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Distribution, Genetic Diversity, Host Defense, and Control of *Varroa destructor* (Acari: Varroidae), a Pest of Honeybees *(Apis Mellifera)* (Hymenoptera: Apidae) in Ethiopia

A Doctoral Dissertation Submitted to the Department of Zoological Sciences, Addis Ababa University in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Zoological Sciences (Insect sciences)

By

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DECLARATION

I, the undersigned, declare that this PhD dissertation entitled **"Distribution, genetic diversity, host defense, and control of *Varroa destructor* (Acari: Varroidae), a pest of honeybees *(Apis mellifera)* (Hymenoptera: Apidae) in Ethiopia"** is my original work, and it has not been submitted or presented anywhere else for any degree. All sources of materials used in this dissertation are duly acknowledged.

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ABSTRACT

Honeybees, *Apis mellifera* (Hymenoptera: Apidae), are the most important social insects that provide vital economic and ecosystem benefits to humans. However, the recent dramatic colony losses and overall bee population declines have jeopardized the apicultural industry while causing high pressures on agricultural crop production and food security. Various biotic and abiotic stressors as well as their synergetic effects thought to be responsible for global colony decline and loss of honeybee populations. The ecto-parasitic mite, *Varroa destructor* (Anderson and Trueman), is one of the most recognized threat to honeybee health, causing dramatic colony losses worldwide. The parasite is known as the most harmful, and globally distributed pest of European honeybees *(A. mellifera)* that successfully infests both managed and wild colonies and play a prominent role in the decline of global honeybee populations. This in turn impacts both ecological and economic benefits of the honeybees, and becoming a threat for global food security. The occurrence of *V. destructor* mites in Ethiopia was reported for the first time in 2010; however, information on the distribution, genetic diversity, and host-parasite interactions of the mite is limited in the country. Therefore, this dissertation was designed to investigate the overall distribution, genetic diversity, host defense mechanisms, and non-chemical control of *V. destructor* in Ethiopian honeybee colonies. For this purpose, field assessments, laboratory diagnostics employing various techniques, and advanced molecular tools were used in this thesis research.

A diagnostic survey was undertaken between October 2020 and July 2022 to determine the prevalence and infestation levels of mites within selected areas across three regions: Oromia, Amhara, and the Southern Nations, Nationalities, and Peoples Region (SNNPR). In total, 360 samples were thoroughly collected from 39 apiaries and analyzed using established protocols of varroa mite research. To elucidate the genetic diversity and population structure of varroa mites, mitochondrial DNA sequencing targeting the *Cytb* and *COX1* gene regions, along with single nucleotide polymorphism (SNP) marker analysis were conducted using the standard procedures of molecular techniques. Genetic diversity assessments, population structure analysis, clustering, and analysis of molecular variance (AMOVA) were subsequently performed to determine variability within varroa genotypes collected from different localities. Furthermore, investigation was undertaken to explore the host behavioral defense mechanisms used by local honeybees

(*A.m. bandasii*) against varroa mites. This involved evaluating key indicators of resistance traits, including hygienic behaviors (HB) and grooming behaviors (GB) of local honeybees. In addition, the potential efficacy of propolis extract in controlling varroa parasitism was tested under laboratory bioassay conditions.

The findings of this study showed that varroa mites were widely distributed in all the three regions of Ethiopia, with prevalence rates of 95.8% in Oromia, 85.2% in Amhara and 71.9% in the SNNP regional states. Mite infestation levels were lower in local hives (2.6 ± 5.9) than in frame hives (5.0 ± 0.9). In addition, the infestation level was significantly higher in brood bees (5.6 ± 0.8) compared to adult bees (1.93 ± 0.17) ($p < 0.001$). Genotyping of varroa samples using mtDNA gene sequencing, combined with 169 *Cytb* and 96 *COX1* sequences from Gene Bank, revealed that only one haplotype, the Korean strain (K1 haplotype), was present in Ethiopia, suggesting a very low mite genetic diversity. SNP markers analysis also revealed that *V. destructor* was the only species detected in the local honeybee colonies. The phylogenetic cluster analysis of SNP markers grouped the mite populations into three hypothetical clusters, all of which shared a common ancestor of the Korean strain. The average allele count, genetic diversity, and polymorphic information content (PIC) values were 1.53, 0.14, and 0.29, respectively. Analysis of molecular variance (AMOVA) showed a very low genetic variation (2%) among varroa populations in Ethiopia, suggesting that the varroa introduced to the country was possibly originated from a single ancestor.

The present study showed that local honeybee, *A. m. bandasii* colonies expressed stronger hygienic behavior (97.6±3.4%) after 48 h. There was a strong negative correlation between the HB and varroa infestation rates ($r = -0.81$, $P = 0.001$), suggesting that HB has potential to reduce the mite population in colonies. Likewise, assessment of GB also indicated higher mean daily fallen mites per colony (16.3±10.2), of which about 80% of the total fallen mites were damaged, and eventually dead. These combined behavioral traits have evolved in African honeybees and have greatly contributed to their survival in the face of the catastrophic effects of the *V. destructor* mite. In this study, the ethanolic extract of propolis was very efficient and could kill 100% of the varroa mites within 4 hr post treatment. This highlights the potential of natural propolis extract in controlling varroa mite infestations.

In general, the results of this thesis provided valuable insights into the current varroa mite parasitism in local honeybee populations in Ethiopia and suggest for regular monitoring and management to mitigate the mites' possible impact on the health and productivity of honeybees. Furthermore, there is a pressing need for awareness creation among local beekeepers, government bodies, and different stakeholders towards designing long-term and sustainable control strategies.

Key words: *Apis mellifera*, brood, infestation, prevalence, *Varroa destructor*, markers, genetic variation, population, grooming behavior, hygienic behavior, varroacidal, propolis, extract, ethanol, mortality

DEDICATION

This PhD dissertation is dedicated to my loving family, for their unreserved support and love, and above all to the Almighty God.

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ACRONMYS AND ABBREVIATIONS

ABPV	Acute Bee Paralysis Virus
AFB	American foulbrood
AmFV	Apis mellifera filamentous virus
AMOVA	Analysis of molecular variance
BecA	Biosciences eastern and central Africa
Bp	Base pair
BQCV	Black Queen Cell Virus
CCD	Colony Collapse Disorder
COI-COII	Cytochrome oxidase subunit I and subunit II
CSA	Central Statistical Agency
DArT	Diversity array technology
DNA	Deoxyribonucleic acid
DWV	Deformed Wing Virus
EFB	European foulbrood
FAO	Food and Agriculture Organization
GB	Grooming Behavior
GPS	Global Positioning System
HB	Hygienic Behavior
HBRC	Holeta Bee Research Center
ICIPE	International Center of Insect Physiology and Ecology
ILRI	International Livestock Research Institute
IPM	Integrated Pest Managment
LSV	Lake Sinai Virus
MoARD	Ministry of Agriculture and Rural Development
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PIC	Polymorphic information content
PVC	Polyvinyl chloride
RNA	Ribonucleic acid
SBV	Sac Brood Virus
SNNPR	Southern Nations, Nationalities and Peoples Region
SNP	Single nucleotide polymorphisms
VSH	Varroa Sensitive Hygiene

CHAPTER ONE

INTRODUCTION

1.1 Back ground of the study

Honeybees (Hymenoptera: Apidae) are social insects that live together in nests or hives. Historically, honeybees have played a significant role in human cultures for centuries. Cave paintings depicting honeycombs, swarms of bees, and honey collecting suggest that humans have been engaged in managing the honeybees since ancient times (Crane, 1999). This enduring practice highlights the critical economic and ecological importance of honeybees in various aspects. The honeybees (*Apis mellifera*) are well known for their commercial hive products, such as honey, beeswax, royal jelly, pollen, and propolis, as source of income and food for many farmers in the world. Thus, the beekeeping sector serves as a significant source of sustenance and employment opportunities for numerous rural households in developing countries (Bradbear, 2009). Beyond providing food and employment, beekeeping plays a pivotal role in rural poverty alleviation, environmental preservation, and the diversification of national economy (King et al., 2011). Consequently, beekeeping significantly contributes to the sustainable livelihoods of developing communities and plays a great role in food security (Martin et al., 2012).

Particularly, honey is a novel hive product that provides notable health benefits, and contributes for nutritional and economic value. Due to its main energy source, honey helps to extend extend nitrogen retention in the digestive system, making it particularly recommended for old people, pregnant women, and for sik people (Kinati et al., 2013). As a result, honey is recognized for its antioxidant, anti-proliferative, and antibacterial properties worldwide and it is believed to possess medicinal properties that can aid in the prevention of chronic diseases (Ranneh et al., 2021). Recent research highlights the significant role of honey in mitigating risks associated with COVID-19, suggesting that honey consumption can boost the immune system fighting against the COVID-19 (Al Naggar et al., 2021; Lima et al., 2021). Furthermore, honey is used for making '*Tej*' a locally made fermented honeywine in Ethiopia. *Tej* is a commonly consumed fermented alcoholic beverage primarily made from crude honey. From the total honey produced in Ethiopia, around 80% is used for the production of *'tej*' and this also plays important roles for job creation and income generation (Fentie et al., 2022).

Apart from economic benefits, bees play important role in pollination services of agricultural and horticultural crops (Hung et al., 2018). They pollinate a wide range of cultivated and wild crops, enhancing agricultural production and playing a key role in the provision of food and nutrition for humans. About one-third of the human diet is assumed to be derived directly or indirectly from pollination services of honeybees (McGregor, 1976; Delaplane et al., 2000), and the economic value of pollination is estimated to be several billion dollars (Hendrikx et al., 2009; Agriculture, 2010). Hence, the economic benefit of honeybee pollination services is highly greater than the value of honey production (Sanjerehei, 2014). In Africa, many high-value crops, such as cash crops, along with many staple food crops, are largely dependent on pollination services of honeybees (Muli et al., 2014). Alebachew (2018) has estimated the economic value of biological pollinators to be about 815.2 million dollars only during the 2015/16 production season in Ethiopia, of which 80% was attributed to honeybee pollination. Furthermore, bees play an important role for ecosystem conservation and stability through their specialized pollination activities. As a result, honeybees are important social insects play crucial role in ensuring global food security and environmental stability (FAO, 2019; Kasina et al., 2009; Venturini et al., 2017).

However, the recently increased mortality and population decline of honeybees have become a concern for beekeepers and many communities in the last few decades (Potts et al., 2010; Van der Zee et al., 2012). Notably, the dramatic losses of honeybee populations have been reported over the past years in the USA, Europe and the Middle East (Aston, 2010; Charrière & Neumann, 2010; Currie et al., 2010). Such losses of honeybees pose a severe threat to the global apicultural industry, imposing high pressure on agricultural crop production and ecosystem services. Although, there are no such significant honey bee colony losses reported in Africa, Australia and South America (Hristov et al., 2020), evidences from decline of colonized hives, reduction in the size of migratory swarms, and a decrease in honey production generally suggests a noticeable concern for global bee decline. Therefore, it is imperative to conserve honeybee species all over the world to sustain the global food security and ecosystem stability.

