Comparative DNA Barcoding of Wheat Crop Insects at the Start and End of the Season: insights from Islamabad, Pakistan

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Comparative DNA Barcoding of Wheat Crop Insects at the Start and End of the Season: insights from Islamabad, Pakistan

A thesis submitted to the Department of Biotechnology, Quaid-i-Azam University Islamabad, in partial fulfillment of the degree of Master of Philosophy in Biotechnology

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CERTIFICATE

It is hereby certified that the research work presented in this thesis entitled "Comparative DNA barcoding of wheat crop insects at the start and end of the season: insights from Islamabad, Pakistan" was conducted by **Ms. Um-e-Aiman Hameed** under the supervision of **Dr. Javaria Qazi**. This thesis is submitted to the department of Biotechnology in partial fulfillment of the requirements for the degree of Master of Philosophy (M.Phil) in **Biotechnology**.

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Chairman _____

Dr. Javaria Qazi

DECLARATION

I declare that the contents of my thesis entitled "Comparative DNA Barcoding of Wheat crop Insects at the Start and End of the Season: insights from Islamabad, Pakistan" has been my own and original creation, carried out in Molecular Virology Lab, Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. The research and write up has been executed by myself and has not been taken from any other source that can be considered as the violation of the international copyright law except where due reference is made and has not been published before.

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Um-e-Aiman Hameed
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Dedicated to my beloved father, mother and Komal Api!

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All praise be to Allah, the most merciful and most beneficent. He, whose glory spans the whole universe and who is there from the first breath till the last. Then I pay my humble regards to the Holy Prophet (P.B.U.H) who is the undisputed symbol of love and mercy for the entire humankind.

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Um-e-Aiman Hameed

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LIST OF ABBREVIATIONS

Abbreviations	Description
µg	microgram
µl	microlitre
µM	micromolar
BIN	barcode index numbers
BOLD	barcode of life data systems database
bp	base pair
$CaCl_2$	calcium chloride
CO1	cytochrome c oxidase subunit 1
Cyt b	cytochrome b
ddH_2O	double-distilled water
DNA	deoxyribo-nucleic acid
dNTP	deoxy nucleotide tri phosphate
HCl	hydrochloric acid
ITS	internal transcribed spacer
mg	milligram
$MgCl_2$	magnesium chloride
ml	milliliter
mM	millimolar
mt-CO1	mitochondrial DNA encoded cytochrome c oxidase subunit 1
PCR	polymerase chain reaction
pH	potential of hydrogen
PK-buffer	proteinase-k buffer
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
TAE	tris-acetate-edta
Taq polymerase	thermus aquaticus polymerase
Tris	tris-aminomethane
UV	ultraviolet
w/v	weight by volume

ABSTRACT

Wheat (*Triticum aestivum*) is a staple crop of Pakistan, and insect infestations can cause significant yield losses and quality deterioration. This study investigates the comparative DNA barcoding of wheat crop insects at the beginning and end of the season in Islamabad, Pakistan. This study's significance lies in enhancing our understanding of wheat crop insect populations and dynamics throughout the growing season. By comparing insect communities, valuable insights into factors influencing abundance and species composition can be gained. The use of DNA barcoding allows for precise species identification, aiding in effective insect surveillance and management, even for cryptic or morphologically similar species.

The study focuses on the importance of employing the COI mitochondrial barcode method to identify wheat crop insects and sheds light on the diversity and composition of these insects. A total of 11 different species of wheat crop insects were morphologically identified *Neoscona adianta, Mycetophila idonea, Auplopus carbonarius, Sogatella furcifera, Coccinella septempumctata, Scirpophaga incertulas, Exitianus indicus, Sphaerophoria philanthus, Psammotettix emarginatus, Exochomus quadripustulatus, Sogatella vibix*. From these insects, 6 species those were common at the start and end season of wheat crop; *Neoscona adianta, Sogatella furcifera, Coccinella septempumctata, Scirpophaga incertulas, Exitianus indicus, Psammotettix emarginatus* were processed for barcode analysis. That involved DNA extraction, PCR amplification using universal primers, sequencing using BLASTn, and the utilization of phylogenetic tree construction and species demarcation tools. This research has significant implications for agricultural practices by enabling targeted pest management and reducing reliance on broad-spectrum pesticides. The DNA barcoding approach facilitates ongoing monitoring for early detection of new insect pests, promoting sustainable agriculture in Pakistan.

CHAPTER NO.1

INTRODUCTION AND LITERATURE REVIEW

1.1 Wheat crop and its significance in Pakistan

The most important cereal crop amidst the field crops is wheat (*Triticum aestivum*), which is not only ancient but also provides a better diet for people. Due to extensive area used for agriculture, great nutritional content, and connections to some of the world's oldest and most significant civilizations, wheat is given a special place among grains. While wheat serves as the main food source of nutrition for a large part of the world, rice is its primary food source (Kundu et al., 2006). About more than 600 million tons harvested each year that's why wheat is considered among "big three" cereal crops. Humans use a lot of wheat, both in the primary producing nations (over 100 according to the production statistics of FAO for the year 2004) and in rest of the nations where it is not farmed (Shewry, 2009).

Triticum tauschii is a species that is diploid (2n=2x=14), and *Triticum turgidum* is a species that is a tetraploid (2n=4x=28), both came from, *Triticum monococcum* and *Aegilops speltoides* related species. Both species are diploid. *Triticum aestivum* is known as common wheat or bread wheat. Bread wheat/common wheat is hexaploid (2n=6x=42), and it is originated in the past 30,000 years. The most adaptable of all agricultural species, wheat can grow in a variety of environmental settings from 47oS to 57oN (Khan et al., 2012).

Wheat is frequently seen as little more than just a calorie source, due to its 60–70% starch content in whole grains and 65 to 75% in white flour—and it's undeniably accurate when it comes to the production of animal feed. Even with the anticipated 60 million tons of soybeans produced each year, wheat still provides big portion of protein for both human and animal consumption (calculated by Shewry, 2000),despite having a relatively low protein concentration (about 8 to 15%). Since bread, noodles, and other foods (such as Bulgar, couscous) may make up a large portion of the diet in countries that are underdeveloped with fewer resources, it is crucial to not underestimate the nutritional significance of wheat proteins. Wheat has been crucial to feeding the planet since prehistoric times. It offers 21% more protein and 19% more calories (Braun et al., 2010). French bread, chapatti, cookies, pasta, macaroni, injera, and porridge are just a few examples of the many food varieties that can be made either by using only wheat flour or combining it with flour from other grains.

In the past, the wheat culture predominated in Central Asia, Australia, North Africa, Europe, America, and West Asia. Due to increased urbanization and dietary shifts, the demand for wheat is rising yearly in all of the different regions, including Australia 2.2%, North Africa 2.2%, South Asia and the Pacific 4.3%, Central and West Africa 4.7%, Central Asia 5.6%, Eastern and Southern Africa 4.8%, and Australia 2.2% (Shiferaw et al., 2013). The majority of the nations in the CWANA and Sub-Saharan African areas are gross importers of wheat. In terms of global wheat imports, Algeria ranks second after Egypt (9 million tonns annually). Each year, 17 billion pounds of wheat are imported by Sub-Saharan African nations. For trades involving 184 million tons of wheat overall the yearly wheat trade value in 2016 was around 36 billion dollars (FAO, 2018).

Wheat is Pakistan's staple food. Wheat is harvested in Pakistan from many centuries. Pakistan's climate and soil are favorable for growing wheat. Compared to previous years, there has been a rise in the national wheat yield. In any case, there are still more opportunities to raise the yield. In 2023, wheat is harvested on 9260 thousand acres, yielding 27 million tonns, according to Pakistan's statistics bureau office. Wheat yields in wealthy nations are ten times higher than Pakistan. The demand for wheat is rising as population continues to rise. In Pakistan, there are two seasons for agricultural cropping: April through June mark the beginning of the first sowing season, or Kharif, which concludes in December with harvest. Rabi, the second sowing season, begins in October and ends in December with harvest in April or May. Wheat, lentil (masoor), gram, tobacco, rapeseed, barley, and mustard are considered to be "Rabi" crops.

The agricultural sector is crucial to Pakistan's economy because it employs more than 45% of the labor force, accounts for around 20% of GDP, and directly or indirectly provides for about 67% of the country's population. The significant crops (wheat, rice, sugarcane, maize, and cotton) provide 4.20% of GDP and 21.73% of the value added by the agricultural industry. According to Pakistan Economic Survey, 2019-2020, other crops are responsible for about 11.53 percent of the value addition in the agricultural sector and 2.23% of GDP. In Pakistan's four provinces, wheat is farmed on a smaller to a larger scale. The provinces that produce the most wheat are listed below: Punjab provided 76% of the wheat, KP produced 5%, Baluchistan province only produced 3% and Sindh produced 16% of the wheat (Iqbal et al., 2022).

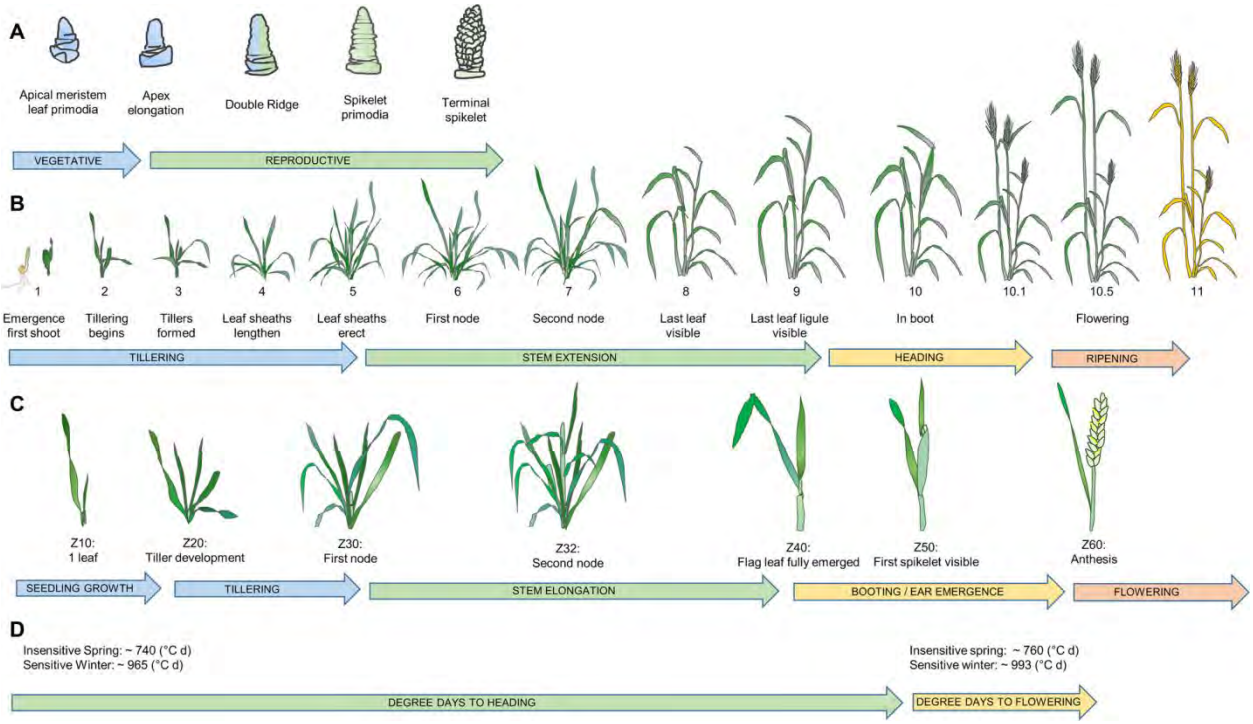

Figure 1 **Phenological growth stages of wheat**

The diagram presents four distinct aspects related to the development stages in wheat. (A) Illustration of the morphological changes in the apex from vegetative to reproductive phases. (B) Feekes Scale, encompassing stages 1-11, provides a standardized classification system for wheat growth and development. (C) Zadoks Decimal Scale, ranging from score 0-100, offers a comprehensive method to assess various growth stages in wheat. (D) An example showcasing the cumulative degree-days required for near-isogenic lines (NILs) with different vernalization or photoperiod requirements, grown in inductive conditions, specifically emphasizing the periods from emergence to heading and emergence to flowering. This information contributes to a deeper understanding of wheat development and its response to environmental stimuli. Image source: (Hyles, Bloomfield, Hunt, Trethowan, & Trevaskis, 2020).

1.2 Classical identification of insect species

Species-level identification of the specimens is required in order to comprehend the diversity of the species, phylogeny, and their evolutionary relationships (Platnick, 2014). Moreover a million species of insects are included in the insect catalogue, yet millions of them remain unknown (Grimaldi and Engel, 2005). Traditional methods for identifying pests such as insects include a variety of morphological traits (Jinbo et al., 2011). However, identifications based on morphology are sometimes difficult and lengthy. Juveniles, early instars, and pupae are examples of immature developmental phases that cannot be distinguished by standard taxonomy because most morpho-taxonomic keys are only used to study adults (Barrett & Hebert 2005). The identifications using morphology are frequently complicated by phenotypic plasticity (Murugan et al., 2016). Using morphology alone to identify cryptic species is equally challenging. Furthermore, extensive skill is needed to use taxonomic keys effectively (Ball and Armstrong, 2006).

It must be acknowledged that conventional methods frequently fail, as in the case of fish fillet species identification (Wong & Hanner, 2008). But even when whole organisms are present, conventional methods frequently fail to identify samples. For instance, Stribling (2006) claims that error rates of 10-15% at the level of genus are regarded acceptable for freshwater benthic invertebrates, creatures that are particularly essential for monitoring water quality, and that error rates of 45% at the level of genus are occasionally recorded. Many of these organisms are young insects that need an adult to be identified at the level of species (Packer et al., 2009).

1.3 Insect pests of wheat crop in Pakistan

Wheat (*Triticum aestivum)* plants sustain significant harm from a variety of arthropods at every stage of their development. Wheat pests are either oligophagous (feeding on only a few plant species) or polyphagous (damaging a great variety of plants), and it is extremely uncommon to find an insect that is monophagous to wheat crops. Prior to the "green revolution," it has been estimated that insect pests caused roughly 5.1% of the world's production losses; however, after the "green revolution," in the 1990s, the losses rose to 9.3%. Pest insects are very adaptable and dynamic in nature. Temperature changes in the environment can alter a species' physiology, behavior, voltinism, and distribution (Farook et al., 2019). Major insects of wheat crop that are globally found are Aphids, Cereal leaf beetle,

Surface grasshopper, Ghujia weevil, Termites, Armyworms, Pod borer, Brown wheat mite, Pink stem borer brown stink bug, white grubs, wire worms, and these are shown in figure 1.3.

Among the most significant cereal crops and sources of basic nutrition worldwide, including in Pakistan, is wheat. With a GDP contribution of 18.9%, the agriculture industry is significant to the economy. Reduced wheat productivity is caused by a variety of biotic and abiotic causes. Numerous insect pests that attack wheat at various stages of the crop inflict damage, and ultimately lower output. Wheat crops are attacked by a number of pests, including aphid, cereal leaf beetle, wheat weevil, wheat midges, grasshopper, white grubs, termites, hessian flies, flea beetles, armyworm, wheat stem sawfly; pink graminous stem borer and *Helicoverpa armigera*. Identification of important insect pest of wheat and their harms to wheat crop are essential for the development of sustainable pest management approaches to reduce pest infestation and production losses in the wheat crop. Figure 1.3 depicts all of the insects listed below.

(A) Aphids

Worldwide, aphids are harmful bugs that attack all cultivated crops. At this time, Pakistan is home to 92 different species of aphid (Irshad, 2001). *Schizaphis graminum, Rhopalosiphum padi and*, two aphid species, worldwide seriously harms wheat crops (Hamid, 1983; Inayatullah et al., 1993). Aphids are soft-bodied, virtually translucent sucking insects. Aphids have the ability to cause leaf yellowing and early mortality when they are present in large enough quantities. They release "honeydew" drips, a sugary liquid, which can leave tiny scorch marks on the foliage. A significant and pervasive pest on cereal crops, aphids. They can do serious harm if they eat in large enough quantities. Additionally, the aforementioned species could serve as BYDV virus vectors that are found in early spring season.

(B) Cereal leaf beetle

Oulema melanopa is the term given to this insect in science. The adult beetles are 4-5 mm long and have a shiny look. They have a black head, a light brown thorax, and wings covered in parallel lines of tiny dots in blue-green color. The faeces that larvae create and pile on their backs give them the appearance of a slimy, spherical, black mass. Larvae are initially a dull to bright yellow color. The presence of prominent, longitudinal stripes on the leaves is the most observable indication of a cereal leaf beetle infestation. Both adult and larval beetles eat on the leaves to produce these stripes. Winter wheat production can be significantly reduced, as can spring wheat grown in the fall.

(C) Hessian fly

Mayetiola destructor is scientific name of this fly. Hessian flies caused the plants to become stunted, create thin stands, lodge, and produce less. The larvae, which eat plant tissues and drink their liquids, are the only ones who do harm. Stems that are infected during jointing frequently break before they reach maturity. The Hessian fly is 3 to 4 mm in length, with a pinkish or yellow-brown abdomen and a black head and thorax. One of the most harmful insect pests on cereals is this one. Hessian flies are mostly a problem for wheat; however they can also harm barley, rye, and other grasses. The majority of wheat-growing regions in the world have received reports of this bug. Each year, there are two generations—one in the autumn and one in the spring. In the middle to end of September, fall-breeding flies typically become active.

