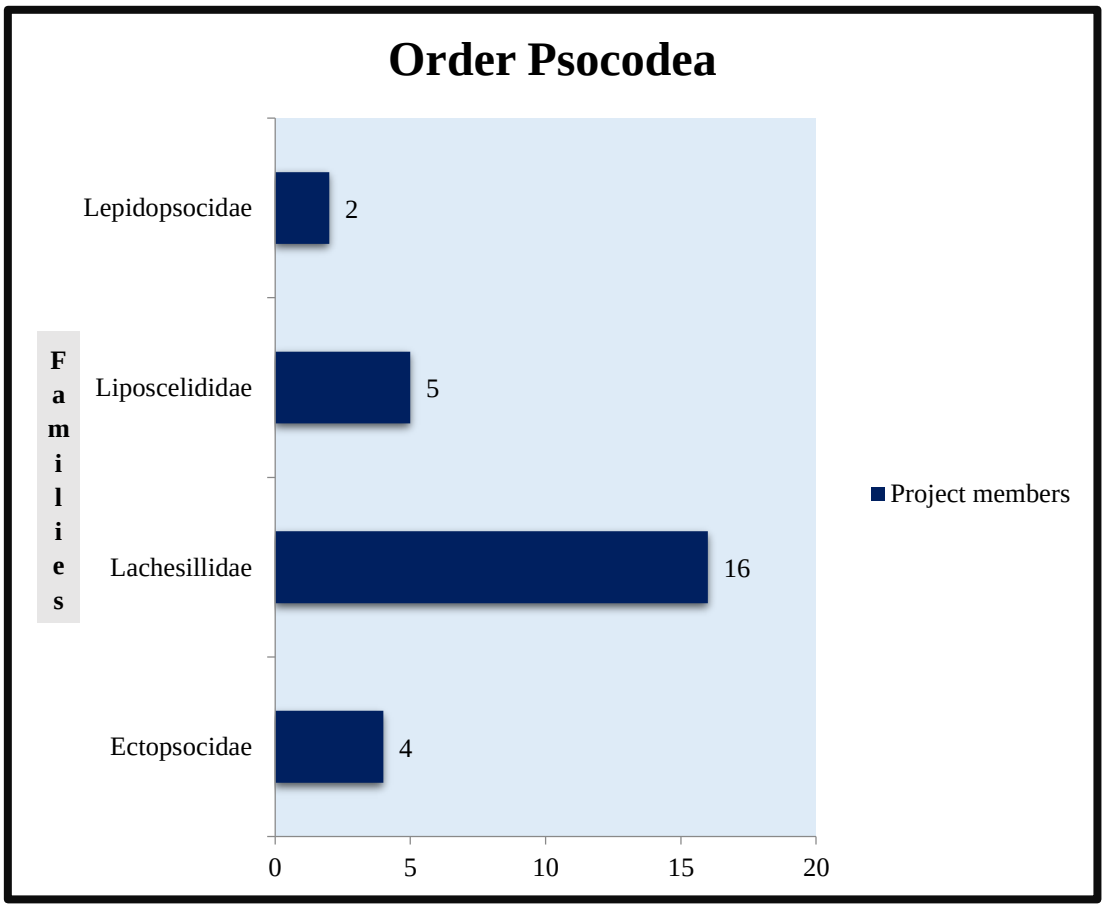

Figure 4.8.1.8: Order Psocodea Showing Families and Members in Projects

4.8.2. Class Collembola

Two orders of class Collembola were reported and its overall 0.98% relative abundance was calculated. Class Collembola had two orders (Entomobryomorpha and Symphypleona) as described in **Table 4.8.2.**

Table 4.8.2: showing total 2 orders in class Collembola and its percentage reported

Class	Order	Project	Frequency
Collembola	Entomobryomorpha	82	0.92%
	Symphypleona	6	0.07%
Total		88	0.98%

Order Entomobryomorpha included two families (Entomobryidea and Isotomidea) and 80 specimens were reported in two families. Two members were identified at the order level. Order Symphypleona had one family and included 5 specimens in it. One member was identified at the order level. Overall 6 specimens of order Symphypleona were reported as shown in **Figure 4.8.2.**

