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Molecular Characterization of Termites from Azad Jammu and Kashmir Using DNA Barcoding

by

Soufia Saeed

A thesis submitted in partial fulfillment for the degree of Master of Science

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Dedicated to Allah Almighty, Hazrat Muhammad (S.A.W.W) and my family life. To my mother and father, who never stopped believing in me and their prayers have always enlightened my way throughout my life to my mother and father, who never stopped believing in me and this can't be possible without their unwavering support, endless love and encouragement throughout my pursuit for education. I hope this achievement will fulfill the dream they envisioned for me.

CERTIFICATE OF APPROVAL

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Soufia Saeed

(MBS223001)

THESIS EXAMINING COMMITTEE

S. No.	Examiner	Name	Organization
(a)	External Examiner	Dr. Malik Badshah	QAU, Islamabad
(b)	Internal Examiner	Dr. Sohail Ahmed Jan	CUST, Islamabad
(c)	Supervisor	Dr. Sahar Fazal	CUST, Islamabad

Dr. Sahar Fazal
Thesis Supervisor
September, 2024

Dr. Syeda Marriam Bakhtiar
Head
Dept. of Bioinfo. & Biosciences
September, 2024

Dr. Sahar Fazal
Dean
Faculty of Health & Life Sciences
September, 2024

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(Soufia Saeed)

Abstract

Termites, belonging to the order *Isoptera*, are cellulose-eating insects. Termites are the present abundance in both numbers and species observed in tropical rainforests. Species identification and phylogenetic analysis through DNA barcodes using mitochondrial *COI* gene (*cytochrome c oxidase subunit I*) was performed for termites collected from District Jhelum valley, and Muzaffarabad, AJK, Pakistan.

Mitochondrial DNA was successfully extracted from termites and region of *COI* gene comprising 650 bps was amplified using universal primers and PCR products were confirmed by 1% agarose gel electrophoresis. Sequencing was carried out by Sanger's method. Sample sequences were analyzed using BLAST at NCBI and BOLD database. BOLD (Barcode of Life Data System) databases were used for molecular taxonomy. Sequence analysis was performed to establish evolutionary relationship of study data.

The sequence analysis of 4 samples using BLAST revealed that the all sample of termites showed similarity with *Heterotermes gertrudae* species. The two samples collected from Muzaffarabad AJK labeled as H3 has percent identity 97.81% and Che 1 with percent similarity 97.79 with query coverage 96% for both. Jhelum valley sample J1 with sequence similarity of 98.18% and J2 98.62% with query coverage 93% and 92% respectively. BOLD database submission showed none of the similarity with the available BINS indicating that these sequences have not been submitted from another part of Pakistan.

Results of phylogenetic analysis revealed that all samples were grouped together with boot strap value of 60 and 98 indicating that these sequences are very closely related and share a common ancestor. It further showed close relationship with *Heterotermes gertrudae* and *H.indicola* whereas i is distant but still within the Heterotermes genus. All the four samples processed were most distantly related to *Reticulitermes virginicus*. The results of current study reveled that DNA barcoding can be used as effective tool in biogeographic and biodiversity of termite species. The *cytochrome c oxidase subunit I* (*COI*) gene is commonly used for DNA barcoding across various species. It provides high-resolution identification. Other

mitochondrial genes and nuclear markers like ITS (Internal Transcribed Spacer) can be used to improve accuracy and robustness. Samples collection from various geographic regions to capture the genetic diversity within and between species can also provide large scale information about biodiversity of different areas

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Abbreviations

AQS	Asexual queen succession
BINS	Barcode index number
BLAST	Basic local alignment search tool
BOLD	Barcode of life data system
CBOL	Consortium for barcode of life
CCDB	Canadian centre for DNA barcoding
CHI buffer	Chloroform isopropyl alcohol
COI gene	ytochrome c oxidase subunit 1 gene
DNA	Deoxy nucleic acid
Elusion buffer	Low Tris EDTA buffer
ITS	Internal transcribe spacer
MerC solution	Mer-capto ethanol
NCBI	National centre for biotechnology information
OTU's	Operational taxonomic units
PCR	Polymerase chain reaction
Reagent D	10% SDS (Sodium Dodecyl Sulfate)
Reagent K	Proteinase-K
TBE buffer	Tris-borate-EDTA
Wash buffer A	Isopropanol
Wash buffer B	70% ethanol
gDNA	Genomic deoxy nucleic acid

Chapter 1

Introduction

The Greek Words Iso (equal) Ptera(wings) are the source of the Order Isoptera which is the scientific classification for termites. Termites are known by their scientific name Isoptera, which refers to their distinctive equal-sized wings [1]. These Social Insects belong to the phylum Arthropoda and the Insect Class Insecta knowing Termites & scientific name aids in creating a common terminology for studying these insects [2].

Termites are known species throughout the world and they are part of the order isoptera. Termites are divided into several families within this order, each with its own features and behaviors. termitidae is the most common family of termites, containing subfamilies such *Macrotermitinae*, *Nasutitermitinae* and *Apicotermitinae*. Termites are divided into groups according to their nesting behavior, eating habits and social structure. Workers, Soldiers, and reproductive (including Kings and Queens) are the three main termite castes [3]. Additional castes of certain termites include nymphs and alates (Winged Reproductive Termites). Termites are divided into several ecological groups, such as drywood termites and subterranean termites. Termites differ from other insects due to their unique features. Their bodies are soft their antennae are straight, and their metamorphosis is incomplete. Termites, who live in organized colonies with specialized castes are known for their sociality [4]. Termites are characterized by complex behaviors including building intricate nests and tunnel systems and they communicate by chemical

signals called pheromones. Comprehending these features facilitates termite identification and management. There are around 3,000 different species of termites, and they can be divided into three main ecological groups: subterranean, dry wood and damp wood termites. The most prevalent and common type of termites are subterranean ones, which depend on soil contact to survive. Drywood termites do not need to come into contact with soil; they live in dry wood [5]. Wood contains damp wood termites, which need high humidity levels. Identificationand control of termites can be aided by knowledge of the many types of termites and their habitats. Termites can cause considerable damage to structures, so finding them early isessential to preventing further damage. Termite infestation is often indicated by damaged wood, discarded wings and mud tubes or tunnels. Additional indicators include swarming behavior, in which winged termites emerge from their nests in search of new territories, and droppings, which are tiny pellets that resemble sawdust. When they notice the first signs of an infestation, homeowners should be cautious and seek professional assistance [6].

Termites are gregarious insects that are vital to ecosystems. They may reside in colonies with a few hundred to a million or more members [7]. A few of the indicators that are used for identification include material degradation, the nitrogen cycles and carbon cycles, the composition of soil structure, and the triggering of microbiological activity at different levels [8]. They promote biodiversity by improving the environment for plants and other living creatures [9]. Different types of termites have a beneficial effect on the ecosystem, but they may also seriously harm the economy by destroying wooden buildings, crops, and forest plantings [10]. Every year, there is an increase in the number of termites attacking landscaping trees in Pakistani cities. Pakistan's four provinces are home to about 52 species of termites [11]. Over the past few decades, major termite infestations in urban trees have been recorded more frequently in Punjab, the most populated province; this condition has been made worse by climate change [12]. The provincial capital of Lahore, formerly dubbed the "Garden City," is today experiencing a serious environmental crisis as a result of climate change, overcrowding, and unsustainable construction projects [13].

It is imperative for the management of horticulture and biodiversity in Lahore's parks and green belts to evaluate the prevalence and extent of termite damage to trees. Manzoor and Mir's study looked at Pakistani construction methods, locally produced building materials, and termite-infested homes. However, termite infestations in urban and landscape forestry were not addressed in this study, which only covered 185 homes from different parts of Punjab [14]. Termites are categorized scientifically as follows:Animalia is the KingdomClass: Arthropoda Class: Insecta Pterygota subclass Insider: Neoptera Classification: Dictyoptera Isoptera is the order [15].

Termites are classified into seven families and fourteen subfamilies, comprising 280 genera and approximately 2,600 species. Lower termites includes *Hodotermitidae, Mastotermitidae, Kalotermitidae, Rhinotermitidae, Termopsidae and Serritermitidae* and higher termites are the two groups of isopterans that are separated phylogenetically [16]. Approximately eighty five percent among all termite genera and seventy percent of all termite species are members of the biggest termite family, the Termitidae [17]. This family is the most advanced group of termites; it lacks symbiotic cellulolytic protists in its gut and is distinguished by a unique microbial flora, a complex social structure, and a variety of eating patterns [18]. The Macrotermitinae subfamily of the over 330 species and 14 genera in the Termitidae family [19].

The subfamily and a Termitomyces-genus basidiomycete fungus have mutualistic relationships formed. The Palaeotropics are home to the Macrotermitinae termites, of which Africa has the greatest diversity [20]. Termites are sometimes confused with ants and are known as "white ants." On closer inspection, though, two characteristics set termites apart from ants: ants have elbowed antennae and a narrow waist, whereas termites have straight antennae and a wide waist [21]. Similarly, Jones and colleagues (2005) noted that merely 3% of species harm crops and structures. India loses approximately $35.12 million year as a result of termites damaging crops, especially maize. The precise economic impact of agricultural losses in southern Africa is yet unknown, however reports range from 3 to 100% [22].

Pakistan's termite species are divided into four families (*Kalotermitidae*, *Hodotermitidae*, *Rhinotermitidae*, and *Termitidae*), with 53 species and 16 genera, according to Ahmad and Akhtar [23]. Termites are found in many tropical regions and a range of terrestrial habitats, making them significant invertebrate decomposers [24].

Termites are gregarious insects with a variety of morphologies that are skilled wood decomposers and have a significant impact on global ecology [25]. They are vital to the carbon cycle and have the potential to yield several ecological benefits. Termites can be categorized into two primary groups based on their ability to reproduce: the castes involved in reproduction or queen castes, and the non-reproductive castes, or worker and soldier castes [26]. Termites are omnipresent throughout the world and play a crucial role in ecosystems across diverse habitats. Within their species, they create intricate connections and are classified as either higher or lower termites [27]. They all have a diverse gut microbiota that is home to numerous prokaryotes and protists. A ubiquitous pest on a global scale, termites excel at breaking down wood. More than 75% of termite species are only members of the apical family and are categorized as top termites. Termitidae [28]. Higher termites differ from lower termites despite having a different prokaryotic population due to the absence of intestinal protists [29]. These termites, which are common and skilled at breaking down wood, are good for the ecosystem and are essential to the carbon cycle [30]. Lower termites are known to have a symbiotic relationship with cellulolytic intestinal protists, whereas higher termites acquire their energy by taking acetate from these protists [31] GHF9 termites, both higher and lower, generate endogenous cellulase enzymes from the stomach or salivary glands, including endo-β-1,4-glucanase and B. β-glucosidase [32]. A system that is easy to use, quick to complete, and precise is necessary to catalog the enormous diversity of insect species. These requirements are met by DNA barcoding, which allows for specimen identification at the species level [33]. Recent molecular investigations have used a variety of markers, such as cytochrome c oxidase subunit 1, to significantly enhance our understanding of the evolutionary relationships among insect species., rDNA internal transcribed spacers region-2, NADH dehydrogenase subunit 1, and cytochrome b [34]. Molecular

scientists employ cytochrome c oxidase subunit 1, one of these markers, extensively throughout the world to identify different kinds of insects [35]. Since DNA barcoding is a new technology, building a reference library of DNA sequences is the first step in any relevant research [36]. In order to build a trustworthy database CO1 sequencing must be performed on specimens that have already been identified by a taxonomist [37]. The most effective methods .for molecular identification are phylogeny and molecular identification utilizing indicators for species identification, especially cytochrome c oxidase 1 from the region of mitochondria [38]. This efficiency can be attributed to a number of things, including the ease of amplification, the availability of conserved areas for the construction of universal primers, and enough heterogeneity to differentiate even closely related species [39]. It is significant to remember that the inheritance patterns in the nuclear and mitochondrial genomes differ [40]. Because mitochondrial DNA is inherited maternally, develops relatively quickly, and most nucleotide substitutions happen at neutral locations, mitochondrial markers are utilized to shed light on the evolutionary relationships of related groups [41]. Sequence data from CO1 marker gene amplification is used to study intra- and inter-phylogenetic interactions using this genetic marker [42]. These associations are inferred with the use of mitochondrial cytochrome c oxidase subunit I gene fragment sequences [43].

Many processes that together constitute concerted evolution are responsible for maintaining relative homogeneity, as mutations swiftly disseminate to all members of the gene family, regardless of where they are on separate chromosomes [44]. This area has been used to identify populations inside a species and shows varied levels of species difference [45].

DNA barcoding does not deal with species demarcation; rather, it attempts to identify preset species. To identify species, it uses a brief, a standardized DNA sequence, typically the 5' end of the mitochondrial cytochrome c oxidase subunit I gene. DNA barcoding accomplishes two key goals: [46] (i) employing a sequence divergence threshold to identify and classify unknown specimens to already documented species; and (ii) assisting in the discovery of new species. DNA taxonomy

can provide a database for DNA barcoding and look into the evolutionary relationships among taxa (e.g., species). It may involve one or more mitochondrial and nuclear DNA regions [47].

In molecular systematics, the CO1 gene is widely utilized as a marker, especially its 5' region, which is employed in the 'Barcoding of Life' project. This 650 nucleotide region's nucleotide sequence acts as a special identification code for every species [48]. For the classification of unclear and unresolved taxa, standardized identification approaches are crucial. Based on statistical taxon separation analysis and tree-based taxon grouping, the molecular data is consistent with morphological ideas [49]. This indicates that species identification based on DNA sequence analysis is viable for the examined taxa.Sophisticated species identification methods, such as minuscule barcodes for archival materials and species-specific primers for mosquito tea bugs, have been developed since DNA barcoding has proven to be a valuable tool for DNA barcoding has shown to be an effective technique for scientists[50] More research has been done on the organization of genes in insect genomes than any other group of invertebrates [51]. Fifteen bug species have had their whole genomes sequenced thus far. The double-stranded circular genomes found in insect mitochondria range in size from 14,503 bp to 19,517 bp [52]. However, comprehensive studies on individual species, their occurrence, and distribution are deficient in Pakistan's different regions. This study looked into the diversity, distribution, and population density of termites in the area under investigation because of their economic significance [53].

1.1 Problem Statement

Although termites are essential to the health of ecosystems, it can be difficult to identify and categorize them remain challenging due to the morphological similarity among species and the lack of reliable diagnostic traits Conventional taxonomy techniques place a lot of emphasis on morphological traits, which can be laborious, arbitrary, and need specific knowledge. Consequently, accurate identification of termite species is often hindered, leading to limitations in ecological studies, pest

management strategies, and biodiversity conservation efforts. To address these challenges, there is a growing interest in utilizing A molecular method DNA barcoding is used for the rapid and accurate identification of termite species.

1.2 Aim

The aim of this research is using DNA barcoding as a molecular technique to quickly and precisely identify termite species.

1.3 Objectives

1. To evaluate the mitochondrial *cytochrome c oxidase subunit 1* (*COI*) gene, for their efficacy in discriminating among termite species collected from District Jhelum valley, District Kotli and District Muzaffarabad, Azad Kashmir, Pakistan.

2. To verify sequenced samples of termites from the BOLD system based on the mitochondrial *cytochrome c oxidase subunit 1* (*COI*) gene.

3. To establish evolutionary relationship of sequenced termite samples using DNA barcodes.

1.4 Scope of Study

Through the use of DNA barcoding, this study can offer a thorough evaluation of termite biodiversity. This could lead to a better knowledge of the biodiversity of the local ecosystem by revealing species that are unknown or have not been recorded before. Studying the diversity and molecular traits of termite species can help us better understand their ecological roles. Termites play an essential role in the breakdown of cellulose and the cycling of nutrients in ecosystems. Understanding how termite diversity impacts soil health and ecosystem functioning

could be aided by this Certain termite species are regarded as pests because they harm crops and wooden buildings. Accurately identifying these species via DNA barcoding could help with the development of focused pest management plans, minimizing environmental damage and lowering financial costs.

Chapter 2

Literature Review

Formerly, termites belonged to the Isoptera order. Based on similarities in their symbiotic gut flagellates, suggestions that they were closely related to wood-eating cockroaches (genus Cryptocercus, the woodroach) were reported as early as 1934.