(D) Wheat stem maggot

When new tillers get attacked in the fall or early spring by stem-boring insects, plants that are infested commonly exhibit the "white head" sign. The adult flies are 6 mm long, light green to yellow in color, and have dark stripes. Around 10-15% of plants may suffer damage in contaminated fields. In some years, damage can be severe, but the bug rarely causes significant harm. However, considerable numbers of the tillers may be eliminated by severe infestations of specific wheat stands. Larvae of wheat stem maggots overwinter in grasses or cereal plants.

(E) Sawfly

Cephus cinctus is its official scientific name. Sawflies can cause the head to prematurely yellow and the grain to shrivel. Later in the crop cycle, lodging is frequent because the larvae have girdled the stem. Sawflies have one generation per year. The larvae pupate in the spring after spending the winter in a straw. From late spring to June, little, fly-like wasps called sawflies emerge. Just behind the crowns of the stems' higher nodes, the females lay tiny white eggs. Sawfly infestations are typically intermittent, but they can cause severe harm. Although wheat is favored, almost all domesticated cereals and native grasses serve as hosts.

F) White grubs

The roots of the host plants might be entirely or partially severed by white grubs. The primary symptoms are wheat plants dying. The largest of these larvae can reach lengths of several centimeters and a thickness of almost one centimeter when completely developed.

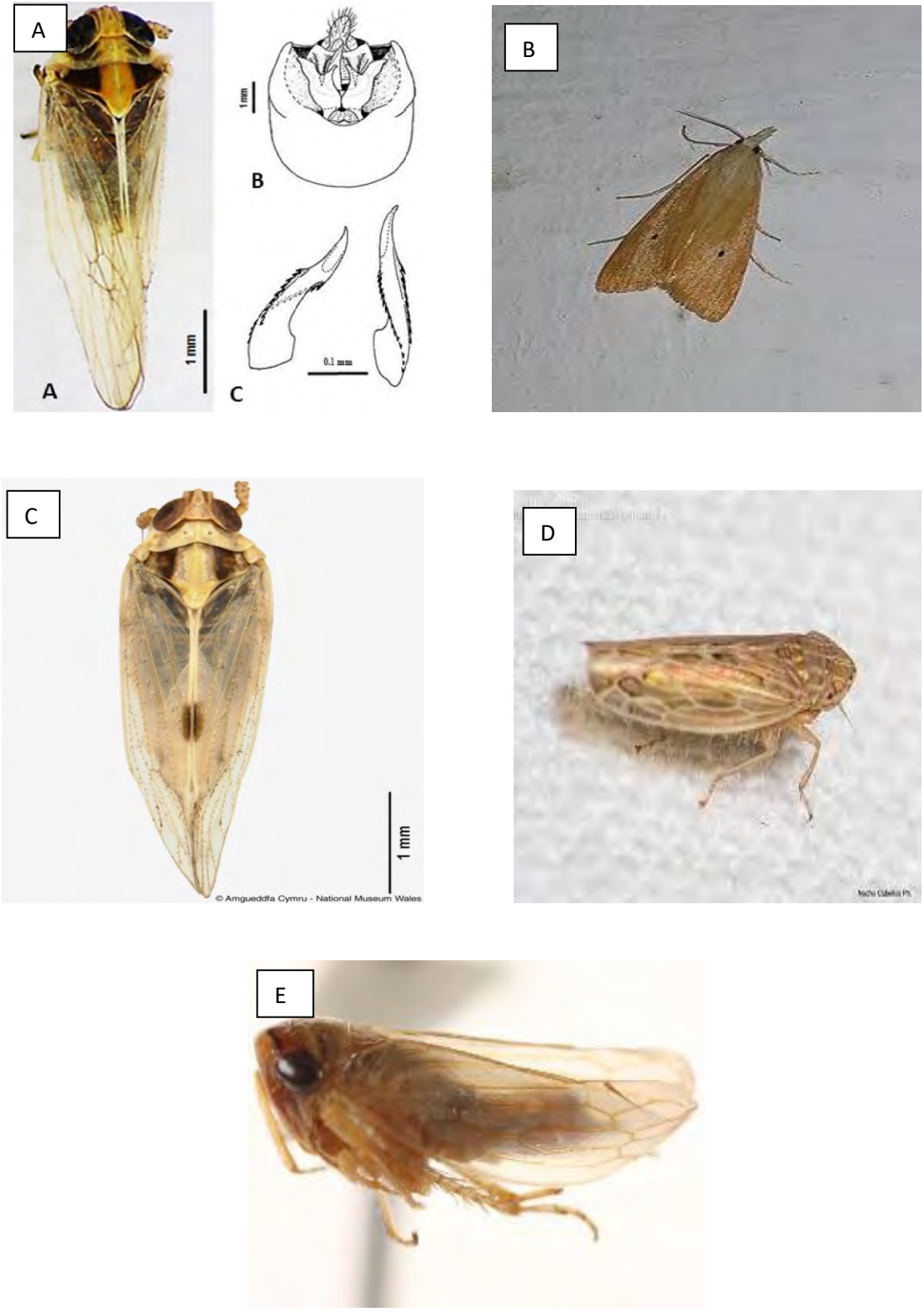

Figure 2 Harmful insects of wheat crop

A. White backed planthopper **B**. Yellow stem borer **C.** Brown planthopper **D**. Notched sand grasshopper **E**. Leafhopper. Image source: Google

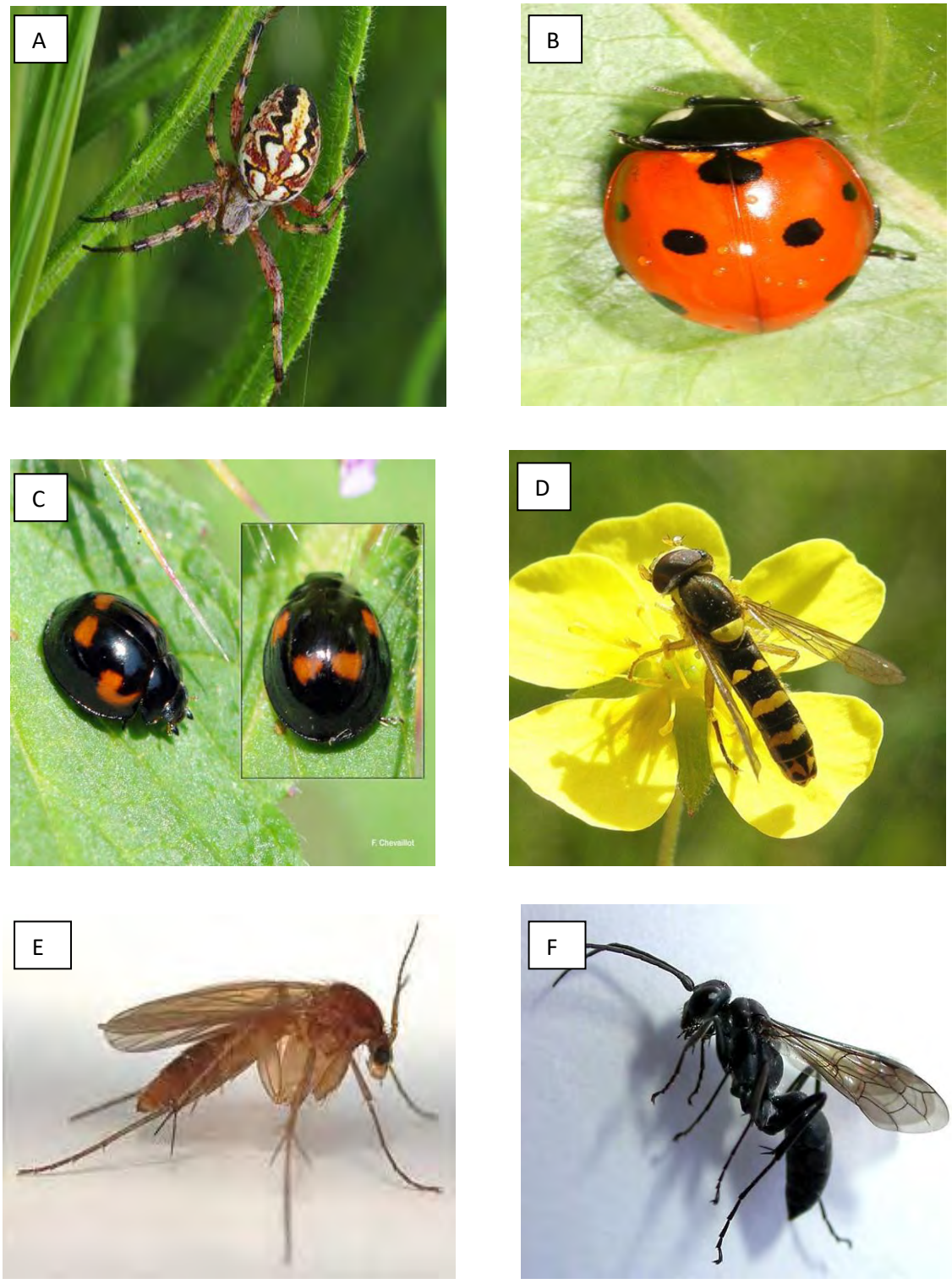

Figure 3 **Useful insects of wheat crop**

A. Orb weaver spider **B.** Lady bird beetle **C.** Pine ladybird **D.** Hover fly **E.** Fungus gnats **F.** Potter spider wasp. Image source: Google

The larvae of May or June beetles are known as white grubs. In the soil, they lay eggs, and when they hatch, larvae live by feeding on roots. From species to species, the larval stage lasts for a different amount of time. The plants may live if the roots are not entirely killed, but they will likely be stunted and unable to produce heads. However, the geography and intensity of the attack vary.

(G) Grasshopper

Grasshoppers, or *Chrotogonus trachypterus*, are naturally polyphagous. They can be found primarily in India, Africa, and the Orient. Gardens and *Triticum aestivum* fields are good places to see and gather them because they usually live on plant surfaces. All phases of development—egg, larvae pupa, and adult—are present, although nymphs and adults—including *Avena sativa*, *Hordeum vulgeum*, and *Gossypium* plant—are particularly hazardous to a number of crops (Abas and Niaz, 2019). Grasshopper harm starts as soon as they hatch, but it gets worse as they get older and more numerous (Farook et al., 2019). In addition to peeling and notched leaf symptoms, they reduce crop yields in fully matured crops. Grasshoppers are a big population of insects; some species like to dwell in swarms like locusts. They include a few of the worst pests for crops, notably wheat. However, species that resemble locusts offer us a more convenient position for cultivation and harvesting. Grasshoppers are valuable commercially since they are utilized as food in many nations. They are regarded as an alternative protein source in Mexico due to their capacity to transform low-land food into food with high nutritional properties. As a result, they are offered in markets for feeding in many different nations (Cerritos and Cano-Santana, 2008).

(H) Ghujia Weevil

The Ghujia weevil, also known as the wheat weevil, may grow up to 7 mm long and is found in Uttar Pradesh, Bihar and Punjab. A female weevil deposits 50 to 70 eggs at a time in soil. Before becoming adults, juvenile weevil larvae, also referred to as grubs, eat the humus of the soil. In June and July, adult wheat weevils begin to appear. Only mature weevils infest crops and destroy seedlings that are just beginning to germinate. Wheat seedlings and leaves sustain substantial damage from wheat weevil (Farook et al., 2019).

1.4 DNA barcoding and its applications in species identification

DNA barcoding is a method used to identify a specimen down to the level of species (Packer et al., 2009). Paul Hebert is the father of DNA barcoding. A DNA barcode, which is a condensed set of nucleotides extracted from the pertinent region of the genome of

an organism, can be applied to identify an organism in a species-specific manner. The differentiation of species is made possible by this sequence's intraspecific variation, which is orders of magnitude less than known inter-specific variation (Hebert et al., 2003).

The use of the rDNA internal transcribed spacer region 2 (ITS-2) (Ashok Kumar et al. 2009), cytochrome c oxidase subunit 1 (COX 1), NADH dehydrogenase subunit 1 (nadh1), and cytochrome b (cytb) markers in current molecular analyses has greatly improved our comprehension of the phylogenetic relationships between species of insects. However, cytochrome c oxidase subunit 1 (COX 1) has been extensively used by molecular scientists all over the world to identify between various insect species.

Since COX 1 sequencing must be done on samples that have already been identified by a taxonomist, DNA barcoding is a modern technique that necessitates the creation of a trustworthy database. Therefore, the technological stage of creating a database of pests of insects and natural enemies in the whole world will be a necessity for genetic research. It is considered effective to use species identification markers from the mitochondrial COX 1 region for molecular identification and phylogeny. The quick collection of molecular data is the primary benefit of DNA barcoding (Monaghan et al., 2005). The energy-producing organelles known as mitochondria are present in almost all cells of virtually all plant and animal species. Because it is found in all eukaryotic creatures and evolves more quickly than nuclear DNA, especially the mitochondrial genome has proven to be quite useful in interpreting the evolution of species. Different inheritance patterns can be seen in nuclear and mitochondrial genomes (Behura, 2006). As mitochondrial DNA (mtDNA) is inherited from the mother, it evolves rather quickly, and the majority of nucleotide alterations occur at sites that are neutral, mitochondrial markers is employed to indicate phylogenetic relationships across related groups. The COX 1 marker gene amplification sequence data was used to study the intra- and inter-phylogenetic interactions related to this genetic marker.

In coordinated evolution, mutations quickly propagate to even if the gene family is spread over multiple chromosomes; relative homogeneity is maintained (Arnheim 1983; Gerbi 1985; Tautz et al., 2002). Although mt-DNA was once thought to serve as an impartial indicator that recorded the ancestry of the species, recent arguments by Ballard and Whitlock (2004) and Bazin et al., (2006) contend that, in contrast to other genomes, mitochondria frequently experience vigorous selection and evolve according to distinct evolutionary principles. According to the hypothesis made by Hurst and Jiggins (2005), selection may have a direct or

indirect impact on mtDNA depending on the equilibrium state of other maternally transmitted DNA.

The majority of life on earth is comprised of insects, which have evolved into an enormous variety of various species. Taxonomists only described 10% of the projected total number of species after approximately 200 years. In this situation, insect identification has been a challenging endeavor that necessitates the availability of more specialists and funds. Naturalists developed the concept of categorizing living things based on taxonomy, a field related to science that enables us to characterize a living organism based on morphological traits, in order to catalogue the enormous number of species.

A novel technique termed DNA profiling, a method of DNA-based taxonomy, is currently being used for identifying known and new species based on the pattern of nucleotide organization in a sample of DNA of a certain species, 250 years after Darwin and Linnaeus (Novotny et al., 2002). In light of the present biodiversity issue, several academics have suggested using species descriptions using DNA profiling in taxonomy (Hebert et al., 2003; Ball and Armstrong, 2006). Wilson (2012) noted that relationship between library barcodes and other data, especially Linnaean names, collecting places, morphology represented by digital photographs, occurs through the voucher specimens from where they originated. This technology is frequently used to compile a list of every species on Earth and is well acknowledged by governmental and nongovernmental organizations as well as hard-core taxonomists and graduate molecular biologists.

Since the development of molecular biology and molecular instruments, it has become simple, fast, and accurate to identify various life forms, including insects. In contrast to morphology-based taxonomy, which may not be able to identify all developmental phases of insects, their food webs, or their biotypes, DNA profiling techniques are a consistent and useful approach of identifying insect species. Morphological data generally take time and require experts. Molecular data do agree with morphological theories, according to statistical taxon separation analysis and tree-based taxon clustering. As a result, it was demonstrated that for the taxa under study, species identification based on DNA sequence analysis was possible. Biologists may find DNA barcoding to be a useful technique. Two examples include the creation of particular primers for tea mosquito bugs and tiny barcodes for archival items of how it has aided in the evolution of many sophisticated tools for species diagnosis (Rebijith et al., 2012).

As these genes are maternal and devoid of recombination, mt-genes are chosen and utilized as universal DNA profiling markers for animals (Birky, 2001). There is no effective recombination because paternal mtDNA is removed prior to, during, or after fertilization (Moses, 1961; Sutovsky et al., 1999; White et al., 2008); as a result, within a species, there are a few variants. A high rate of CO1 mutation results in intra-specific diversity, which facilitates the identification and delimitation of species recognition (Hlaing et al., 2009; Wheat & Watt, 2008; Williams & Knowlton, 2001). Additionally, each cell contains several copies of mitochondria rather than just one copy of each parent's nuclear DNA (nDNA) (Randi, 2000). Additionally, mt-DNA can be extracted from damaged or tiny samples (Stoeckle & Hebert, 2008; Waugh, 2007).

In plants, polyploidy and hybridization are prevalent, making it challenging to differentiate between different species (Fazekas et al., 2009; Rieseberg et al., 2006). Due to frequent recombination and a low mutation rate, (Palmer et al., 2000) the mt-genome is not ideal for profiling of DNA in plants (Cowen et al., 2006; Fazekas et al., 2008; Kress et al., 2005). The rate of additional nucleotide substitution in plant mt-DNA is 40 to 100 times less than that in mt-DNA of animal (Cho et al., 2004; Mower et al., 2007). The plastid genes for maturase (mat-K) and ribulose bisphosphate carboxylase (rbcL) are regarded as advanced plant barcode genes. As a complimentary area, the DNA internal transcribed spacer (ITS) region is employed (Chase et al., 2005; CBOL plant working group 2009; Consortium for the barcode of life, 2009; Kress et al., 2005). The ITS region is regarded as a universal barcode of DNA in the case of fungi (Nilsson et al., 2006; Seifert et al., 2007; Seifert, 2009).