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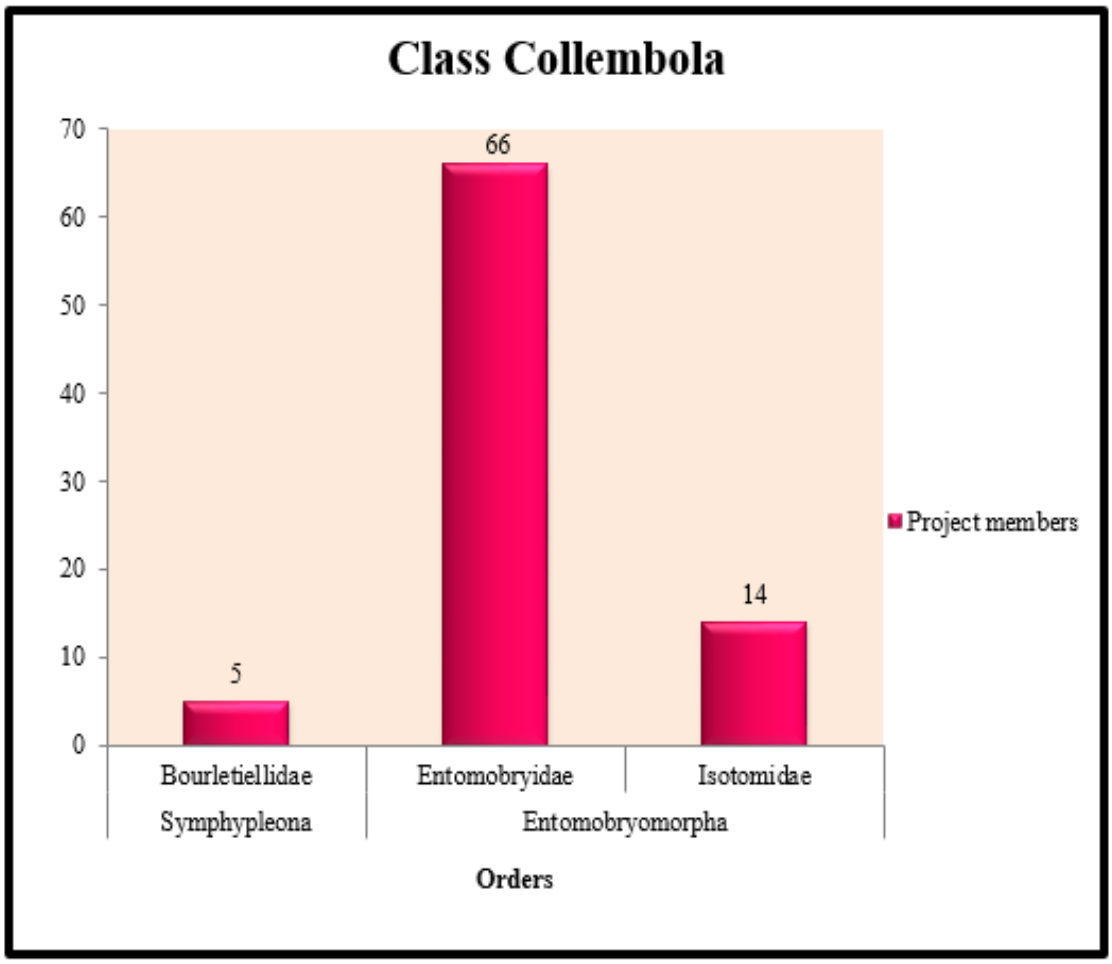

Figure 4.8.2: Class Collembola Showing Families and Members in Projects

4.8.3. Class Archnida

Four orders of class Arachnida were reported and its overall 0.78% relative abundance was calculated. Seventy four specimens were reported. Class Arachnida had four orders (Araneae, Sarcoptiformes, Mesostigmata and Trombidiformes). Sixty four specimens included in overall families. Four specimens were identified at the order level. Six specimens of order Mesostigmata were identified at the order level as shown in **Figure 4.8.3.**