Various biotic and abiotic stressors and their synergetic effects have been attributing to the global decline and losses of honeybee populations (Nazzi et al., 2012; Lin et al., 2023). Widely reported evidences indicate that parasites and pathogens, exposure to pesticides, habitat loss, food shortage, climate change and their synergetic interactions have largely contributed to the

global losses and decline of honeybee species (Smith et al., 2013; Giacobino et al., 2015; Traynor et al., 2016). Honeybee colonies face several pests and parasites that can significantly impact their health and productivity worldwide. Some of the major honeybee pathogens and parasites include: *Varroa destructor*, *Nosema Apis*, American foulbrood, European foulbrood, Chalk brood, honeybee viruses etc. (Van Engelsdorp et. al., 2010). In Ethiopia, honeybee colonies face several pests and parasites such as ants, wax moths, small hive beetels, varroa mite, wasps and different birds that can significantly impact their health and productivity (Begna, 2015). These pests and pathogens can individually or collectively weaken honeybee colonies, leading to decreased honey production, reduced pollination services, and in severe cases, colony collapse (Genersch et. al., 2010). The ecto-parasitic mite, *Varroa destructor* (Anderson & Trueman, 2000), is among the factors causing the most devastating effect to the Western honeybees *(A. mellifera*), and has becoming a serious threat for global apiculture industry (Hristov et al., 2020; Traynor et al., 2020). Although *V. destructor* is not the only species in the varroa genus, this haplotype is recognized for causing devastating damage to honeybee colonies. Hereafter, all mention of varroa in this thesis will exclusively refer to *V. destructor* unless otherwise specified.

Since the varroa mite has shifted its original host from the Eastern honeybees, *A. cerana* to the Western honeybees, *A. mellifera*, about 50 years ago, it has instigated severe damages on the *A. mellifera* honeybee colonies (Rosenkranz et al., 2010). Currently, the mite is recognized as a serious and widely distributed parasite causing losses of honeybee populations worldwide. The mite feeds on the bees' fat body and hemolymph (Ramsey et al., 2019), and its ability to transmit and spread multiple viruses throughout colonies made it a global threat to honeybee populations (Tentcheva et al., 2004; Wilfert et al., 2016). Consequently, the majority of beekeepers in the Northern hemisphere largely depend on chemical treatment of their colonies to control the devastating effect of the mite. Nevertheless, varroa has developed resistance against chemical acaricides and it remained spreading and highly complex parasite of honeybees posing significant challenges to the apiculture industry (Higes et al., 2020; Mitton et al., 2022). Except a few subspecies of African and Africanized honeybees, *A. mellifera* colonies lack some adaptive mechanisms that limit varroa parasitism in their colony (O'Shea-Wheller et al., 2022). However, the impact of the mite depends on the infestation threshold, which is determined by the susceptibility of specific bee races originated from different geographic origins (Locke, 2016;

Mendoza et al., 2020). Some *A. mellifera* colonies in Africa and African-derived populations in North America demonstrated varying levels of tolerance or resistance to mite infestations without chemical treatment (Locke, 2016; Castilhos et al., 2023). Although there have been no reported instances of colony losses attributed to varroa infestations in Africa, it's important to note that heavy infestations can weaken and kill entire colonies over time. For example, Pirk et al. (2014) observed a notable bee population crash (29.6%-46.2%) in South Africa once varroa infestation reached a certain threshold level. This highlights the need for regular assessment and early monitoring of mite infestation levels in Africa in specific honeybee subspecies to prevent the potential impacts caused by the mite.

In Ethiopia, varroa mite was first detected in 2010 in Tigrai region (Begna, 2014). Since then, few subsequent studies confirmed the widespread occurrences of the parasite in different regional states of the country owing to factors such as bee colony trading and movement, colony migration, natural swarming and other beekeeping activities. However, comprehensive data on the mite's periodic distribution and infestation levels, genetic diversity and its host-parasite interactions are very limited in the country. Furthermore, no research attempts have been done so far on the management of the mite in order to mitigate its potential impacts on the local honeybees and their products. Therefore, this thesis study aimed at investigating the current distribution patterns, genetic diversity, host defensive behavior, and non-chemical control test of *V. destructor* mite in Ethiopian local honeybees.

1.2 Statement of the problem

V. destructor mite is a globally distributed honeybee pest affecting the apiculture industry at large scale. Controlling the parasite presents numerous challenges, including that mites have developed resistance to several chemical treatments, the contamination of chemicals with hive products, the mites transmit secondary patogens and the lack of universal control methods. As a result, varroa has continued spread all over the world and crippling honeybees worldwide. Addressing these challenges requires international collaboration, research into alternative control methods, adaptation of IPM strategies to local conditions, and increased education and support for beekeepers worldwide.

Since the first detection of varroa mite in Ethiopia (Begna, 2014), scientific data elucidating the wider varroa distribution, and contributing factors to the mite's prevalence across various parts of

the country is generally scarce. The detrimental impacts of the parasitic mite on honeybee populations are still a matter of public concern and a subject of ongoing research. Existing information on varroa mite prevalence and infestation levels is very limited to specific locations and highly fragmented in Ethiopia. Furthermore, the Ethiopian government has currently promoting modern beekeeping to enhance honey production in the country. However, there has been a lack of consideration given to mitigating the transmission of honeybee pathogens and parasites, such as the *V. destructor* mite, no precautions are taken during colony distribution. Therefore, comprehensive assessment studies on the varroa distribution patterns and infestation levels in the country is very crucial to create awareness among beekeepers, government, and other stakeholders before the parasite could potentially cause a devastating impact on honeybee health and the beekeeping industry. It is necessary to take lessons from the experiences of Western countries such as the USA and Europe where the parasite was previously considered as minor pest until it has caused unexpected losses of both wild and managed honeybee colonies in the past decades (Le Conte et al., 2010). In addition, there is a lack of information about the genetic diversity and population structure of the parasite infesting local honeybee populations in Ethiopia. Understanding the genetic profile of the mite is very essential to determine the adaptation of the parasite in response to host defensive behaviors of local honeybee populations and for marker-assited host selection for breeding.

On the other hand, Ethiopian honeybees have been surviving the mite infestation for the past 14 years without beekeepers' interventions, although that varroa mite is known to weaken and cause honeybee population collapse at certain infestation thresholds. However, the reason behind their survival without treatment is not known. Understanding the survival mechanisms of local honeybees against the mite infestation is essential to determine the host defensive behaviours against the mite parasitism pressure. The combination of social and behavioral traits, including hygienic and grooming behaviors could be attributed in local honeybee colonies to fight against the parasitism of *V. destructor*, and these have to be explored through standard evaluation techniques.

So far, there was no attempt to control the varroa mite infestations in Ethiopia, despite the widespread of the parasite across the country. Thus, we aimed in this research to test the varroacidal effect of natural propolis extracts in the laboratory bioassay as a control mechanism to mitigate the parasite infestation in local honeybees.

1.3 Significance of the study

This thesis research emphasizes the importance of gaining accurate information on the status of *Varroa destructor* mite in Ethiopia before it inflicts substantial harm on local honeybee colonies. The study evaluates the prevalence and infestation rates of the parasite across three regional states of the country, including Oromia, Amhara and SNNP regions. In this study, random and stratified sampling techniques, sample diagnosis, and counting mite samples were employed to determine the mite infestation levels. The findings of the study aim to raise awareness among local beekeepers, government bodies and other stakeholders about the varroa parasitism in local honeybee populations, providing a crucial foundation for designing long-term monitoring and management strategies.

In addition, this study determined the genetic diversity and population dynamics of *V. destructor* mite using mtDNA sequencing and high-throughput SNP marker analysis for the first time in Ethiopia. This would enhance our understanding of distinct varroa genetic profile parasitizing the local honeybees and potentially explain mite co-adaptation in different geographical regions and rapid spread across the country. Moreover, the study evaluates major behavioral defenses of local honeybees, offering insights into host-parasite interactions and lay foundation for the selection of honeybee breeds in future colony-breeding programs. Furthermore, investigating alternative management approaches, such as using natural products like propolis extract, can ensure the safety of honeybees while controlling the impact of varroa parasitism.

Overall, the findings of this thesis research offer essential insights for establishing baseline data crucial for the implementation of effective varroa monitoring and control measures, which also include adaptation of IPM strategies to local conditions in Ethiopia. This information will help to facilitate the conservation, genetic enhancement, and efficient utilization of local honeybee subspecies, contributing to the sustainability of beekeeping practices in Ethiopia.

1.4 Objectives of the study

1.4.1 General objective

The general objective of this study was to investigate the distribution, genetic diversity, behavioural host defences, and management of *V. destructor* mite in Ethiopia.

1.4.2 Specific objectives

The specific objectives of this study included:

- To determine the distribution and infestation status of varroa mites in local honeybees across three regional states of Ethiopia.
- To investigate the genetic and population diversity of the *V. destructor* mite infesting local honeybees
- To study the predominant defensive behaviours exhibited by local honeybees to combat varroa mite infestations
- To test the acaricidal effects of ethanolic extracts of propolis against *V. destructor* in laboratory settings

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of honeybees (*Apis mellifera*)

Honeybees belonging to the genus *Apis* originated in tropical Africa and spread from Southern Africa to northern Europe and East into India and China (Otis, 1990). Being attributed to wide variations in environmental, topographic and climatic conditions, the genus *Apis* exhibits a wide range of geographical distribution throughout the world, which has caused the evolution of highly divergent subspecies (Winston, 1991; Michener, 2000). It is believed that there are more than ten existing species for the genus *Apis* worldwide (Raffiudin & Crozier, 2007). These are known taxonomically and classified in the Order: *Hymenoptera*, family: *Apidae,* subfamily: *Apinae*, genus: *Apis* (Ruttner, 1988). At least four species are commonly recognized among the genus *Apis*, including the Asian honeybees (*A. cerana*), the European honeybees (*A. mellifera*), the dwarf or midget bees (*A. florea*), and the giant bees (*A. dorsata*) (Ruttner, 1988). With the exception of *A. mellifera,* all the species listed are currently restricted to Asia, and the lineage raised to *A. mellifera* is commonly assumed to evolve from Africa and expanded to Asia and Europe (Han et al., 2012). *A. mellifera is* the most classified species into subspecies through common taxonomic analysis, including morphometric and phylogenetic analysis. It consists of about 28 subspecies or races, of which four are commonly used for beekeeping, namely: *A. m. ligustica*, *A. m. carnica*, *A. m. caucasica* and *A. m. mellifera* (Ruttner, 1988; Engel, 1999; Alqarni et al., 2011). These subspecies of *A. mellifera* are the most important and widely studied bees, and they have been introduced into the world long period ago (Suwannapong et al., 2012). According to Ruttner (2013), the species *A. mellifera* generally exhibits significant intraspecific variability in both morphology and behaviors, which attribute to adaptive responses to diverse ecological conditions in the world. Consequently, this polytypic species naturally inhabits a wide range of geographical areas and spreads across Europe, Africa, America, and Western Asia (Crane, 1999; Parker et al., 2010). However, the current anthropogenic effects of modern beekeeping, such as commercial breeding, colony trading and migratory beekeeping, have been affecting the genetic variability of these honeybee populations, leading to genetic admixture.

2.2 The biology of honeybees

Like many other insects, bees undergo complete metamorphosis, and their development is divided into four phases (egg, larva, pupa, and adult). These developmental stages for honeybees proceed through, spanning about three weeks in total, before reaching adulthood: three days as an egg, a period of active embryogenesis, about 5-7 days as a feeding larva, during which an outstanding increase in size is observed, and the remaining time as a pro-pupa stage, during which metamorphosis occurs within a sealed cell (Sammataro & Avitabile, 1998; Amdam & Page Jr, 2010). The duration of development from egg to adult eclosion differs considerably among queens, workers, and drones, spanning 16, 21, and 24 days, respectively (Wheeler, 1986; Adjare, 1990).