Due to their complex evolutionary past, protists have a genetic makeup that is extremely diversified. There are various genetic markers utilized in protists for various populations. These markers are mitochondrial CO1, ribulose1,5-bisphosphate carboxylase-oxygenase (rbcL), ribosomal ITS1/ITS2, D1-D2/D2-D3 sections at 50 end of 28S ribosomal DNA, ribosomal ITS1/ITS2, chloroplastic 23S rRNA, and spliced leader RNA genes (Barth et al., 2006; Chantangsi et al., 2007; Decelle et al., 2012; Evans et al., 2007; Gentekaki & Lynn, 2009; Gile et al., 2010; Hagino et al., 2011; Hamsher et al., 2011; Kosakyan et al., 2012; Kucera & Saunders, 2008; Liu et al., 2009; Mann et al., 2010; McDevit & Saunders, 2009; MacGillivary & Kaczmarska, 2011; Moniz & Kaczmarska, 2010; Nassonova et al., 2010; Robideau et al., 2011; Saunders, 2008; Schoch et al., 2012; Sherwood & Prestling, 2007; Stern et al., 2010, 2012; Trobajo et al., 2010; Votypka et al., 2010). The Consortium for the Barcode of Life Protist Working Group (CBOL ProWG) is attempting to develop standards

for a barcode that is unique or universal for protists (Moritz & Cicero, 2004; Taylor & Harris, 2012).

Researchers may also employ additional alternative loci as makers for species identification in the event that there is insufficient data to delimit species using CO1 alone (Vences et al., 2005). Other mt-gene areas that can be employed as tracers are cytochrome b (Bradley & Baker, 2001; DeSalle et al., 2006; Hajibabaei et al., 2007; Pfunder et al., 2004), 12S rDNA, and 16S rDNA. Nijman &Aliabadian (2010) concluded that after contrasting CO1's efficiency in identifying species with that of 16S rRNA and cytochrome b, it was shown that 16S rRNA genes were less effective at designating species in DNA barcoding (Doyle & Gaut, 2000). Nicolas et al. (2012) used three mitochondrial genes (16S, cytb, and cytc) to identify species in the Praomyini tribe (Rodentia: Muridae), with a success rate of up to 99%.The 16S gene's discriminating power is lower since it has 2.5% less variation than cytb and cytc, they added. Soil nematodes and other tiny organisms were identified by Blaxter (2004) using the 18S (nuclear) gene. According to Skerratt et al. (2002), the *Sarcoptesscabiei* population marker for wombats, dogs, and people in Australia was the mitochondrial 12S rRNA.

In 2011, Luo et al. evaluated the efficiency of the other protein-coding genes that are mitochondrial and the global CO1 barcoding area in eutherian species. They recommended that, at least for eutherian species identification, preference should be given on mitochondrial protein-coding genes for the standard DNA barcode. In this investigation, the species recovery rate was greater than 90%. Instead of Cytochrome b, 12S rDNA and 16S rDNA, it is preferable to use fragment of 650 bp of the 50 end of the mt-gene Cytochrome C Oxidase 1 (Doyle & Gaut, 2000). They contain indels that make sequence alignment difficult. In terms of evolution, CO1 is three times more significant because of its greater rate of substitutions at the position of third nucleotide (Knowlton & Weigt, 1998). All eukaryotes contain the CO1 gene, which is readily amplified and sequenced due to its relatively small length and the availability of credible universal primers and it is used by scientists despite the fact that there are no restrictions on choosing a particular gene (Folmer et al., 1994; Simmons & Weller, 2001; Zhang & Hewitt, 1997). Due to its elevated mutation rate and variety, the CO1 gene tends to be used to distinguish between species that are geographically dispersed and those that are closely related. It has also been demonstrated that shorter CO1 fragments are useful for identifying species with damaged DNA (Cox & Hebert, 2001; Moritz & Cicero, 2004;

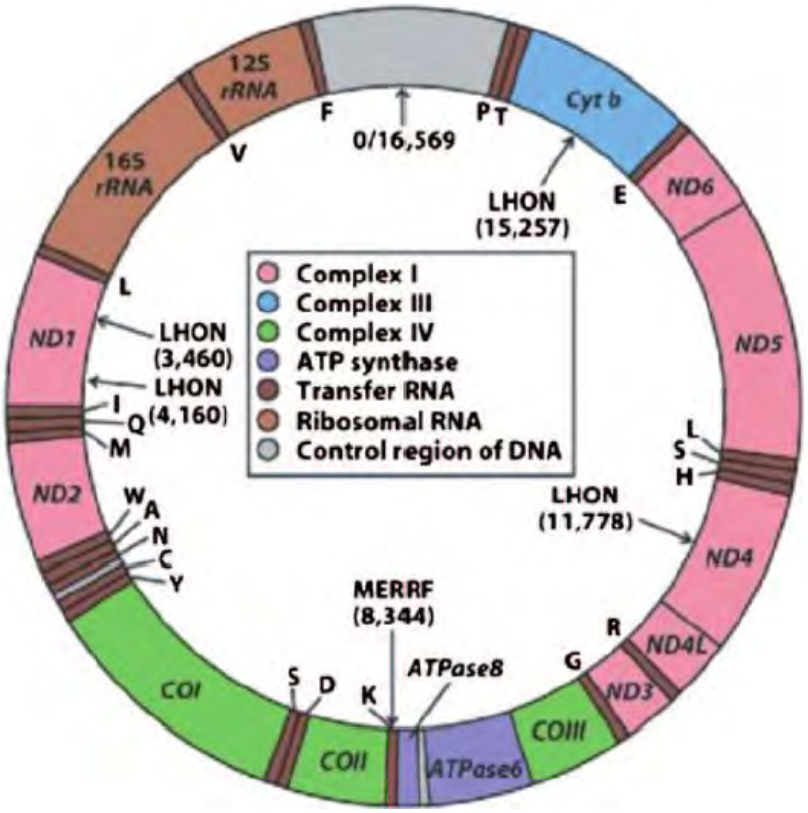

Figure 4 **Organization of insect mitochondrial genome**

The regions encoding for cytochrome b (Cyt B), various subunits of NADH-coenzyme Q reductase (ND), cytochrome c oxidase (COX), ATPase, and ribosomal RNAs (rRNA) are indicated on mitochondrial DNA, which indicates its circular structure and comprises of two strands—the outer a heavy and the inner a light strand. Image source: (Connor et al., 2017).

Wares & Cunningham, 2001). By employing a mini-barcode, or 100-base fragment Hajibabaei et al. (2006b) were able to effectively spot the museum samples of moths and wasps.

With millions of species and vast diversity in their life stages, accurate identification becomes a difficult problem for taxonomy. DNA barcoding, a new method of DNA-based taxonomy, is used to identify both recognized and unidentified species based on the sequence of nucleotide organization in a sample of DNA from the target species (Moritz and Cicero 2004; Hebert and Gregory 2005; Schindel and Miller 2005). DNA barcoding provides the fastest way for taxonomists to sort specimens and helps in species identification.

Some applications of species identification by using DNA barcoding are mentioned here as identification of species is the core component of describing biodiversity. The majority of animal species are classified according to morphological characteristics, with insects being the most prevalent group. Utilizing a standardized section of their genome, DNA barcoding is a substitute method that is more trustworthy and accurate, quicker and more effective for identifying and differentiating between species. The prompt detection of alien unwanted bug species is essential for preserving biosecurity in any country due to their economic importance. Furthermore, insect pests pose a serious challenge for farmers everywhere, making accurate pest identification essential.

With millions of species and wide differences in their life stages, taxonomy finds it difficult to make the accurate identification. However, because to recent scientific developments, it is now possible to distinguish between known and undiscovered species using a technique called DNA barcoding, which is based on DNA-based taxonomy. Taxonomists can classify specimens and identify species more quickly due to DNA barcoding. To make sure that a variety of biological materials may be quickly and precisely identified, Paul Hebert (Jinbo et al., 2011) devised this method in 2003. It involves employing a primer designed to amplify the mt cytochrome-c oxidase subunit 1 (COI) gene over a 648 base pair (bp) region (Hebert et al, 2003). Based on numerous investigations using this area on numerous taxa, the 5' fragment of the COI gene has been regarded as the universal/standard barcoding region in mammals. Regarding evolution and speciation, this area of the COI gene has been seen as being extremely instructive.

The fundamental requirement of biology is the discovery, description, naming, and identification of various species or taxa (Blaxter & Floyd, 2003; Carvalho et al., 2008; Lee,

2002; Lipscomb et al., 2003; Meier et al., 2006; Moritiz & Cicero, 2004; Prendini, 2005). Without understanding and recognizing the species, scientists working in the field of biology are unable to discuss their findings and results (Wilson, 2004). There are many species on the waiting list to be found, identified, and described.

A quick and precise procedure that can play a revolutionary role in altering the established taxonomy and accelerating work is needed in such a situation (Godfray, 2002; Hebert et al., 2003b; La Salle et al., 2009; Tautz et al., 2003; Wheeler, 2007). Since 2003, many researchers have had success separating the species via barcoding. Birds (Kerr et al., 2007), butterflies (Lukhtanov et al., 2009), spiders (Hebert & Barrett, 2005), and plant species (Kress et al., 2005) identification and recognition have all benefited greatly by DNA barcoding. Nearly 40700 spider species and subspecies from 109 families and 3694 genera have been identified worldwide (Sharma, 2014).

DNA barcoding base identification is particularly helpful for such diversity. For different taxa, there are different genetic variations between two barcode sequences. Some taxa do not allow for simple barcode identification of species. This is due to the complexity of speciation events, the genetic system's wide range of activities, natural selection, and evolutionary time. Inter-specific divergence generally outweighs intra-specific diversity in importance (Meyer &Paulay, 2005). However, in earlier investigations, divergence values below 2% were also noted (Sbordoni, 2010).Typically, species delimitation considers divergence values of 2% or less. (Carvalho et al., 2011; Hubert et al., 2008; Mabragana et al., 2011; Pereira et al., 2011a, b; Ward, 2009; Ward et al., 2009).

When it comes to identifying spiders, DNA barcoding is very effective. With intra-specific divergence of 1.4% and inter-specific divergence of 16.4%, Barrett & Hebert (2005) All specimens were accurately classified to their respective species with 100% accuracy. There have been attempts to determine the trophic interactions/relationships between the organisms using DNA amplification from the contents of the gut or waste materials (Dunshea, 2009; Farrell et al., 2000; Fournier et al., 2008; Gariepy et al., 2007; King et al., 2010; Matheson et al., 2007; Sheppard & Harwood, 2005;Weber& Lundgren, 2009). A study was done on the red bat, *Lasiurus borealis*, and its prey. Bats, with the exception of Arctiidae, primarily hunt on Lepidopterans, as revealed by the DNA barcode amplified from their faeces. This investigation also provides information or hints about more sophisticated defense systems in Arciinae (Clare et al., 2009). There have been attempts to use DNA amplification from the food contents of stomach or waste products to identify the feeding relationships among the

organisms. The red bat, *Lasiurus borealis*, and its prey have been the subject of research. According to the DNA barcode generated from bat faeces, except for *Arctiidae*, bats mostly hunt on Lepidopterans. This study also offers details or suggestions concerning more advanced Arciinae protection systems.

This method is widely applicable for determining the authenticity of food. This method has shown to be very successful for seafood to be traced (Filonzi et al., 2010). Through barcoding, poisonous puffer fish (illegally imported) at a store in Chicago was incorrectly labeled as headless monkfish and that was discovered in a case of food poisoning (J. Deeds, personal communication, 13 November 2007). Similar to this, Barbuto et al. (2010) found instances of Shark slices with different species sold in Italy under the slang name "palombo."

In surveillance efforts for diseases spread by vectors, accurate and thorough parasite and vector identification is crucial. Because their hosts and vectors rarely possess morphological competence, many parasites make morphometrics extremely challenging or perhaps impossible (Besansky et al., 2003). Non-taxonomists may recognize these vectors thanks to DNA barcoding, which contributes to our understanding of and ability to control infections and pests that spread disease (www. http://barcoding.si.edu/PDF/CBOL). Identification of vectors that spread disease, like mosquitoes, is made easier because of DNA barcoding. Using DNA barcoding, Wang et al. (2012) identified the primary mosquito (Alcaide et al., 2009; Ashfaq et al., 2014; Kumar, 2013; Townzen et al., 2008) species in China. Insect pests, as well as their predators and parasitoids, are being accurately and quickly identified by agricultural experts using DNA barcoding. Lepidoptera: Insecta pests belonging to the Noctuidae family were identified using DNA barcoding by Li et al. (2012). DNA barcoding procedures created for the controlled arthropods, nematodes, fungi, bacteria, and phytoplasmas of the European Union have been shown to be highly helpful for identifying plant pests and pathogens for the quarantine organism's barcoding of life (QBOL) project (Vossenberg et al., 2013).

Microgastrine wasps are one of the most varied and numerous parasitoids of lepidopterans, with DNA tag sequences having been produced for over 20 000 collections from 75 different countries, covering 50 genera with over than 1700 species, according to Smith et al. (2013). This will undoubtedly quicken the process of this group's species identification. When examining semi-processed or visually indistinguishable animal products or when conducting commercial trade monitoring of protected species, DNA barcoding is useful to wildlife authorities (Eaton et al., 2012; Panday et al., 2014). Nougoue (2012) investigated surveillance

of wildlife trade using DNA profiling and came to the conclusion that when used in this context, DNA barcodes offer a quick and accurate approach for species identification.

Forensics is an important key application of DNA profiling (Shen et al., 2013). In addition to their natural significance in decomposition, such as in the case of meat flies (Diptera: *Sarcophagidae*), captured insects from crime scenes can also be utilized in forensic inquiries as proof (Catts & Goff, 1992; Erzinclioglu, 1983).The barcoding of DNA is also utilized for identifying specific animals in more advanced animals such as birds, fish, reptiles, mammals, and amphibians, as well as to distinguish across species. In Panama (Crawford et al., 2011), a new *Eleuotherodatylus* species (Anura: *Eleutherodactylidae*) was found and named. A barcode data source repository for Korea's reptiles and amphibians (68%) was developed by Jeong et al. in 2012. A uniform identification framework for monitoring purposes was established by this study.

1.5 Major drawbacks of barcoding and future prospective

Although DNA barcoding is becoming more and more common in taxonomy, it still has significant drawbacks and critics when it comes to identifying and defining species. Due to the need for certain laboratory processes like tissue extraction, specimen preservation, and sequencing, this technology is not available to the general public (Cameron et al., 2006). Additionally, it costs $5 US to calculate one specimen's sequences. It is not a cost-effective technology because it requires 10 or more specimen sequences of a species for effective results, which increases the cost (Cameron et al., 2006; Hajibabaei et al., 2005; Meyer & Paulay, 2005). Insufficient geographic sample makes it impossible to identify recently divergent species, related to this; scientists ignore the unknown biodiversity in favour of studying populations that are genetically related to them (Sperling, 2003). How species are defined it depends upon the difference of intra-specific and inter-specific divergence levels. For a few species, like birds, (Hebert et al., 2004b), spiders (Barrett & Hebert, 2005), fish (Ward et al., 2005), and butterflies (Hajibabaei et al., 2006a), it is true. In a select few other species (such as cowries, Meyer &Paulay, 2005; amphibia, Rubinoff et al., 2006; Vences et al., 2005), it was found that the intra and inter-specific divergence values overlapped.

The economic advantages, rapid identification, and precision of DNA barcoding, according to its proponents, outweigh the expenses and costs (Hebert & Gregory, 2005). About nearby species delimitation, DNA barcoding offers information. It only defines the limits of taxa that differ genetically. The species is not entirely described by it (Hebert et al., 2004b; Gregory

2005; Ward et al., 2005). By geographically and globally sampling many different taxonomic groups, the problem of limited sampling can be resolved (Robinson et al., 2009). As a result, cryptic, allopatric, and recently divergent species will be identified. DNA sequences are available to the general public, whereas traditional taxonomy requires knowledge for species identification (Coyne & Orr, 2004). DNA barcode sequences are available online because DNA analysis is inexpensive.

Despite negative criticism of DNA barcoding from some experts, the technology is becoming more and more popular because it is user-friendly. The promise of DNA barcoding as a method for identifying, differentiating, and defining species has been demonstrated (Robinson et al., 2009). It is now simple to identify cryptic species that were previously difficult or nearly impossible to identify using morphological characteristics thanks to DNA barcoding (Dasmahapatra et al., 2010; Decaens & Rougerie, 2008; Hausmann et al., 2011; Hebert et al., 2004b; Janzen et al., 2005; Mitchell & Samways, 2005; Pfenninger et al., 2007; Smith et al., 2006; Vaglia et al., 2008; Wheat & Watt, 2008). The projected barcoding of 25,000 species in IBOL by a working group for agricultural and forestry pests and their parasitoids include aphids, thrips, real fruit flies, scale insects, saw flies, and gall wasps.

1.6 Significance of DNA barcoding

Significant food and financial losses are brought on by insects and other pests. The insects that harm crops of wheat causes significant, albeit temporally and spatially varied, harm and are apparently responsible for 10–50% (on average) of losses to wheat crops globally (e.g., Oerke, 2006; Savary et al., 2019). Even though farmers had taken preventive measures by applying herbicides to 71% of their wheat acreage, fungicides to 30%, and insecticides to 7% of the cropped area (in 2017) (USDA-NASS, 2018, authors' calculations), $209 million was paid out to wheat farmers in the U.S. on insurance claims for crop losses attributable to insects, diseases, and weeds between 2010 and 2020 (USDA-RMA, 2021, authors' calculation).