More proof for that theory surfaced in the 1960s when F. A. McKittrick pointed that Some termites and Cryptocercus nymphs share comparable physical traits [54]. In 2008, it was determined by DNA study of 16S rRNA sequences that termites belonged in the same evolutionary tree as the cockroaches, or order Blattodea. With the most phylogenetically similarity to termites, In 2008, it was determined by DNA study of 16S rRNA sequences that termites belonged in the same evolutionary tree as the cockroaches, or order Blattodea. With the most phylogenetically similarity to termites the cockroach genus Cryptocercus is believed to represent a sister group to termites.

There are morphological and behavioural similarities between termites and Cryptocercus. For instance, although most cockroaches lack social skills, Cryptocercus tends to its young and demonstrates additional social behaviors including allogrooming and trophallaxis [55].

FIGURE 2.1: Map showing the distribution of termites worldwide[57]

It is believed that termites are descended from the Cryptocercus genus. A more cautious approach, recommended by some scholars, would be to keep termites classified as members of the cockroach order's epifamily, the Termitoidae, which maintains termites' classification at the family and lower levels.

A more cautious approach, recommended by some scholars, would be to keep termites classified as members of the cockroach order's epifamily, the Termitoidae, which maintains termites' classification at the family and lower levels. Fossils of social insect nests resembling termite nests are also found in the Morrison Formation. The oldest known faecal pellets were found in West Texas during the Upper Cretaceous, which is also thought to be the source of the oldest termite nest ever found [56].

There has been debate about claims that termites first appeared.for example, F. M. Weesner said that fossilised wings discovered in Kansas' Permian layers resemble the wings of Mastotermes, the oldest ancient living termite, and that the Mastotermitidae termites may have evolved 251 million years ago in the Late Permian. It's even plausible that term'ites evolved in the Carboniferous Period. It's even plausible that term'ites evolved in the Carboniferous Period [57].

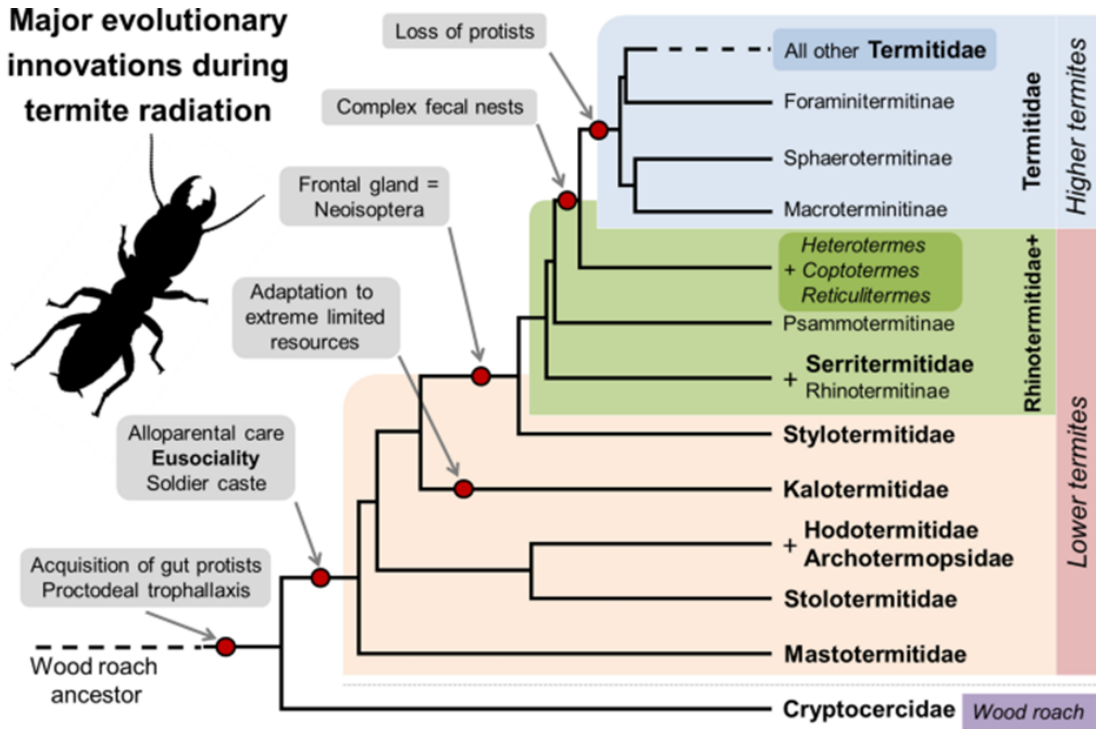

FIGURE 2.2: Termites evolution [58]

Since only Mastotermes has folded wings that resemble the folded wings of the fossil wood roach Pycnoblattina, which are organised in a convex pattern between segments 1a and 2a, all Paleozoic and Triassic insects tentatively classified as termites are thought to be unrelated to termites and should be removed from the Isoptera.

Other studies suggest that termites have a more recent origin, having split out during the Early Cretaceous, from Cryptocercus. Since only Mastotermes has folded wings that resemble the folded wings of the fossil wood roach Pycnoblattina, which are arranged in a convex pattern between segments 1a and 2a, all Paleozoic and Triassic insects tentatively classified as termites are thought to be unrelated to termites and should be removed from the Isoptera [58]. Other studies suggest that termites have a more recent origin, having split out from Cryptocercus sometime during the Early Cretaceous. Several cockroach-like traits are unique to the primordial giant northern termite (*Mastotermes darwiniensis*), including its ability to lay eggs in rafts and its anal lobes on its wings [59].

It has been suggested that the clade "Xylophagodea" include the Isoptera and Cryptocercidae. Though they are frequently called "white ants," termites and ants share a basic evolutionary trait in that they are social insects. Convergent evolution made termites the first social insects to create a caste structure more than 100 million years ago. In comparison to other insects, termite genomes are typically rather large. Zootermopsis nevadensis's genome was the first to be completely sequenced and was released in the journal Nature Communications.

Zootermopsis nevadensis's genome was the first to be completely sequenced and was released in the journal Nature Communications. It is approximately 500 Mb in size. Two subsequent published termite genomes are of *Macrotermes* natalensis. and Cryptotermes secundus, which weigh about 1.3 gigabytes more [60].

2.1 Distribution of Termites

Termites are found around the world, mostly in tropical rainforests close to the equator, and they can be found in both northern and southern latitudes. The Eastern Hemisphere has a higher diversity of termite species than the Northern Hemisphere, and some of these species are found in hilly areas up to 2000 meters above sea level, suggesting a wider range than the Western Hemisphere [61].

With the exception of Antarctica, termites are found on every continent, demonstrating their extensive worldwide existence. They grow well in tropical and subtropical climates with warm temperatures, as well as warm, humid lowland and coastal locations [62]. Equatorial rainforests usually exhibit the highest termite diversity, with diversity falling as latitude increases.

Termite species vary in terms of their diversity and propagation significantly between continents: North America: Approximately 50 species. Europe: Only ten recognized species. South America: Over 400 recognized species. Asia: 435 species, predominantly found in subtropical and tropical regions south of the Yangtze River. Africa: Over 1,000 of the 3,000 identified termite species, exhibiting a diverse ecological distribution [63].

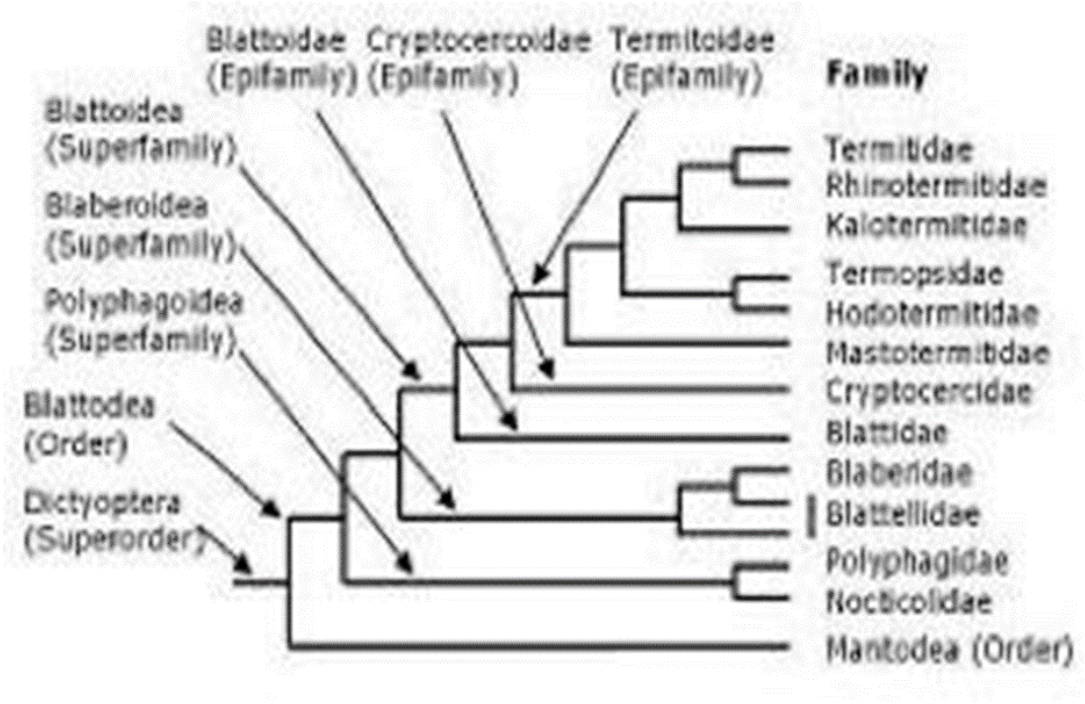

FIGURE 2.3: Classification of temites [16]

Based on ecological distribution and species composition, seven *termitesgenera* *Macrotermes*, *Microtermes*, *Odontotermes*, *Amitermes*, *Angulitermes*, *Microcerotermes*, and *Trinervitermes* have been found in Ethiopia's central rift valley. *Trinervitermes* and *Angulitermes* were uncommon and restricted to particular areas, but the first five taxa were widely distributed over the whole study area [64].

The global distribution and diversity of termites underscore their adaptability to various environments. This extensive distribution pattern is crucial for understanding their ecological roles and impacts across different regions.

This study investigates termite populations across various land use types, the study conducted in westren Ethiopia revealed the distribution through cropland, rangeland and protected areas, using defined belt transects and corn stalk baits [65]. The level of human cultivation differed among these land use types, with protected areas being the least disturbed, followed by cropland and rangeland. This variability raises concerns about the potential decline of certain termite species due to agricultural and animal grazing practices.

The ecological characteristics of the sampled areas influenced the frequency of termite occurrences, and the forms of land use impacted the distribution of specific termite genera. *Macrotermes* was detected 20 times (32.8%) out of 61 occurrences, with 10 of these occurrences (16.4%) specifically on rangelands. Each land use type hosted *Macrotermes*, *Microcerotermes*, *Amitermes*, and *Microtermes*. *Microtermes* were more prevalent in croplands, *Microtermes* in protected areas, and *Macrotermes* in pastures [66].

TABLE 2.1: Percentage of occurance of different termite species in seven forest of Punjab [67]

Names of species	**Percentage**
H indicola	6
C heimi	2
O obesus	15.5
O hora	10
O gurdaspurensis	5
O guptai	10.5
O assmuthi	3.5
M pakistanicus	7.5
M mycophagus	13.5
M unicolar	5.5
M obesi	19
B beesoni	2

In the Manasibu region of western Ethiopia, the research aimed to determine the prevalence of termites and their impact on key pastures and crop s. The study revealed that 40 percent of the 150 samples examined were *Microtermes* species. Specifically, 45 termite samples showing damage were collected from 15 different corn fields. About 15 termite samples from six genera *Trinervitermes*, Ancistrotermes, *Macrotermes*, *Microtermes*, *Odontotermes*, and *Trinervitermes* were collected from pastureland [67].

The study the impact of termite diversity on land use and distribution. The findings suggest that agricultural and grazing practices may impact termite populations, with potential implications for ecosystem health and agricultural productivity. Understanding these patterns is crucial for developing sustainable land management strategies that protect termite diversity and ecosystem functions [68].

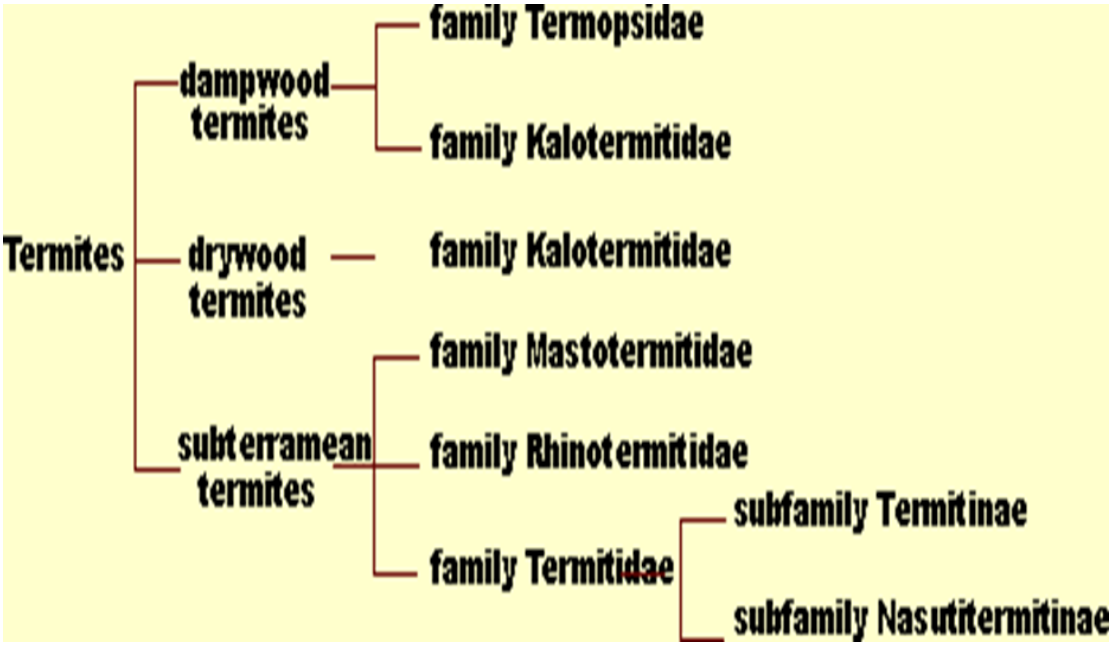

FIGURE 2.4: Termite classification based on habitats [28]

Microtermes termites are commonly found infesting matu re teff roots, with an occurrence rate of 37.5%. They are also prevalent in the roots and stems of corn and sorghum, with occurrence rates of pseudodacanthotherms at 17.8% and macroterms at 24.4%. *Microtermes* termites are characterized by their absence of visible mounds and exhibit a seasonal pattern, becoming more active during the cold dry season and emerging from the ground as the dry season is coming to a close and the rains start. Their tunnels and feeding sites may be disrupted by intense rainstorm events, which helps to maintain control over them [69].

2.2 Species Distribution

Termites are commonly distributed worldwide, particularly in tropical rainforests near the equator, spanning both northern and southern latitudes [70]. The Eastern

FIGURE 2.5: Types of termites based on food [23]

Hemisphere harbors a greater diversity of termite species compared to the Northern Hemisphere. In the Northern Hemisphere, numerous termite species have been identified, with biodiversity levels higher than those found in the Southern Hemisphere. Some species have even been discovered at elevations up to 2000 meters in Eastern Hemisphere mountains, where their dispersion is more extensive than in the Western Hemisphere [71].

Termites are found on every continent except Antarctica, indicating a widespread global distribution of species [72]. They thrive predominantly in tropical regions, subtropical zones, and areas with warm climates, favoring warm and moist lowlands as well as coastal regions . Their highest diversity of species is typically observed in equatorial rainforests, with their presence generally decreasing as one moves towards higher latitudes.

The diversity and distribution of termite species vary significantly between continents and even among countries. In Europe, for instance, there are ten identified species, whereas North America hosts around 50 species [73]. South America stands out with a particularly high diversity, boasting over 400 known species. In Asia, specifically in China, there are 435 termite species, predominantly found in mildly regions that are tropical or subtropical south of the Yangtze River. Of the roughly 3,000 species of termites that are identified worldwide, about 1,000 are found in Africa, which indicates a varied ecological distribution of their mounds [74].