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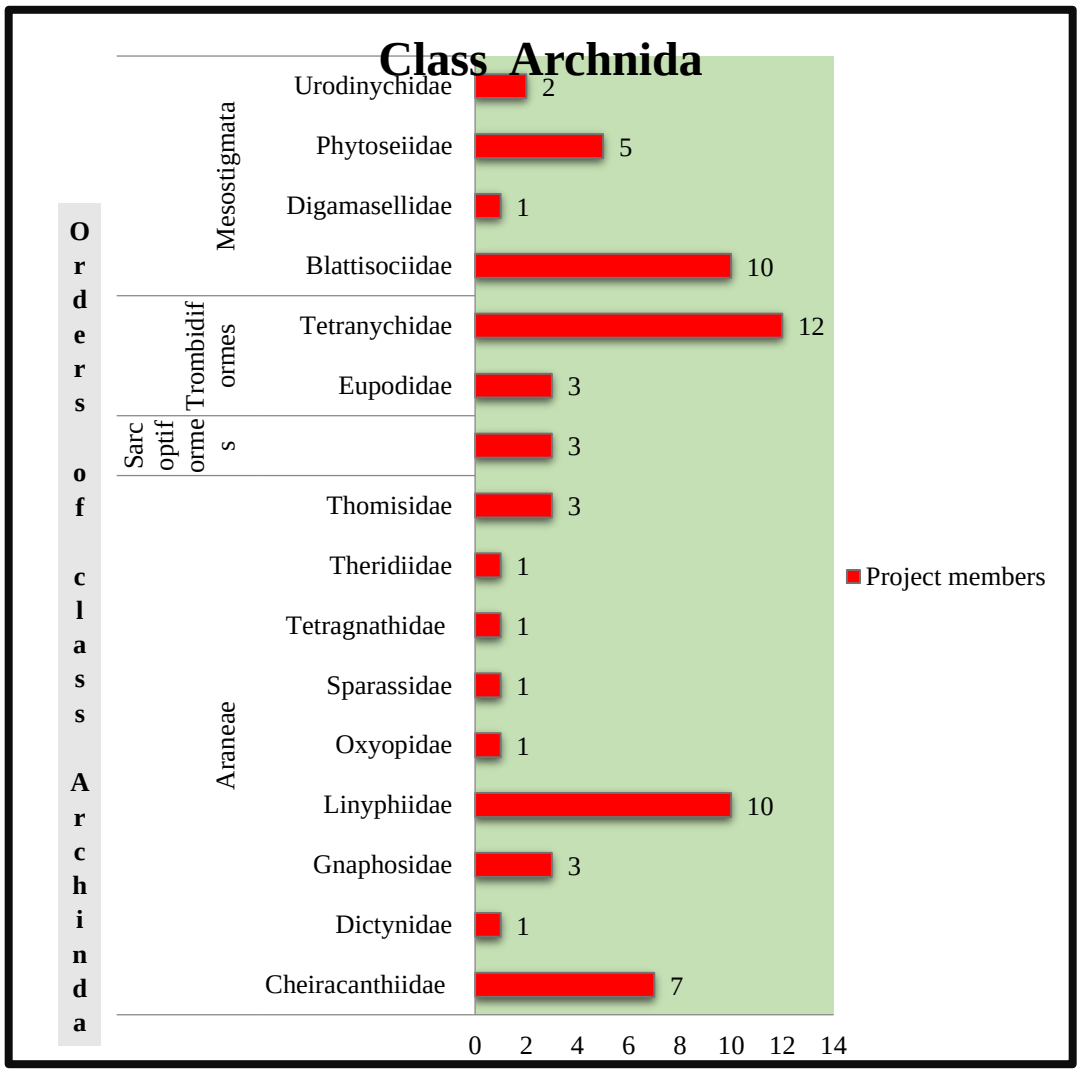