The reproductive biology of honeybees exhibits under a haplodiploid system, in which the fertilized eggs develop in to diploid females, while unfertilized eggs give rise to haploid male offspring through a process called parthenogenesis (Wheeler, 1986). The ultimate developmental trajectory of fertilized eggs into workers or a queens is predominantly determined by the nutritional quality of the diet it feeds during the larval development phase (Wang et al., 2015). Bee larvae intended to become queens are nourished with surplus royal jelly until they undergo metamorphosis, while larvae designated as workers consume a mixture of royal jelly, honey, and pollen during their later larval period (Jay, 1964).

The social organization of honeybee colonies distinctly characterized by a complex structure that encompasses overlapping generations with specialized labor divisions. A single colony consists of three distinct castes: female worker bees, whose population can range from 20,000-80,000 individuals, depending on seasonal variations; a few hundreds of male drones usually present in the spring; and one reproducing female queen (Moritz & Fuchs, 1998). Several thousand worker bees cooperate in nest building, food collection, and brood rearing. Each member has a definite task to perform, related to its adult age; however, surviving and reproduction take the combined efforts of the entire colony.

In the division labor of a colony, the female worker bees perform all the tasks of the colony (except for egg laying), but the temporal labor division depends on their age. According to Johnson (2010), the younger workers undertake nest cleaning, building comb, and attending

developing broods, at which point they are called nurse bees. After two to three weeks, the adult workers will begin foraging for pollen to feed their broods, and for nectar to concentrate into honey and store in the hive as the supplementary food. The queen bee lays all the eggs for the colony at a rate of 2000 per day on average (Ratnieks, 1993). The queen mates once in her life with several drones and is able to store all the sperm in her spermathecal for her entire life of about three years. The drones perform only to mate with virgin queens. Upon copulation the drone will die while any drones that did not manage to mate will be expelled from the colony in the autumn (Rangel & Fisher, 2019). Workers and drones have a life span of about six weeks with the exception that worker bees sometimes can survive up to eight months in a winter season.

2.3 Importance of honeybees

Humans have been long managed the honeybees for crucial economic and ecological importance. Their vital contributions in production of valuable commodities including honey and other hive products, as well as their indispensable role in pollinating cultivated crops and wild plants would make honeybees the most beneficial insect in the world (Saunders et al., 2018). They provide tremendous significances for humans through high value products, including honey, bees wax, propolis, royal jelly and bee venom, which play significant role for income generation for the beekeepers. In modern agriculture, bees are among critical bio-components in the course of their leading pollination service (Aizen & Harder, 2009; Khalifa et al., 2021). As a result, beekeeping significantly contributes to sustainable livelihoods of developing community and play great role in the global food security and environmental stability (Bradbear, 2009). In particular, in Africa beekeeping provides additional employment opportunity and helps in financial security for poor and landless farmers. However, the essential and valuable contributions of honeybees depend upon the healthy populations.

2.3.1 Economic importance

The Western honeybees (*A. mellifera*) are the most important economically managed species in the world, followed by the Asian honeybees (*A. cerana*), which is primarily found in Asia (Caron & Connor, 2013). Apart from pollination services, honeybees are well known for their commercial hive products, such as honey, beeswax, royal jelly, pollen, and propolis, as a source of food, medicine and income generation for many rural farmers, particularly in Africa. For

many rural communities in Afria, beekeeping enhances farmers' resilience by alleviating credit constraints during times of adversity. Income generated from beekeeping can finance investments in agricultural inputs, fund children's education, and ensure stable consumption patterns. As a result, beekeeping is an emerging agricultural sub-sector as a viable economic activity, which acts as a safety net by offering additional income through the sale of honey and other various bee-related products (Abro et al., 2022). As a result, bees significantly contribute to the sustainable livelihoods of developing communities and play a great role in global food security (Martin et al., 2012). The honey industry and other commercial products of honeybees provide economic opportunities by providing employment opportunities to youths in most African countries. Importantly, engagement in the honey and wax production value chain has been advocated as a means of creating employment opportunities for young individuals. For example, around 2 million people, including youth, have made their livelihood in the beekeeping industry in Ethiopia (Amulen et al., 2017). Moreover, beekeeping is actively promoted in various African communities as a tool for gender empowerment, enabling women to enhance their social standing through income generation (Crane, 1997).

Beekeeping is also believed to contribute to the national economy by generating foreign exchange revenue through the sale of high-valued organic products such as honey and beeswax in global markets. This export of honey and other beekeeping products presents a promising economic avenue for several African nations (Sarka, 2017). Obviously, there is a significant potential for Ethiopian honey in the global market, providing the country with opportunities to export honey and beeswax to various countries, including the Europe, the United States, and the Middle East (Desalgne, 2012). According to a report by the Food and Agriculture Organization (FAO), Ethiopia exported over 5,500 metric tons of honey in 2018, valued at approximately $11.5 million USD. This export volume underscores the economic importance of beekeeping, providing sustainable income opportunities for rural communities and contributing to the country's overall economic resilience (FAO, 2020). urthermore, the expansion of Ethiopia's beekeeping sector has been supported by initiatives aimed at improving honey production practices, enhancing product quality, and promoting exports to international markets. These efforts not only boost foreign exchange earnings but also contribute to poverty reduction and agricultural diversification in Ethiopia.

On the other hand, the economic value of honeybees can be explained through their indispensable pollination services. Both managed and wild bees contribute and make an important contribution directly to crop and food production through pollination services. Consequently, bees are the most economically important pollinators for sustaining global food security and environmental stability (Van der Sluijs & Vaage, 2016). It is estimated that around 90% of the world's food supply is pollinated by bees (Bailes et al., 2015). According to Khalifa et al. (2021), the value of pollination provide by honey bees is estimated to be approximately 11 billion USD only in the USA per year and Gallai et al. (2009) also estimated the global value of pollination over €153 billion Euro. In Africa, many high value crops such as cash crops, along with many staple food crops are largely dependent on pollination services from honey bees (Muli et al., 2014). In Ethiopia, the economic value of biological pollinators has been estimated to be 815.2 million dollars per year, of which about 80% is attributed to honeybee pollination services (Alebachew, 2018).

2.3.2 Ecological importance

Apart from food production and economic contributions, honeybees play an important role in ecosystem conservation, stability, and genetic variation in plant biodiversity through their specialized pollination activities (Abrol, 2011; Patrício-Roberto & Campos, 2014). As a major insect pollinator, honeybees contribute to the conservation of ecosystem diversity and maintain the balance between plant and animal species. In most parts of Africa, beekeeping has been highly integrated with forest conservation and water shade management strategies due to the positive impact of honeybees' pollination services, which directly contributes to the preservation of entire ecosystems rather than solely benefiting a single crop or species (Bradbear, 2009). During their natural foraging behavior, bees significantly support the reproduction of various plant species and promote the plant biodiversity. This can ensure the reproductive success and survival of numerous plant species, which, in turn, also supports the animals that rely on those plants for food and habitat (Khalifa et al., 2021). In regards, bees play a crucial role in the conservation of natural ecosystems for sustainable biodiversity and food security. However, little recognition has been given to the roles of bees in sustaining most terrestrial ecosystems and environmental biodiversity. Consequently, beekeepers are always aware of planting and

conserving the natural vegetation than non-beekeepers, and this can help to balance the effects of ongoing global ecosystem depletion and climate change.

2.4 The honeybees of Ethiopia

2.4.1 Diversity and geographic distributions of Ethiopian honeybees

The variable agro-ecological conditions and availability of diverse floral resources in Ethiopia makes the country one of the very suitable place for the existence of large and unique biodiversity of bee species (Nuru, 2002; Admassu et al., 2014). The country sustains about 10 million honeybee colonies, of which 70% of the colonies are managed and the remaining exists in forests as wild species (MoARD, 2007). Until recently, morphological description has been widely used for describing, quantifying and classifying the diversity of honeybee species on the earth and has given rise to many successful research activities. Hence, scientific evidence on the taxonomic classification and identification of bee species in Ethiopia also majorly depends on morphometric analysis. Accordingly, the first honeybee race reported to occur in Ethiopia was *A. m. monticola* (Smith, 1961), which was previously known to exist in the mountains of Kenya and Tanzania. Subsequently, Ruttner (1975) identified the presence of *A. m. scutellata* and *A. m. jemenitica* in Ethiopia. Kassaye (1990) later expanded the classification to include five honeybee races: *A. m. jemenitica*, *A. m. monticola*, *A. m. litorea*, *A. m. adansonii*, and *A. m. abyssinica* in the country. Contrastingly, Hepburn et al. (1998) reported the existence of only three honeybee races in the country: *A. m. bandasii*, *A. m. sudanensis,* and *A. m. jemenitica*, but later they suggested the first two subspecies could be categorized under a single local population of *A. m. jemenitica*. After six years, Amssalu et al. (2004) conducted a comprehensive multivariate morphometric analysis and reported the existence of five distinct honeybee races, including *A.m. bandasii, A. m. scutellate*, *A. m. jemenitica, A. m. monticola* and *A.m. weyi gambella* of each inhabiting different agro-ecologies. However, Meixner et al. (2011) employed a similar morphometric analysis on samples from comparable study regions, and contrarily argued that all Ethiopian honeybee populations are statistically indistinguishable, which they represented as a single subspecies of *A. m. simensis*. Recently, Hailu et al. (2021) integrated both morphometric and genetic studies to elucidate the controversial classification of Ethiopian honeybees, and they found that Ethiopian honey bees belong to subspecies *A. m. simensis*. Apart from morphometric analyses, a few molecular studies also highlighted the distinctive genetic differences of Ethiopian honeybees from neighboring honeybee subspecies. A molecular approach to phylogenetic

analysis in honeybees used the mitochondrial marker found in the intergenic region between cytochrome oxidase subunit I and subunit II (*COI-COII*) and served as a straight forward method for identifying evolutionary lineages (Cornuet & Garnery, 1991; Garnery et al., 1992). Based on this technique, Franck et al. (2001) reported for the first time that Ethiopian honeybees are uniquely represented by an evolutionary lineage Y. Subsequently, Hailu et al. (2020) employed the same COI-COII analysis, and revealed the presence of a maternal lineage O populations in the northern region of Ethiopia. More recently, Hunde et al. (2023) conducted a comprehensive mitochondrial DNA sequence analysis for a large sample size collected from all the regions of the country and reported the clear presence of three well-separated evolutionary linages (lineages Y, O and A) within Ethiopian honeybee populations (Figure 2.1). However, to elucidate the exact placement of Ethiopian honeybee populations, further studies that involve the application of genome-wide single nucleotide polymorphism (SNP) analysis are required.