Crop pest losses are anticipated to be significant for smallholder farmers in developing nations, especially given the low adoption of contemporary seed types and the even lower usage of agricultural pesticides that can lessen these losses (Sheahan and Barrett, 2017; Pardey et al., 2022). Maintaining the world's food supply depends on determining how pests and illnesses affect yield losses. However, it is notoriously difficult to estimate wheat yield losses quantitatively. Researchers present a quantitative experiment-based estimate of wheat

yield losses in China from 2000 to 2018 brought on by pests and diseases. Researchers discovered that losses to regional yield from pests and pathogens were 16.29%, 7.46%, 11.71%, 12.64%, 6.54%, and 4.84%, respectively, in the Yellow and Huai River valleys, the middle and lower reaches of the Yangtze River, the Southwest China, the Loess Plateau, the Northeast China, and the Xinjiang province.

In terms of overall production, area, and yield per acre, Pakistan ranks as the tenth greatest producer of wheat in the world. In Pakistan, the average annual per capita consumption of wheat is around 125 kg, making almost 60% of the average person's daily calorie intake. Wheat is a key component of the population's diet and plays a major role in the government's agricultural policies (Shahid, 2003). The agricultural sector is crucial to Pakistan's economy because it accounts for 20% of the country's GDP, employs more than 45% of the labour force, and provides food directly or indirectly for around 67% of the population. Any internal or external shock to agriculture is likely to have an impact on the performance of the nation's economic growth as well as a significant portion of its people. Wheat, Pakistan's main food staple, adds around 13% to the value added in agriculture and 2.8% to the country's gross domestic product (GOP, 2009).

The study on comparative DNA barcoding of wheat crop insects at the start and end of the season has significant implications for the field of agriculture and entomology. Here are some of the reasons why:

- **Identification of insect species**

  The process of DNA barcoding entails the sequencing of a specific DNA region to identify a species. This method was employed by the researchers in this study to distinguish between wheat crop insects at the beginning and end of the growing season. The diversity and abundance of insect species in a given area can be estimated using this information.

- **Crop health**

  Insects can spread diseases that can harm the health of crops. The potential for disease transmission and the spread of disease during the crop season may be revealed by changes in the genetic diversity and genetic makeup of insects.

- **Ecosystem health**

  Changes in genetic diversity of insects, which play significant roles in ecosystems, may have effects on the overall health of ecosystems. For instance, if pollinator insect genetic variety declines over the growing season, it may have an impact on plant reproduction and ultimately biodiversity in the surrounding environment.

- **Monitoring pest populations**

  Wheat crop insects have the potential to seriously harm crops, costing farmers money. Insects' DNA may be compared at the beginning and end of the season, allowing researchers to track changes in pest populations and implement the necessary controls.

- **Understanding insect behavior**

  Insects have a complicated life cycle, a number of variables, such as the environment and the availability of food, might have an impact on their behaviour. Researchers can learn more about how insects behave and how that behaviour might affect agricultural yields by examining the DNA of insects at various periods of the season.

- **Developing targeted pest management strategies**

  Broad-spectrum insecticides with the potential to harm the environment and non-target creatures are frequently used in traditional pest management techniques. Researchers can create focused pest management tactics that are more effective and environmentally benign by employing DNA barcoding to identify specific insect species.

- Overall, this work has significant implications for raising crop yields and lowering the costs and harm caused by insect infestations in wheat crops on the economy and environment.

1.7 Aims and objectives

1. Collection of wheat crop insects from various locations across Islamabad, Pakistan
2. Extraction and amplification of DNA from the collected insect samples
3. Sequencing of the amplified DNA fragment
4. Morphological and molecular identification and comparative analysis of wheat crop insects obtained from diverse host plants across Islamabad, Pakistan, at the beginning and end of the crop season, utilizing the mt-CO1 gene as a DNA barcode

5. Phylogenetic analysis and construction of a matrix to assess the genetic relationships among the insect specimens.

CHAPTER NO.2

Materials and Methods

This chapter includes the materials and methods to study wheat crop insects at the start and end of the crop season. This includes sample collection, morphological identification of insects, DNA extraction, PCR, gel electrophoresis, DNA sequencing, phylogenetic analysis and Specie demarcation tool.

2.1 Sample collection

Wheat crop insects were sampled using the sweep net technique, targeting various host wheat crops across distinct geographical areas within Islamabad named Chak Shehzad, National Institute of Health (NIH), National Agriculture Research Center (NARC) and Quaid-i-Azam University. Subsequently, all collected insect samples were promptly immersed in 95% ethanol as a preservation medium. The samples were then transferred to the Molecular Virology Laboratory situated in the Department of Biotechnology at Quaid-i-Azam University for subsequent DNA isolation procedures. To maintain sample integrity, the preserved specimens were securely stored at a temperature of -20°C in 95% ethanol, ensuring their viability for future utilization.

2.2 Morphological Identification of collected insects

Sogatella vibix and Sogatella furcifera can be identified using the morphological key of Dupo and Barrison, 2009 (Dupo & Barrion, 2009). *Scirpophaga incertulas* can be identified using morphological key of Hattori and Siwi, 1986 (Hattori & Siwi, 1986). *Psammotettix emarginatus* can be identified using morphological key of Greene, 1971 (Greene, 1971). *Sphaerophoria philanthus*is can be identified using morphological key from USDA (USDA, 1862). *Exitianus indicus* can be identified by using morphological key of Khatri, Rustamani, Ahmed, & Sultana, 2014 (Khatri, Rustamani, Ahmed, & Sultana, 2014). *Exochomus quadripustulatus*is can be identified using morphological identification key from Nature spot (Nature spot, 2009). *Neoscona adianta* can be identified using morphological identification key of Preston-Mafham, 1998 (Preston-Mafham, 1998). *Coccinella septempunctata* can be identified using morphological identification key of Abdalla et al., 2022 (Abdalla et al., 2022). *Mycetophila idonea* can be identified using morphological identification key from North Carolina State University. *Auplopus carbonarius* can be identified using morphological identification key of Buck, 2012 (Buck, 2012). These insects are shown in figure 2 and 3.

2.3 DNA extraction from insect specimen

Organic DNA extraction method using 2X CTAB was used for the extraction of DNA from wheat crop insects. In a 1.5ml eppendorf tube or centrifuge tube, a single whole insect or an insect's head /abdominal part was taken and crushed in micro mortar and pestle. 150µl of 2X CTAB was added to the crushed wheat crop insect in the tube and 5µl of Proteinase K (1%) was also added to the centrifuge tube. The centrifuge tube containing the reaction sample was incubated for one hour at 55°C in a water bath. The reaction sample was shaken at regular intervals after 10-15 minutes. After withdrawing from the water bath, the reaction sample was cooled for 2 minutes and then 150µl of chloroform: isoamyl alcohol was added and shaken gently for the cloudy appearance of reaction sample. The tube was opened in order to release the pressure in tube. Then the tube with reaction sample was centrifuged for 10 minutes at 14000 rpm. After centrifugation, the supernatant was transferred to a new tube and pellet was removed. 150µl of l00% ethanol and 30µl of sodium acetate was added to the supernatant in centrifuge tube. Then the reaction sample was placed in freezer at -20°C for 2 hours. After cooling, the reaction sample was again centrifuged at 14000 rpm for 10 minutes. After centrifugation supernatant was discarded and the tube was place upside down on paper to dry out. Then 150µl of 70% ethanol was added to the tube. After adding 70% ethanol, the sample was once again centrifuged at 14000 rpm for 5 minutes. The resultant supernatant was discarded, and the tube was left open to dry out at room temperature. The pellet was then dissolved in 20µl of ddH_2O and the extracted DNA sample was then stored at 4°C for further use.

2.4 PCR (Polymerase chain reaction)

All the PCR tubes were labeled. An Eppendorf tube was taken to make the PCR master mix manually. Then, added all the PCR components of PCR reaction mix including dNTPs, $MgCl_2$, buffer, Taq polymerase, ddH2O and forward primer, reverse primer. A final reaction volume of 25ul was prepared using 2.5ul diluted DNA template. Thermal cycling conditions and primer sequences and their names are enlisted in Table 3 and 4.

2.5 Gel electrophoresis:

For confirmation of the PCR results, 1% agarose gel was prepared by using 50ml of 1X TAE buffer and 0.5g of agarose. Addition of 5µl of Ethidium bromide to the gel and the mixture was transferred in to gel tray and gel comb was placed for formation of wells. Around 5µl of

each DNA samples, mixed well with 2µl of loading dye were then loaded in the wells after solidification of the gel. A 1.5µl of 1kb DNA ladder was also loaded as a standard for measuring the size of DNA samples. It was then run across a voltage of 80 volts for 35 minutes. The DNA bands were observed under UV light in an ultraviolet (UV) trans-illuminator. The results were compared with the ladder to identify the size of DNA sample.

2.6 DNA sequencing

The positive PCR products were sequenced unidirectional using Sanger sequencing method (Macrogen, South Korea) using Universal primers LCO 1490 and HCO 2198. The total numbers of samples sequenced were 6 those were common in both seasons and we got 4 accurate sequences of different types of insect species.

2.7 Phylogenetic analysis

Sequences were trimmed and aligned using BLAST tool in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify sequence similarities. Closely related sequences were retrieved from databases in FASTA format and aligned using MUSCLE implemented in MEGA ver. X (Saitou and Nei, 1987) and phylogenetic tree was constructed using 1000 bootstrap values by maximum likelihood method (Saitou and Nei, 1987) where *Bemisia tabaci* MF289534.1 was used as an out-group.

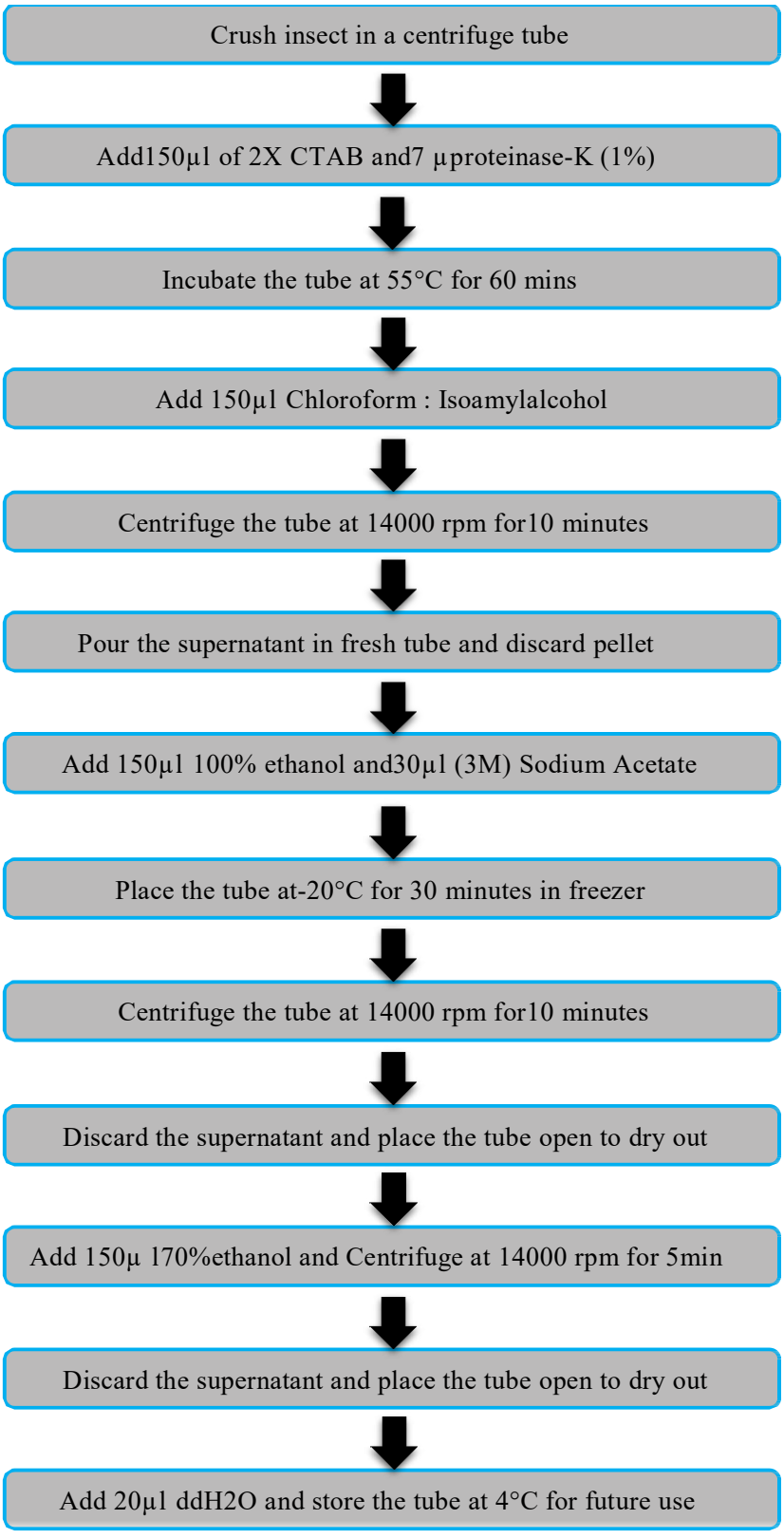

Figure 5 Steps involved in the process of Insect DNA using 2X CTAB

Table 1 Preparation of solutions used for DNA extraction and analysis

Solutions for DNA extraction		
Name of solution	**Composition of solution**	**Preparation and storage of working solutions**
2X CTAB (50ml)	Tris. HCl (pH.8) =100mM EDTA (pH.8)= 20mM NaCl = 1.4M CTAB = 2% PV-40= 1%	CTAB buffer was prepared by dissolving all the reagents in dH_2O and then autoclaved. Buffer was stored at room temperature for further use.
Proteinase-K (1%)	20mg/ml	In a 1.5ml tube, 0.02g of Proteinase-K powder and 1ml of PK buffer were added and mixed well. Then 200µl of glycerol was added in the tube. Finally, the prepared 1% Proteinase-K solution was stored at -20°C for further use.
Chloroform: isoamylalcohol (24:1)	24ml chloroform + 1ml isoamylalcohol	Mixed 24ml chloroform with 1ml isoamylalcohol to prepare 24:1 chloroform: isoamylalcohol solution.
Sodium acetate (3M)	40.8g Sodium acetate 100ml dH_2O	Added 40.8g sodium acetate in 100ml dH_2O to prepare 3M solution of sodium acetate
Solutions for DNA analysis (Gel electrophoresis)		
50X TAE buffer	50X	In a beaker, 121.14g of tris-base (2M), 9.306g of EDTA (50mM) and 28.59ml glacial acetic acid (1M) were added in 200mldH_2O. The solution was mixed very well. The final volume of the solution was adjusted at 500ml by adding more dH_2O.
1% Agarose gel	1% w/v agarose Ethidium bromide (0.5µg/ml)	0.5g of agarose was dissolved in 50ml (1%) of TAE buffer and boiled to properly dissolve it. Allowed it to cool down. Before gel casting, 5µl of Ethidium bromide (0.5µg/ml concentration) was added.

Table 2 Master Mix preparation for 25µl PCR reaction mix

Reagents	Stock concentration	Working concentration	Working volume
DNA template	-	-	2.5µl
dNTPs solution	2mM (each)	200µM	2.5µl
Taq buffer	200mM	20mM	2.5 µl
$MgCl_2$	25mM	1.5mM	1.5uL
Forward primer	10µM	0.2µM	0.5µl
Reverse primer	10µM	0.2µM	0.5µl
Taq polymerase	5 U/µl	1.25 Units	0.25µl
ddH_2O	-	-	14.75µl

Table 3 Primers for mt-CO1 gene of wheat crop insects DNA samples

Primer	Target gene	Reading direction	Sequence 5'-3'	Ann temp range
LCO1490	COI	Forward	GGTCAACAAATCATAAAGATATTGG	48-55°C
HCO2198	COI	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	48-55°C

Table 4 PCR conditions for wheat insects DNA amplification

Step	Temperature °C	Number of cycles	Time (min)
Initial denaturation	94°C	1	10 min
Denaturation	94°C	35	30 sec
Annealing	49°C		30 sec
Extension	72°C		45 sec
Final extension	72°C	1	10 min

CHAPTER NO.3

RESULTS

This chapter includes the study of wheat crop insects at start and end of the crop season by morphological identification of insects, description of insects, their features and geographical location in the tabular form, amplification of insect barcode region, sequence analysis, phylogenetic analysis and species demarcation tool SDT.

3.1 Insect Identification based on morphology

(A) *Sogatella vibix*

Sogatella vibix, also known as the white-backed planthopper, has forewings transparent and unmarked; the face is white with dark brown genae; parameres have a slim and petiolated base; the apex is strongly cleft with the apico-outer side obliquely truncate and the apico-inner sides acute and converging (Dupo & Barrion, 2009).

(B) *Sogatella furcifera*

Brown planthopper is the popular name for *Sogatella furcifera*, which may be identified morphologically by its prominent pterostigma; Frons, gena, and clypeus are entirely black, with the exception of a whitish carina in the frons and clypeus. Parameres have a bulbous sub basal inner edge, an unequally cleft apex with a little inner spine, and a more apically rounded outer section (Dupo & Barrion, 2009).

(C) *Scirpophaga incertulas*

Scirpophaga incertulas, also known as the yellow stem borer, can be identified by its morphological characteristics, which include hind wings that are ochreous brown in males and orange yellow in females with black spots on them. Frons is absent, and male fore wings measure 8 to 9 millimeters in length while female fore wings measure 11 to 13 millimeters (Hattori & Siwi, 1986).