In Ethiopia's central rift valley, the ecological distribution and species composition of termites revealed the presence of there are seven genera: *Microtermes*,

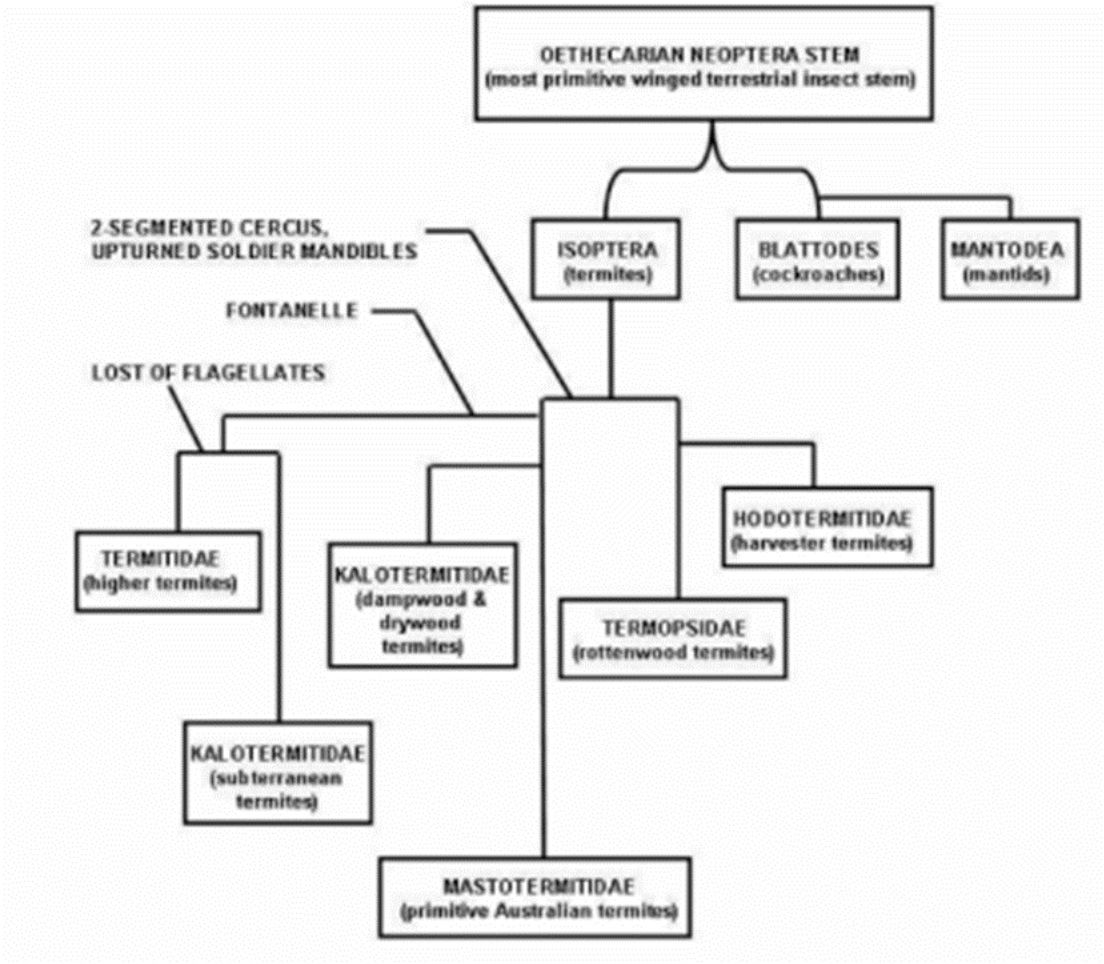

FIGURE 2.6: Isopteran phylogenetic tree [73]

Odontotermes, *Amitermes*, *Microcerotermes*, *Trinervitermes*, and *Macrotermes*. *Trinervitermes* and *Angulitermes* were found in limited numbers and restricted to specific locations, whereas the first five taxa were widely distributed across the surveyed area [75].

Researchers retrieved termites from a variety of land-use types, including farmlands, rangelands, and protected lands, using defined belt transects and maize stalk baiting. Humans managed these land-use groups to varying degrees; protected lands saw the least amount of disturbance, followed by rangelands and farmlands. This differentiation suggests that certain termite species could be experiencing declines due to agricultural cultivation and grazing by livestock. According to a theory, termite colonies might face starvation if the grass and straw consumed by livestock are removed [76].

The abundance of termites varies significantly based on the ecological characteristics of the regions studied, particularly influenced by the type of land use. Among the 61 occurrences recorded, *Macrotermes* was found 20 times (32.8%), with 10 of these occurrences (16.4%) in rangelands in particular, out of a total of 20 instances . All land-use kinds included *Macrotermes*, *Microcerotermes*, *Amitermes*, and *Microtermes*. *Macrotermes* were predominantly observed in rangelands, *Microtermes* in agricultural areas, and *Microcerotermes* in protected regions [77].

TABLE 2.2: Taxanomical classification of termites [78]

Sr no.	**Particulars of classification**	**Name of the Order/Family**
1	Order	*Balttoda*
	Infraorder	*Isoptera*
	Family	*Cratomastotermitidae*
		Mastotermittidae
2	Parvorder	*Euisoptera*
		Termopsidae
		Archotermopsidae
	Family	*Hodotermitidae*
		Stolotermitidae
		Kalotermitidae
3	Nanorder	*Neoisoptera*
		Archeoorhinotermitidae
		Stylotermitidae
	Family	*Rhinotermitidae*
		Serritermitidae
		Termitidae

In Manasibu district, west Ethiopia, the impact of termites on important farm crops and rangelands was evaluated. Among 150 samples assessed, *Microtermes* species accounted for 40%. Specifically, 45 termite samples were collected from damaged maize in fifteen fields, while 15 termite samples were obtained from

rangelands. These samples represented six genera: *Trinervitermes*, Ancistrotermes, *Macrotermes*, *Microtermes*, *Odontotermes*, and *Trinervitermes* [78].

Microtermes is frequently found infesting the roots and stems of maize, as well as the stalks of sorghum, with an occurrence rate of 37.5% at the base of matured teff roots. *Trinervitermes* and *Macrotermes* have occurrence rates of 24.4% and 17.8%, respectively, in these crops.

Microtermes, being moundless termites, exhibit a preference for seasonal activity especially during the beginning and closing phases of the wet and dry seasons, respectively, particularly in colder climates.

Following the initial raindrops, these termites swiftly emerge from the soil. Intense rainstorms can effectively control termite populations by washing away their tunnels and feeding sites [79].

Reticulitermes flaviceps was first discovered and named in Taipei, Taiwan. Subsequent evaluations, taking into account factors like population size, behavior, and colony structure, have identified it as the most populous species [80].

R. flaviceps is distributed across several provinces in China, including Anhui, Hubei, Yunnan, Sichuan, Shaanxi, Guizhou, Zhejiang, Guangdong, Hunan, Guangxi, Jiangxi, and Zhejiang [81].

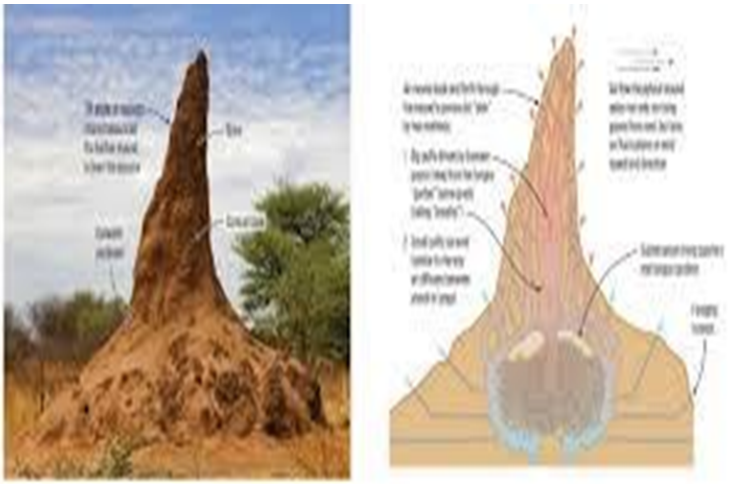

FIGURE 2.7: Termites Mounds showing the infestation of termites [27]

2.3 Bibology/Morphology

From a phylogenetic viewpoint, termites can be classified into two main groups: primitive and metamorphic, distinguished by their external features [82]. Despite variations in the appearance of soldiers, which at times resemble scorpions, the fundamental morphology of their thorax and head remains largely unchanged among workers and reproductive members. Termites are generally small, varying in length from 4 to 15 mm, and display a range of colors from white to tan, occasionally appearing black [83].

Changes in morphology, particularly in the heads and thoraxes of soldiers, serve as significant indicators for classification and recognition [84]. For example, the transparent upper lip of Reticulitermes chinensis is sharply pointed, resembling a needle, whereas in *R. flaviceps* it is slender, akin to a snail. Alates, the winged reproductive individuals of *R. flaviceps*, display a gray-yellow structure on the anterior thorax [85].

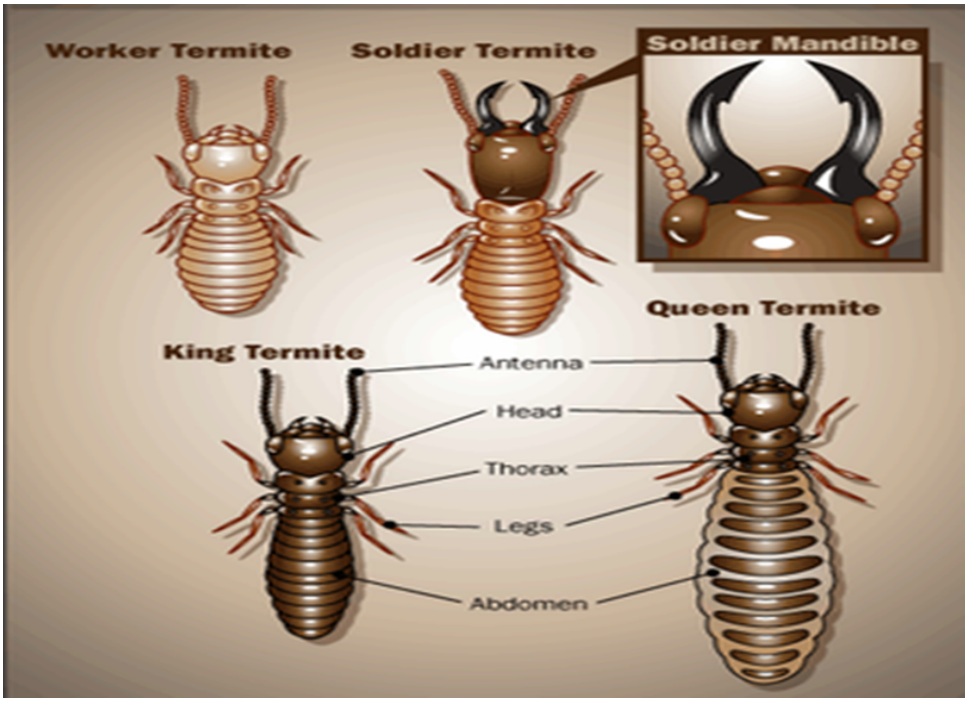

FIGURE 2.8: Morphology of termites [87]

Termites stand out among social insects for their unique characteristic of having both female and male members. Some develop wing buds that gradually grow

larger. Nymphs mature into fully winged adults, destined to become upcoming monarchs and queens. These termites' range in color from black to pale brown, with opaque grey to black on their wings. Events that swarm occur at varying times depending on the species but generally follow rainfall. Termites, along with the extensive mud structures they construct, are prominent in tropical environments. These formations function as natural air-conditioning systems and can house millions of individual termites [86].

Termites can be categorized based on their reproductive roles into two groups: non-reproductives, which include soldiers and workers, and reproductive, consisting of the queen and king. The distinguishing features of the primary reproductive are used as criteria for their classification [87].

On the other hand, secondary reproductives, which originate from older adult nests, differ from primary reproductives. Because they reproduce and lay eggs after molting (ecdysis), they are essential to the colony's growth [88].

2.4 Ecology of Termites

Termites are categorized into three primary ecological groups: subterranean, drywood, and dampwood termites, each exhibiting unique ecological behaviors and habitat preferences. Australia recognizes Cryptotermes Brevis as an invasive species of drywood termites. Both drywood and subterranean termites feed on decomposing plant debris, such wood and leaf litter structures, soil, crops, forests, and plantations, utilizing their gnawing mouthparts. These termites pose risks to various objects including buildings, documents, artworks, literature, flooring, carpets and clothing [89].

Subtereanen termites, social insects that establish colonies underground, primarily consume dead tree matter and avoid living trees. They dominate tropical and subtropical habitats between approximately 50 degrees north and south latitudes, boasting significant biomass. Termites are adept at constructing diverse mound

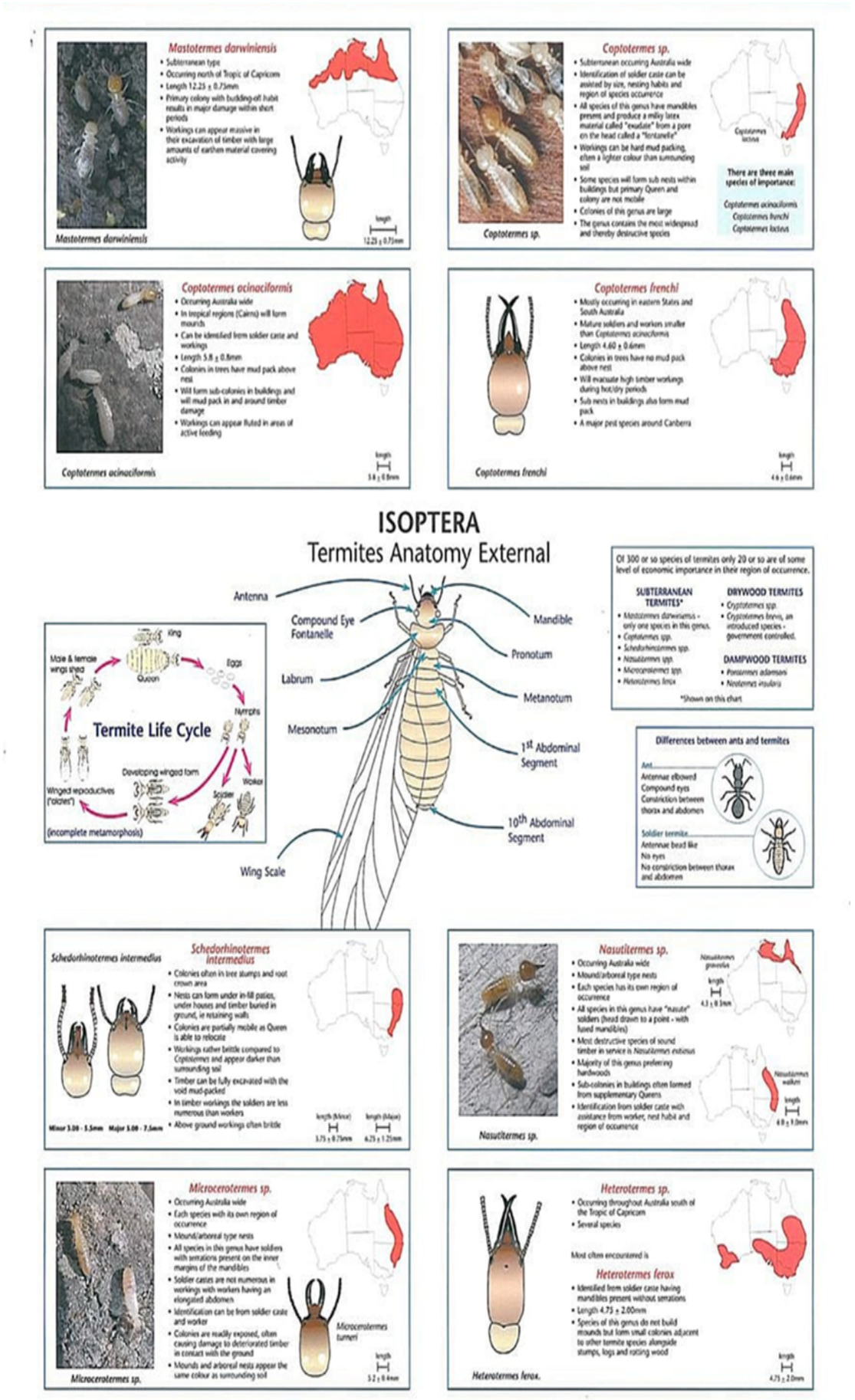

FIGURE 2.9: Identification keys of subtereanen termites[90]

and nest structures to support their colonies. Ground-dwelling termites build tall mounds, while arboreal termites nest in trees.