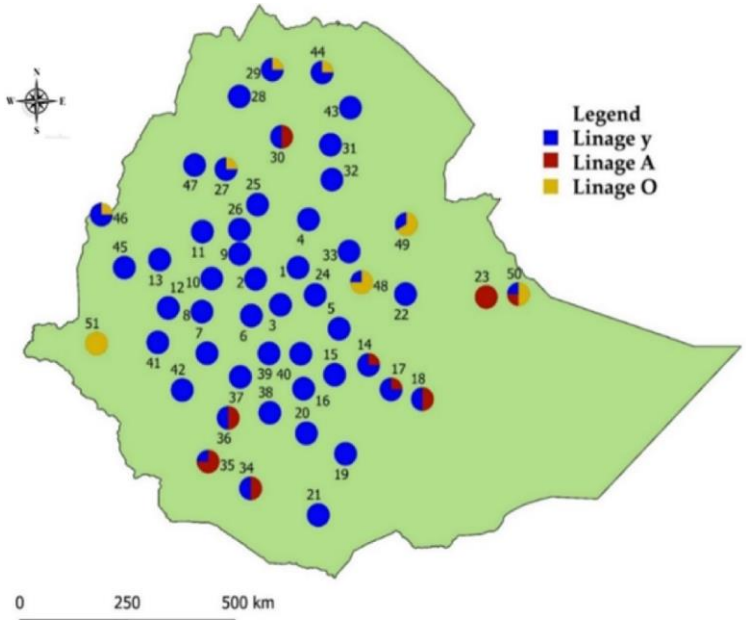

Figure 2.1 Distribution of mitochondrial evolutionary lineages of Ethiopian honeybees (Source: Hunde et al. (2023))

2.4.2 Behaviors of Ethiopian honeybees

In general, African honeybees *(Apis mellifera)* are recognized for their distinctive behavioral characteristics, such as aggressiveness, nervousness, and high migration tendency (Hepburn et al., 1998). Furthermore, they display prolific brood production, featuring numerous queen cells that often mark a strong inclination towards swarming behaviors. Due to some naturally evolved behavioral traits, African honeybees are notably defensive against various pests and pathogens

and considered to have mainly resistance or tolerance without chemical treatment (Fazier et al., 2010; Muli, et al., 2014). However, such natural traits considerably vary based on bees' genetic factors and environmental conditions, as well as prevailing pathogens and parasites (Meixner et al., 2015). Several studies suggest that hygienic and grooming behaviors play key roles in defense mechanisms and enable honeybee populations to survive the effects of brood diseases and parasitic mites in Africa (Nganso et al., 2017; Khan & Ghramh, 2021;). Particularly, Nganso et al. (2017) observed that African savanna honeybees in Kenya have displayed significantly stronger hygienic and grooming behaviors than their European counterparts.

The distinctive behavioral responses and innate tendencies observed across localized Ethiopian honeybee races markedly influenced by their respective geographic contexts (Hailu et al., 2021). For example, previous studies indicated that the subspecies *A. m. jemenitica* and *A. m. scutellata* exhibit higher migratory behavior, defensiveness, and reproductive swarming tendencies compared with *A. m. monticola* and *A.m. bandasii* honeybee races (Amssalu, 2003; Nuru, 2002). These behavioral traits are thought to have evolved as an adaptive response to a variety of biotic and abiotic threats, such as anthropogenic disturbances, pathogens, pest and predators, environmental stressors like drought and climate change.

2.5 Beekeeping in Ethiopia

Historical records preserved in Egyptian hieroglyphic inscriptions illustrate a rich inheritance of traditional (basket) beekeeping practices in Ethiopia, dating back approximately 5000 years (Gratzer et al., 2021). These ancient documents demonstrated trade relations involving honey and beeswax with the kingdom known as Abyssinia, the historical exonym for present-day Ethiopia (Kritsky, 2010). Hence, beekeeping is a long-standing and deep-rooted practice that provides additional employment opportunities and helps in financial security for poor and landless communities in Ethiopia. About ten million honeybee colonies are expected to exist in the country, of which more than seven million are managed (CSA, 2020). Although there is no comprehensive data indicating colony distribution across the regions, Oromia, Amhara and Southern Nations Nationalities People Region (SNNPR) are known to be dominant honey-producing regions with average production of 54.3%, 19.5% and 16.6%, respectively of total honey production (CSA, 2020). With this resource, more than 1.8 million farmers are believed to be involved in beekeeping sector earning various levels of income from the industry (Gupta et

al., 2014). Currently, Ethiopia is the leading honey and beeswax producer in Africa and belongs to the top ten ranked in the world (Gratzer et al., 2021). Recognizing the significance of beekeeping and the imperative apiculture resource in the country, a proclamation entitled as "Apiculture Resources Development and Protection" (Federal Parliament, 2009) was initiated in Ethiopia, which aims to enhance the apiculture resource development and protection of honeybee populations (Hailu et al., 2022).

2.5.1 Practices and prospects of beekeeping in Ethiopia

Unlike other agricultural enterprises, beekeeping is a relatively low-cost and low-labor intensive practice that demands minimum land resources to start (Jacobs et al., 2006). Consequently, beekeeping is adopted in regions where alternative forms of land use are less dependable, requiring minimal investments in terms of labor, time, and capital (Vinci et al., 2018). This inherent beekeeping practice plays crucial role in rural poverty alleviation, environmental conservation and diversification of income through additional employment opportunities. In Ethiopia, many rural households are actively involved in beekeeping for the production of honey and beeswax as sources of income generation. Currently, the Ethiopian government is actively undertaking initiatives to transform traditional beekeeping to an advanced level through promoting the adoption of box hives, providing comprehensive training programs for beekeepers, practical demonstrations, and the widespread dissemination of technology, all aimed at enhancing the production and productivity of honey and beeswax (Tulu et al., 2020). As a result, beekeeping has been recognized as the potential sector providing more employment opportunities to several communities, and for its role in poverty reduction. Generally, beekeeping production in Ethiopia is classified into three main categories based on the hive types: traditional (fixed-comb hives), transitional (intermediate hives), and modern (movable frame hives) beekeeping.

2.5.1.1 Traditional beekeeping

Traditional beekeeping belongs to the oldest agricultural activities in Ethiopia and still is a major integral component in today's apiculture economy of the country. It is the predominant and most ancient form of beekeeping, primarily relying on various indigenous hive designs which are smaller in size and usually made of locally available materials such as wood, bamboo, straw,

clay, or mud (Hailu & Tesfay, 2012; Andaregie & Astatkie, 2021; Gebewo et al., 2023). Although the construction design and size of traditional hives vary according to local areas, the majority of hives typically exhibit a cylindrical shape (about 1-1.5 meter in length and 30-50 cm width) with a single chamber fixed comb (Gratzer et al., 2021). The honeybees construct the combs vertically by fixing the upper side to the inner walls of the hive in horizontal arrangements. Whilst, there has been an ongoing transition to modern beekeeping in recent years, the majority of beekeepers (about 96%) are still practicing traditional beekeeping in Ethiopia (Gratzer et al., 2021). The average honey yield of traditional beekeeping is about 7g/per colony/per harvest depending on the hive size, availability of bee forage and the level of beekeeping management (Nuru, 2007). In general, traditional beekeeping is of two types: forest and backyard beekeeping. Forest beekeeping is mainly practiced by hanging a number of traditional hives on trees in the dense forest where there is high vegetation cover and high honeybee colonies. Whereas. backyard beekeeping is practiced around residence areas, particularly in areas with little forest coverage and where intensive agricultural practices carried out (Tarekegn, 2022).

2.5.1.2 Transitional beekeeping

Transitional (intermediate) beekeeping is an intermediate between traditional and modern beekeeping system. Transitional beekeeping is widely promoted in many rural areas of East African countries, including Kenya, Tanzania, Uganda and Ethiopia and appropriate for resource poor beekeepers with low income and skills of bee management (GDS, 2009). It is one of the improved methods of beekeeping practices by which the hives can be constructed from timber, mud, or locally available materials called “Chefeka” (Nuru, 2007). The majority of beekeepers in rural areas of Ethiopia are not yet in a position to use modern hives because of technical and economic reason, and this condition makes transitional beekeeping as an appropriate beekeeping for them. The use of transitional hives in Ethiopia started around 1985 (Nuru, 2007). The transitional beekeeping method offers several advantages compared to traditional beekeeping. These include that the hives can be constructed by the beekeepers or local carpenters, the hives can be opened easily and quickly for inspection, the top bars are easily removable and suitable for bees to build combs, harvesting honeycombs from the hives can be done without disturbing combs containing broods, and so on (Ababor & Tekle, 2018). However, the adoption rate of transitional beekeeping so far is very low, and the household survey estimated that the number of

transitional hives in Ethiopia was around 34,552 (Serda et al., 2015). The average honey yield obtained from intermediate beekeeping ranges between 15-20 kg/year, which is much higher than the traditional hive (Gratzer et al., 2021; Andaregie et al., 2022).

2.5.1.3 Modern beekeeping

Modern beekeeping is characterized by movable frame hives with advanced level of beekeeping equipment and tools. Movable frame hives allow multiple honey harvests per year and help to obtain maximum yield without causing damage to bee colonies (Nicola, 2002). Moveable frame hive beekeeping was introduced to the country in 1978 through the Ethiopian Rural Development Extension program (MoARD, 2007). Although the productivity of movable frame hive is higher as compared to the other two types (traditional and transitional beekeeping), its adoption rate by the beekeepers is still very low due to its high initial cost and expenses of accompanying equipment. The number of movable frame hives in Ethiopia until 2009 was estimated at 100,843 (GDS, 2009). The productivity of the modern box hives is significantly higher than the traditional and intermediate beekeeping, which is estimated to 30kg/annum/hive (Gratzer et al., 2021). However, in potential areas and well managed colonies, far more than the average yield can be harvested as reported from the northern and south western parts of the country (Gemechis, 2016). Furthermore, movable frame hives enable advanced colony management and the utilization of sophisticated technologies, leading to higher yields of quality honey. However, it is constrained due to it requires higher investment costs and demand skilled workers.

2.5.2 Challenge and constraints of beekeeping in Ethiopia

Despite the long-standing tradition of beekeeping in Ethiopia, the sector faces numerous challenges that hinder its full production potential. Although the country is ranked among the top honey and beeswax producers; however, the current honey and beeswax stands only for about 10% of its production potential. These lower productions attribute to various constraints as highlighted by several studies. These include shortage of bee forages (especially during droughts), habitat degradation, unwise application of pesticides, swarming and absconding behaviors of bees, the prevalence of pests and predators, limited knowledge in beekeeping practices and management, and insufficient storage and marketing facilities (Gidey & Kibrom, 2010; Yirga et al., 2012; Fikru, 2015; Gratzer et al., 2021). Moreover, beekeepers and

agricultural experts in Ethiopia have been frequently reporting the widespread decline and massive death of bee colonies and there are indicatives of some endangered bee species such as stingless bees in the country. Although there has not been reported colony losses in the country, the emergence of new novel pests, pathogens and parasites like *V. destructor* could be potential risk to the sustainability of the country's apiculture industry (Begna, 2015). Therefore, it is crucial to promptly enforce control measures and regular monitoring of honeybee stressors (including pathogens, pests and predators and invasive parasites) across potential beekeeping regions.

2.6 Factors affecting the health of honeybees

The vital contributions of honeybees in human economy and ecosystem balance are highly dependent on the healthy performance of their populations. The health of honeybees is one of the most important factor given due attention in apiculture research in recent years (Genersch, 2010). Obviously, honeybee health can be influenced by several biotic and abiotic factors, and understanding these elements is crucial for sustaining bee populations and ensuring successful beekeeping practices. Over the last two to three decades, considerable research has been dedicated to the health of honeybees, driven mainly by significant global colony losses (Moritz et al., 2010; Potts et al., 2010; Pirk et al., 2016). A large spectrum of stressors can synergistically affect bees while operating in diverse ecosystems (Lin et al., 2023). Frazier et al. (2024) currently reviewed the presence of diverse factors contributing to honeybee colony losses, such as pathogens, pests, parasites, and habitat degradation and malnutrition. In addition, the widespread exposure of bees to several pesticide pollutants is not only threatening the life of individual bees, but also intimidating the survival of the colony through contamination of nectar and pollen being shared throughout the hive (Sanchez-Bayo & Goka, 2016; Abay et al., 2023).