Figure 4.8.3: Class Archnida Showing Families and Members in Projects

4.8.3.1. Order Araneae

It included nine families (Cheiracanthiidae, Dictynidae, Gnaphosidae, Linyphiidae, Oxyopidae, Sparassidae, Tetragnathidae, Theridiidae and Thomisidae) and its overall 0.31% relative abundance was reported. Order Sarcoptiformes was not identified at the family level. It included 27 specimens calculated in overall families as presented in **Figure 4.8.3.**

4.8.3.2. Order Trombidiformes

It contained two families (Eupodidae and Tetranychidae) and its overall 0.17% relative abundance was calculated. It contained 15 specimens in overall two families as depicted in **Figure 4.8.3.**

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4.8.3.3. Order Mesostigmata

It reported four families **(**Blattisociidae, Digamasellidae, Phytoseiidae and Urodinychidae) and its overall 0.27% relative abundance were calculated. Eighteen members covered in overall four families. Overall 24 specimens were reported in order Mesostigmata as shown in **Figure 4.8.3.**

5. DISCUSSION

We have surveyed total insect diversity at Jinnah Garden Lahore, Pakistan. For this purpose, we have used Malaise trap because it has high efficiency for the collection of insects and qualitative data. Malaise trap have suitable for the collection a wide range of genera's. I have used active and passive method for the collection of specimens at the family level. Sample was collection from the Jinnah Garden, Lahore for the morphological and molecular identification in the gene variation. After collection identification of insects was done with reference to key (Laboratory procedure and essentials of entomology by W. A. Foster, 2005), insect identification with casual behavior and with reference to Pakistan insect museum as shown in **Figure 4.2.**

DNA isolation, Polymerase chain reaction, amplification and DNA sequencing, DNA bar-coding were performed for the molecular identification of insects. Following molecular techniques were performed to investigate the COI gene by extracting mitochondrial DNA of insects for the molecular identification. After it we have determined intraspecific variation in 658 bp in CO1 gene among the insect diversity.

Total 242,226 specimens were testified for DNA isolation through manual method. The DNA of 354 local insects and 8605 exotic insect species were examined as explain in **Figure 4.3.** Amplified DNA of 242,226 specimens by using forward (LCO1490) and reversed (HCO2198) primers, targeted the CO1 gene as explain in **Figure 4.4**.DNA barcodes were recovered from 242,226 of the 244499 specimens analyzed as descried in **Figure 4.6.** A total number of 1362 BINs was recovered. Our data reported that total number of 4 classes of phylum Arthropda. A total 242,226 insect species were reported An overall total 4 classes (Insecta, Collembola, Arachinda, and Malacostraca) and 17 orders (Diptera, Coleoptera, Lepidoptera, Hemiptera, Hymenoptera, Thysanoptera, Odonata, Orthoptera, Psocodea, Neuroptera, Entomobryomorpha, Symphypleona, Araneae, Sarcoptiformes,Mesostigmata, Trombidiformes, and Isopoda)of the local **(Table 4.7.1.)** and exotic insect species **(Table 4.7.2.)** were recognized and reported. But in previous study 19 orders (Blattodea, Coleptera, Dermaptera, Diptera, Embioptera, Hymenoptera, Hemiptera, Lepidoptera, Megaloptera, Mantodea, Neuroptera, Odonata, Orthoptera,Psocodea, Phasmatodea, Strepsiptera, Thysanoptera, Trichoptera and Zygentoma) were reported (Ashfaq *et al*., 2022). This study was done by DNA bar-coding (Ritter *et al*., 2019; Pentinsaari *et al*., 2020).

During entire time period of my study, 242,226 insect species were collected by Malaise trap class insects was most abundant and class collembola was least abundant (Rare).

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Overall in our study of local and exotic insect species, order Diptera was the most abundance and order Nuetroptera was least abundance in the data collection. In the previous study, order Diptera was most major order and it was considered a most important order of insect species (Larson *et al*., 2001).

Order Diptera showed 66.80% of the insect diversity in the Jinnah garden so that it showed most relative abundance of the diversity. Insects helped in pollination flower's seed and kept the stable environment of the Jinnah garden.