(D) *Psammotettix emarginatus*

The common name for *Psammotettix emarginatus* is the "notched sand grasshopper." Males range in length from 3.00 to 3.40 mm, while females range from 2.95 to 3.40 mm. Males have heads that are 0.94 to 0.98 mm wide, while females are 0.92 to 1.00 mm wide, with a crown that has a median sulcus that extends anteriorly 0.61 to 0.79 median lengths. Pronotum

color varies whereas circular pits are restricted to the region above the anterior border of the scutum (Greene, 1971).

(E) *Sphaerophoria philanthus*

Sphaerophoria philanthus is commonly known as hover fly and has black and yellow stripes on body, long slender body with transparent wings (USDA, 1862).

(F) *Exitianus indicus*

Exitianus indicus, often known as the Asian rice grasshopper, has a vertex that typically has a transverse brown band with an arcuate shape. Basal triangles on the scutellum are a light brown color. Male bears 2-3 apical brown or black macrosetae on pygofer side. Strong lateral compression of the aedeagal shaft, a big gonopore with a rim that forms a concave border, and two tiny dorso basal processes are present (Khatri, Rustamani, Ahmed, & Sultana, 2014).

(G) *Exochomus quadripustulatus*

*Exochomus quadripustulatus*is commonly named as pine ladybird and it is black in color with four red spots, 4.5 mm in length (Nature spot, 2009).

(H) *Neoscona adianta*

Neoscona adianta, also known as the orb weaver spider, has the morphological characteristics of a brown to red abdomen that is decorated with a sequence of black-bordered white or cream triangles. The male is considerably smaller than the female, measuring just about 9 millimeters (0.35 in) in length (without the legs) (Preston-Mafham, 1998).

(I) *Coccinella septempunctata*

Coccinella septempunctata is commonly known as seven spotted ladybird and it is orange-red in color and has seven black colored spots on its body (Abdalla et al., 2022).

(J) *Mycetophila idonea*

Mycetophila idonea, often known as the fungus gnat. Long, multi-segmented antennae, a hump-backed thorax, long legs with long coxae, huge bristles, and apical spurs on the tibiae are morphological characteristics that help identify the body color is dull yellow or dark gray (North Carolina State University).

(K) *Auplopus carbonarius*

The potter spider wasp, or *Auplopus carbonarius*, is black in color with a large thorax and broad wings that measure about 10 millimeters in length. The male can be identified by the ivory-colored maculae next to the eyes (Buck, 2012).

3.2 Amplification of insect barcode region

For the optimization of PCR conditions for wheat crop insects DNA amplification, annealing temperature adjustment was applied ranging from 48°C to 52°C. Optimum results were obtained when annealing temperature was adjusted at 49°C, as displayed by the sharply bright bands in gel on UV trans-illuminator after undertaking gel electrophoresis of PCR product in 1% agarose gel at 80V for 35 minutes. Strong and bright bands of desired size i.e. ~700bp were visible on the gel. The size of band was calculated by comparing its position with 1kb DNA ladder (Fermentas) as shown in Figure 6 and 7.

3.3 Sequence analysis

For sequence analysis a total of 6 DNA amplified products were sent for sequencing (macrogen South Korean). The obtained sequences from macrogen were refined and aligned using BLASTn to find and compare with similar sequences in the NCBI database. Alignment result showed 4 different insects those have accurate sequences. Remaining samples were identified morphologically by using morphological identification keys. Detail of the both season samples is present in the table 5 and 6. Based on the sequence similarity, 7-9 sequences including out- group sequence were downloaded for each sequence of this study from NCBI GenBank with accession number, for phylogenetic tree reconstruction and analysis. For the construction of phylogenetic tree, all the sequences were aligned on MEGA X software using MUSCLE. We constructed phylogenetic tree with method of maximum likelihood using the kimura-2 model of MEGA X with 1000 bootstraps replicates.

3.4 Sequence Demarcation tool (SDT) analysis

For the validatation of phylogenetic analysis, SDT analysis of the sequences were also performed using sequence demarcation tool (SDTv1.2) as shown in Figure 9, 10 and 11 representing four clusters in *Sogatella furcifera* and seven clusters in *Scirpophaga incertulas, Exitianus indicus,* and *Exitianus indicus* varifying our tree. The SDT similarity score ranged between 95-100% for Sogatella furcefera, *Scirpophaga incertulas, Exitianus indicus, and Exitianus indicus.*

Table 5 Insects collected after sowing of wheat crop and their geographical locations

Serial number	Common name	Scientific name	Location	Morphological identification
1	White-backed planthopper	*Sogatella vibix*	Chak Shehzad/NARC/NIH/ QAU University	Slender, elongated body with a typical planthopper shape, body is relatively flat and laterally compressed. About 6-7 mm in length, pale to yellowish colored.
2	Yellow stem borer	*Scirpophaga incertulas*	NARC/QAU University	The Yellow Stem Borer has a relatively slender body with a typical moth-like shape, wingspan ranging from 20 to 30 mm, primarily yellow or golden-brown, with darker brown or blackish-brown markings, six legs, he hind wings are paler and more uniformly colored.
3	Brown planthopper	*Sogatella furcifera*	Chak Shehzad/NARC/NIH/ QAU University	Brownish body color, about 3 to 4 mm in length, Its body is elongated and slender.
4	Notched sand grasshopper	*Psammotettix emarginatus*	NARC/QAU University	The body may be brown, green, or a combination of these colors, 5 to 7 millimeters in length, has six legs. The legs are adapted for jumping and are often long and slender.
5	Leafhopper	*Exitianus indicus*	Chak shehzad, QAU University	Variable colors, the body may be pale yellow, greenish-yellow, or a combination of these colors. The wings may have patterns or markings, such as spots or lines, 5 to 6 millimeters in length.
6	Orb weaver spider	*Neoscona adianta*	NARC/ QAU University	5mm in length, brown in color, marking on abdomen
7	Lady bird beetle	*Coccinella septempunctata*	Chak Shehzad/NARC/NIH/ QAU University	Orange in color, seven spot ladybird

Table 6 Insects collected near harvest of wheat crop and their geographical location

Serial Number:	Wheat crop Insects	Scientific Names	Location	Key identification features
1	Orb weaver spider	*Neoscona adianta*	NARC/ QAU University	5mm in length, brown in color, marking on abdomen
2	Fungus gnats	*Mycetophila idonea*	Chak Shehzad/NARC/NIH/QAU University	Brown abdomen, 2.2 mm in length, head and thorax are somewhat darker.
3	Potter spider wasp	*Auplopus carbonarius*	NARC/QAU University	Black body with, big thorax region and broad wings
4	Brown planthopper	*Sogatella furcifera*	Chak Shehzad/NARC/NIH/QAU University	Brownish body color, about 3 to 4 mm in length, Its body is elongated and slender.
5	Lady bird beetle	*Coccinella septempumctata*	Chak Shehzad/NARC/NIH/QAU University	Orange in color, seven spot ladybird,
6	Yellow stem borer	*Scirpophaga incertulas*	NARC/QAU University	The Yellow Stem Borer has a relatively slender body with a typical moth-like shape, wingspan ranging from 20 to 30 mm, primarily yellow or golden-brown, with darker brown or blackish-brown markings, six legs, he hind wings are paler and more uniformly colored.
7	Leafhopper	*Exitianus indicus*	Chak shehzad, QAU University	Variable colors, the body may be pale yellow, greenish-yellow, or a combination of these colors. The wings may have patterns or markings, such as spots or lines, 5 to 6 millimeters in length.
8	Hover fly	*Sphaerophoria philanthus*	Chak Shehzad/NARC/NIH/QAU University	Black and yellow stripes on body, long slender body with transparent wings
9	Notched sand grasshopper	*Psammotettix emarginatus*	NARC/ QAU University	Overall coloration of is variable. The body may be brown, green, or a combination of these colors, 5 to 7 millimeters in length, has six legs. The legs are adapted for jumping and are often long and slender.
10	Pine lady bird	*Exochomus quadripustulatus*	Chak shehzad, QAU University	Black in color with four red spots, 4.5 mm in length

Table 7 Comparison of insects after sowing and before harvest of wheat crop

<table>
<tr><th colspan="2">Common insect specimen at the start and end season of wheat crop</th><th>Variable insects at the start and end season of wheat crop</th></tr>
<tr><td>Wheat Crop Insects during the Early November of 2022</td><td rowspan="2">1.Brown planthopper
2.Yellow stem borer
3.Leafhopper
4.Notched sand grasshopper
5.Orb weaver spider
6. Ladybird beetle</td><td>1. Brown plant hopper
2.Yellow stem borer
3. Leafhopper
4.Notched sand grasshopper
5.White-backed planthopper
6.Orb weaver spider
7.Ladybird beetle</td></tr>
<tr><td>Wheat crop Insects during late February to early March 2023</td><td>1.Orb weaver spider
2.Fungus gnats
3.Potter spider wasp,
4.Brown planthopper
5. Ladybird beetle
6.Yellow stem borer,
7.Leafhopper
8. Hover fly
9.Notched sand grasshopper
10. Pine ladybird</td></tr>
</table>

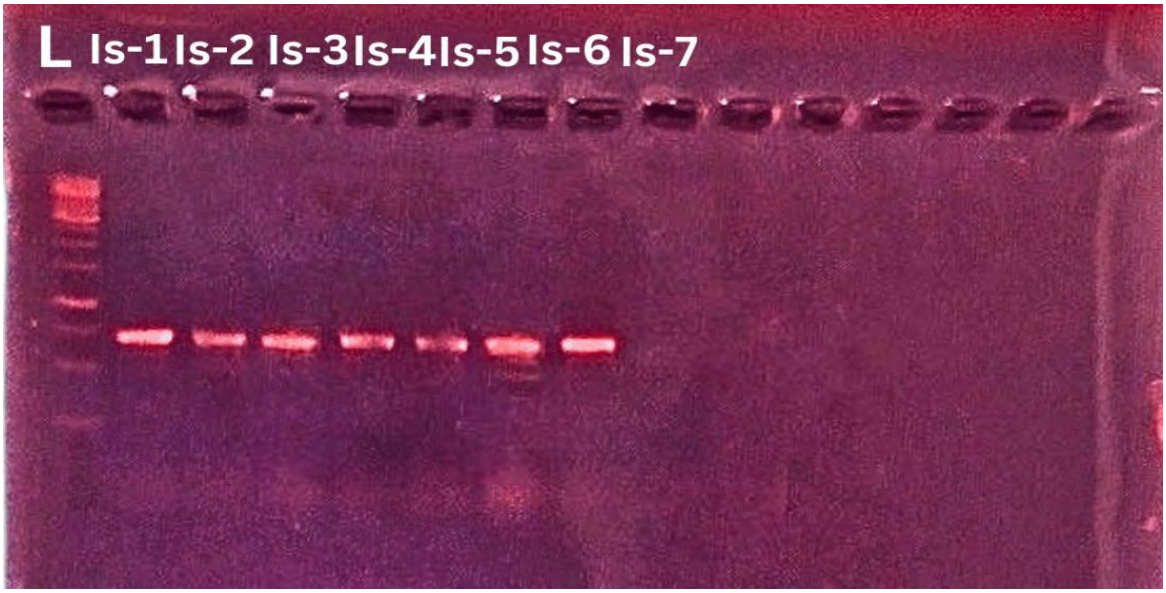

Figure 6 **PCR product illustrated by UV trans-illuminator after gel electrophoresis**

Lane 1 shows the ladder while bands in lane 2-7 shows amplified mt-COI fragments of wheat crop insects sampled at the start of the wheat crop season.

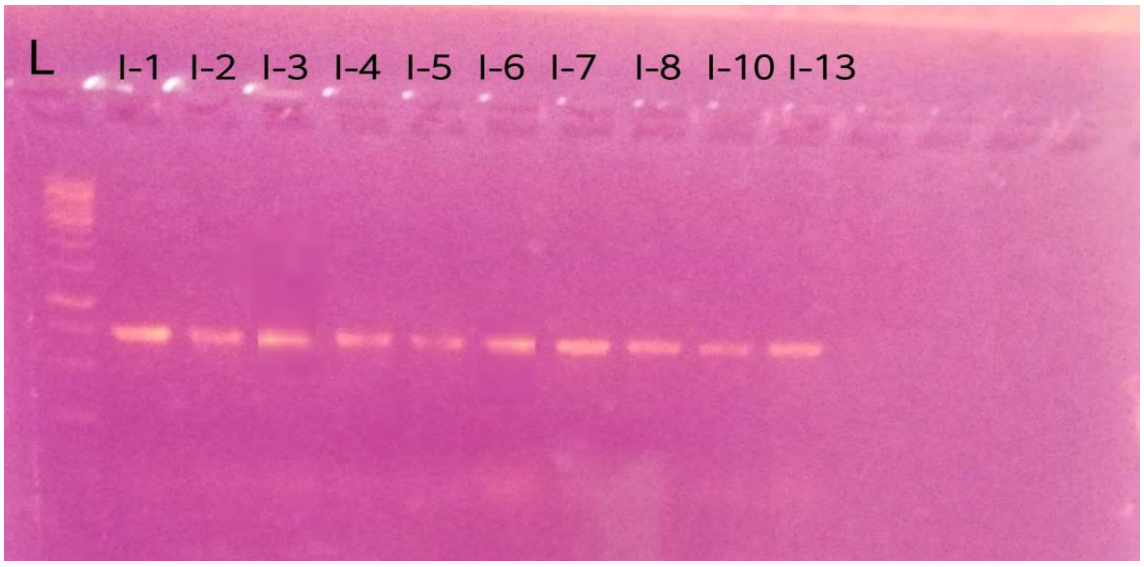

Figure 7 **PCR product illustrated by UV trans-illuminator after gel electrophoresis**

Lane 1 shows the ladder while bands in lane 2-10 shows the amplified mt-COI fragments of wheat crop insects sampled at the end of the wheat crop season.

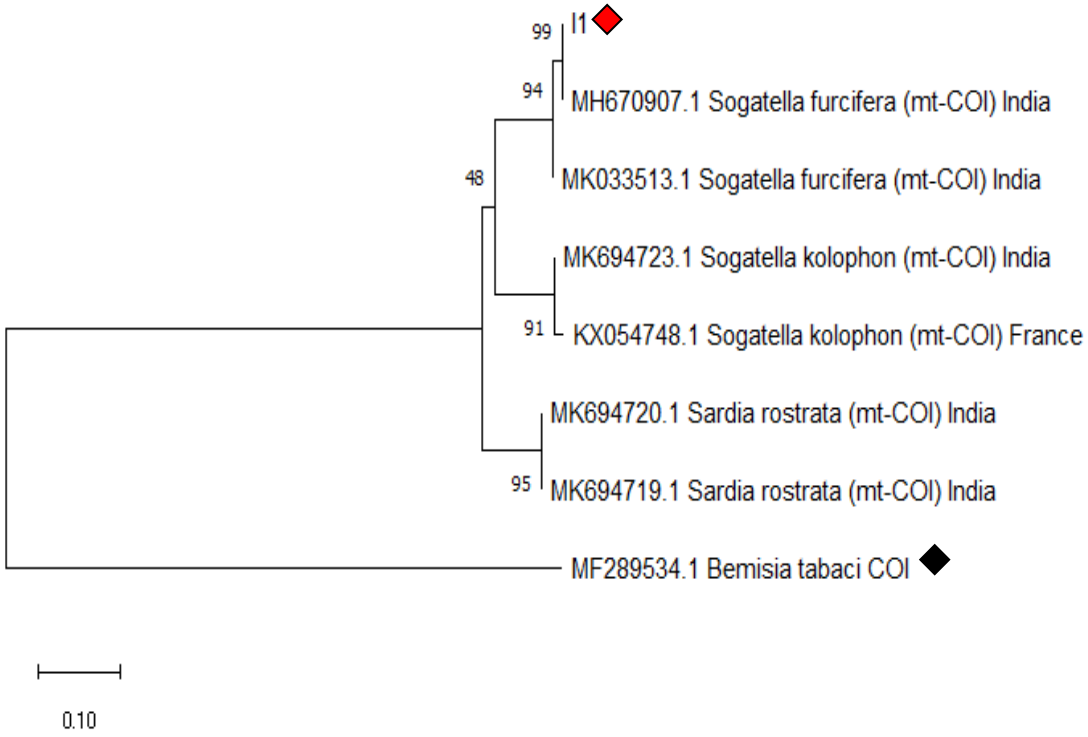

Figure 8 **Phylogenetic analysis of *Sogatella furcifera***

The above figure shows a phylogenetic tree reconstructed through maximum likelihood method after sequence alignment of our sequences with those sequences downloaded from NCBI GenBank. Sequence labeled with red rhombus represents the sequence of this study while the black rhombus sequence at the bottom of tree represents out-group. This phylogenetic tree was reconstructed using MEGA X version software.