Termites are adept at constructing diverse mound and nest structures to support their colonies. Ground-dwelling termites build tall mounds, while arboreal termites nest in trees, using their structures to control condensation, conserve water, and shelter eggs and developing larvae in deep brood chambers [90].

Termite mounds in tropical savannahs exhibit considerable size and shape variation. Some species construct exceptionally large mounds reaching up to nine meters in height, predominantly found in densely forested areas with typical cone-shaped structures measuring two to three meters in diameter. These mounds display diverse morphologies, including sculpted hard earth forms, irregular domes or cones adorned with woody plants and grasses [91].

2.5 Life Cycle

The termite life cycle encompasses three castes: workers, soldiers, and reproductives. Originating from eggs, termites progress through developmental stages including larvae, nymphs, soldiers, and eventually reproductive adults, facilitating a division of labor typical in social insect societies.

Pheromonal, environmental and social cues collectively influence caste differentiation, determining whether a larva develops into a worker, soldier, or secondary reproductive. Larvae undergo multiple molts before adulthood, typically three times. Newly emerged individuals from termite eggs are known as larvae, which bifurcate into workers or soldiers [92].

The termite larva has two potential paths that determine its life cycle outcome. It may mature into a worker or soldier, fulfilling its role until death. Alternatively, it can develop into a secondary reproductive or reproductive alate, which may then become a queen or king to initiate a new colony. Queen termites have the longest lifespan, averaging about 25 years. In contrast, the lifespans of other termite castes range from 12 to 24 months [93].

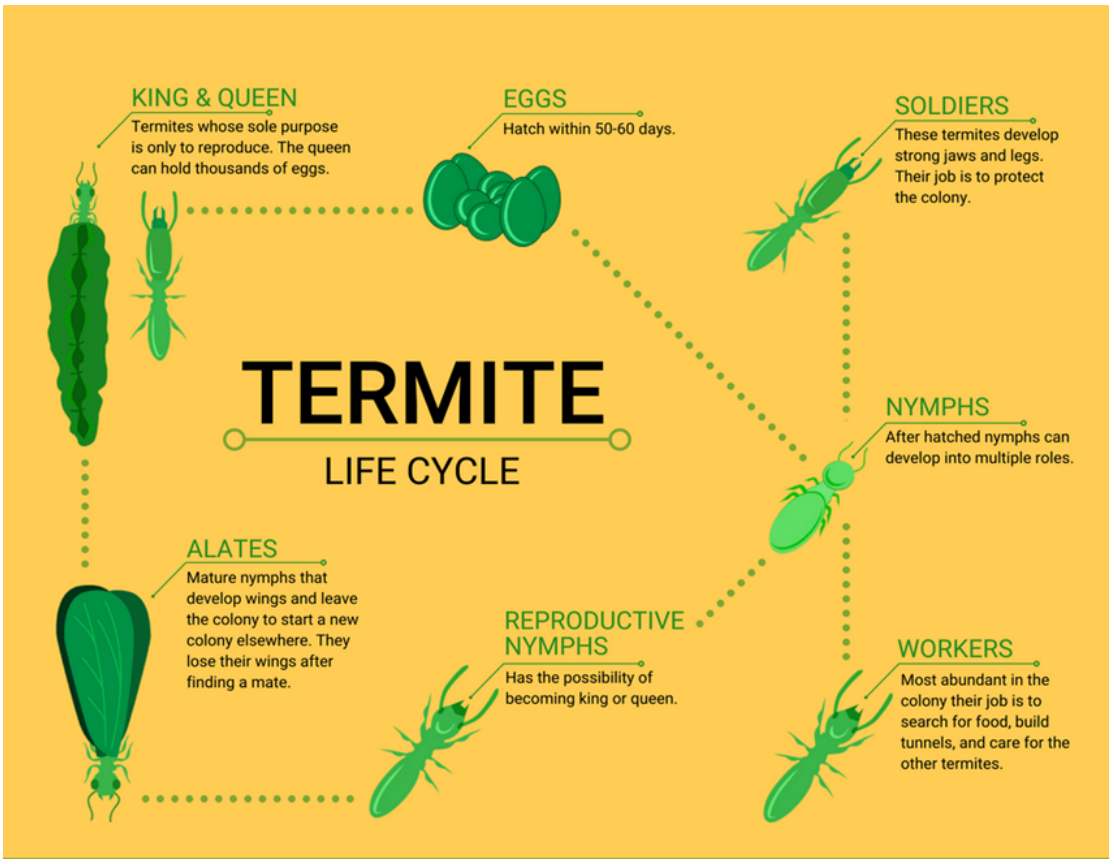

FIGURE 2.10: Life Cycle of termites [93]

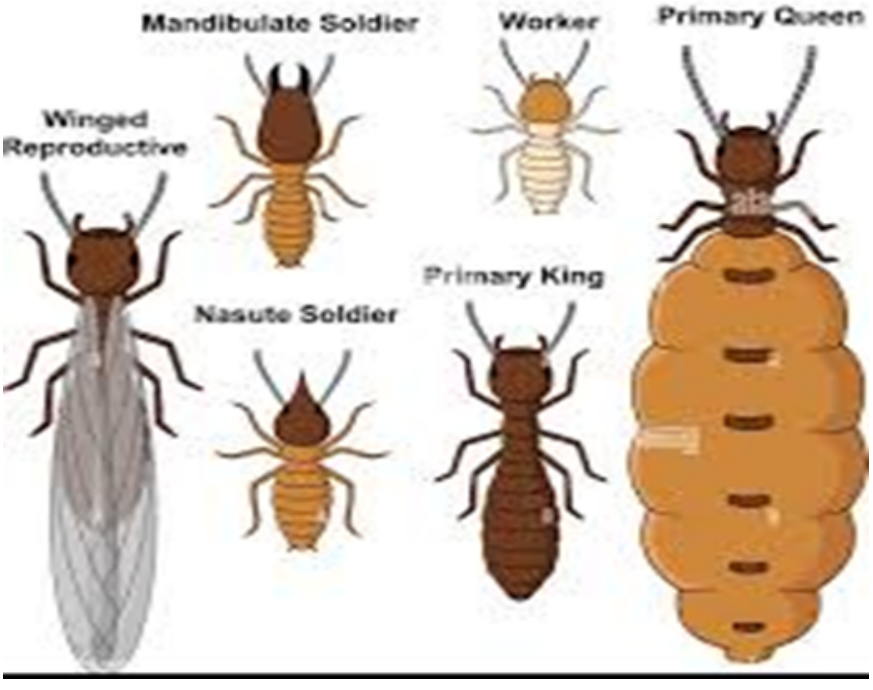

FIGURE 2.11: Queen, Soldier and worker in termites [94]

2.6 Reproduction of Termites

Termites, like many other species, reproduce to ensure the continuity of their generations. Thousands of termites gather in huge groups during the summer months, led by the king and queen, in search of a mate [94]. The king and queen perform a courtship dance after selecting a suitable companion before establishing their individual colony. When the fertilized queen is ready to lay eggs and start creating new termites, the male, or king, helps her. During the queen can lay during her first year of laying hundreds or thousands of eggs every day [95]. The termite king and queen care for the colony's initial generations jointly until there are sufficient young animals or workers. The pheromones and temperature conditions that termites are exposed to determine how they will develop into soldiers or workers when they hatch [96]. Because they are responsible for feeding young, maintaining colony calm, and foraging, workers are crucial to the division of labor within the colony.

Soldiers and workers who are infertile and unable to reproduce can be either male or female [97]. The population of the termite colony gradually increases over a period of five years. At this point, the queen gives birth to new members of the colony, who become the first reproductive adults. When they reach adulthood, these reproductive salads prepare to swarm and leave the nest in the summer to start new colonies. Through this reproductive cycle, termite colonies continue to spread and become established [98].

2.7 Termite Mating Behavior

Termites undertake group flights as adults to reproduce, leaving their original colony to establish new ones where they lay eggs and raise additional offspring [99]. The dispersal process is influenced by several variables including humidity, temperature, barometric pressure, and seasonal variations. Male reproductive termites usually accompany females on these flights as they shed their wings. The

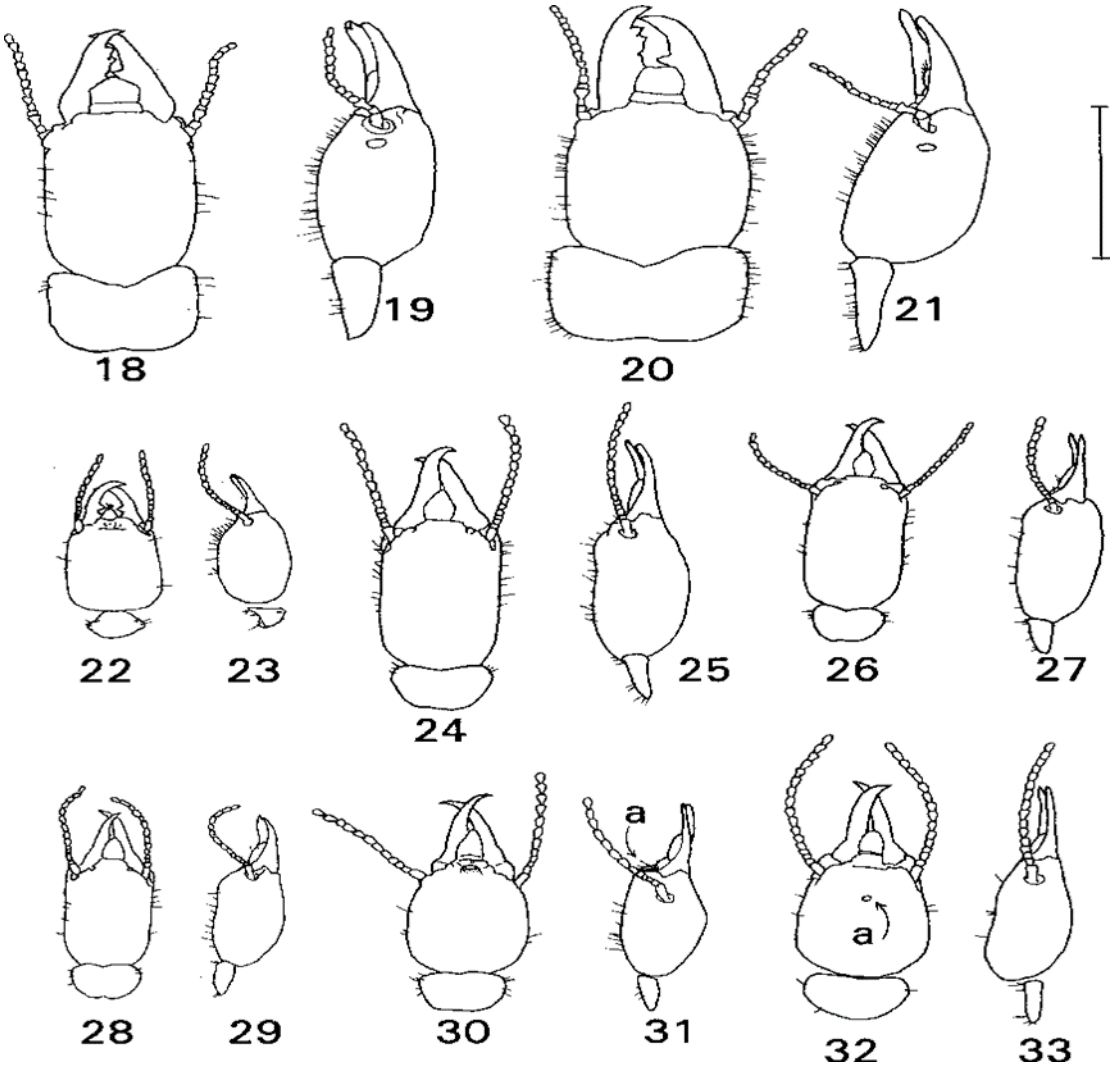

FIGURE 2.12: Taxonomical features of solider termites [98]

formation of tandem pairs of kings and queens is a crucial genetic behavior for termites in establishing new colonies [100].

When the king builds a new nest, the queen is crucial in choosing a good place to build the nest. Each year, hundreds of individuals are released from established colonies by *R. flaviceps* . Once a colony is established, alates locate females, form partnerships, lose their wings after flight, and engage in tandem activities to reproduce, lay eggs, and hatch. Through contact with the female's abdomen, the male initiates the tandem activity. To separate and begin building the first colony, the female independently looks for a suitable nesting site. It begins with dispersal flights and matures over an indefinite period [101].

2.8 Termite Parthenogenesis

During sexual reproduction, insect eggs are activated when meiosis is completed, and the sperm and egg nuclei fuse [102]. In some insects, parthenogenesis allows eggs to develop without fertilization [103]. Successful bonding between male and female is crucial for the regular functioning of the colony and directly impacts the survival rate of female offspring [104]. Although parthenogenetic reproduction has adaptive advantages, sexual reproduction is often considered more beneficial because it achieves similar results with twice the effort [105]. Genetic and developmental factors limit parthenogenesis resulting in generally lower offspring survival compared to sexual reproduction [106].

Certain reproductive termites also use asexual reproductive techniques, although parthenogenesis is only present in a small number of isopteran species [107]. Asexual queen succession (AQS), a distinct process of termite parthenogenesis, has been observed in several termite species, including *R. verginicus*, *R. verginicus*, *Cavitermes tuberosus*, and *Embiratermes neotenicus*. Parthenogenesis is referred to as "terminal fusion" in lower termites but as "central fusion" in higher termites [108]. *R. verginicus* exhibits the phenomenon of asexual queen succession, which leads to the production of unfertilized eggs despite the lack of an egg incubation phase [109].

Many studies revealed the external morphology, cleavage, and Using laser scanning and a digital microscope, the development of embryo of fertilized and unfertilized eggs in two termite species *R. verginicus* and *R. aculabialis* was studied. Within 24 to 48 hours, significant differences in the cleavage, number of nuclei, size, breadth, and density were seen, offering insight into both of these termite species' egg development processes [110].

Using laser scanning and digital imaging, researchers examined the embryonic development of viable and unfertilized eggs in *R. aculabialis* and *R. verginicus*. The findings, which shed light on both types of egg formation, revealed striking variations in breadth, size, number of nuclei, volume, and cleavage within 24 to 48 hours [111].

2.9 The Hybridization of Termites

Hybridization, a reproductive behavior involving the genetic mixing of offspring from two termite species with different genetic backgrounds, results in the offspring inheriting traits from both parents. This genetic diversity, demonstrated by hybrid compounds' ecological compatibility and success, aligns with emerging economic factors [112]. Hybrid populations in heterozygous regions show favorable ecological and evolutionary outcomes compared to their parental populations [113]. For instance, the union of the two species of fire ants, *Solenopsis richteri* and *Solenopsis invicta* in the southern United States results in progeny with dominant traits contributing to their wide distribution [114].

In plants, hybridization enhances adaptability to challenging conditions, producing offspring that are more resilient to stress, develop, adapt, and survive better than their parents [115]. Hybridization typically occurs at the boundaries of neighboring populations in different biological zones, supported by dispersal flights and the spread of the hybrid genome during shared periods [116]. This results in new phenotypes adapted to their natural habitats, especially in restricted and mixed areas where interbreeding is common [117].

Gene connections between local populations, including parasite interactions between herbivorous arthropods, have been documented [118]. This interaction can involve an invading species and a native species or two native species [119]. The combination of genes through hybridization produces unique individuals distinct from their ancestors, increasing the likelihood of adaptation and survival for parental populations [120]. Reproductive isolation, including prezygotic and postzygotic barriers, plays a crucial role. Prezygotic isolation factors, such as morphology, diet, breeding season, location, and ecology, determine compatibility for mating and fertilization [121]. Postzygotic hurdles include gamete isolation, developmental problems, early embryonic mortality, infertility in hybrids, and challenges in acclimatization and adaptation to hybrid life.

In termite colonies, limited interaction between soldiers and workers from different colonies has been observed, leading to resource hostility and interspecies rivalry

[122]. Termites with proliferating wings, called alates, compete with other species [123]. Many organisms, including termites like *R. verginicus* and Zootermopsis nevadensis, undergo hybridization; the timing of the dispersal flight season is crucial for the outcomes of these hybridization events [124].

2.10 Swarming

Adult winged termites, disperse at fixed distances from established colonies to establish termite colonies [125]. After molting, alates spend some time in specific chambers outside the nest until they take to the skies, typically signaled by high humidity combined with climatic conditions unique to their species [126]. Alates are attracted to light during mating flights, are protected by soldiers at exit holes, and rely on the wind to move, mate, shed wings, and establish new colonies [127].