In the previous study local and exotic insect species were counted. Exotic insect species were known as tourist species. Now, our study indicated that local as well as exotic insect species have increased day by day in Jinnah garden. Exotic insect species (N= 8948) have greater in number as compared to local insect species (N= 343). Insects captured in our study, have played important role in pollination, stable the environment, act as predator for exotic species and pests etc. Insects are beneficial for our ecology. Sometime insects play important roles in ecosystem as decomposer, recycle the nutrients, scavenger and act as food source for human because insect have high amount of protein, nutrients and amino acids.

Maina *et al*. (2012) reported that insects play roles for the stability of ecology and ecosystem environment. Our study reported that extra richness of the insects in the Jinnah garden so that insects act a disease vector for many diseases, insects damage the plant leaves and spread the allergy for the citizens.

Van Dyck (2008) reported that insect diversity increased in the hot months but in the cold month's insect diversity decreased of year. The Current study aimed to estimation of the species richness of Jinnah garden Lahore, Pakistan by using DNA bar-coding with BIN system. Overall 242,226 specimens were sequenced. After it a total 1362 barcode were generated.

In previous French Polynesia study reported that orders recovery Diptera (91%) and Colepteran (63%) (Ramage *et al*., 2017). Similarly in Canadian study showed that order Diptera (95%), Hemiptera (77%) and Hymenoptera (74%) reported at high abundance in range (de Waard *et al*., 2019a). In our study reported that overall 19 orders of phylum Arthropoda. It represented that highest abundance of order Diptera 66.80% and Hymenoptera 15.87%. Overall 99% specimens have orders that are reported. Fourteen most diverse order where species assignment 7.34% (Coleptera: 2.72%, Lepidoptera: 1.44%, Thysanoptera: 0.92%. Odonata: 0.06%, Orthoptera: 0.06%, Psocodea: 0.34%, Nueroptera: 0.01%, Entomobryomorpha: 0.92% Symphypleona: 0.07%, Araneae: 0.31%, Sarcoptiformes: 0.03%, Mesostigmata: 0.27%, Trombidiformes: 0.17%, and Isopoda: 0.03%) as shown in **Table 4.7.3.**Four specimens of class Arachnida were not showing at order level. The present study classified insects in 4 classes, 19 orders, 173 families and 1362 BINs of overall 9007 specimens.

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In previous study while 19 orders were reported. Five Oder's (Coleptera, Hemiptera, Diptera, Lepidoptera, and Hymenoptera) were dominant. But in our study only two orders (Diotera and Hymenoptera) are dominant from overall 17 orders.

The selection of a trap depends upon the research's goals; Malaise traps may manage insect orders and are less likely to catch insects from close regions than light traps look after, making it possible to estimate the area being examined and region more simply for a Malaise trap as compared to a light trap.

6. CONCLUSION

The Present study is conducted from November (2022) to June (2023) in the Jinnah Garden, Lahore. DNA bar-coding is a molecular technique for the identification of differences between species. CO1 gene was a molecular marker for phylogenetic analysis and taxonomical classification. It is concluded that Jinnah Garden has rich exotic insect biodiversity as compared to local insect biodiversity.

This work has classified and also documented at the family level for the first time, insect samples collection by using Malaise trap. When the temperature decreases and humidity increases then insect diversity increases. Three orders represented 92.56% of the specimens: Diptera (66.80%), Hymenoptera (15.87%), and Hemiptera (9.92%).This study is not beneficial for further research but also helpful for further investigation of insect diversity in Jinnah garden, Lahore. We can improve forensic entomological research by using the different DNA bar-coding regions to investigate mutation in genetic diversity and evolution.

7. RECOMMENDATIONS

The following suggestions are helpful for the collecting of a variety of beneficial insect species:

- Additional research should be carried out utilizing other sampling techniques and extending the geographical area of the research.
- There is a need to also study the genus and species level of insect species. To check the prevalence of exotic insect species in Jinnah Garden, Lahore.
- To collect a wide variety of insect species, several traps should be used.

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