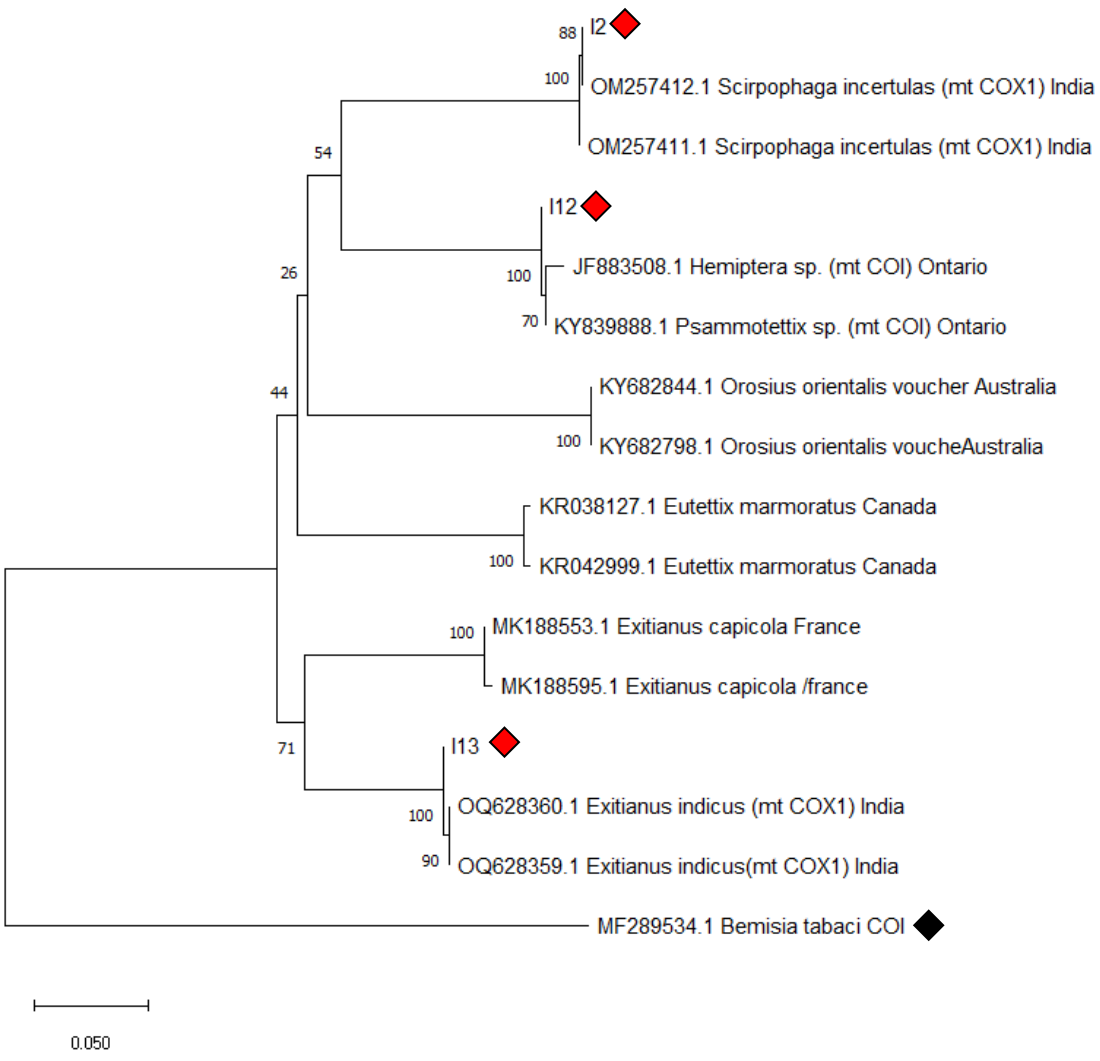

Figure 9 **Phylogenetic analyses of *Scirpophaga incertulas, Exitianus indicus, Psammotettix emarginatus***

The above figure shows a phylogenetic tree reconstructed through maximum likelihood method after sequence alignment of our sequences with those sequences downloaded from NCBI GenBank. Sequence labeled with red rhombus represents the sequence of this study while the black rhombus sequence at the bottom of tree represents out-group. This phylogenetic tree was reconstructed using MEGA X version software.

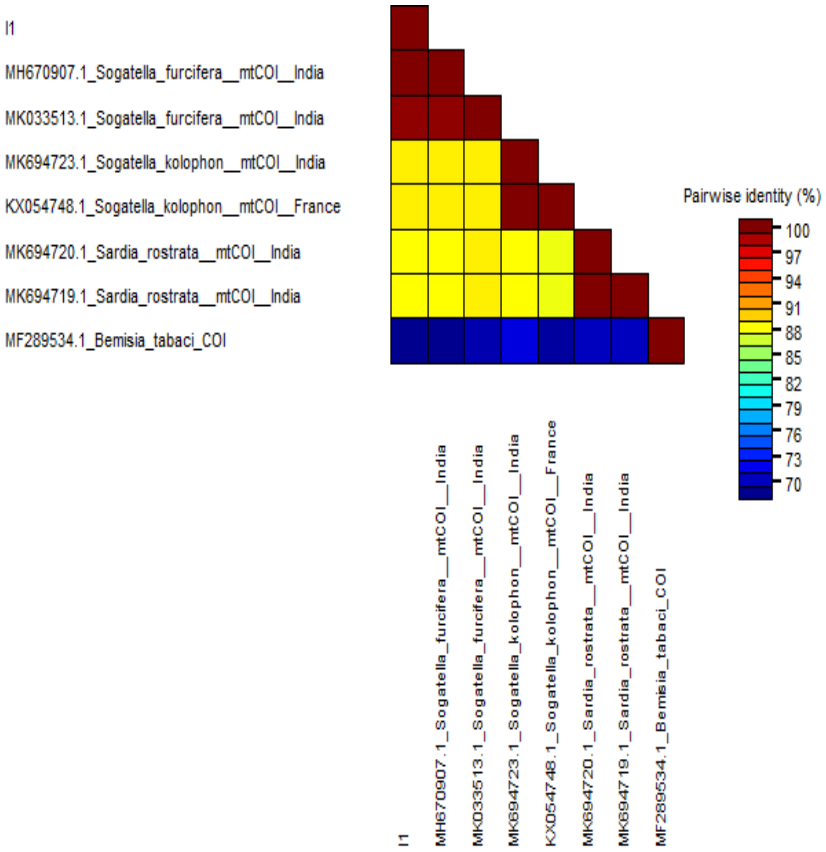

Figure 10 SDT colour coded matrix of *Sogatella furcifera*

The above figure represents homology index among sequences of this study for *Sogatella furcifera* and those sequences downloaded from NCBI GenBank. A percentage similarity score between two sequences is represented by each square that is colored. Software known as Sequence Demarcation Tool Version 1.2 (SDT 1.2) was used to create this figure.

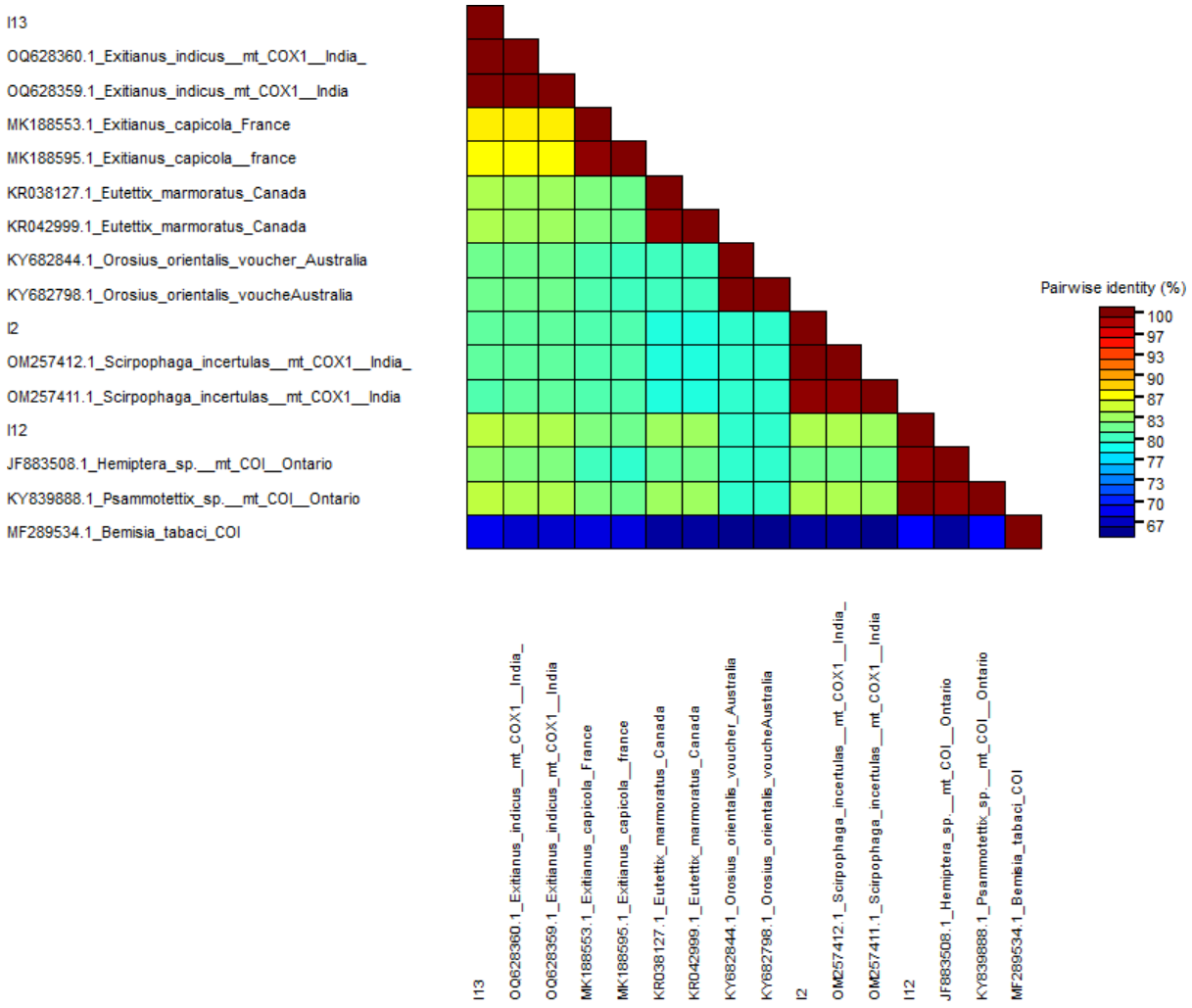

Figure 11 **SDT colour coded matrix of *Scirpophaga incertulas, Exitianus indicus, and Psammotettix emarginatus***

The above figure represents homology index among sequences of this study for *Scirpophaga incertulas, Exitianus indicus, Psammotettix emarginatus* and those sequences downloaded from NCBI GenBank. A percentage similarity score between two sequences is represented by each square that is colored. Software known as Sequence Demarcation Tool Version 1.2 (SDT 1.2) was used to create this figure.

CHAPTER NO.4

DISCUSSION

Wheat holds immense agricultural significance as the primary staple food crop in Pakistan. However, throughout its developmental stages, wheat plants face substantial challenges caused by various arthropod pests. These pests can be categorized as either oligophagous, feeding on a limited number of plant species, or polyphagous, causing harm to a very large range of plants. This is exceptionally rare to encounter an insect species that exclusively targets wheat crops (monophagous). Before the advent of the "green revolution," which entailed the introduction of crop cultivars with high yields, and modern agricultural practices, it was estimated that insect pests caused approximately 5.1% of global wheat production losses. However, following the implementation of these new agricultural approaches in the 1990s, the losses attributable to pest insects escalated to 9.3%.

This increase in pest-related losses highlights the adaptability and dynamic nature of these insects in response to environmental factors. Temperature fluctuations within the environment exert notable effects on the physiology, behavior, reproductive patterns (voltinism), and geographic distribution of insect pest species. Changes in temperature can influence the developmental rates of insects, their metabolic processes, and the synchrony of life cycle events. Consequently, temperature variations can directly impact the abundance and distribution of insect populations, potentially leading to increased pest pressure on wheat crops (Farook et al., 2019).

It is necessary to study and comprehend the dynamics of insect pests in wheat cultivation to develop effective strategies for their management. By employing scientific methodologies such as DNA barcoding, researchers can accurately identify and classify insect species associated with wheat crops. This knowledge contributes to a deeper comprehension of the biodiversity and ecological interactions within the wheat agro-ecosystem. Given the critical role of wheat as a major food source, it is crucial to mitigate the impacts of pest insects on its production. Sustainable pest management practices that integrate biological control, cultural practices, and judicious use of insecticides can help minimize yield losses caused by insect pests while promoting ecological balance and reducing environmental risks.

In this study, we employed a PCR-based method for the identification of various wheat crop insects collected from diverse regions within Islamabad, Pakistan. The DNA was extracted

using 2XCTAB methods. The quantity of DNA extracted from 2XCTAB methods was good so it was adapted as the primary protocol for all extractions. The DNA obtained from Wheat crop insects were detected through PCR. Samples from different locations of Islamabad showed positive results for mt-COI primers. The annealing temperature was tested at 48˚C, 49˚C and 50˚C. The band size of positive PCR products was approximately 700bp. Total 6 samples were sequenced from Macrogen, South Korea. Out of 6 samples 4 were accurate sequences. The sequences were then aligned with the other sequences present in NCBI through BLASTn tool. Then phylogenetic analyses were performed and phylogenetic tree was constructed using MEGA X software.

In conclusion, the utilization of DNA barcoding, an established methodology involving a standardized DNA region for species identification, has gained considerable traction across diverse scientific disciplines, including entomology. However, the comparative DNA barcoding analysis of wheat crop insects specifically within the agricultural milieu of Islamabad, Pakistan, remains an uncharted territory. In view of the paucity of research in this area, understanding the genetic diversity, species composition, and population fluctuations of these insects during distinct phases of the growing season assumes paramount importance for devising effective and sustainable pest management protocols. This study seeks to address this research gap by undertaking a comprehensive comparative DNA barcoding analysis of wheat crop insects at the inception and culmination of the growing season in Islamabad, Pakistan.

CHAPTER NO.5

REFERENCES

Abdalla, H. A., Gamal Fiteha, Y., Abdel-Salam Rashed, M., Mohamed Ali, R., Mohamed Abdel-Salam Hammad, A., Saber Mohamed Bream, A., & Magdy, M. (2022). Molecular Identification of Ladybird Beetles (Coccinella: Coccinellidae) Using DNA Barcodes. *Journal of Scientific Research in Science*, *39*(2), 179-193.

Akhtar, M. (1971). Laboratory feeding tests with Chrotogonus trachypterus (Blanchard) (Orthoptera, Acrididae). *Pakistan journal of zoology*.

Alcaide M, Rico C, Ruiz S, Soriguer R, Munoz J, Figuerola J. (2009). Disentangling vector-borne transmission networks: A universal DNA barcoding method to identify vertebrate hosts from arthropod bloodmeals. PLoS One 4:e7092

Aliabadian M, Kaboli M, Nijman V, Vences M. (2009). Molecular identification of birds: Performance of distance-based DNA barcoding in three genes to delimit parapatric species. PLoS ONE 4:e4119.

Arnheim N (1983) Concerted evolution in multigene families. In: Nei M, Koehn R (eds) Evolution of genes and proteins. Sinauer, Sunderland, pp 38–61

Ashfaq M, Hebert PDN, Mirza JH, Khan AM, Zafar Y, Mirza S. (2014). Analyzing mosquito (Diptera: Culicidae) diversity in Pakistan by DNA barcoding. PLoS One. 9:e97268.

Ashok Kumar G Jalali SK Venkatesan T Stouthamer R Niranjana P Lalitha Y (2009) Internal transcribed spacer-2 restriction fragment length polymorphism (ITS-2-RFLP) tool to differentiate some exotic and indigenous trichogrammatid egg parasitoids in India. Biol Control 49(3):207–213

Ball SL, Armstrong KF. 2006. DNA barcodes for insect pest identification: a test case with tussock moths (Lepidoptera: Lymantriidae). *Can J Forest Res*. 36:337–350.

Ballard JW, Whitlock MC (2004) The incomplete natural history of mitochondria. Mol Ecol 13(4):729–744

Barbuto M, Galimberti A, Ferri E, Labra M, Malandra RP, Galli P, Casiraghi M. (2010). DNA barcoding reveals fraudulent substitutions in shark seafood products: The Italian case of ‘‘palombo’’ (Mustelus spp.). Food Res Int 43:376–81.

Barrett RDH, Hebert PDN. (2005). Identifying spiders through DNA barcodes. Can J Zool 83:481–91.

Barth D, Krenek S, Fokin SI, Berendonk TU. (2006). Intraspecific genetic variation in Paramecium revealed by mitochondrial cytochrome C oxidase I sequences. J Eukaryot Microbiol 53:20–5.

Bashir, M. K., Mehmood, Y., & Hassan, S. (2010). Impact of agricultural credit on productivity of wheat crop: Evidence from Lahore, Punjab, Pakistan. *Pakistan Journal of Agricultural Science*, *47*(4), 405-409.

Bazin E, Glemin S, Galtier N (2006) Population size does not influence mitochondrial genetic diversity in animals. Science 312(5773):570–572

Becker S, Hanner R, Steinke D. (2011). Five years FISH-BOL: Brief status report. Mitochondrial DNA 22:3–9.

Behura SK (2006) Molecular marker systems in insects: current trends and future avenues. Mol Ecol 15(11):3087–3113

Besansky NJ, Severson W, Ferdig T. (2003). DNA barcoding of parasites and invertebrate disease vectors: What you don't know can hurt you. Trends Parasitol 19:545–6.

Birky CW. (2001). The inheritance of genes in mitochondria and chloroplasts: Laws, mechanisms, and models. Annu Rev Genet 35: 125–48.

Bradley R, Baker R. (2001). A test of the genetic species concept: Cytochrome-b sequences and mammals. J Mammal 82:960–73

Braun HJ, Atlin G, Payne T.2010**.** Multi-location testing as a tool to identify plant response to global climate change. In: Reynolds MP, editor. Climate change and crop production. Wallingford (UK): CABI Publishers;

Brower AVZ. (2006). Problems with DNA barcodes for species delimitation: 'Ten species' of Astraptesfulgerator reassessed (Lepidoptera: Hesperiidae). Syst Biodiv 4:127–32.