Copulation and sperm storage take place in a nuptial chamber created by couples and sealed with feces and saliva [128]. Termite colonies then produce worker and soldier castes from young nymphs, which build complex social structures based on the demands of the colony, the environment and nutrition. Given the differences in colony sizes between termite groups, the transition from reproductive care to worker and soldier tasks is critical to understanding termite colony dynamics [129].

2.11 Organization of Insect Mitochondrial Genome

The mitochondrial genomes of insects have been more extensively studied than those of any other group of invertebrates. So far, the complete mitochondrial genomes of 15 insect species have been sequenced. Circular, double-stranded DNA molecules, these genomes range in size from 14,503 bp to 19,517 bp [130] (Fig. 2.12).

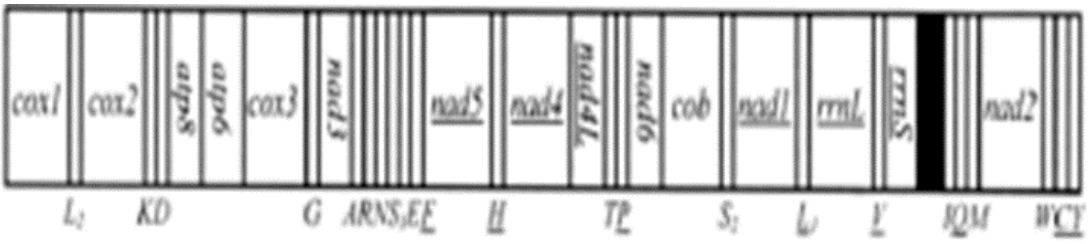

FIGURE 2.13: Structure of the Mitochondrial Genome of Insects [120]

Standard techniques were used to barcode a total of 60,273 insects [131]. With sterile forceps, a larger specimen's leg was extracted and put in a well that had been loaded with 30 μl of 95% ethanol. Vouchers were retrieved following DNA extraction from smaller versions already mounted on plates and ready for examinations [132].

At the Canadian Centre for DNA Barcoding (CCDB), DNA extraction, PCR amplification, and sequencing were carried out in accordance with recognized methods. Sanger sequencing was used to sequence 73% of the specimens, and SMRT sequencing on a Sequel platform was used to examine the remaining specimens. The Big Dye Terminator Cycle Sequencing Kit (v3.1) was used for Sanger sequencing on an Applied Biosystems 3730XL DNA Analyzer [133].

Before being submitted to BOLD, sequences were put together, aligned, and modified via CodonCode Aligner. SMRT sequencing according to Hebert et al. (2018)'s guidelines. Following sequence trimming, quality filtering, de-replication, and identification, the resulting sequences were uploaded to mBRAVE for the creation of operational taxonomic units (OTUs) and modification [134]. After editing, the sequences were exported to BOLD for reference library construction and BIN assignment. The "DS-INSCTPAK" collection contains the specimen records, sequencing data, electropherograms, and primer details. Every DNA extract is kept in the CBG's DNA archive facility [135].

2.12 Termites as Edible and Medicinal Values

Complex interactions, including harmonic and antagonistic ties, between humans and other animals have been documented from ancient times [136]. This condition is exemplified by termites, which can have substantial economic harm in both rural and urban areas. In addition, they are used as a source of popular medicine and as sustenance for humans and livestock by people from all over the world. Given that insects make up the largest group of species in nature and are therefore a major source of food for humans, Significant biomass is not surprising [137]. Insects are considered as essential natural resources and constitute a staple of meals for humans and other animals [138].

They are also frequently utilised as a source of pharmaceuticals [139]. The history of human nutrition in Africa, Asia, and Latin America has been significantly influenced by entomophagy, or the practice of consuming insects [140].

Insects are also used extensively by humans for medical purposes, including entomotherapy. The widespread negative image of termites can occasionally conceal their ecological significance as intermediaries in the breakdown of organic plant matter and as agents influencing soil formation, energy, and nutrient flows—particularly in tropical forests [141]. It is important to note that termites are frequently employed insects in traditional popular medicine from a utilitarian standpoint [142].

They are used to treat many different human diseases, such as whooping cough, sinusitis, tonsillitis, influenza, bronchitis, asthma, and hoarseness [143]. Furthermore, because they are considered a nonconventional meal with significant economic and social value, these creatures have traditionally been an essential source of food that may help improve human diets, especially for those who suffer from malnutrition owing to a lack of protein [144].

In many parts of the world, they have been ingested for centuries; in recent years, this practice has become more and more common [145]. Although termites are

beneficial to humans and livestock in terms of nutrition and medicine, their most well-known function is that of a plague, with very little practical use [146].

TABLE 2.3: Termite species used as food or feed [147]

Species	**Use**	**Country/Region**
Hodotermes mossambicus	Feed	Botswana
Macrotermes michaelseni	Food	Malawi
Kalotermes flavicollis	Food	Brazil, Thailand
Coptotermes formosanus	Food	China
Microhodotermes viator	Food	South Africa
Reticulitermes tibialis	Food	Mexico
Cubitermes atrox	Food	Indonesia
Labiotermes labralis	Food	Colombia
Macrotermes acrocephalus	Food	China
Reticulitermes flavipes	Food	Thailand
Macrotermes barneyi	Food	China
Macrotermes bellicosus	Food	Many African countries
Macrotermes falciger	Food	Southern Africa
Macrotermes gabonensis	Food	Congo
Macrotermes annandalei	Food	China
Macrotermes herus	Food	Tanzania
Macrotermes lilljeborgi	Food	West Africa
Macrotermes gilvus	Food	Southeast Asia
Macrotermes natalensis	Food	Central Africa
Macrotermes nobilis	Food	West Central Africa
Macrotermes renouxi	Food	Cameroon
Macrotermes subhyalinus	Food	Sub-Saharan Africa
Macrotermes vitrialatus	Food	Zambia
Nasutitermes surinamensis	Food	Venezuela
Odontotermes feae	Food	India
Microcerotermes dubius	Food	Malaysia
Microcerotermes serrula	Food	Malaysia

Table 2.3 continued from previous page

Species	**Use**	**Country/Region**
Nasutitermes corniger	Food	Venezuela
Nasutitermes ephratae	Food	Venezuela
Nasutitermes macrocephalus	Food	Venezuela
Odontotermes badius	Food	Southern Africa
Odontotermes capensis	Food	South Africa
Macrotermes muelleri	Food	West Central Africa
Odontotermes formosanus	Food	Asia
Odontotermes kibarensis	Food	Uganda
Odontotermes yunnanensis	Food	China
Pseudacanthotermes militaris	Food	East and Central Africa
Pseudacanthotermes spiniger	Food	Central and East Africa
Syntermes spinosus	Food	South America
Syntermes aculeosus	Food	South America
Syntermes parallelus	Food	Colombia
Syntermes tanygnathus	Food	Colombia
Termes fatalis	Food	Guyana, Indonesia

Termites serve a significant purpose, as evidenced by their usage as a medicinal resource. Evidence of the separated products' antibacterial action from these creatures have been documented, including peptides that had antifungal and antibacterial properties that were extracted from *Pseudocanthotermes spiniger*, such as termicine and espinigerine [148].

Based on the data that have been obtained, Nasutitermes macrocephalus (Silvestri, 1903) is widely utilised to treat a wide range of ailments. Antimicrobial peptide synthesis is a capability of Nasutitermes termites, according to research on their molecular biology [149].In this regard, termites suggest that these creatures should be further studied from a pharmacological standpoint because to their extensive usage in traditional medicinal systems globally.

Termites' medicinal activity may help to elevate these creatures, whose detrimental traits linked to the harm they inflict on people are frequently emphasised. Nasutitermes corniger and its nest are potentially useful natural materials for antibacterial treatment, according to Chaves et al. [150]. From a wider angle, insects have great promise as a source of compounds with medical use [151].

TABLE 2.4: Termite species used in traditional folk medicines [152]

Termite Species/-Family	**Use**	**Treated Diseases**	**Country**
Hodotermes mossambicus (Hagen)	Child malnutrition	Not specified	Zambia
Macrotermes nigeriensis (Sjoestedt)	Various (medicine, rituals)	Not specified	Nigeria
Macrotermes bellicosus (Smeathman)	Wound treatment	Suturing wounds	Somalia
Microcerotermes exiguus (Hagen)	Respiratory	Asthma, bronchitis, influenza, whooping cough	Brazil
Nasutitermes corniger (Motschulsky)	Respiratory and Antibacterial	Asthma, cough, flu, sore throat	Brazil
Nasutitermes macrocephalus (Silvestri)	Respiratory	Asthma, coughs, sinusitis, sore throat	Brazil
Pseudacanthotermes spiniger (Sjoestedt)	Antibacterial and Antifungal	Not specified	Brazil
Odontotermes feae (Wasmann)	Respiratory	Asthma	India
Odontotermes formosanus (Shiraki)	Various (medicine and health)	Ulcers, pain relief, rheumatism	India

2.13 DNA Barcoding

DNA barcoding was first described by Hebert et al. (2003). It is based on amplification of a 648-base pair (bp) section of the mitochondrial gene *cytochrome c oxidase subunit 1* (*COI*) using certain primers [153]. This method leverages genetic diversity within this region to swiftly identify and classify organisms. This makes it possible to identify a variety of biological materials quickly and accurately [154]. Following that, the Barcode of Life initiative was put up to promote DNA barcoding as an international standard for eukaryotic sequence-based identification.

The Consortium for the Barcode of Life (CBOL) was formally founded in 2004 with the goal of generating a comprehensive DNA barcode library and standardizing DNA barcoding technology [155]. The standard barcodes for plants are the two parts of chloroplast DNA known as maturase K and mature-bisphosphate carboxylase; other regions are called supplementary barcodes. The mitochondrial genome contains a variety of markers that are used to differentiate between different species. The *COI* region has proven to have enough data to categorize organisms down to the species level [156]. This study primarily focused on the molecular characterization of different species of Passeriformes from various regions of Pakistan using the *COI* gene [157].

The *COI* region of the mitochondrial genome is a short segment (∼600-800 base pairs) located at the 5' end of the mitochondrial gene. It serves as a DNA barcode for identifying animal species [158]. This region is highly effective for extracting information from small samples because each cell contains a large number of mitochondrial copies [159]. A eukaryotic cell harbors over 1,000 mitochondria, each containing approximately 10 copies of the mitochondrial genome. This contrasts sharply with the nuclear genome, suggesting that a cell may possess more than 10,000 mitochondrial genome copies [160].

Because of this, it is easier to recover enough mitochondrial DNA (mtDNA) even from small amounts of tissue, including non-invasive samples or materials that are dried or decayed in the field. Therefore, a small quantity of mitochondrial DNA sequence obtained from decayed, deceased, or denatured specimens is adequate for

amplifying the *COI* sequence to differentiate between species [161]. The *COI* gene can be efficiently t amplified with universal primers and Polymerase Chain Reaction (PCR) that are compatible across diverse taxa [162]. In DNA-based species identification systems, standardized techniques for DNA extraction, gene amplification (PCR), and sequencing are employed to identify any unknown organism using an extensive collection of all Earth's known creatures [163].

With a short, homogeneous part of the genome sequenced, the molecular barcoding approach rapidly and accurately identifies species by focusing on the mitochondrial gene *cytochrome c oxidase subunit 1* (*COI*) [164]. Insects may be identified using this DNA-based method at any stage of development, from egg to adult. Complementing traditional morphological taxonomy. In agricultural settings, DNA barcoding enables real-time detection of invasive species through regular monitoring, facilitating timely implementation of control and prevention measures before populations become uncontrollable [165]. DNA barcoding also establishes a standardized identification system for commercially used biocontrol agents, ensuring accuracy in species identification and consistency among agents marketed as belonging to the same species [166]. Insect tissue samples are obtained for DNA sequencing in order to set up a DNA barcoding system, and morphological vouchers in the form of finely detailed pictures of original specimens are used. DNA barcode sequences are safely kept in a dedicated database along with these photos and the related identification and collection data [167]. A reference library of *COI* barcode sequences associated with names and photos of known species is produced as a result of this approach. By cross-referencing unknown specimens' *COI* sequences with the database, it is possible to identify them rapidly. Building a DNA barcoding system requires having an extensive reference library with all known sequences of *cytochrome c oxidase subunit 1* [168]. DNA barcode data can be found universally in the Barcode of Life Data Systems (BOLD) web-based platform, which also offers data analysis tools. Strict uniformity of methodology and data management procedures is essential for the long-term viability of DNA barcoding projects [169].

In Pakistan, which spans diverse biomes and intersects major biogeographic realms,

its varied physiography and climate foster a rich fauna diversity. Despite hosting over 5,000 insect species, comprehensive taxonomic assessments have been limited by a shortage of taxonomists and the presence of many undescribed species [170]. Traditional morphological approaches face challenges in scaling up to accurately estimate species diversity in Pakistan, a global issue [171].

To address these challenges, DNA barcoding was employed to assess insect diversity across Pakistan. Between 2010 and 2019, specimens from 1,858 sites were analyzed for sequence variation in the cytochrome c oxidase 1 (*COI*) gene's 658 bp barcode region [172].

Through sequence analysis, we were able to classify about 49,000 specimens into 6,590 Barcode Index Numbers (BINs), which are proxies for species identification. For the bulk (88%) of these BINs, sample photos were available in the Barcode of Life Data System (BOLD) [144]. Each BIN was allocated to one of 19 orders by combining morphological evaluations and barcode comparisons on BOLD; nearly all (99.8%) of them were also grouped into one of 362 families. However, just 40% of the BINs (1,375) could be identified at the genus level, and 21% (1,364) could be identified at the species level [173].

Coleoptera, Diptera, Hemiptera, Hymenoptera, and Lepidoptera were the five orders that accounted for the majority of specimens and BINs (92%) [174]. currently, Pakistan stands as the sole nation with over half of the world's BINs (59%) while reports of BINs have also come from Bangladesh (13%), India (12%), and China (8%). As the first comprehensive DNA barcode assessment of insect fauna in any South Asian country, this work provides the groundwork for a thorough inventory of Pakistan's insect species. It expands the global DNA barcode reference library with important new data [175].

2.14 Applications of DNA Barcoding

Providing a shared pool of DNA sequences for the community to utilize for taxonomic classification, identification, and organism discrimination is the aim of

DNA barcoding. [176]. This technique identifies and differentiates an organism from specific species, variations, or even inter-varieties using a brief genetic marker found in its DNA. DNA barcoding uses an existing categorization to identify unknown samples, in contrast to molecular phylogeny, which finds patterns of evolutionary relationships [177].

The mitochondrial gene cytochrome c oxidase I (*COI*) has been identified as the cornerstone of an international system of animal bio-identification. DNA barcoding for plants uses internal transcribed spacer (ITS) sequences from the nuclear ribosomal cistron and chloroplast sequences. [178]. DNA barcoding is based on a simple principle: identify one or a few DNA regions that can distinguish between the majority of Earthly species, and sequence these regions from a range of samples to create a comprehensive reference library containing all known species. DNA barcoding offers a novel method to biodiversity studies by utilizing both existing molecular biology techniques and recent advances in bioinformatics [179]. Numerous uses for this technique exist, such as safeguarding biodiversity from harm caused by alterations in the ecosystem and the illicit trade in wildlife [180].

Using a section of (*COI*) has shown extremely effective at differentiating species in a variety of groups like fish, insects and birds, first DNA barcoding investigations were conducted on animals [181]. A brief DNA sequence found in the *COI* gene has recently been used to propose and show on a wide scale, the identification of animal biodiversity using molecular markers [182]. These ”DNA barcodes” provide a useful, standardized method for species-level identification, supporting ecological and life cycle research, forensic analysis, and biodiversity assessment [183]. Kress and Hebert et al. have made strong cases for the use of DNA barcoding as a useful foundation for specimen identification [184].

A specific region of the mitochondrial *COI* gene was selected for animal identification when DNA barcoding was first proposed [185], and later pilot investigations have shown that it is broadly applicable in animal systems [186]. Species-level molecular investigations have shown that the internal transcribed spacer (ITS) region of the nuclear ribosomal cistron (18S-5.8S-26S) is the most commonly sequenced locus in plants [187]. This area has been proposed as a possible plant

barcode locus and is extensively utilized by fungi and photosynthetic eukaryotes (apart from ferns) [188]. Many phylogenetic investigations using ITS have proven species-level differentiation and simplicity of use because of the quantity of sequence data currently available (36,000 angiosperm sequences were in Gene Bank as of December 2004 [189].