2.6.1 Honeybee pathogens

Bees exhibit a high level of sophistication through their intricate social organization, making them particularly susceptible to various pathogenic stressors. They commonly face threats from a range of pathogens, including bacteria, protozoa, viruses, and fungal infections (Genersch, 2010; Grobov et al., 1987). These pathogenic agents impact multiple aspects of honeybee biology, resulting in various detrimental effects such as reducing immunity, reduced reproductive capacity, body malformations, hindered development, tremor paralysis, impaired cognition and

homing ability, loss of navigation systems, and a shortened lifespan (Alberoni et al., 2016; Ullah et al., 2021). Although many of the pathogens may kill the individual bees they infect, their consecutive effects on the colony can lead to reduced hive populations, and severe infections may even result in the colony collapse (Evans & Schwarz, 2011). Forsgren (2009) suggested that some of the microorganisms causing honeybee diseases are more pathogenic, causing more infections, apparently leading to colony losses. Nevertheless, the effects of pathogens are often specific to certain ages of honeybees; some primarily affect adult bees (referred to as adult bee diseases), while others target the immature stages of bees (known as brood diseases). Additionally, certain pathogens like bee viruses can affect both stages of honeybee development. Unlike western honeybees, African bee populations are less susceptible to most pathogens due to their behavioral and immunological factors evolved from various conditions (Fazier et al., 2010; Muli et al., 2014).

In Ethiopia, the prevalence of commonly known honeybee microbial diseases, such as varroosis, nosemosis (*Nosemaapis*), amoeba (*Malpighamoeba mellificae*), and chalk brood (*Ascosphaera apis*) were reported so far in local honeybees (Bezabeh et al., 2013; Begna, 2015). Recently, Gebremedhn et al. (2020) employed metagenomics next-generation sequencing of bee samples and identified five commonly known RNA viruses, including deformed wing virus (DWV), sac brood virus (SBV), black queen cell virus (BQCV), *Apis mellifera* filamentous virus (AmFV), and lake sinai virus (LSV) and 25 a typical viral species in *A. mellifera simensis* colonies. Luckily, the two devastating brood diseases, such as European foulbrood (EFB) and American foulbrood (AFB), which affect honeybee colonies in most Western countries have not been reported so far in Ethiopia (Bezabeh et al., 2013). Overall, regular assessment and monitoring for emerging honeybee pathogens and diseases is very crucial for safeguarding the native honeybee populations.

2.6.2 Honeybee Pests and Predators

In tropical and sub-tropical countries, the infestation of honeybee colonies by pests is much greater than the impact of bee diseases and pathogens. Unlike European honeybees, African honeybees are less susceptible to pathogens due to behavioral and immunological adaptations evolved from various conditions (Fazier et al., 2010; Muli et al., 2014). As a result, it is the honeybee pests and predators that are challenging the health of bees and beekeeping production

in Africa than bee pathogens (Pirk et al., 2016). With this understanding, the existing literature indicated that a number of assessments were conducted in different parts of the continent at different times with the objectives of identifying honeybee pests along with their distribution ranges and kinds of products they affect. Pirk et al. (2016) reviewed the widely known honeybee pests and predators in Africa, including hive beetles, flies, wax moths, ants, wasps, spiders, pseudo-scorpions (Ellingsenius spp.), bee-invasive species., birds, and mammals.

In Ethiopia, different survey and diagnostic studies indicated the occurrence of more than 15 honeybee pests and predators, which either attack the bees themselves or their products. These includes different ants, wax moths (greater and lesser wax moths), mice, birds (different types), honey badger, wasps, death's head hawks, bee lice (*braula coeca*), beetles (different types), lizards, toads/frog, prey-mantis, spiders, pseudo scorpions (*chelifer species*) and etc.(Gidey et al., 2012; Kinati et al., 2012; Bezabeh et al., 2013; Tesfay, 2014; Begna, 2015). The devastative effects of these prominent honeybee pests and predators, either on the bees themselves or on their products, have been identified, apparently influencing the pollination services of the bees and the overall beekeeping industry in the country.

2.6.3 The parasites of honeybees

Honeybees face numerous threats from various parasites that can negatively affect their health and overall hive productivity. Some major honeybee parasites include the Varroa mite (*V. destructor*) (Anderson & Trueman, 2000), the tracheal mite (*Acarapis woodi*) (Garrido-Bailón et al., 2012), the bee louse (*Braul acoeca*) (Alfallah & Mirwan, 2018), and the microsporidian parasites (*Nosema apis* and *Nosema ceranae*) (Higes et al., 2009). The Varroa and tracheal mites (Acaria, Acarpis) are among the most widely distributed parasites and pose significant threats to *A. mellifera* populations across the world. However, their ability to adapt successfully on their host underscores the severity of the challenges that they inflict on the apiculture industry. Particularly, the varroa mite currently represents substantial risks to honeybee health and is potentially associated with global colony collapse in the absence of human interventions (Rosenkranz et al., 2010; Traynor et al., 2020). Therefore, several reports indicate that the widespread losses of wild and feral *A. mellifera* populations in the Northern Hemisphere are mainly attributed to the infestation of *V. destructor* mite, and this emphasizes the need for effective parasite control and monitoring strategies (Bruckner et al., 2023).

2.7 The Varroa mites (Mesostigmata: Varroidae)

Varroa mite is an ecto-parasitic mite of honeybees parasitizing both the brood and adult honeybees. The parasite was first reported in Asia, specifically in Java, in 1904 on *A. cerana,* honeybee species (Oudemans, 1904). Particularly, the species *V. destructor* is the most widespread and economically damaging species, especially the lineage of the Korean haplotype (Lin et al., 2018). It inflicts substantial harm to honeybee colonies by feeding on the body fluids of both adult bees and developing larvae (Figure 2.2), then weakening the overall colony health and productivity (Dietemann et al., 2012). Apart from this, several studies confirmed that varroa serves as a vector for various viral, bacterial and fungal pathogens exacerbating serious negative impact on honeybee populations (Martin et al., 2012; Locke et al., 2021; Mendoza et al., 2020).

Given the devastating effects on honey bee colonies *(A. mellifera), V. destructor* has emerged as a central focus of scientific studies in the field of apiculture and various management efforts have been implemented over the past several years (Dietemann et al., 2019; Traynor et al., 2020; Lin et al., 2021b). Therefore, understanding the biology (morphology, taxonomy and life cycle), behavior, genetics, and distribution of this parasitic mite is imperative for mitigating its adverse impact on honeybee populations and ensuring the sustainability of beekeeping practices worldwide.

2.7.1 Morphology, Taxonomy and Life cycle of *V. destructor* mite

2.7.1.1 Morphology

The Varroa mite is an external parasite, which can be easily visible with the naked eye, exhibits distinctive characteristics. The female mite, ranging in color from brown to reddish-brown, measures about 1.1 to 1.2 mm in length and 0.7 to 1.6 mm in width, approximately the size of a pinhead (Figure 2.2). Conversely, the males are smaller than females, measuring about 0.8 mm by 0.7 mm (Rosenkranz et al., 2010).

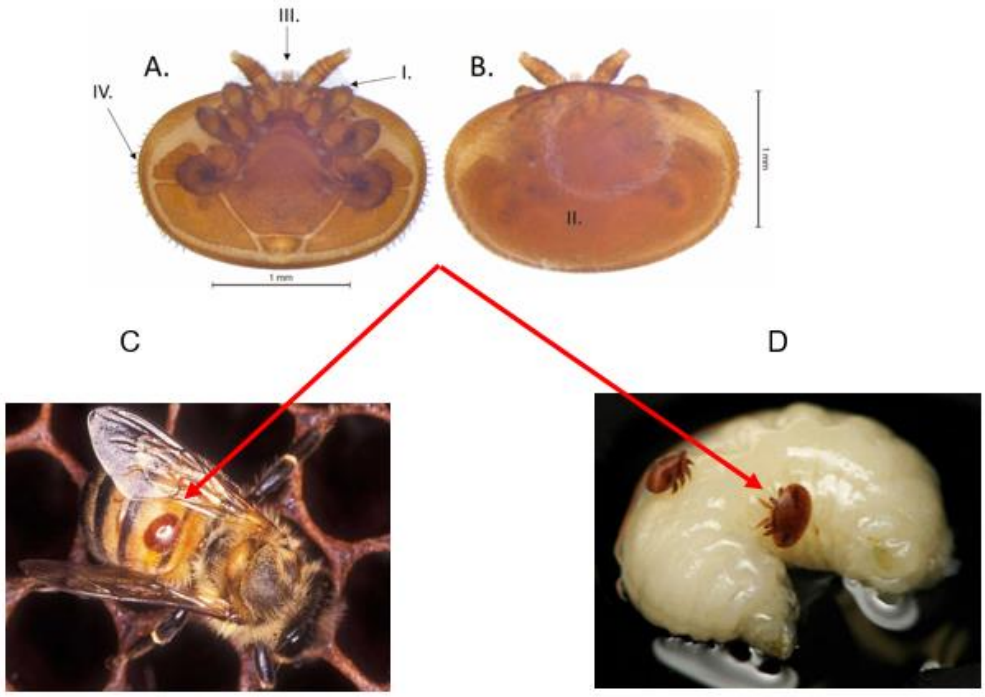

Figure 2. 2 Varroa morphological view*:* ventral view (A), dorsal view (B). The arrows show the feeding nature of the mite on the fat tissues of an adult bee (C) and on an immature (larva) (D) (Source; Boncristiani et al..2020)

The mite morphology is finely adapted to attach to the host, as illustrated in Figure 2.2. Both male and female mites share a common feature: the division of the body into two distinct parts, namely the idiosoma and gnathosoma. In female mites, the idiosoma is flattened and ellipsoidal, with greater width than length. Additionally, the female mite's legs are short and feature a specialized structure known as the opoteles, facilitating attachment to the host (Sammataro et al., 2000).

2.7.1.2 Taxonomy

In the early 19th century, the genus varroa was named as *V. jacobsoni* and known for parasitizing Asian honeybees (Oudemans, 1904). This species was later classified into a distinct genus of Varroa and family Varroidae (Delfinado & Baker, 1974). However, scientific investigations employed different techniques to identify the diversity within varroa mite species, of which all have contributed to the present understanding of varroa taxonomy. The most common and simple technique used for classifying the varroa species is the body measure of physical traits (morphometric analysis). Based on this, the current varroa taxonomy is characterized as:

Kingdom: *Animalia;* Phylum: *Arthropoda;* Class: *Arachnida;* Subclass: *Acari;* Order: *Mesostigmata;* Family: *Varroidae;* Genus: *Varroa* (de Guzman & Rinderer, 1999).

Furthermore, molecular techniques have significantly enhanced varroa taxonomy, particularly in determining the species diversity within varroa genus and unveiling cryptic species (Anderson & Trueman, 2000; Evans & Lopez, 2002). The genus varroa consists of four recognized species and these include: *Varroa jacobsoni* (Oudemans, 1904), *Varroa underwoodi* (Delfinado-Baker & Aggarwal, 1987), *Varroa rindereri* (de Guzman & Rinderer, 1999), and *Varroa destructor* (Anderson & Trueman, 2000). *V. jacobsoni* and *V. destructor* were the only species known to affect honeybees and originally confined to their natural host, the Eastern honeybees, *A. cerana* (Oldroyd, 1999). Among these, *V. destructor* is known only to infest the European honeybees, *A. mellifera*, and the other three species remain originally infesting the Asian honeybees, *A. cerana* (de Guzman & Rinderer, 1999; Genersch & Aubert, 2010). Although *V. jacobsoni* can be found on *A. mellifera*, it is only known to reproduce when living in *A. cerana* colonies. According to Anderson and Trueman (2000), noted that a significant phenotypic contrast between *V. jacobsoni* and *V. destructor* lies in the greater body size evident in specimens of *V. destructor*.