Buck, M. (2012). Two introduced spider wasps (Hymenoptera: Pompilidae) new to Canada, with notes on nesting habits and the incidence of introductions (abstract). Canadian Entomologist, e137(3), 278-282.

Cameron S, Rubinoff D, Will K. (2006). Who will actually use DNA barcoding and what will it cost? Syst Biol 55:844–47.

Carman GM, Packer L (1996) A cryptic species allied to Halictusligatus Say (Hymenoptera: Halictidae) =]

Carvalho DC, Oliveira DAA, Pompeu PS, Leal CG, Oliveira C, Hanner R. (2011). Deep barcode divergence in Brazilian freshwater fishes: The case of the Sao Francisco River Basin. Mitochondrial DNA 22:1–7.

Carvalho MR, Bockmann FA, Amorim DS, Brandao CR. (2008). Systematics must embrace comparative biology and evolution, not speed and automation. Evol Biol 35:150–57.

Catts EP, Goff ML. (1992). Forensic entomology in criminal investigations. Annu Rev Entomol 37:253–72.

Chantangsi C, Lynn DH, Brandl MT, Cole JC, Hetrick N, et al. (2007). Barcoding ciliates: A comprehensive study of 75 isolates of the genus Tetrahymena. Int J Syst EvolMicrobiol 57:2412–25.

Chase MW, Salamin N, Wilkinson M, Dunwell JM, Kesanakurthi RP, Haidar N, Savolainen V. (2005). Land plants and DNA barcodes: Short-term and long-term goals. Philos Trans R Soc Lond, B, Biol Sci 360:1889–95.

Cho Y, Mower JP, Qiu YL, Palmer JD. (2004). Mitochondrial substitution rates are extraordinarily elevated and variable in a genus of flowering plants. Proc Natl Acad Sci USA 101:17741–6.

Clare EL, Fraser EE, Braid HE, Fenton MB, Hebert PDN. (2009). Species on the menu of a generalist predator, the eastern red bat (Lasiurus borealis): Using a molecular approach to detect arthropod prey. Mol Ecol 18:2532–42.

Clare EL, Lim BK, Engstrom MD, Eger JL, Hebert PDN. (2007). DNA barcoding of Neotropical bats: Species identification and discovery within Guyana. Mol Ecol Notes 7:184–90.

Cognato AI. (2006). Standard percent DNA sequence difference for insects does not predict species boundaries. J Econ Entomol 99: 1037–45.

Connor, T. M., Hoer, S., Mallett, A., Gale, D. P., Gomez-Duran, A., Posse, V., ... & Maxwell, P. H. (2017). Mutations in mitochondrial DNA causing tubulointerstitial kidney disease. PLoS genetics, 13(3), e1006620.

Cowen RK, Paris CB, Srinivasan A. (2006). Scaling of connectivity in marine populations. Science 311:522–7.

Cox AJ, Hebert PDN. (2001). Colonization, extinction and phylogeographic patterning in a freshwater crustacean. Mol Ecol 10: 371–86 Coyne JA, Orr HA. (2004). Speciation. Sunderland, MA: Sinauer Associates.

Crawford AJ, ALonso R, Jaramillo C, Sucre S, Ibanez R. (2011). DNA barcoding identifies a third invasive species of Eleutherodactylus (Anura: Eleutherodactylidae) in Panama City, Panama. Zootaxa 2890: 65–7.

Danforth BN (1999) Phylogeny of the bee genus Lasioglossum (Hymenoptera: Halictidae) based on mitochondrial COI sequence data. Systematic Entomology, 24, 377–393.

Danforth BN, Mitchell PL, Packer L (1998) Mitochondrial DNA differentiation between two cryptic Halictus (Hymenoptera: Halictidae) species. Annals of the Entomological Society of America, 91, 387–391.

Dasmahapatra KK, Elias M, Hill RI, Hoffman JI, Mallet J. (2010). Mitochondrial DNA barcoding detects some species that are real, and some that are not. Mol EcolResour 10:264–73.

Decaens T, Rougerie R. (2008). Descriptions of two new species of Hemileucinae (Lepidoptera: Saturniidae) from the region of Muzo in Colombia – evidence from morphology and DNA barcodes. Zootaxa 34–52.

Decelle J, Suzuki N, Mahe F, de Vargas C, Not F. (2012). Molecular phylogeny and morphological evolution of the Acantharia (Radiolaria). Protist 163:435–50.

Desalle R. (2006). Species discovery versus species identification in DNA barcoding efforts: Response to Rubinoff. Conserv Biol 20:1545–7.

Dhaliwal GS. Jindal V, Dhawan AK. Insect pest problems and crop losses: changing trends. Indian Journal of Ecological sciences. 2010; 37:1-7

Doyle JJ, Gaut BS. (2000). Evolution of genes and taxa: A primer. Plant Mol Biol 42:1–6

Dunshea G. (2009). DNA-based diet analysis for any predator. PLoS One 4:e5252.

Dupo, A. L. B., & Barrion, A. T. (2009). Taxonomy and general biology of delphacid planthoppers in rice agroecosystems. *Planthoppers: new threats to the sustainability of intensive rice production systems in Asia*, 3-155.

Eaton MJ, Meyers GL, Kolokotronis S-O, Leslie MS, Martin AP, Amato G. (2012). Barcoding bushmeat: Molecular identification of Central African and South American harvested vertebrates. Conserv Genet 11:1389–404.

Edwards D, Horn A, Taylor D, Savolainen V, Hawkins JA. (2008). DNA barcoding of a large genus, Aspalathus L. (Fabaceae). Taxon 57: 1317–27.

Ekrem T, Willassen E, Stur E. (2007). A comprehensive DNA sequence library is essential for identification with DNA barcodes. Mol PhylogenetEvol 43:530–42.

Erzinclioglu YZ. (1983). The application of entomology to forensic medicine. Med Sci Law 23:57–63.

Evans KM, Wortley AH, Mann DG. (2007). An assessment of potential diatom ‘‘barcode’’ genes (cox1, rbcL, 18S and ITS rDNA) and their effectiveness in determining relationships in Sellaphora (Bacillariophyta). Protist 158:349–64.

FAO/WHO. 2001. Human vitamin and mineral requirements. In: Report of a joint FAO/WHO expert consultation. Bangkok, Thailand: Food and Nutrition Division, FAO, Rome, Italy.

Farook, U. B., Khan, Z. H., Ahad, I., Maqbool, S., Yaqoob, M., Rafieq, I., ... & Sultan, N. (2019). A review on insect pest complex of wheat (Triticum aestivum L.). *J EntomolZool Stud*, *7*(1), 1292-1298.

Farrell LE, Roman J, Sunquist ME. (2000). Dietary separation of sympatric carnivores identified by molecular analysis of scats. Mol Ecol 9:1583–90.

Fazekas AJ, Kesanakurti PR, Burgess KS, Percy DM, Graham SW, Barrett SC, Newmaster SG, et al. (2009). Are plant species inherently harder to discriminate than animal species using DNA barcoding markers? Mol EcolResour 9:130–39.

Filonzi L, Stefania C, Marina V, Francesco NM. (2010). Molecular barcoding reveals mislabelling of commercial fish products in Italy. Food Res Int 43:1383–8.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Marine Biol Biotechnol 3:294–9.

Fournier V, Hagler J, Daane K, de Leon J, Groves R. (2008). Identifying the predator complex of Homalodisca vitripennis (Hemiptera: Cicadellidae): A comparative study of the efficacy of an ELISA and PCR gut content assay. Oecologia 157:629–40.

Galimberti A, De Mattia F, Losa A, Bruni I, Federici S, Casiraghi M, Martellos S, Labra M. (2013). DNA barcoding as a new tool for food traceability. Food Res Int 50:55–63.

Gariepy TD, Kuhlmann U, Gillott C, Erlandson M. (2007). Parasitoids, predators and PCR: The use of diagnostic molecular markers in biological control of Arthropods. J Appl Entomol 131:225–40.

Gentekaki E, Lynn DH. (2009). High-level genetic diversity but no population structure inferred from nuclear and mitochondrial markers of the peritrichous ciliate Carchesiumpolypinum in the Grand River basin (North America). Appl Environ Microbiol 75:3187–95.

Gerbi SA (1985) Evolution of ribosomal DNA. In: MacIntyre RJ (ed) Molecular evolutionary genetics. Plenum, New York, pp 419–517

Godfray HCJ. (2002). Challenges for taxonomy. Nature 417:17–8.

Greene, J. F. (1971). A revision of the nearctic species of the genus Psammotettix (Homoptera: Cicadellidae).

Grimaldi D, Engel MS. 2005. *Evolution of the Insects*. New York: Cambridge University Press.

Hagino K, Bendif EIM, Young JR, Kogame K, Probert I, et al. (2011). New evidence for morphological and genetic variation in the cosmopolitan coccolithophore Emilianahuxleyi (Prymnesiophyceae) from the cox1b-ATP4 genes. J Phycol 47:1164–76.

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN. (2006a). DNA barcodes distinguish species of tropical Lepidoptera. Proc Natl Acad Sci USA 103:968–71. Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W. (2004a). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptesfulgerator. Proc Natl Acad Sci USA 101:14812–7.

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN. (2006a). DNA barcodes distinguish species of tropical Lepidoptera. Proc Natl Acad Sci USA 103:968–71.

Hajibabaei M, Smith MA, Janzen DH, Rodriguez JJ, Whitfield JB, Hebert PDN. (2006b). A minimalist barcode can identify a specimen whose DNA is degraded. Mol Ecol Notes 6:959–64.

Hamsher SE, Evans KM, Mann DG, Poulickova A, Saunders GW. (2011). Barcoding diatoms: exploring alternatives to COI-5P. Protist 162: 405–22.

Hattori, I. S. O. K. O., & Siwi, S. S. (1986). Rice stemborers in Indonesia. *Japan Agricultural Research Quarterly*, *20*, 25-30.

Hausmann A, Haszprunar G, Hebert PDN. (2011). DNA barcoding the geometrid fauna of Bavaria (Lepidoptera): Successes, surprises, and questions. PLoS One. 6:e17134.

Hebert PD, Gregory TR. 2005. The promise of DNA barcoding for taxonomy. Systematic biology 54:852-859.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. Proc R Soc B 270:313–321

Hebert PDN, Ratnasingham S, deWaard JR. (2003b). Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. Proc R Soc Lond B Biol Sci 270:S96–9.

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM. (2004b). Identification of birds through DNA barcodes. PLoS Bio l2:1657–63.

Hlaing T, Tun-Lin W, Somboon P, Socheat D, Setha T, Min S, Chang MS, Walton C. (2009). Mitochondrial pseudogenes in the nuclear genome of Aedes aegypti mosquitoes: Implications for past and future population genetic studies. BMC Genet 10:11.

Hollingsworth PM. (2007). DNA barcoding: Potential users. Genomics Soc Policy 3:44–7.

https://www.fao.org/land-water/databases-and-software/crop-information/wheat/en/

https://www.fs.usda.gov/wildflowers/pollinators/pollinator-of-the-month/syrphid-fly.shtml

https://www.naturespot.org.uk/species/pine-ladybird

Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, Burridge M, Watkinson D, et al. (2008). Identifying Canadian freshwater fishes through DNA barcodes. PLoS One 3:e2490.

Hurst GD, Jiggins FM (2005) Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. Proc R Soc B 272:1525–1534

Hyles, J., Bloomfield, M. T., Hunt, J. R., Trethowan, R. M., & Trevaskis, B. (2020). Phenology and related traits for wheat adaptation. *Heredity*, *125*(6), 417-430.

Iqbal, E., Kazi, F., & Raza, S. (2022). Status of Wheat Production and Associated Nematode Pests in Pakistan. *Pakistan Journal of Nematology*, *40*(1).

J.S. (Pat) Heslop-Harrison, Polyploidy ,Reference Module in Life Sciences ,Elsevier, 2017, ISBN 9780128096338.

Janzen DH, Hajibabaei M, Burns JM, Hallwachs W, Remigio E, Hebert PDN. (2005). Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. Philos Trans R Soc Lond, B, Biol Sci 360:1835–45.

Jeong TJ, Jun J, Han S, Kim HT, OH K, Kwak M. (2012). DNA barcode reference data for the Korean herpetofauna and their applications. Mol EcolResour 13:1019–32.

Jinbo U, Kato T, Ito M. 2011. Current progress in DNA barcoding and future implications for entomology. *Entomol Sci*. 14:107–124.

Kappner I, Bieler R. (2006). Phylogeny of venus clams (Bivalvia: Venerinae) as inferred from nuclear and mitochondrial gene sequences. Mol PhylogenetEvol 40:317–31.

Kerr KCR, Stoeckle MY, Dove CJ, Weigt LA, Francis CM, Hebert PDN. (2007). Comprehensive DNA barcode coverage of North American birds. Mol Ecol Notes 7:535–43.

Khan, A. M., Khan, A. A., Afzal, M., & Iqbal, M. S. (2012). Wheat crop yield losses caused by the aphids infestation. *J BiofertilBiopestici*, *3*(4), 2-7.

Khatri, I., Rustamani, M. A., Ahmed, Z., & Sultana, R. (2014). Genus Exitianus (Auchenorrhyncha, Cicadellidae, Deltocephalinae, and Chiasmini) in Tando Jam, Sindh, Pakistan. *Journal of Insect Science*, *14*(1), 235.

King RA, Vaughan IP, Bell JR, Bohan DA, Symondson WOC. (2010). Prey choice by carabid beetles feeding on an earthworm community analysed using species and lineagespecific PCR primers. Mol Ecol 19: 1721–32.

Knowlton N, Weigt LA. (1998). New dates and new rates for divergence across the Isthmus of Panama. Proc R Soc Lond B 265:2257–63

Kosakyan A, Heger TJ, Leander BS, Todorov M, Mitchell EA, et al. (2012). COI barcoding of nebelid testate amoebae (Amoebozoa: Arcellinida): Extensive cryptic diversity and redefinition of the Hyalospheniidae Schultze. Protist. 163:415–34.

Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. (2005). Use of DNA barcodes to identify flowering plants. Proc Natl Acad Sci USA 102:8369–74

Kucera E, Saunders GW. (2008). Assigning morphological variants of Fucus (Fucales, Phaeophyccae) in Canadian waters to recognized species using DNA barcoding. Botany 86:1065–79.

Kumar NP, Krishnamoorthy N, Sahu SS, Rajavel AR, Sabesan S, Jambulingam P. (2013). DNA Barcodes indicate members of the Anopheles fluviatilis (Diptera: Culicidae) species complex to be conspecific in India. Mol EcolResour 13:354–61.

Kundu PK. Acharjee TK, Mojid MA. Growth and yield of wheat under irrigation by sugar mills wastewater. Progress Agric. 2006; 24(1-2):211-218.

La salle J, Wheeler Q, Jackway P, Winterton S, Hobern D, Lovell D. (2009). Accelerating taxonomic discovery through automated character extraction. Zootaxa 2217:43–55.

Landi M, Dimech M, Arculeo M, Biondo G, Martins R, et al. (2014). DNA barcoding for species assignment: The case of Mediterranean marine fishes. PLoS One 9:e106135.

Lee MSY. (2002). Online database could end taxonomic anarchy. Nature 417:787–8.

Li J, Han H, Gao Q, Jin Q, Chi M, WU C, Zhang A. (2012). Species identification of Noctuidae (Insecta: Lepidopera) with DNA barcoding of support vector machine and neighbor-joining method. J Biosafety 21:308–14.

Lipscomb D, Platnick N, Wheeler Q. (2003). The intellectual content of taxonomy: A comment on DNA taxonomy. Trends Ecol E 18:65–6.

Liu H, Probert I, Uitz J, Claustre H, Aris-Brosou S, et al. (2009). Extreme diversity in noncalcifying haptophytes explains a major pigment paradox in open oceans. Proc Natl Acad Sci USA. 106:12803–8.

Luo A, Zhang A, Ho SYW, Xu W, Zhang Y, Shi W, Cameron SL, Zhu C. (2011). Potential efficacy of mitochondrial genes for animal DNA barcoding: A case study using eutherian mammals. BMC Genomics 12:84

Mabragana E, Astarloa JMD, Hanner R, Zhang J, Castro MG. (2011). DNA barcoding identifies Argentine fishes from marine and brackish waters. PLoS One 6:e28655.

MacGillivary ML, Kaczmarska I. (2011). Survey of the efficacy of a short fragment of the rbcL gene as a supplemental DNA barcode for diatoms. J EukaryotMicrobiol 58:529–36.

Mann DG, Sato S, Trobajo R, Vanormelingen P, Souffreau C. (2010). DNA barcoding for species identification and discovery in diatoms. Cryptogamie Algo l31:557–77.

Maralit BA, Aguila RD, Ventolero MFH, Perez SKL, Willette DA, Mudjekeewis D, Santos MD. (2013). Detection of mislabeled commercial fishery by-products in the Philippines using DNA barcodes and its implications to food traceability and safety. Food Control 33: 119e125.

Matheson CD, Muller GC, Junnila A, Vernond K, Hausmanne A, Millere MA, Greenblattb C, Schleinb Y. (2007). A PCR method for detection of plant meals from the guts of insects. Org Divers E 7:294–303.

McDevit DC, Saunders GW. (2009). On the utility of DNA barcoding for species differentiation among brown macroalgae (Phaeophyceae) including a novel extraction protocol. Phycological Res 57:131–41.

Meier R, Shiyang K, Vaidya G, Ng PKL. (2006). DNA Barcoding and taxonomy in Diptera: A tale of high intraspecific variability and low identification success.

Mitchell A, Samways MJ. (2005). DNA evidence that the morphological ‘forms’ of Palpopleuralucia (Drury) are separate species (Odonata: Libellulidae). Odonatologica 34:173–8.