Because of their extreme variability, insects especially those belonging to holometabolous orders—have been the subject of multiple attempts to use molecular markers to associate life stages [190]. Apart from the characteristics of standard non-barcode molecular markers, DNA barcoding provides benefits including primer universality, information accumulation over a broad spectrum of taxonomic groups, and interaction with taxonomy [191]. utilising non-barcode molecular markers, as investigated by Kathirithamby et al. (2010) [192]. The investigation on ecologically significant insect phenomena, like aphid host plant alternation and the high sexual dimorphism and heterotrophic heteronomy of Strepsiptera, may benefit from these benefits [193]. Useful for identifying prospective biological control agent candidates and evaluating their dangers is DNA barcoding [194]. Growing international trade has made it more likely that non-native pests may invade, which emphasises the need of biological control agents that either consume or infest pests [195]. Long-term feeding studies have historically been necessary for the purpose of finding, vetting, and assessing the dangers associated with these drugs [196]. However, using DNA barcoding to identify agents based on the contents of their stomachs can streamline and optimise this process (Symondson) [197].

Because higher plants evolve their cytochrome c oxidase I genes at a far slower rate than animal species, the *COI* sequence is inappropriate for the majority of plant species [198]. A better genomic area for blooming plants' DNA barcoding—or that of the broader class of land plants—was found through experiments. In 2005, some researchers advised using the nuclear internal transcribed spacer area and the plastid trnH-psbA intergenic spacer, while others recommended regions like matK [199]. A sizable consortium of experts in plant DNA barcoding suggested in 2009 that the standard plant barcode be derived from the combination of two chloroplast genes, matK and rbcL [200]. It is expected that a *COI*-based identification system

will yield improved taxonomic resolution when compared to morphological studies; nonetheless, guidelines will be needed to address such concerns [201]. By storing their knowledge before they leave the field, creating *COI* profiles can assist lessen the loss of morphological taxonomists [202]. The identification of adult male and female insects can be confirmed by DNA barcoding, which can also provide insight into the basic ecology of vector insects, including their habitats and larval diets [203].

Chapter 3

Materials and Methods

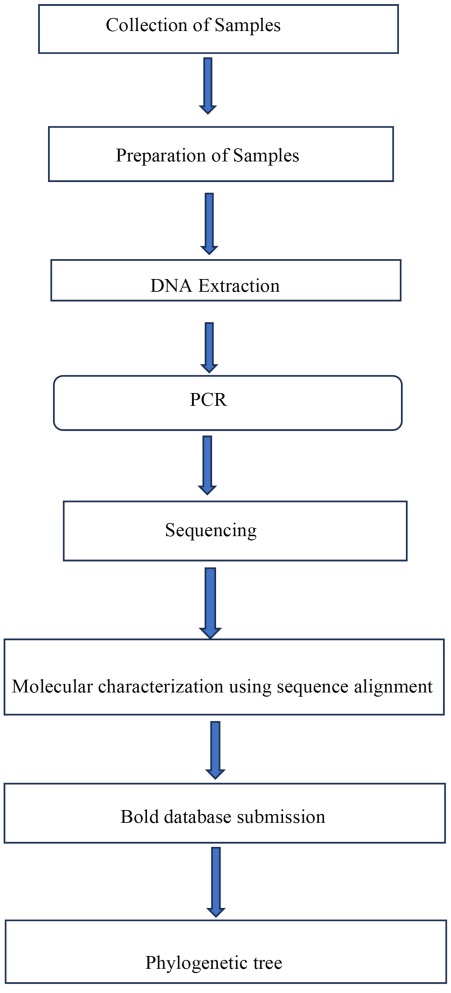

FIGURE 3.1: Methodology Flow Chart

DNA barcoding of termites involves using a specific region of DNA to identify and differentiate termite species.

The standard region used for DNA barcoding in animals is the *cytochrome c oxidase subunit 1* (*COI*) gene. Here's a detailed methodology for DNA barcoding of termites:

3.1 Methodology

DNA barcoding of termites involves using a specific region of DNA to identify and differentiate termite species.

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3.1.1 List of Equipment

Vortex, sample storage bottles, PCR thermocycler, Microscope, Shaker, centrifuge, homogenizer.

3.1.2 List of Apparatus

Petri dishes, micro-pipette, glass rods, Eppendorf, Reagent bottles, micro-pipette tips, filter paper, para-film tape, gloves, test tubes.

3.1.3 List of Chemicals

Agarose Gel, Elusion Buffer, ChI Buffer, lysis buffer, MerC Solution, Wash Buffers A&B, 70% Alchohol, Ethanol, TBE buffer, Ethedium Bromide.

3.2 Sample Collection

The specified location is situated at an elevation of 737 meters (2,418 feet) above sea level. The residential area with the infestation of termites was identified by visual inspection. Termites were collected in September, which, while slightly outside the peak swarming season of late spring to early summer, still provided valuable insights.

3.2.1 Exterior Inspection

Foundation, walls, and external wood structures for signs of termite damage were checked by looking for mud tubes, hollow-sounding wood, blistering in wood flooring, and visible swarms of termites.

3.2.2 Interior Inspection

Once above-mentioned signs were confirmed then baseboards, walls, wooden furniture inside the house for similar signs of termite activity were also inspected. Termite specimens from various locations of area Tehsil Hattian Bala, District Jhelum, valley Muzaffarabad, Azad Kashmir were collected in glass bottles.

Table 3.1: Tags for labelling of termite sample

No.	Samples	Area	Sample ID
1	Sample 1	Muzaffarabad	H3
2	Sample 2	Muzaffarabad	Che1
3	Sample 3	Kotli	1
4	Sample 4	Kotli	2
5	Sample 5	Jhelum valley	J1
6	Sample 6	Jhelum valley	J2

Bottles were properly labelled with collection sites, date and area. Samples were preserved in 95-100% ethanol/freeze them at -20^{o}C or -80^{o}C until DNA extraction.

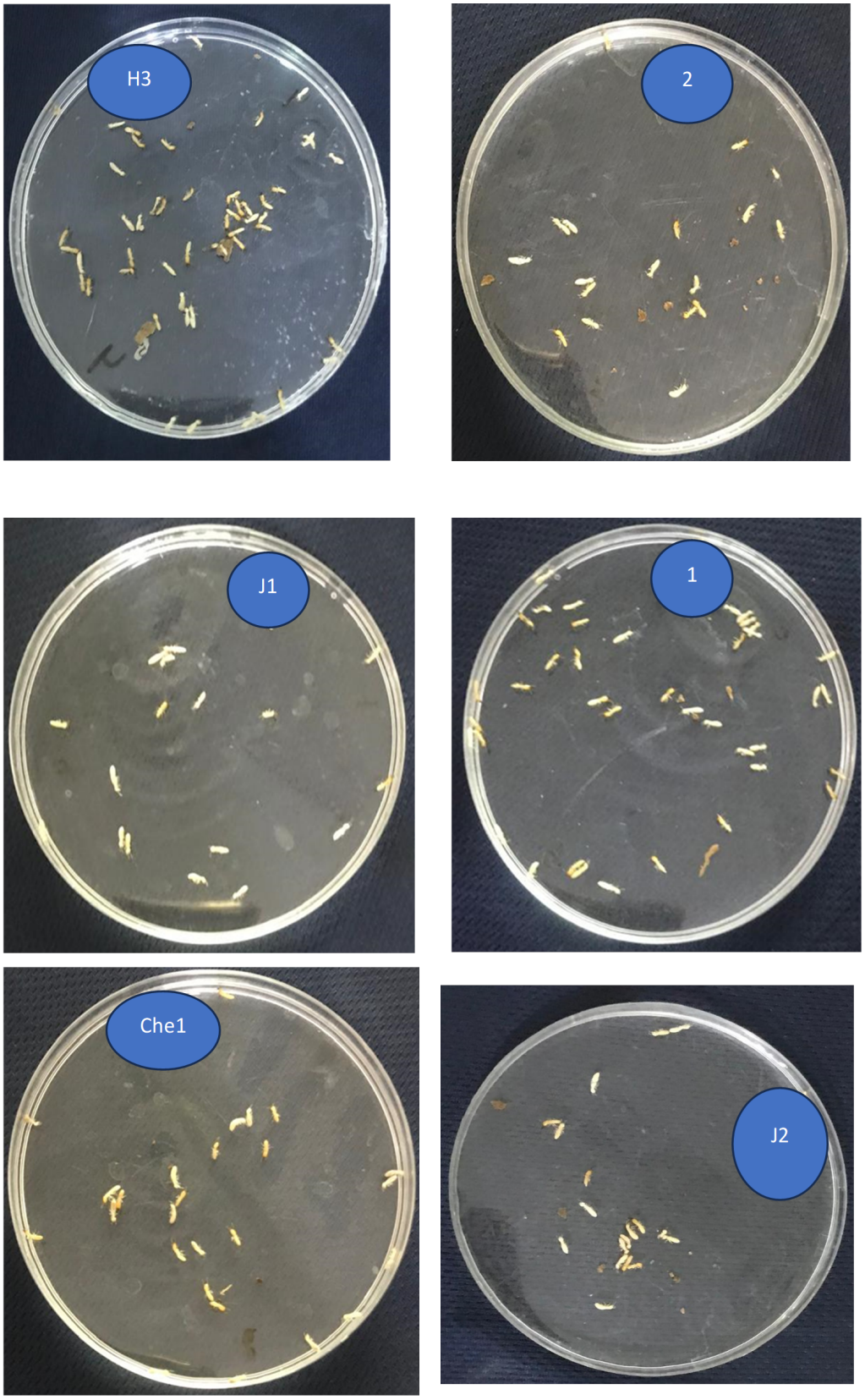

FIGURE 3.2: Specimen of termites used for Sequencing

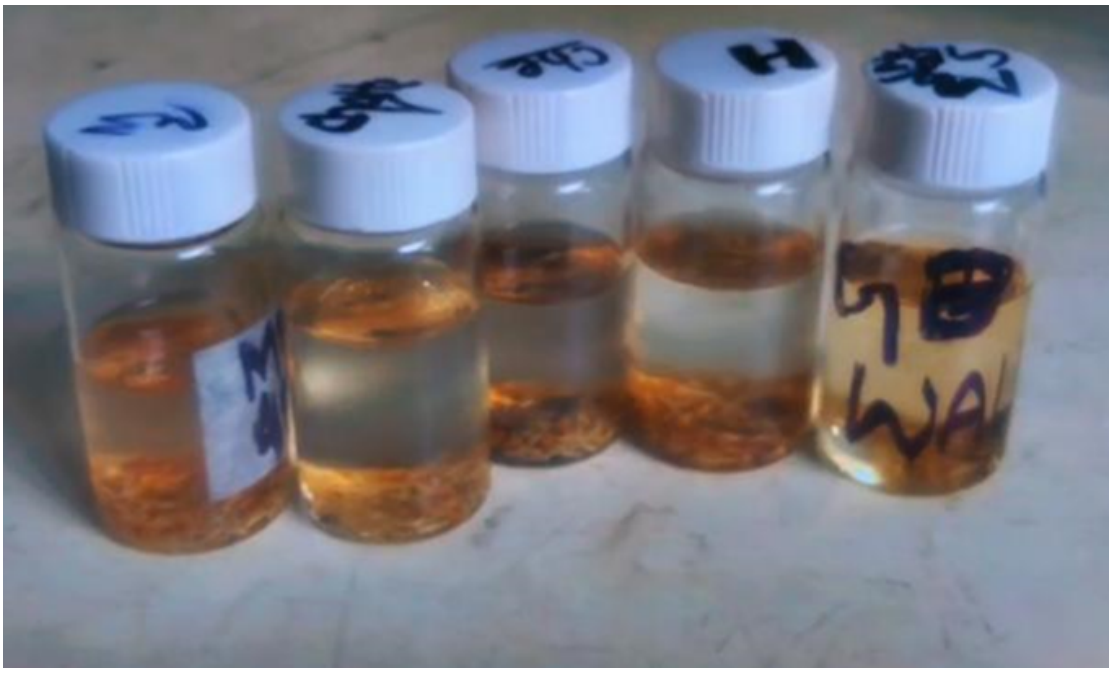

FIGURE 3.3: Termites specimen

3.3 DNA Extraction

Termites were washed well with distilled water. After that, 5-6 termites were picked using a sterilized wire loop and thoroughly crushed with a tissue homogenizer in an Eppendorf tube.500 μL of pre-warmed lysis buffer (60-65^{o}C) was added, followed by 10 μL of Reagent D, 20 μL of Reagent K, and 1.6 μL of MerC Solution.The sample was incubated at 60-65^{o}C for 1-2 hours for bacterial samples, and overnight for tissue samples.700 μL of ChI Buffer was added, and the mixture was centrifuged at 13,000 rpm for 10 minutes.

The aqueous layer was collected in a new tube and 950 μL of Wash Buffer A was added. After another centrifugation at 13,000 rpm for 15 minutes, the supernatant was discarded, and 500 μL of Wash Buffer B was added to the pellet. The sample was then centrifuged at 8,000 rpm for 5-10 minutes. The supernatant was discarded, the pellet was air-dried, and 40-45 μL of Elution Buffer was added. The pellet was incubated for 10-15 minutes at 60^{o}C to dissolve. The DNA was then stored at -20^{o}C.

3.4 Quantification of DNA

The extracted DNA was quantified using a Nanodrop spectrophotometer to determine the amount of DNA in the samples. As organic compounds exhibit characteristic absorption, the nitrogenous bases in DNA show strong absorption at a wavelength of 260 nm.

Table 3.2: Quantification of DNA

S. No.	Sample ID	ng/μL	260/280
1	H3	279	1.77
2	Che1	504	1.76
3	1	261	1.67
4	2	249	1.96
5	J1	376	1.95
6	J2	312	1.21

3.5 PCR Amplification

3.5.1 Primer Selection

Universal *COI* primers for insects, given below was used:

F' ACGAAATCCACAACCCAACA

R' GTGTGGCGTCTGTTTTCACT

Following PCR reaction mixture was used

- Template DNA: 1-2 μL
- Forward primer (10 μM): 1 μL
- Reverse primer (10 μM): 1 μL
- PCR buffer (10X): 2.5 μL

- dNTP mix (10 mM each): 0.5 μL
- Taq DNA polymerase: 0.25 μL
- MgCl2 (25 mM): 1.5 μL
- Nuclease-free water: up to 25 μL
- Total Master mix: 19.7 μL

3.5.2 PCR Cycling Conditions

- Initial denaturation: 94^{o}C for 3 min, 35 cycles of:
- Denaturation: 94^{o}C for 30 sec
- Annealing: 48-52^{o}C for 30 sec
- Extension: 72^{o}C for 1 min
- Final extension: 72^{o}C for 5 min
- Hold: 4^{o}C

3.6 Gel Electrophoresis

Purified PCR products were run on 1.5% Agarose gel. 30ml of 1.5% gel was prepared by adding 0.45g of Agarose in 1× TBE buffer. Solution was then heating for 1 minute in microwave and cooled down a bit before adding 5μl Ethidium Bromide. 2μl PCR purified Samples were then loaded on the gel and Ladder was used for recognition.

3.7 Sequencing

The PCR products were subjected to Sanger sequencing by sending the samples to a sequencing facility.

3.8 Molecular Characterization Using Sequence Alignment

After sequencing, the data quality check was performed and low-quality ends and ambiguous bases from the sequences were removed. The edited sequences were then aligned using the Basic Local Alignment Search Tool (BLAST) with sequences of the same or related genera retrieved from the nucleotide database (PubMed) of the National Center for Biotechnology Information (NCBI).

3.9 Bold Database Submission

The aligned sequences of DNA were submitted to the DNA barcode databases BOLD (Barcode of Life Data Systems) to identify the termite species. The Barcode of Life Data System (BOLD) is a comprehensive online platform designed to support the generation and application of DNA barcodes. It provides a repository for barcode records, a suite of tools for analysing barcode data, and a reference library for species identification [204].

3.10 Phylogenetic Analysis

A phylogenetic tree was constructed using the neighbour-joining method, with evolutionary distances computed using the p-distance method and a bootstrap consensus of 1000 replicates (Tamura and Nei). The constructed phylogenetic tree was visualized using a tree viewer program [205].

Chapter 4

Results

4.1 Genomic DNA (gDNA) Extraction

Genomic DNA (gDNA) was successfully extracted from six samples of termites of district jahalam valley, and Muzafarabad AJK, using the phenol-chloroform protocol. From crushed sample, a portion (*COI* gene) of the mitochondrial genome from its first half was amplified and using universal primer *COX1*_F and *COX1*_R was sequenced.