2.7.1.3 Varroa life cycle

There are two distinct phases in the life cycle of varroa mite: the reproductive and the dispersal or phoretic phase (Rosenkranz et al., 2010). The reproductive phase of the mite greatly depends on the development of drone or worker broods, permitting only a narrow time period for reproductive success (Rosenkranz et al., 2010). The reproductive adult female mite enters the fifth instar brood cells of the honeybee, just before cell capping. Once inside the cell, the female mite hides underneath the larva´s food until the cell capping and she starts feeding on the 5^{th} instar larval fat body, or hemolymph and initiates oogenesis within a few hours (Rosenkranz & Garrido, 2004). About three days later, the female mite starts laying an unfertilized haploid male egg, and followed by fertilized diploid female eggs in 30 h intervals (Frey et al., 2013). In normal condition, the first unfertilized egg is usually laid about 70 hours after cell capping and eventually develops into a male mite. As soon as the first female reaches sexual maturity, the male mates with the female within the brood cells, and this is triggered by female sex pheromones (Häußermann et al., 2015). The next female also follows the same process when she is getting maturity. Accordingly, a female mite can lay 5-6 eggs in single honeybee brood cells that will hatch into nymphs, which go through two nymphal stages, the protonymph and

deutonymph before molting into adult mites (Rosenkranz et al., 2010). At this phase, the mites again feed on the fat body and hemolymph of the last instar larvae, pupae, and adult bees, causing significant weight loss and shortening the lifespan of honeybees (Navajas et al., 2008; Ramsey et al., 2019). Finally, the adult females released following the host bee hatching and can be transmitted between individual honeybees within the same colony or might even be spread to a new host colony through foraging and drifting bees as well as through human beekeeping activities (Rosenkranz et al., 2010).

During the phoretic phase, female mites attach themselves to adult bees and parasitize them, which allow them to be transported to worker or drone brood cells, where they breed. Then, the mites can spread across colonies through parasitized robber or drifting bees, as well as through beekeeping practices (Figure 2.3) (Pirk et al., 2016). At the higher varroa infestation level, the colony become weak and robbing by foragers from other colonies is common. During this time, mites can jump hosts from infested colony to the robbing bees and vice versa from robbers to other workers and infest a new colony. Furthermore, mites can also attach to foragers of their original colony and, in the event those foragers accidentally drift, mites gain access to other colonies (Peck & Seeley, 2019). Additionally, beekeepers inadvertently introduce mites into foreign colonies when they transfer supplies and bees between hives (Figure 2.3), for example, during movement of frames and exchange of combs.

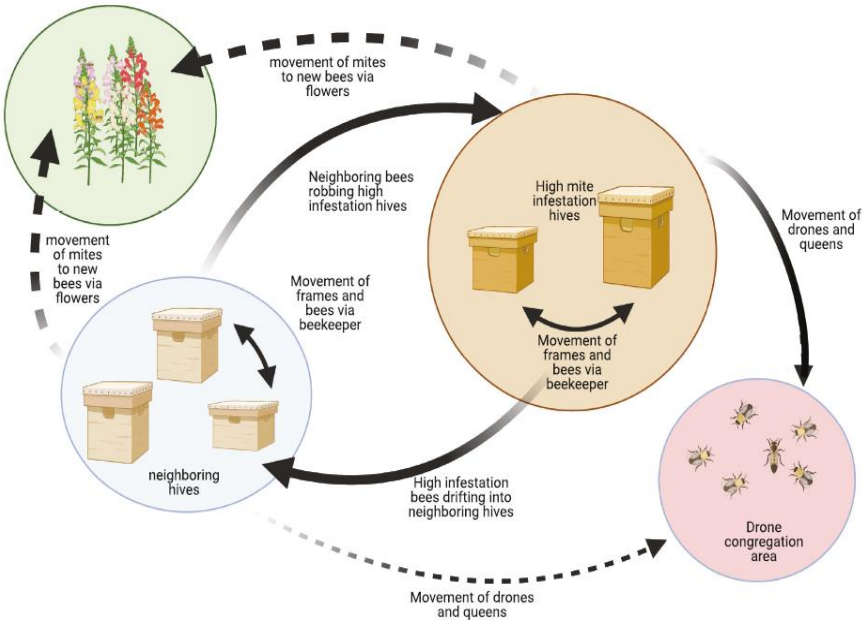

Figure 2. 3 Diagram depicting the potential ways in which Varroa mites can move between honey bee colonies (Source: Reams & Rangel (2022)).

2.7.2 Global distribution of *V. destructor* mite

Originally, *V. destructor* was well adapted to its native host, the Eastern honeybees, and later it shifted its host to the Western honeybees (Oldroyd, 1999), and this led to the worldwide spread of the parasite. During the mid-nineteenth century, the European honeybee colonies were transported from Europe and the USA to Asia for the purpose of beekeeping, and this sympatry provided an opportunity for the *V. destructor* to parasitize and quickly spread to *A. mellifera* honeybees (Chantawannakul & Ramsey, 2018; Anderson & Trueman, 2000). Following this host shift, *V. destructor* exhibited a rapid spread across the world, reaching Europe in early 1970s and achieving global distribution approximately in 2000 (Traynor et al., 2020). Studies suggested human-mediated factors, such as global bee trade and movement of colonies were the main attributing factors for the global spread of the parasite (Lin et al., 2018; Moro et al., 2021). Furthermore, the lower individual behavioral defenses of *A. mellifera* as compared to *A. cerana* against the mite have also contributed for the mite rapid spread across the world. Together with the worldwide decline of natural pollinators, the varroa mite may exacerbate future problems for food security (Flores et al., 2021). Today, *V. destructor* is the most established parasite of *A. mellifera* and is known to be globally distributed in Europe, the USA, New Zealand, Africa, and the Middle East from southern and southwestern Asia, except in Australia (Figure 2. 4) (Traynor et al., 2020).

In Africa, the parasite was first reported in 1997 in South Africa (Allsopp et al., 1997; Martin & Kryger, 2002), and later in 2009 in Kenya, Tanzania, Uganda, and Ghana (Fazier et al., 2010) and Ethiopia in 2010 (Begna, 2014). However, despite its wide occurrence and rapid spread, severe colony losses attributed to *V. destructor* have not been extensively reported in Africa. In Ethiopia, there was a notable gap in understanding the current prevalence and infestation rates of the mite across diverse geographical regions until the present study. Thus, detailed studies on the overall distribution and infestation levels of the mite in Ethiopia are very crucial.

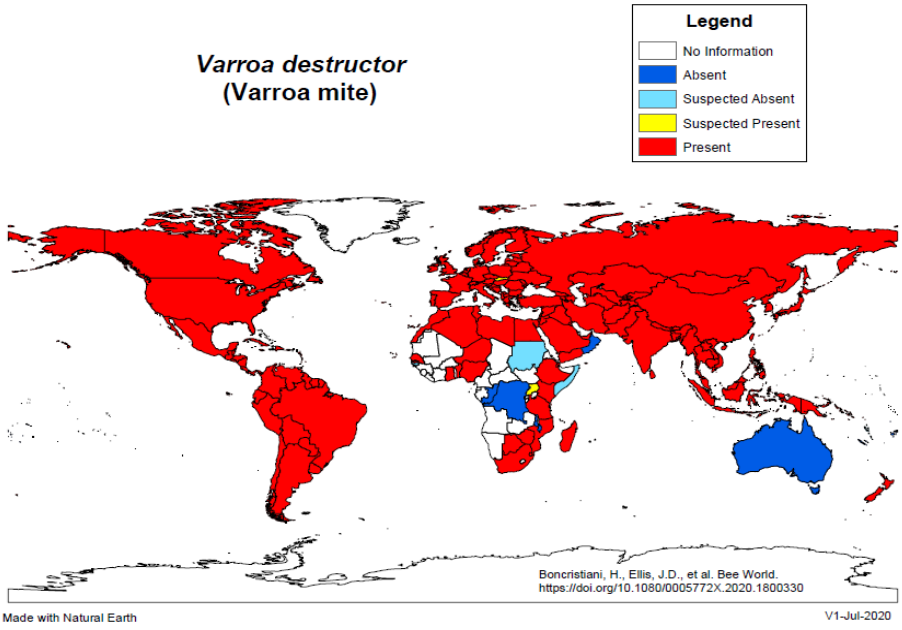

Figure 2. 4 Global distribution of *V. destructor* mite (source; Boncristiani et al. (2020))

2.7.3 Genetic diversity of Varroa mite (*V. destructor*)

Varroa is the only genus in the family Varroidae, and currently consists of four recognized species: *V. destructor*, *V. jacobsoni*, *V. rindereri* and *V. underwoodi* (Anderson and Trueman, 2000). Among these four species, variants of *V. jacobsoni* and *V. destructor* have been well studied and both categorized into multiple groups. The first sequence of mitochondrial DNA cytochrome oxidase subunit I (COI/CO-I/COXI) by Anderson and Trueman (2000) discovered twelve *V. jacobsoni* and four *V. destructor* haplotypes in *A. cerana* honeybees. On the other hand, they found two haplotypes of *V. destructor* (the Korean and Japanese haplotypes) in *A. mellifera* honeybees and there were also unidentified mite haplotypes in both honeybee species as described by Morfin et al. (2023). Each haplotype was named after the country or island in which it was first discovered, as indicated in Figure 2.5. The Japanese haplotype is restricted to Japan, Thailand, French Guyana, Chile, and Brazil, whereas the Korea haplotype is the most prevalent and virulent globally (Traynor et al., 2020) (Figure 2.5).

However, in addition to COX-I analysis, Lin et al. (2021) examined sequence variation in cytochrome b(Cytb) analysis and identified two variants for the Korea haplotype in china and Giuffre et al. (2019) discovered five variants of the same Korean haplotype in Serbia both of

which were associated with *A. mellifera*. Furthermore, analysis of sequence variation in Cytb, COX1, ATP synthase subunit 6(ATP6), cytochrome c oxidase subunit III (COX3) discovered four variants of the Japanese-Thailand haplotype, and three variants of the Korean haplotype, all of which are found in northeast Asia associated with *A. cerana* honeybees (Navajas et al., 2010) (Figure 2.5). In contrast, Ogiharaet al. (2020) did not find any variation within the Korean haplotype in Japan following the same set of gene analysis.

Another alternative method for assessing genetic variation in *V. destructor* involves the examination of microsatellite polymorphisms. Analysis of the *V. destructor* transcriptome identified a substantial number of potential microsatellite loci, and among a set of 60 randomly selected microsatellite loci, six were confirmed to exhibit polymorphism in *V. destructor* samples collected from five locations in China (Duan et al., 2020). This suggests that while *V. destructor* contains numerous potential microsatellites, only a fraction may display polymorphic characteristics. For example, Navajas et al. (2010) did not identify any single nucleotide polymorphisms (SNPs) in the Cytb gene within the Korea haplotype of *V. destructor* infesting *A. mellifera* in Asia.