Moniz MB, Kaczmarska I. (2010). Barcoding of diatoms: Nuclear encoded ITS revisited. Protist 161:7–34.

Moritz C, Cicero C. 2004. DNA barcoding: promise and pitfalls. PLoS Biol 2:e354.

Moses MJ. (1961). Spermiogenesis in the crayfish (Procambarusclarkii) II. Description of stages. J BiophysBiochem Cytol 10:301–33.

Mower JP, Touzet P, Gummow JS, Delph LF, Palmer JD (2007). Extensive variation in synonymous substitution rates in mitochondrial genes of seed plants. BMC Evol Biol 7:135. doi:10.1186/1471-2148-7- 135.

Muhammad Nabeel Saleem ., et al. "Wheat Crop Protection in Pakistan". Acta Scientific Agriculture 3.5 (2019): 67-69

Murugan K, Vadivalagan C, Karthika P, Panneerselvam C, Paulpandi M, Subramaniam J, Wei H, Alsalhi MS, Devanesan S, Nicoletti M, et al.. 2016. DNA barcoding and molecular evolution of mosquito vectors of medical and veterinary importance. *Parasitol Res.* 115:107–121.

Nassonova E, Smirnov A, Fahrni J, Pawlowski J. (2010). Barcoding Amoebae: Comparison of SSU, ITS and COI genes as tools for molecular identification of naked lobose Amoebae. Protist 161: 102–15.

Nicolas V, Schaeffer B, Missoup AD, Kennis J, Colyn M, Denys C, Tatard C, et al. (2012). Assessment of three mitochondrial genes (16S, Cytb, CO1) for identifying species in the Praomyini tribe (Rodentia: Muridae). PLoS One. 7:e36586. doi:10.1371/journal.pone.0036586.

Nijman V, Aliabadian M. (2010). Performance of distance-based DNA barcoding in the molecular identification of Primates. C R Biol. 333: 11–6.

Nilsson RH, Ryberg M, Kristiansson E, Abarenkov K, Larsson KH, Koljalg U. (2006). Taxonomic reliability of DNA sequences in public sequence databases: A fungal perspective. PLoS One. 1:e59. doi:10.1371/journal.pone.0000059.

North Carolina State University. (n.d.). General Entomology. Raleigh, NC 27695.

Nougoue AR. (2012). DNA barcoding as a tool for the identification of illegally traded wildlife products [M.Sc. thesis]. Quebec, Canada: Department of Biology, University of Concordia, Montreal.

Novotny V, Basset Y, Miller SE, Weiblen GD, Bremer B, Cizek L, Drozd P (2002) Low host specificity of herbivorous insects in a tropical forest. Nature 416:841–844. (Proc Biol Sci 274(1619):1731–1739)

Oerke, E.-C. (2006). Crop losses to pests. J. Agric. Sci. 144 (1), 31–43. doi: 10.1017/S0021859605005708

Packer, L., Gibbs, J., Sheffield, C., & Hanner, R. (2009). DNA barcoding and the mediocrity of morphology. *Molecular Ecology Resources*, *9*, 42-50.

Palmer JD, Adams KL, Cho Y, Parkinson CL, Qiu YL, Song K. (2000). Dynamic evolution of plant mitochondrial genomes: mobile genes and introns and highly variable mutation rates. Proc Natl Acad Sci USA 97: 6960–6.

Panday R, Jha DK, Thapa N, Pokharel BR, Aryal NK. (2014). Forensic wildlife parts and their product identification and individualization using DNA barcoding. Open Forensic Sci J 7:6–13.

Pardey, P. G., and Beddow, J. M. (2013). Agricultural innovation: The united states in a changing global reality (CCGA Report Chicago Council on Global Affairs). doi: 10.1126/science.122970

Pereira LHG, Maia GMG, Hanner R, Foresti F, Oliveira C. (2011). DNA barcodes discriminate freshwater fishes from the Paraiba do Sul River Basin, Sao Paulo, Brazil. Mitochondrial DNA 22:71–9.

Pereira LHG, Pazian MF, Hanner R, Foresti F, Oliveira C. (2011). DNA barcoding reveals hidden diversity in the Neotropical freshwater fish Piabina argentea (Characiformes: Characidae) from the Upper Parana Basin of Brazil. Mitochondrial DNA. 22:87–96

Pfenninger M, Nowak C, Kley C, Steinke D, Streit B. (2007). Utility of DNA taxonomy and barcoding for the inference of larval community structure in morphologically cryptic Chironomus (Diptera) species. Mol Ecol 16:1957–68.

Pfunder M, Holzgang O, Frey J. (2004). Development of microarraybased diagnostics of voles and shrews for use in biodiversity monitoring studies, and evaluation of mitochondrial cytochrome oxidase I vs. cytochrome b as genetic markers. Mol Ecol 13:1277–86.

Platnick NI. 2014. The world spider catalog, version 10.0. American Museum of Natural History. [accessed 2018 September 10].

Prendini L. (2005). Comment on identifying spiders through DNA barcodes. Can J Zoo l83:481–91.

Preston-Mafham, K. (1998). Spiders: Compact Study Guide and Identifier. Angus Books.

Randi E. (2000). Mitochondrial DNA. In: Baker AJ, editor. Molecular methods in ecology. Malden: Blackwell Science.

Rebijith KB, Asokan R, Krishna V, Krishna Kumar NK, Ramamurathy VV (2012) Development of speciesspecific markers and molecular differences in mitochondrial and nuclear DNA sequences of Aphis gossypii and Myzuspersicae (Hemiptera: Aphididae). Florida Entomol 95:674–682.

Rieseberg LH, Wood TE, Baack EJ. (2006). The nature of plant species. Nature 440:524–7.

Robideau GP, De Cock AW, Coffey MD, Voglmayr H, Brouwer H, et al. (2011). DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Mol EcolResour 11:1002–11.

Robinson EA, Blagoev GA, Hebert PDN, Adamowicz SJ. (2009). Prospects for using DNA barcoding to identify spiders in speciesrich genera. ZooKeys 16:27–46.

Roonwal ML. Termite injuring crops, plantations and fruit and forest trees and their control. In termite life and termite control. Publ. by Scientific Publication, Jodhpur, 1979, 24.

Rubinoff D. (2006). Utility of mitochondrial DNA barcodes in species conservation. Conserv Biol 20:1026–33.

Sahid M, (2003). “Wheat in Pakistan”. Pakistan agricultural research council. www.PARC.com

Saitou N. and Nei M. (**1987**). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**:406-425

Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., and Nelson, A. (2019). The global burden of pathogens and pests on major food crops. Nat. Ecol. Evol. 3 (3), 430. doi: 10.1038/s41559-018-0793-y

Sbordoni V. (2010). Strength and limitations of DNA barcode under the multidimensional species perspective. In: Nimis PL, Vignes Lebbe R, editors. Tools for identifying biodiversity: Progress and problems. Italy: EUT. p 271–6

Schindel DE, Miller SE. 2005. DNA barcoding a useful tool for taxonomists. Nature.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, et al. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc Natl Acad Sci USA 109: 6241–6.

Seifert KA, Samson RA, Dewaard JR, Houbraken J, Levesque CA, Moncalvo JM, Louis-Seize G, Hebert PDN. (2007). Prospects for fungus identification using CO1 DNA barcodes, with Penicillium as a test case. Proc Natl Acad Sci USA 104:3901–6.

Seifert KA. (2009). Progress towards DNA barcoding of fungi. Mol EcolResour 9:83–9.

Sharma S. (2014). A study on spiders as predators in the agro ecosystems. Mun Ent Zool 9:80–3.

Sheahan, M., and Barrett, C. B. (2017). Ten striking facts about agricultural input use in Sub-Saharan Africa. Food Policy 67, 12–25. doi: 10.1016/ j.foodpol.2016.09.010

Shen YY, Chen X, Murphy RW. (2013). Assessing DNA barcoding as a tool for species identification and data quality control. PLoS One 8:e57125.

Sheppard SK, Harwood JD. (2005). Advances in molecular ecology: Tracking trophic links through predator-prey food-webs. FunctEcol 19:751–62.

Sherwood AR, Prestling GG. (2007). Universal primers amplify a 23S rDNA plastid marker in eukaryotic algae and cyanobacteria. J Phycol 43:605–8.

Shewry, P. R. (2009). The HEALTHGRAIN programme opens new opportunities for improving wheat for nutrition and health.

Shiferaw B, Smale M, Braun HJ, Duveiller E, Reynolds M, Muricho G. Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. Food Secur. 2013;5:291-317.

Simmons RB, Weller SJ. (2001). Utility and evolution of cytochrome b in insects. Mol PhylogenetEvol 20:196–210

Skerratt LF, Campbell NJH, Murrell A, Walton S, Kemp D, Barker SC. (2002). The mitochondrial 12S gene is a suitable marker of populations of Sarcoptesscabiei from wombats, dogs and humans in Australia. Parasitol Res 88:376–9.

Smith MA, Fernandez-Triana JL, Eveleigh E, Gomez J, Guclu C, Hallwachs W, Hebert PDN, et al. (2013). DNA barcoding and the taxonomy of Microgastrinae wasps (Hymenoptera, Braconidae): Impacts after 8 years and nearly 20000 sequences. Mol Ecol Res 13: 168–76.

Smith MA, Wood DM, Janzen DH, Hallwachs W, Hebert PDN. (2007). DNA barcodes affirm that 16 species of apparently generalist tropical parasitoid flies (Diptera, Tachinidae) are not all generalists. Proc Natl Acad Sci USA 104:4967–72.

Smith MA, Woodley NE, Janzen DH, Hallwachs W, Hebert PDN. (2006). DNA barcodes reveal cryptic host-specificity within the presumed polyphagous members of a genus of parasitoid flies (Diptera: Tachinidae). Proc Natl Acad Sci USA 103:3657–62.

Steinke D, Vences M, Salzburger W, Meyer A. (2005). TaxI: A software tool for DNA barcoding using distance methods. Philos Trans R Soc Lond, B, Biol Sci 360:1975–80.

Stern RF, Andersen RA, Jameson I, Kupper FC, Coffroth MA, et al. (2012). Evaluating the ribosomal internal transcribed spacer (ITS) as a candidate dinoflagellate barcode marker. PLoS One 7:e42780.

Stern RF, Horak A, Andrew RL, Coffroth MA, Andersen RA, et al. (2010). Environmental barcoding reveals massive dinoflagellate diversity in marine environments. PLoS One 5:e13991.

Stoeckle M. (2003). Taxonomy, DNA, and the bar code of life. Bioscience 53:2–3.

Sun XQ, Zhu YJ, Guo JL, Peng B, Bai MM, Hang YY. (2012). DNA barcoding the Dioscorea in China, a vital group in the evolution of Monocotyledon: Use of matK gene for species discrimination. PLoS One 7:e32057.

Sutovsky P, Moreno RD, Ramalho-Santos J, Dominko T, Simerly C, Schatten G. (1999). Ubiquitin tag for sperm mitochondria. Nature 402: 371–2.

Tadesse W, Sanchez-Garcia M, Assefa SG, Amri A, Bishaw Z, Ogbonnaya FC, Baum M. Genetic Gains in Wheat Breeding and Its Role in Feeding the World. Crop Breed Genet Genom. 2019;1:e190005.

Tautz D, Arctander P, Minelli A, Thomas RH, Vogler AP (2002) DNA points the way ahead in taxonomy. Nature 418(6897):479

Taylor HR, Harris WE. (2012). An emergent science on the brink of irrelevance: A review of the past 8 years of DNA barcoding. Mol EcolResour 12:377–88.

Townzen JS, Brower AVZ, Judd DD. (2008). Identification of mosquito bloodmeals using mitochondrial cytochrome oxidase subunit I and cytochrome b gene sequences. Med Vet Entomol 22:386–93.

Trobajo R, Mann DG, Clavero E, Evans KM, Vanormelingen P, et al. (2010). The use of partial cox1, rbcL and LSU rDNA sequences for phylogenetics and species identification within the Nitzschia palea complex (Bacillariophyceae). 45:413–25.

USDA-NASS (2018). 2017 agricultural chemical use survey: Wheat. no 2018-5 (Washington, D.C: USDA, National Agricultural Statistical Service). Available at: https://www.nass.usda.gov/Surveys/Guide_to_NASS_Surveys/Chemical_Use/ 2012_Wheat_Highlights/.

USDA-RMA (2021). United states department of agriculture risk management agency summary of business (Washington, D.C: USDA, Agriculture Risk Management Agency). Available at: https://www.rma.usda.gov/ SummaryOfBusiness.

Vaglia T, Haxaire J, Kitching IJ, Meusnier I, Rougerie R. (2008). Morphology and DNA barcoding reveal three cryptic species within the Xylophanesneoptolemus and loelia species-groups (Lepidoptera: Sphingidae). Zootaxa 1923:18–36.

Van de Vossenberg BTLH, Westenberg M, Bonants PJM. (2013). DNA barcoding as an identification tool for selected EU-regulated plant pests: An international collaborative test performance study among 14 laboratories. EPPO Bulletin 43:216–28.

Van Velzen R, Bakker FT, Van Loon JJA. (2007). DNA barcoding reveals hidden species diversity in Cymothoe (Nymphalidae). Proc NethEntomol Soc Meet 18:95–103.

Vences M, Thomas M, Bonett RM, Vieites DR. (2005). Deciphering amphibian diversity through DNA barcoding: Chances and challenges. Philos Trans R Soc Lond, B, Biol Sci 360:1859–68.

Vences M, Thomas M, Van der Meijden A, Chiari Y, Vieites DR. (2004). Performance of 16S rRNA in DNA barcoding of amphibians. Integr Comp Bio l44:657–657.

Votypka J, Maslov DA, Yurchenko V, Jirku M, Kment P, et al. (2010). Probing into the diversity of trypanosomatid flagellates parasitizing insect hosts in South-West China reveals both endemism and global dispersal. Mol PhylogenetEvol 54:243–53.

Wang G, Li C, Guo X, Xing D, Dong Y. (2012). Identifying the main mosquito species in china based on DNA barcoding. PLoS One 7:e47051. doi: 10.1371/journal.pone.0047051.

Ward RD, Hanner R, Hebert PDN. (2009). The campaign to DNA barcode all fishes, FISH-BOL. J Fish Biol 74:329–56.

Ward RD, Zemlak ST, Innes BH, Last PR, Hebert PDN. (2005). DNA barcoding Australia's fish species. Philos Trans R Soc Lond, B, Biol Sci 360:1847–57.

Ward RD. (2009). DNA barcode divergence among species and genera of birds and fishes. Mol EcolResour 9:1077–85.

Wares JP, Cunningham CW. (2001). Phylogeography and historical ecology of the North Atlantic intertidal. Evolution 12:2455–69

Waugh J. (2007). DNA barcoding in animal species: Progress, potential and pitfalls. BioEssays 29:188–97

Weber DC, Lundgren JG. (2009). Detection of predation using qPCR: Effect of prey quantity, elapsed time, chaser diet, and sample preservation on detectable quantity of prey DNA. J Insect Sci 9:1–12.

Weigt LA, Baldwin CC, Driskell A, Smith DG, Ormos A. (2012). Using DNA barcoding to assess Caribbean reef fish biodiversity: Expanding taxonomic and geographic coverage. PLoS One 7:e41059.

Wheat CW, Watt WB. (2008). A mitochondrial-DNA-based phylogeny for some evolutionary-genetic model species of Colias butterflies (Lepidoptera, Pieridae). Mol PhylogenetEvol 47:893–902.

Wheeler QD. (2004). Taxonomic triage and the poverty of phylogeny. Philos Trans R Soc Lond, B, Biol Sci 359:571–83.

Wheeler QD. (2007). Invertebrate systematics or spineless taxonomy? Zootaxa 1668:11–8.

White DJ, Wolff JN, Pierson M, Gemmell NJ. (2008). Revealing the hidden complexities of mtDNA inheritance. Mol Ecol 17:4925–42.

Whitworth TL, Dawson RD, Magalon H, Baudry E. (2007). DNA barcoding cannot reliably identify species of the blowfly genus Protocalliphora (Diptera: Calliphoridae). Proc Biol Sci 274:1731–9.

Will KW, Rubinoff D. (2004). Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. Cladistics 20:47–55.

Williams ST, Knowlton N. (2001). Mitochondrial pseudogenes are pervasive and often insidious in the snapping shrimp Genus Alpheus. Mol Biol Evol 18:1484–93.

Wilson EO. (2004). Taxonomy as a fundamental discipline. Philos Trans R Soc Lond, B, Biol Sci 359:739. doi:10.1098/rstb.2003.1440

Wilson JJ (2012) DNA barcodes of insects. Methods Mol Biol 858:17–46

Wong EHK, Hanner RH (2008) DNA barcoding detects marketsubstitution in North American seafood. Food Research Interna-tional, 41, 828–837

Wong EHK, Hanner RH. (2008). DNA barcoding detects market substitution in North American seafood. Food Res Int 41:828–37.

Yang Q, Zhao S, Kucerova Z, Stejskal V, Opit G, Qin M, Cao Y, Li F, et al. (2013). Validation of the 16S rDNA and COI DNA barcoding technique for rapid molecular identification of stored product Psocids (Insecta: Psocodea: Liposcelididae). J Econ Entomol 106:419–25.

Zhang D-X, Hewitt GM. (1997). Assessment of the universality and utility of a set of conserved mitochondrial primers in insects. Insect Mol Biol 6:143–50

Zhang, Q., Men, X., Hui, C., Ge, F., & Ouyang, F. (2022). Wheat yield losses from pests and pathogens in China. *Agriculture, Ecosystems & Environment*, *326*, 107821.