The same primers have been used to amplify *COI* region in different arthropods, mentioned in literature. Extracted gDNA was confirmed using agarose gel (1.5%) on gel electrophoresis apparatus.

4.2 DNA Quantification

DNA quality was assessed by measuring A260/A280 ratio was assessed which ranges from 1.76 to 1.21 ratios for genomic DNA of all extracted from three replicates for each sample.

The wavelength ratio A260 was measured for purified DNA quantification. For genomic DNA the quantity ratio ranges from 504 to 312 ng /μL of extracted genomic DNA samples.

FIGURE 4.1: Gel electrophoresis for gradient PCR

4.3 PCR Amplification

Genomic DNA (gDNA) was successfully extracted from six samples of Termites of district Jahalam Valley, and Muzafarabad AJK, Pakistan.

Photographs of gels with good quality DNA bands were saved with proper reference to be amplified by PCR. The PCR reaction for the extracted DNA of samples was carried out by using universal primer as mentioned in chapter 3.

The optimum annealing temperature was found to be 48-52^{o}C to amplify *CO1* gene. In analysis of samples, 677 bp of *CO1* gene was amplified.

The optimum annealing temperature was found to be 48-52^{o}C to amplify *CO1* gene. In analysis of samples, 677 bp of *CO1* gene was amplified. The representative gel results of 650bp segment are shown in the Figure 4.2. The samples from the Kotli were not amplified and thus were excluded for further processing.

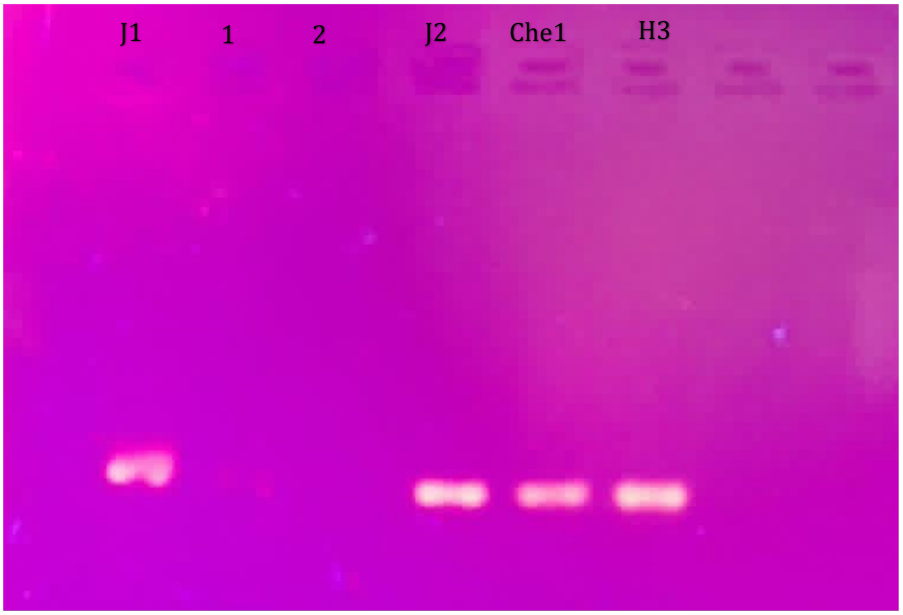

FIGURE 4.2: Gel electrophoresis for DNA amplification analysis

4.4 DNA Sequencing

The Sanger's sequencing of the PCR products was carried from Macrogen Korea, and sequences were then converted into FASTA format.

These sequences of samples were further analyzed for the molecular identification along with phylogenetic analysis.

Following sequences were obtained:

1. >H3

NNNNNTTGGGCACNATGATACTGAAGCTACGAATACTCAGACTTCACAAAACTAGAGTTTGACTCATACATAGTCC
CACAAGAAGAGCAACAAACAAGAACATTTCGACTGCTAGACACAGACAACCGAGTCGTCCTACCAATAAACTCAC
CAATCCGATTAATCGTTACAGCAGCAGACGTATTACACTCATGAACCATTCCAAGACTAGGAGTGAAAACAGACGC
CACACACG

2. >J1

NNNNNTNGGGNACAATGNTACTGAAGCTACGAATACTCAGACTTCACAAAACTAGAGTTTGACTCATACATAGTC
CCACAAGAAGAGCAACAAACAAGAACATTTCGACTGCTAGACACAGACAACCGAGTCGTCCTACCAATAAACTCA
CCAATCCGATTAATCGTTACAGCAGCAGACGTATTACACTCATGAACCATTCCAAGACTAGGAGTGAAAACAGACG
CCACACACG

3. >J2

NNNNNTNGGGCNCNNTGATACTGAAGCTACGAATACTCAGACTTCACAAAACTAGAGTTTGACTCATACATAGTC
CCACAAGAAGAGCAACAAACAAGAACATTTCGACTGCTAGACACAGACAACCGAGTCGTCCTACCAATAAACTCA
CCAATCCGATTAATCGTTACAGCAGCAGACGTATTACACTCATGAACCATTCCAAGACTAGGAGTGAAAACAGACG
CCACACAA

4. >Che1

NNNNNNNGGGCACNATGATACTGAAGCTACGAATACTCAGACTTCACAAAACTAGAGTTTGACTCATACATAGTC
CCACAAGAAGAGCAACAAACAAGAACATTTCGACTGCTAGACACAGACAACCGAGTCGTCCTACCAATAAACTCA
CCAATCCGATTAATCGTTACAGCAGCAGACGTATTACACTCATGAACCATTCCAAGACTAGGAGTGAAAACAGACG
CCACACAA

4.5 Molecular Characterization Using Sequence Alignment

The sequence analysis of four samples using BLAST revealed that the all sample of termites showed similarity with *Heterotermes gertrudae* species. The two samples collected from Muzaffarabad AJK labeled as H3 has percent identity 97.81% (Fig. 4.3) and Che 1 with percent similarity 97.79 (Fig: 4.4) with query coverage 96% for both. Jhelum valley sample J1 with sequence similarity of 98.18%.

Jhelum valley sample J1 with sequence similarity of 98.18% (Fig: 4.5) and J2 98.62% (Fig: 4.6) with query coverage 93% and 92% respectively. BOLD database submission showed none of the similarity with the available BINS indicating that these sequences have not been submitted.

BOLD database submission showed none of the similarity with the available BINS indicating that these sequences have not been submitted from another part of Pakistan (Table 4.1).

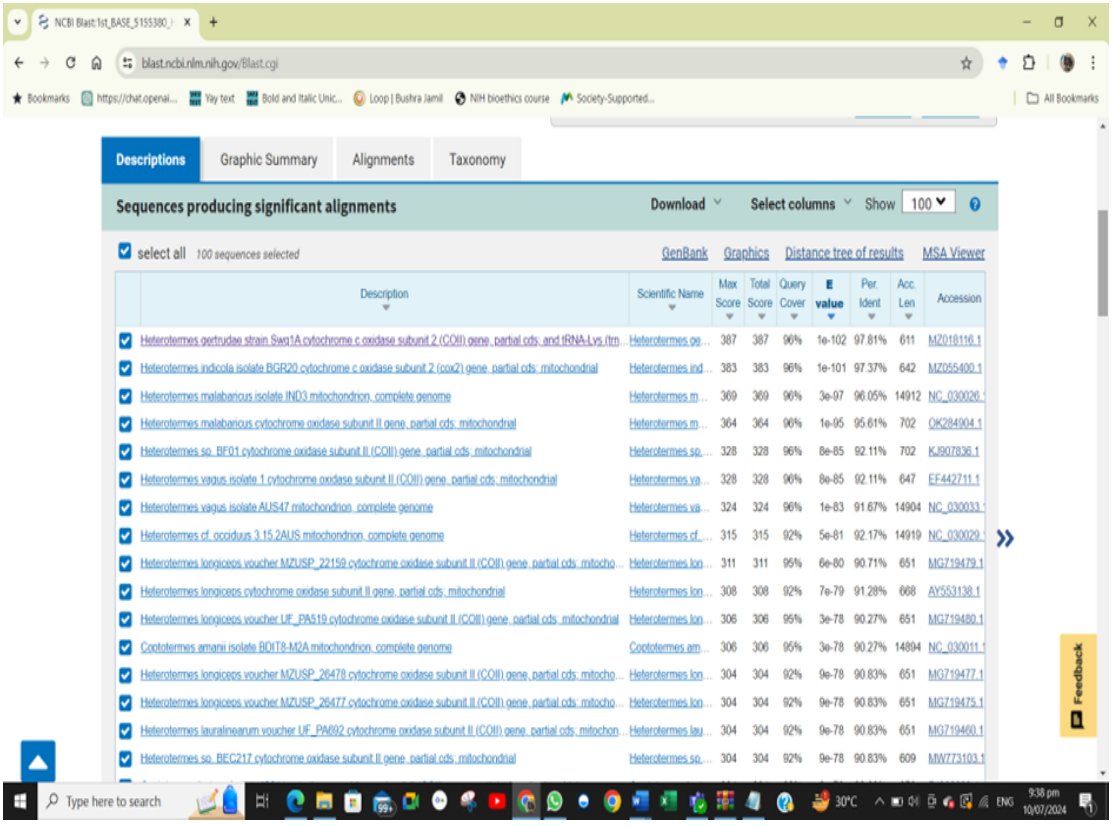

FIGURE 4.3: BLAST results of sample H3

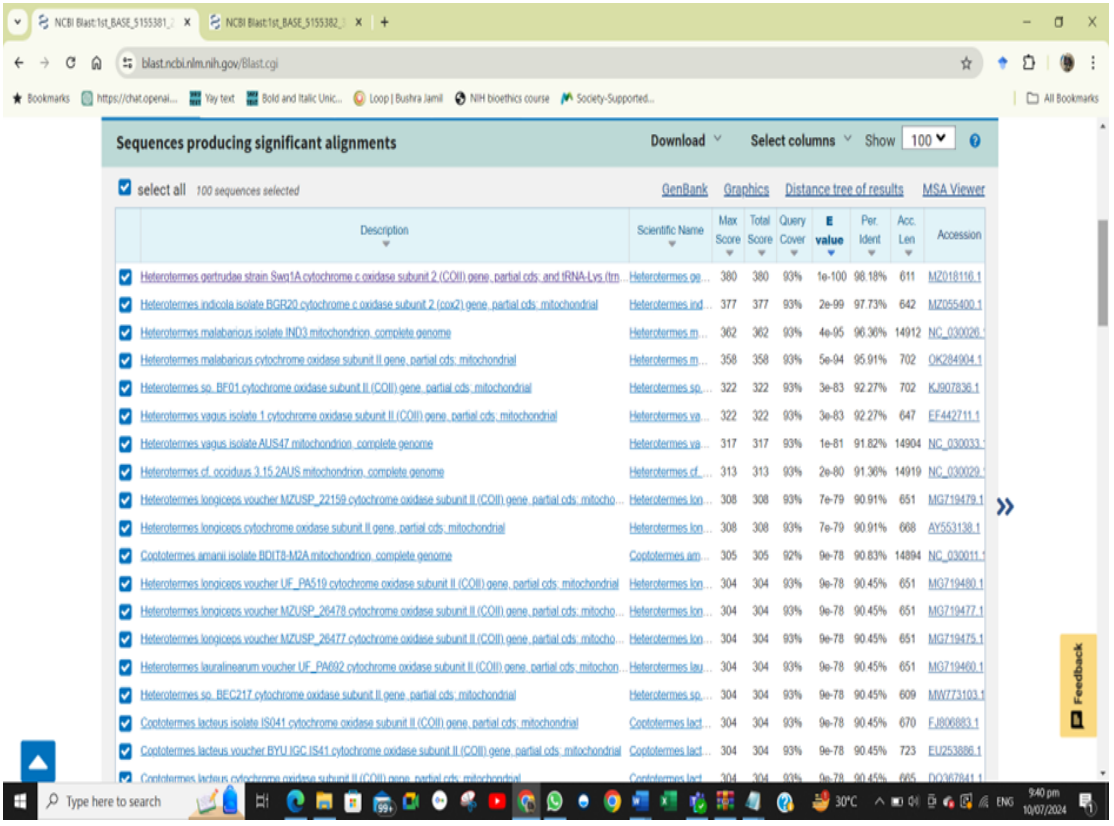

FIGURE 4.4: BLAST results of sample J1

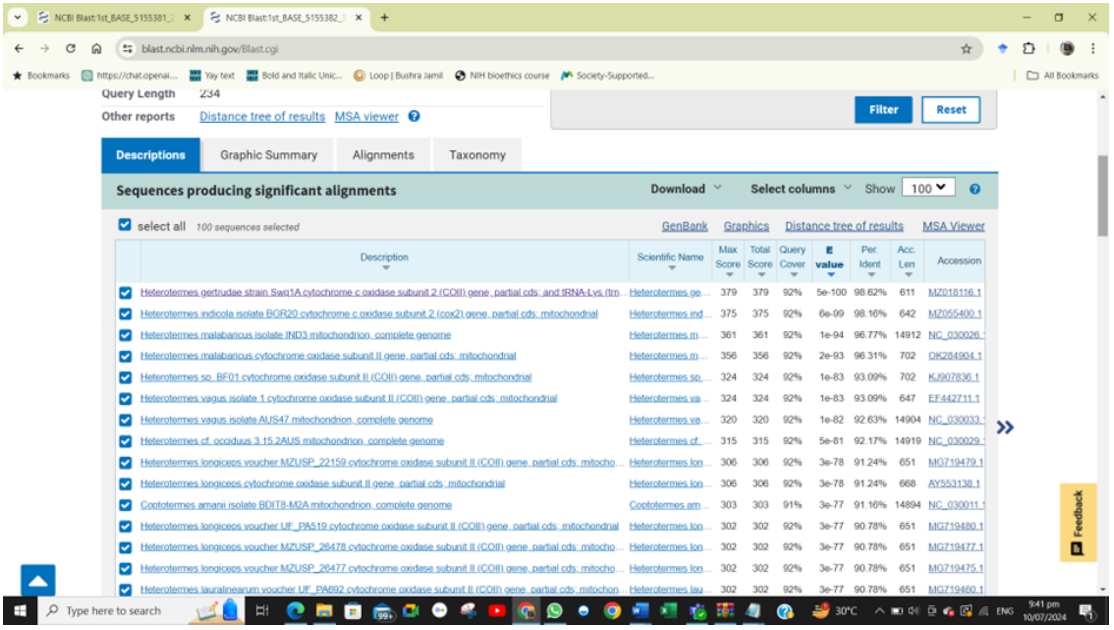

FIGURE 4.5: BLAST results of sample J2

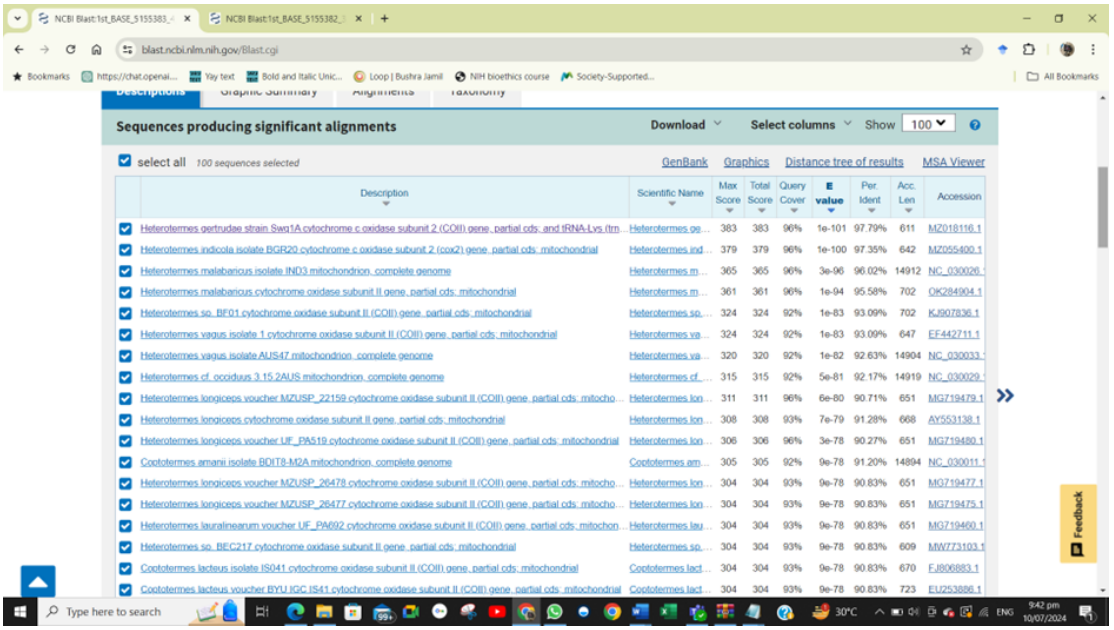

FIGURE 4.6: BLAST results of sample Che1

TABLE 4.1: Molecular characterization of sequenced Samples

Sr no.	Sample id	Scientific name	Query cover	Percentage identity
1	1	Che1	96%	97.79%
2	3	H3	96%	97.81%
3	4	J1	93%	98.18 %
4	5	J2	92%	98.62%

4.6 BOLD Database Submission

The BINs system clusters COI gene sequence data into operational taxonomic units (OTUs) known as BINs (Barcode Index Numbers). This system helps confirm the similarity (concordance) between barcode sequence clusters for species designations. The analysis validates input records against others within the same BIN, including those submitted by other users, by comparing their taxonomy. This analysis was conducted using the BOLD systems database.