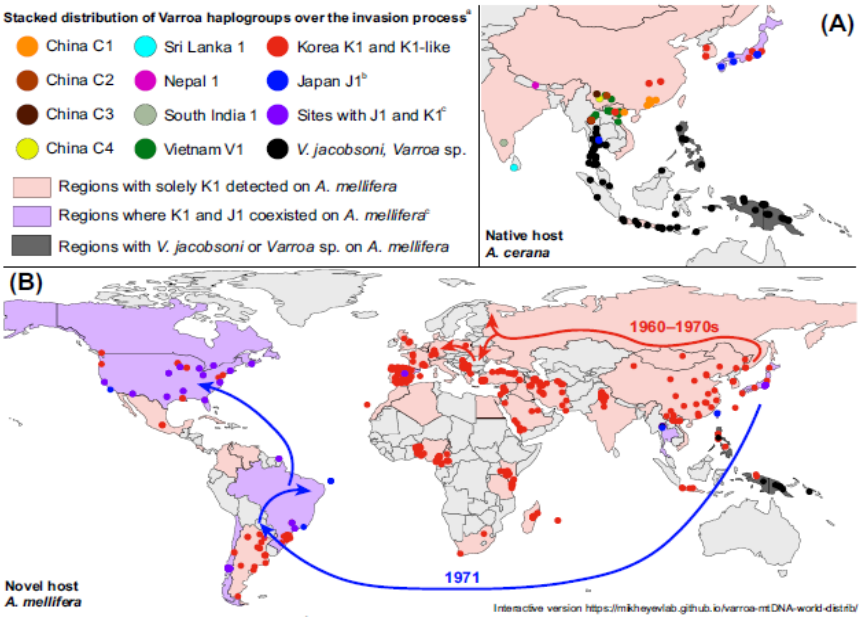

Figure 2.5 Global distributions of varroa haplotypes, determined by mtDNA COX-I, 458 nucleotide identity on *A. cerana* (A) and *A. mellifera* (B) (Source: Traynor et al., 2020).

2.7.4 Economic importance of *V. destructor* mite

The invasive ecto-parasitic mite, *V. destructor* is one of the most economically important and widely distributed honey bee parasite, attacking mainly European honeybees, *Apis mellifera*. The trajectory of host shifts from its native Asian honeybees *(A. cerana)* to the European honeybees (*A. mellifera*) has made the *V. destructor* to become the most virulent and threat to the *A. mellifera* colonies (Traynor et al., 2020). Locke et al. (2012) indicated that most *A. mellifera* honeybees lack specific genetic adaptations that support them to fight against the mite infestations, unlike those of *A. cerana* bees. Thus, *V. destructor* is recognized as the most harmful, and globally distributed pest of European honeybees that successfully infests both managed and wild colonies and play a prominent role in the dramatic losses and decline of global honeybee populations (Rosenkranz et al., 2010; Traynor et al., 2020).

The mite is an obligate honeybee parasite, which feeds on the hemolymph and fat bodies of brood and adult bees (Ramsey et al., 2019; Cournoyer et al., 2022). Consequently, the infestation by *V. destructor* has a strong negative effect on the fitness of honeybees both at the individual and at the colony level. Depending on the level of mite infestation, bee pupae undergo significant nutritional deficiencies during their development, so that both adult female mites and their offspring feed on the pupae's fat tissue. This feeding behavior of the mite can ultimately reduce the survival of honeybees, by altering the host physiology and reducing the immuno-competence of the bees (Aronstein et al., 2012; Stanimirović et al., 2019). The disease symptom caused by high varroa infestation threshold in honeybees is called ‘varroosis’ (Ramsey et al., 2019). When the mite infestation threshold reaches the level of varroosis, the condition leads to the phenomenon called “Colony Collapse Disorder” (CCD) (Flores et al., 2021). Such a heavy mite infestation, causing CCD has a longstanding threat to the economic importance of honeybees and also disturb the ecological balance of various ecosystem (Bartlett, 2022). Thus, this parasitic mite has been classified as a notifiable parasite by the International Organization for Animal Health (OIE) (Aufauvre et al., 2012).

In general, the parasitism of *V. destructor* involves two different mechanisms: one by affecting the development of honeybees on which it feeds and reproduces (Rosenkranz et al., 2010), while the other entails by transmitting secondary pathogens, including bee viruses, bacteria, and fungal diseases (Shen et al., 2005; Cox-Foster et al., 2007; Di Prisco et al., 2011). For example, the

DWV and ABPV are among the most common pathogenic viruses transmitted by this ecto-parasite, and usually the mite is linked to higher viral loads in infected colonies (Wilfert et al., 2016; Piou et al., 2022). Its parasitism, coupled with its vector role in propagating viral diseases, contributes to reduced lifespan of honeybees and is linked to the current losses and decline of colony populations worldwide (Le Conte et al., 2010; Dainat et al., 2012). Furthermore, honeybees exposed to varroa mite infestation demonstrate changes in the expression of genes linked with stress response, immunity, nervous system function, metabolism, and behavioral maturation (Zanni et al., 2017). This in turn impact both ecological and economic benefits of the honeybees, and becoming a threat for global food security (Potts et al., 2010). Consequently, the impacts of *V. destructor* on honeybee populations and beekeeping industry have been well documented in the Northern hemisphere and a subject of several studies (Rosenkranz et al., 2010; Traynor et al., 2020; Morfin et al., 2023). Thus, *V. destructor* is known as the leading threat to the survival of honeybees and has persistently pose significant challenges to the apiculture industry, as described in the latest review by Warner et al. (2023). Beside its combined impact on bee health and potential beekeeping production, varroa also reduces colony ability for pollination services which in turn has serious impact on crop production for food security (De Jong et al., 1982).

2.7.5 Effect of Varroa on African honeybees

The devastating effects of *V. destructor* depend on the levels of mite infestations determined by the susceptibility of specific bee races that originated in different geographic regions (Locke, 2016a; Mendoza et al., 2020). The effect of varroa and its associated viruses on Western honeybees (*A. mellifera*) is being increasingly reported from different parts of the world, including Europe, the USA and the Middle East (Forsgren, 2009; McMenamin & Genersch, 2015; Pirk et al., 2016). In contrast, African honeybees and a few populations of Africanized honeybees in Europe have evolved natural adaptations to survive against the effects of this parasitic mite without human intervention (Pirk et al., 2016; Strauss et al., 2016; Nganso et al., 2017; Gebremedhn et al., 2019; Tibatá et al., 2021). These "surviving populations' have increased the interest of scientific communities to investigate the mite evolutionary history in response to novel honeybee defensive traits and determine genetic variations among the varroa populations in different areas. Sainsbury et al. (2022) indicated that there were more significant genetic changes in the mite-resistant honeybee colonies than mite-susceptible colonies,

suggesting that resistant bees are fast responding to mite infestation pressure in a coevolutionary arms race.

In general, African honeybee subspecies are less likely to be threatened by the mite infestation, and the impact of varroosis on African bee populations is rather limited (Muli et al., 2014; Pirk et al., 2016). Various factors, such as behavioral, genetic, and reproductive characteristics, have been identified as contributing to the ability of African honeybees to tolerate mite parasitism. Consequently, African bees have coexisted with mites for prolonged periods, approximately 15 years, without the use of chemical treatment. However, this may not always true and does not ensure the sustainable resilience of African honeybee populations in the long-term process of changing climate, and the potential adaptation of varroa mites in the future. For example, Pirk et al. (2014) observed a population decline of honeybee colonies ranging from 29.6%-46.2% in South Africa in the years between 2009 to 2011 due to sever infestations of *V. destructor*. Similarly, Allsopp (2001) reported a reduction of approximately 14% in the pollination efficiency of colonies that were heavily varroa-infested in the same country. This underscores the potential threat posed by varroa mites to African honeybees under specific circumstances and highlights the importance of regular monitoring and early intervention measures to control mite infestations. Despite the absence of reported colony losses in Ethiopia at present, awareness creation, designing, and implementing evidence-based interventions are very imperative.

2.8 Behavioral responses of honeybees against *V. destructor*

Honeybees are social insects living in overcrowded populations and due to this phenomenon; they are vulnerable to various pathogen and parasite transmissions. On the other hand, bees have evolved a variety of defense mechanisms to combat against specific pathogen and parasite infestations at both individual and social levels (Kurze et al., 2016). A number of potential resistance mechanisms were evolved by different honeybee populations to fight against the varroa mite. Some defense mechanisms of honeybees against the mite include, hygienic behavior, grooming behavior, reduced post-capping stage duration of worker brood, brood recapping and suppressed mite reproduction as reviewed by Büchler et al. (2020). Apart from their unique behavioral defense, co-existing genetic traits that enable honeybees to tolerate the mite infestation have also been identified in some honeybee species (Strauss et al., 2016; Nganso et al., 2018). However, the effectiveness of each response varies depending on the mite

infestation level, the honeybee species, and their geographical origins (Mondet et al., 2020). Hygienic and grooming behaviors are among the well-documented behavioral defenses of honey bees in response to *V. destructor* parasitism.

2.8.1 Hygienic behavior

The hygienic behavior is a heritable trait of honeybees in which the worker bees are detecting targeting, opening, and removal of diseased, parasitized, or dead broods (Spivak et al., 2009; Unger & Guzman-Novoa, 2010). The early removal of infected broods from the nest before the pathogen becomes infectious would help to minimize the contagious infestation below the injury level and can enhance the survival of the colony. Hygienic worker bees have the ability to identify and uncap comb cells containing larvae infected with bacteria, fungi, or parasitized by *V. destructor* (Guzman-Novoa & Morfin, 2019). Particularly, the infestation and reproduction of varroa in honeybee brood trigger the hygienic behavior of worker bees and expressed as the Varroa Sensitive Hygiene (VSH) behavior (Mondet et al., 2015). In response to this infestation, the parasitized larva may either be removed from its cell (Boecking & Marla Spivak, 1999; Boecking et al., 2000) or the cell may be subsequently recapped (Hawkins & Martin, 2021), thereby disrupting the mite's life cycle that limits the number of mite offspring. The number of dead broods removed within 24 hours is efficient selection criteria to improve hygienic behavior, as evidenced by a model that includes the genetic effects of queen and workers being separate (Guichard et al., 2020).

The heritable trait of hygienic behavior enables certain honey bee strains exhibiting greater hygienic tendencies than others (Spivak & Danka, 2021). It is well documented that African honeybees in general display higher hygienic behavior within their hives than European honeybees (Muli et al., 2014; Nganso et al., 2017; Mondet et al., 2020). The hygienic worker bees usually use their antennae to detect chemical odorants from varroa infested broods, such as hydrocarbons and oleic acid (Mondet et al., 2015; McAfee et al., 2018). These chemical cues elicit hygienic responses in the bees and help them to identify and remove infected brood or pupae that are hosting varroa mites and prevent the mites from reproducing and spreading within the colony. Notably, strains expressing the VSH trait are usually known to reduce mite reproduction and minimize its infestation level in a colony (Harbo & Harris, 2009). Furthermore,

hygienic behavior is an important behavioral mechanism for African bees allowing them to resist the infestation by pathogens like fungal, bacterial, and viral infestations (Cheruiyot et al., 2018)

2.8.2 Grooming behavior

Grooming behavior is also an important defense mechanism of honeybee populations used to protect themselves against parasitic infestations (such as varroa mites and tracheal mites). This behavior involves the meticulous dislodging and damage of parasites from bees' bodies either by themselves (auto-grooming) or by their nest mates (allo-grooming) (Bąk & Wilde, 2015; Traynor et al., 2020)., According to Mondet et al. (2020) honeybee workers typically exhibit grooming behavior in response to *V. destructor* infestations, which enables them to effectively reduce the parasite population within their colony below the danger threshold level. Parasitized worker bees employ their legs and mandibles to remove the parasites from their bodies, sometimes biting and inflicting injuries upon the parasites in the process (Boecking & Marla Spivak, 1999). The presence of varroa mites within a colony, along with the wounds they cause to bee bodies, typically triggers irritations in the bees, and these stimulate adult bees to engage in grooming responses (Morfin et al., 2019). However, grooming behavior is recognized as a heritable trait, and the expression of this behavior varies among different genotypes and strains of honeybees (Rinderer et al., 2001; Morfin et al., 2020).

Interestingly, the higher intensity of grooming behavior and injuries that the bees inflict on the body of the varroa mites can significantly influence the mites' reproduction cycle and population growth in varroa-surviving colonies (Dadoun et al., 2020; Russo et al., 2020). In fact, African honeybees are well recognized to display stronger grooming responses to varroa infestation compared to European honeybees, and this trait may has attributed to the survival and resistance of African honeybee colonies against varroa mite infestations (Muli et al., 2014; Pritchard, 2016; Nganso et al., 2017).