Sequence identification through the BOLD identification engine revealed that the sequences of sequenced samples of termites were not present in the BOLD database from any other part of the world and have been submitted as a singleton specimen from our study.

FIGURE 4.7: J1 J2 che1 H3

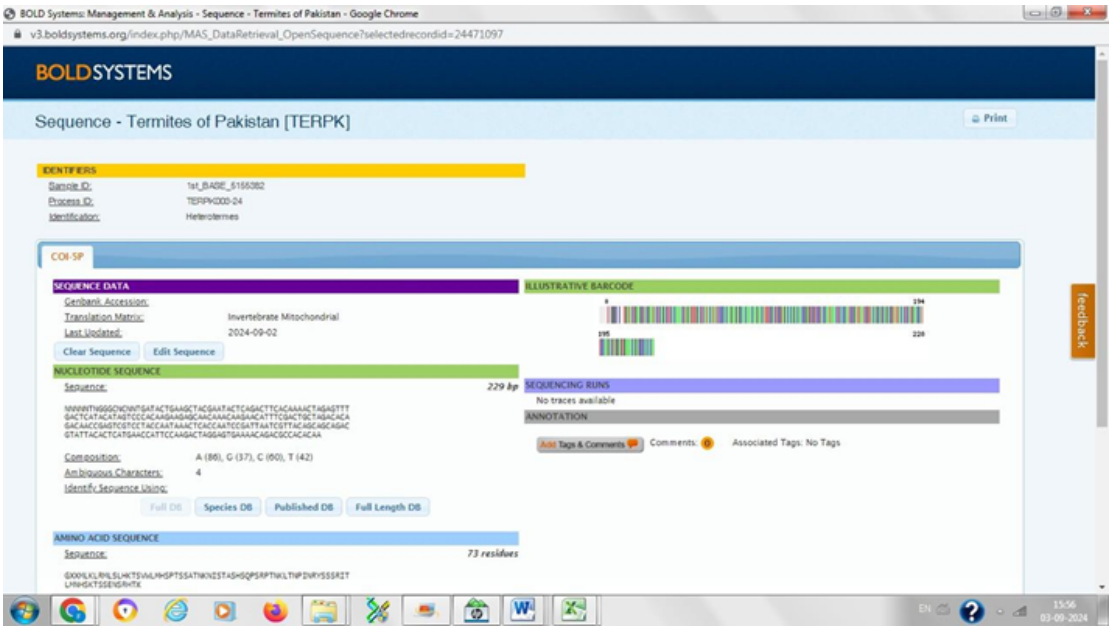

FIGURE 4.8: Submission to BOLD database

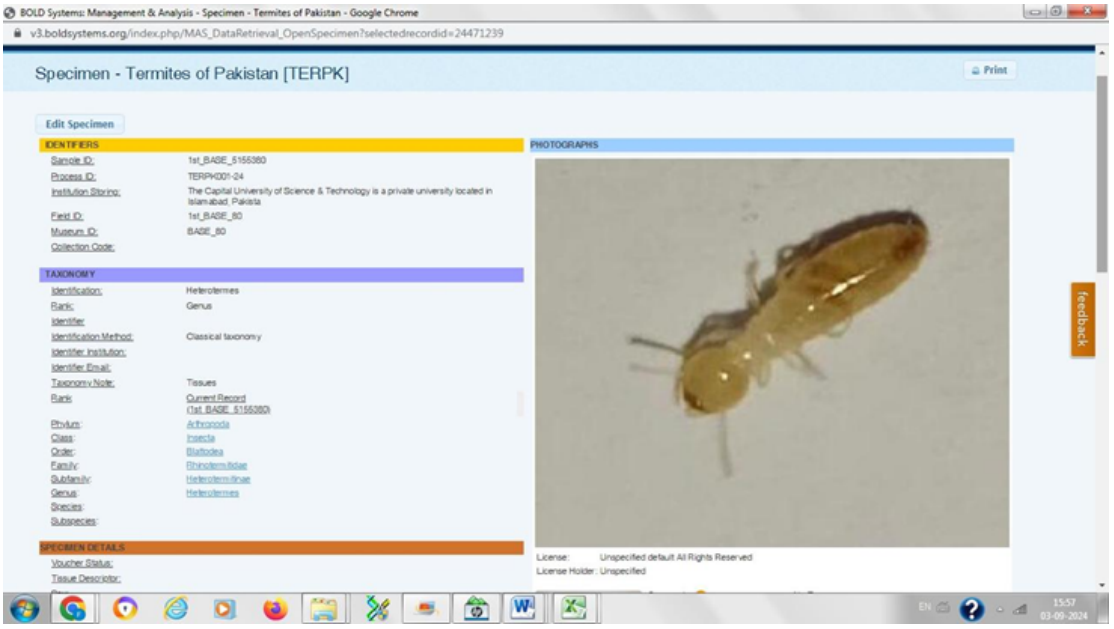

FIGURE 4.9: Submission to BOLD database

4.7 Phylogenetic Analysis

Figure 4.3 shows the evolutionary relationships among different sequences based on their similarities and differences in the *cytochrome c oxidase subunit i* (*COI*) gene. Sequences H3, che1, J1 and J2 are grouped together with high bootstrap values

(60 and 98). This indicates that these sequences are very closely related. They likely to share a common ancestor. *Heterotermes gertrudae* Swq1A *cytochrome c oxidase subunit 1* (*COI*) gene sequence was slightly more distant from the sample sequences, with a bootstrap value of 83 connecting it to the previous group. This suggests a close, but slightly more distant, relationship to the study sequence. *Heterotermes indicola* isolate BGR20 *cytochrome c oxidase subunit 1* gene was closely related to the *Heterotermes gertrudae* sequence, with a high bootstrap value of 87 indicating strong support for their common ancestry. *Heterotermes malabaricus* cytochrome oxidase subunit II gene partial cds mitochondrial branches off next with a bootstrap value of 44. This suggests a less confident, but still notable, relationship to the previous sequences, indicating it is a bit more distantly related.

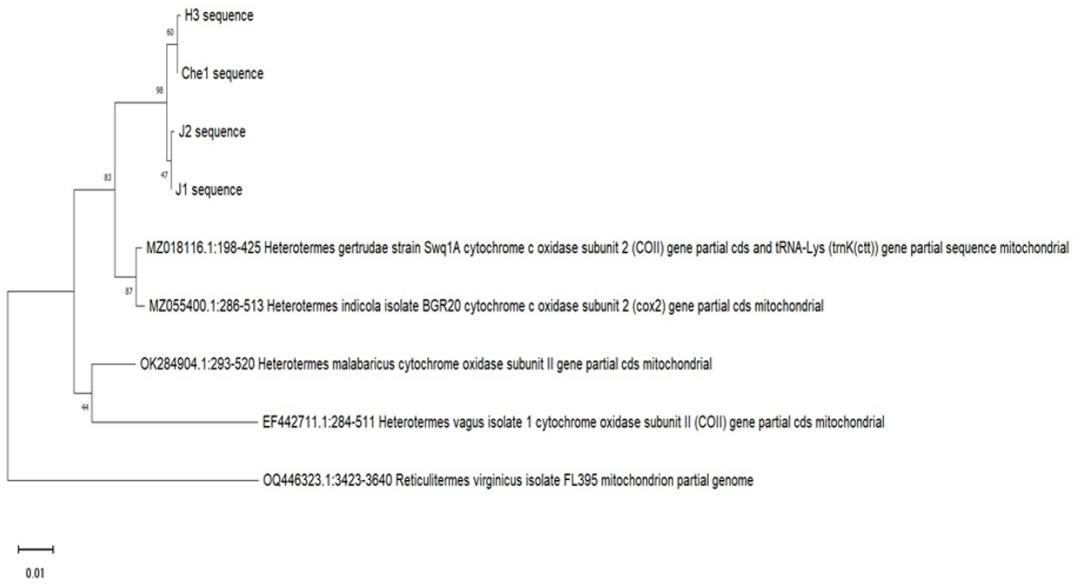

FIGURE 4.10: Phylogenetic Tree with the evolutionary relationship of four samples(H3, Che1 from Muzaffarabad and J1 and J2 from Jhelum valley)

Chapter 5

Discussion

Termites are social insects that are divided into worker, soldier, and classes inside colonies that are reproductive.

They are classified into seven families (order *Blattodea*) [206]. 3106 termite species are known to exist, 363 of which pose a serious threat to human health status [207]. Termites are linked to significant losses in forestry, agriculture, and housing, but they also play a significant role in a number of ecological engineering procedures such as the decomposition and recycling organic matter, manure elimination, soil formation, loosening, fertility, pollination, greenhouse gas emissions, and the provision of food for humans, livestock, and wildlife [208].

Japan, Thailand, Indonesia, France, USA, and Australia have reported 1.5, 11, 1, 0.5, 0.5, and 0.8–1 billion US dollars were lost to termites each year, respectively [209]. Although termite attacks are more likely to affect older trees in forest plantations, other effects include ring and root debarking , range lands, and seedlings and saplings [210].

An estimated 15–25% of the maize crop in India is lost due to crop failure, causing southern Africa to lose thirty five million US dollars a year. Crop losses varying from three to hundred percent have been recorded documented, however it's unclear how much money was lost annually. In Malaysia, reports of damages to

residential, industrial, and commercial structures have been made in percentages of 70%, 20%, and 10%, respectively [211].

Four termite pest genera, including *Odontotermes* (Macrotermitinae: *Termitidae*), damage crops, ornamental plants, grasslands, and fruit trees in Pakistani agro-ecosystems.

Particularly, losses have been recorded as 2–43.5% for tobacco, 8–12% for wheat, 90–100% for sugarcane, and 1-33% for ground nuts [212]. Termites severely damage wood 6–8 months old *Pinus roxburghii* and *Acacia arabica* in structures, but it has been shown that *Cedrus deodara* trees older than 100 years are somewhat resistant [213].

Pakistan is a varied ecological zone for both flora and animals because of its high-altitude ranges, varied latitudes, and geological past [214]. Recent studies on termites in Pakistan revealed financial inadequacies in a few areas of Khyber Pakhtunkhwa, formerly known as the NWFP [215].

However, several areas remain unexplored, including the districts of Buner, Swabi, and Haripur (the eastern area). According to Iqbal et al. [216], the families *Rhinotermitidae* and *Termitidae* are home to majority of the 53 termite species that are inflicting harm in Pakistan. The eastern region's *Termitidae* have been thoroughly examined by [217], with one of the most prevalent and varied genera being *Odontotermes*, which damages forests, crops, and structures in a variety of agro-ecological zones [218]. How widespread and varied this genus is in Swabi, Haripur and Buner, is still unclear.

While morphology plays a significant role in termite taxonomy, [219]. There are some drawbacks. Unknown variety within taxa can make identification more difficult, and variations in phenotypic features have the potential to cause misidentification [220]. Correct identification based only on morphology frequently necessitates the knowledge of experts and proficient technicians conversant with the morphological traits of the species [221].

On the other hand, molecular approaches are a potential tool that have already progressed insect systematics because they do not require specialized understanding [222]. Specifically, DNA barcoding has been utilised to examine enigmatic species, discover novel species, and identifying categorise unknown specimens to species [223].

13 species were found during the survey, including *Odontotermes obesus, Odontotermes guptai, Odontotermes gurdasurensis, Odontotermes horai, Heterotermes indicola, Microtermes obesi, Microtermes mycophagus, Eremotermes paradoxalis,* and *Coptotermes heimi*. *Heterotermes indicola, Microtermes obesi*, and *Microtermes mycophagus* had the 3 species that most significantly affected the economy and caused infestations in residential timber constructions. *Odontotermes obesus* was the most common termite species found in residential lawns and gardens, followed by *Coptotermes heimi*, which was found in soil and trees When several termite species were examined for seasonal variations, it was discovered that *H. indicola* and *Microtermes* were more persistent, usually remaining active all year round [224].

While the predicted divergence between distinct species is usually larger than 3%, genetic diversity between individuals of the same species can vary from 0.0 to 0.51% [199, 200], according to [225] and other researchers. Sequencing does not have rigorous standards for species identification, however matches with a lower percentage than 98% are probably considered unique [226]. Four samples' sequences did not match the BLAST search or sequences exactly. The sequences in the current study exhibited 97% to 98% similarity with *Heterotermes*, demonstrating the genetic diversity among termite species that has been uncovered by sequencing. 67% of the termites belonged to the genus *Heterotermes*, 17% to the genus Microcerotermes, and 16% to the genus Odontotermes, per a Tanveer study (Diversity of Termite Fauna in District Swabi, Tanveer Ahmad*).

The termite genus *Heterotermes* is closely linked to the genus *Reticulitermes* and is a member of the Rhinotermitidae family. Due to their propensity to establish massive colonies, some *Heterotermes* species are regarded as pests. Their main food source is cellulose, which they get from dead tree logs, stumps, and branches

as well as from wooden objects manufactured by humans, such books, paper, and buildings.

Chapter 6

Conclusion and Future Recommendation

The over 3,000 termite species can be categorized into three main ecological types: subterranean, dry wood, and damp wood termites. Subterranean termites are the most prevalent and widespread, requiring soil contact for survival. Drywood termites, in contrast, live in dry wood and do not need soil contact. Damp wood termites thrive in moist wood and need high humidity levels. Understanding these termite types and their habitats is essential for their identification and control.

In current study, *COI* gene has been use for DNA barcoding by collecting the samples of termites from 3 districts of Azad Kashmir. The first objective of this study was the molecular characterization of the termites species collected from District Jhelum, District, Kotli and District Muzafarabad, AJK. Samples collected from these locations showed genetic variation. The sequence analysis of 4 samples using BLAST revealed that the all sample of termites showed similarity with *Heterotermes gertrudae* species. The two samples collected from Muzaffarabad AJK labeled as H3 has percent identity 97.81% and Che 1 with percent similarity 97.79 with query coverage 96% for both. Jhelum valley sample J1 with sequence similarity of 98.18% and J2 98.62% with query coverage 93% and 92% respectively.

The second objective of this study was the BOLD database submission for species verification and to check its presence in system by matching unknown specimens

to known species by comparing their DNA sequences. BOLD (Barcode of life Data Systems) is a database designed and devoted specifically for generation of sequences and DNA barcode data application. It also provides an online platform for analysing DNA sequences . BOLD assign showed none of the similarity with the available BINS indicating that these sequences have not been submitted from another part of Pakistan.

The third objective of the study was to establish the evolutionary relationship of sequenced termites samples with already reported termite species. It was concluded that that all samples were grouped together with boot strap value of 60 and 98 indicating that these sequences are very closely related and share a common ancestor. It further showed close relationship with *Heterotermes gertrudae* and *H.indicola* whereas *H. vagus* is distant but still within the *Heterotermes genus*. All the four samples processed were most distantly related to Reticulitermes virginicus.

The *cytochrome c oxidase subunit I* (*COI*) gene is commonly used for DNA barcoding across various species. It provides high-resolution identification. Other mitochondrial genes and nuclear markers like ITS (Internal Transcribed Spacer) can be used to improve accuracy and robustness.

Samples collection from various geographic regions to capture the genetic diversity within and between species can also provide large scale information about biodiversity of different areas.

Moreover, multiple individuals from different colonies to account for intra-species genetic variability can be sequenced to understand the gentic diversity trends, variations and evolutionary relationship.

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