2.8.3 Social immunity

Species that live in colonies are more susceptible to infectious diseases and parasite infestations due to frequent contact among conspecifics (Harwood et al., 202; Conroy & Holman, 2022). It has been hypothesized that highly social species have developed more robust immune systems compared to solitary species. As a result, social immunity refers to a set of behavioral, physiological, and organizational adaptations aimed at hindering the entrance, establishment, and

spread of pathogens and parasites in the colony (Cremer et al., 2007). Social immunity in insects encompasses a range of strategies employed by social insect colonies to combat the threat of pathogens and parasites. These strategies involve not only individual immune responses but also collective behaviors and social interactions in the colony (Moret & Schmid-Hempel, 2000).

Honeybees are highly social insects living in a colony, and they develop social immunity as a result of cooperation of individual bees within the colony to minimize the potential risk of disease transmission, evolving through selective pressures at both individual and colony scales (Simone-Finstrom, 2017; Jones et al., 2018). In most cases, honeybees depend on colony level adapted behaviors for defense against major bee pathogens and parasites. For example, exposure to *V. destructor* mites can activate the honeybee immune system, leading to immune priming. This process enhances the immune response of individual bees and can confer resistance to subsequent mite infestations (Richard et al., 2012). In general, social behaviors such as hygienic, grooming, and migration (swarming and absconding) have evolved in honeybees to defend against the catastrophic effects of various pathogens and parasites. Nevertheless, different honeybee species exhibit various levels of adaptive defense traits in order to combat the negative impact of pests and pathogens. Particularly, African honeybee populations are known to evolve strong behavioral defense mechanisms to reduce the transmission of pathogens between colonies as well as to limit the infestation level of pests and parasites in a colony (Fries & Camazine, 2001; Mondet et al., 2020).

2.9 The occurrences of Varroa mite in Ethiopia

The occurrence of parasitic mite of *V. destructor* in Ethiopia was first reported in 2010 in the northern part of Ethiopia, Tigray region (Begna, 2014). However, a few years' later, the mite has been rapidly spread across the entire country, supposed to be due to colony movement and various beekeeping activities. This has created national awareness in different parts of the country and some studies indicated the prevalence, temporal distribution and effect of varroa on honeybee colonies across different beekeeping areas (Godifey, 2015; Begna et al., 2016; Mezgabu et al., 2016). Although these reports are limited to specific apiary locations, very recent studies, but in specific areas, showed the fast spread of the mite in the country with more than 95% prevalence rate (Shegaw et al., 2022; Robi et al., 2023). However, information on the current threshold infestation level at which the mite causes potential effect on local honeybee

colonies, in specific beekeeping sites is lacking. Many colonies exhibiting severe varroa infestations have been observed surviving and well performing during the course of this study, and it is still unknown how acutely the mites can impact the honeybee population of Ethiopia. Gebremedhn et al. (2023) recently identified the higher gene expression of odorant binding protein, OBP14, in the antennae of Ethiopian bees, which could potentially be attributed to resilience against varroa infestation.

2.10 Management of *V. destructor* mites

Varroa destructor is undoubtedly the most important and damaging pathogen among honey bee *(Apis mellifera)* pests worldwide. To overcome the impact of varroa parasitism on honeybee health and its threat to apiculture industry, several efferts including, cultural, mechanical, biological, and chemical control strategies have been used against varroa infestation globaly. Particularly, beekeepers in the northern hemisphere largely depend on the regular monitoring and chemical treatments against the parasite (Rosenkranz et al., 2010). However, effective control of the mite is still challenging due to the limitations of each control strategy. For example, the overuse and mismanagement of synthetic acaricides such as parathion, amitraz, coumaphos, and taufluvalinate have led to widespread resistance among varroa populations against these acaricides (Sammataro et al., 2005; Higes et al., 2020). Moreover, some chemicals pose potential hazardous effects to bee health and human well-being (Jack & Ellis, 2021), while the accumulation of chemical residues in hive products presents significant challenges (Bogdanov, 2006; Martel et al., 2007). These challenges have prompted beekeepers to adopt Integrated Pest Management (IPM), an environmentally sound and sustainable approach to pest control. IPM is systemic approach to pest management that combines various strategies to minimize risks on human health and the environment (Jack et al., 2021; Bubnič et al., 2024). An effective IPM program involves establishing economic thresholds, monitoring pest populations, implementing preventive measures, and applying targeted treatments as necessary (Flint, 2012).

Another approach for varroa management is the use of soft acaricides or organic compounds such as formic acid, oxalic acid, and Lactic acid for the last many years (Rosenkranz et al., 2010; Jack & Ellis, 2021). The use of such organic compounds has also been restricted due to the potential residue left in hives and hive products intended for human consumption (Jack and Ellis, 2021). Furthermore, the applications of entomo-pathogenic fungi such as *Metarhizium anisopliae*

and *Beauveria bassiana* as biological control have becoming effective for control of varroa mite at different spatial scales (Rodríguez et al., 2009; Sinia & Guzman-Novoa, 2018). These fungi infect and kill the mites, providing a natural and environmentally friendly alternative to chemical pesticides. According to Han et al. (2021) these entomo-pathogenic fungi can be applied directly to the hive or incorporated into integrated pest management strategies, contributing to sustainable beekeeping practices while minimizing harm to the bees and their products.

Furthermore, the use of botanical extracts and essential oils is currently becoming an alternative approach to the control of varroa mite (Bava et al., 2023). The application of such plant derivatives such as thyme (thymol) has been recognized as the best alternative approach for varroa control over synthetic acaricides in maintaining the safety and health of honeybees and bee products (Damiani et al., 2014; Tutun et al., 2018). Many researchers have evaluated the efficacy of some botanical extracts against *V. destructor,* suggesting the potential advantages of botanical pesticides over synthetic acaricides for the safety of bees while controlling mite infestations below the economic injury levels (Lindberg et al., 2000; Damiani et al., 2011; Qayyoum et al., 2013). Propolis is also among the plant resins collected by worker honeybees from different plant parts and has been assessed for the control of *V. destructor* in different countries (Garedew et al., 2002; Simone-Finstrom et al., 2017). However, its control effects on varroa mites in Ethiopia context has not been evaluated so far, until the present study revealed its potential varroacidal effect.

CHAPTER THREE

Distribution and infestation levels of Varroa mite (*V. destructor*) in honeybee *(Apis mellifera)* colonies of Ethiopia

3.1 Introduction

The honey bee, *Apis mellifera*, is a well-adapted insect with significant economic and ecological values that exists in a variety of geographical conditions around the world (Ivanova et al., 2010; Boncristiani et al., 2020). They are economically important because of their role as pollinators of numerous agricultural crops and as producers of honey and various hive-related commodities such as beeswax, propolis, royal jelly, and pollen. Approximately about 90% of the world's agricultural production of human food depends on bees' pollination (Bailes et al., 2015). Furthermore, honey and beeswax production is a source of income and financial security for many rural communities in Africa.

Despite the vital role that bees play in sustaining ecosystem and ensuring food security through pollination services, there has been a concerning global trend of honeybee losses and colony decline (VanEngelsdorp et al., 2009; Potts et al., 2010; Zattara & Aizen, 2021). Many interacting factors, including parasites and pests, pathogens, exposure to pesticides, food shortages and climate change have been proposed for the ongoing threat to the bees (VanEngelsdorp et al., 2008; Henry et al., 2012; Reddy et al., 2012; Van der Zee et al., 2012; Smith et al., 2013). The ecto-parasitic mite *Varroa destructor* (Anderson & Trueman, 2000) is one of the most economically important and widely distributed honey bee parasites in the world. The mite feeds on the fat bodies of the bees (Ramsey et al., 2019) and also transmits other secondary pathogens like bee viruses and bacteria infestations (Le Conte et al., 2010; Traynor et al., 2020). These synergetic effects of varroa parasitism and the transmission of other pathogens, such as deformed wing virus (DWV) (De Miranda et al., 2013), can result in weakening the immune system of honey bees and ultimately lead to colony collapse (Noël et al., 2020; Traynor et al., 2020). As a result, the parasite has a devastating impact on the apiculture industry and also reduces pollination dependent agricultural production, especially in the western countries. To this fact, beekeepers in the northern hemisphere have relied on acaricide treatments for decades to control varroa mite infestations. However, effective control of the mite is more challenging due to the spread of mite resistance to acaricides and the potential risks of acaricide residues (Sammataro et

al., 2005; Higes et al., 2020; Mitton et al., 2022). Hence, varroa remains a highly intricate parasite that poses a significant challenge to honey bees and is continuing spreading globally.

In contrast, honey bee populations in Africa appear to be less likely threatened by mite infestations when compared to European honey bees. Studies suggest that African honey bees have developed some biological, behavioral, and genetic resistance that enable them to cope with the stress caused by the mite (Strauss et al., 2016; Nganso et al., 2017; Gebremedhn et al., 2019). This could be evidence that African beekeepers do not use any acaricide treatments to control the varroa infestations in their colonies. However, the pest has continued to spread rapidly across the continent, even in countries where it was not previously reported. So far, the occurrence of this parasitic mite was recognized in many African countries including Kenya, Tanzania, Uganda, Ghana, Ethiopia and other countries (Dietemann et al., 2009; Fazier et al., 2010; Begna, 2014; Chemurot et al., 2016).

The varroa mite was first detected in 2010, in Ethiopia in the Tigrai region, Ethiopia (Tigray Agricultural Bureau, unpublished data). Later in 2014, it was reported that varroa is an invasive pest of honeybees that had widely spread across the same region (Begna, 2014). This situation raised awareness in the other regional states of the country, and consecutive studies recognized the presence of the mite in different locations of Ethiopia (Begna, 2015; Godifey, 2015; Mezgabu et al., 2016; Ayele et al., 2020). Later, the study by Gebremedhn et al. (2019) indicated the resistance behavior of Ethiopian honeybee colonies by investigating the low reproductive capability of female mites to produce adult male offspring in *A. m. simensis* colonies again in the Tigrai region. However, these data are limited to specific locations and are very fragmented. Comprehensive study indicating the distribution pattern and infestation level of the mite as well as factors contributing to the mite spread across the wider regions of the country is lacking. Thus, this study aimed to determine the current distribution pattern of varroa mites in Ethiopia by analyzing mite prevalence and infestation levels at different bee developmental stages, hive types, and various locations across the wide regional states of the country. This will provide a better understanding of the current parasitism in the country and help to design and develop sustainable monitoring and control strategies.

3.2 Material and Method

3.2.1 Study area description

The study was carried out in three administrative regions of Ethiopia (Oromia, Amhara and SNNP), from September 2020 to June 2022. The three administrative regions were purposively selected based on their beekeeping potential and accessibility. From each administrative region, apiary sites were also purposively selected. Accordingly, a total of 39 apiary sites (27 in Oromia, 7 in Amhara and 5 in SNNP) were selected for colony sampling. Global Positioning System (GPS) data was recorded for each apiary site and presented on a map (Figure 3.1), along with detailed descriptions provided in supplementary material (Table S-1, Appendix 1). The altitude of the sampling locations ranged from 1109m to 3366m above sea level, representing the arid agro-ecology of Rayitu district (East Bale zone, Oromia region) to the afro-alpine ecology of Anza-Guassa district (North Shoa zone, Amhara region). In this assessment, the bee colonies were randomly sampled both from traditional hives (local hives) and from modern hives (movable frame hives).

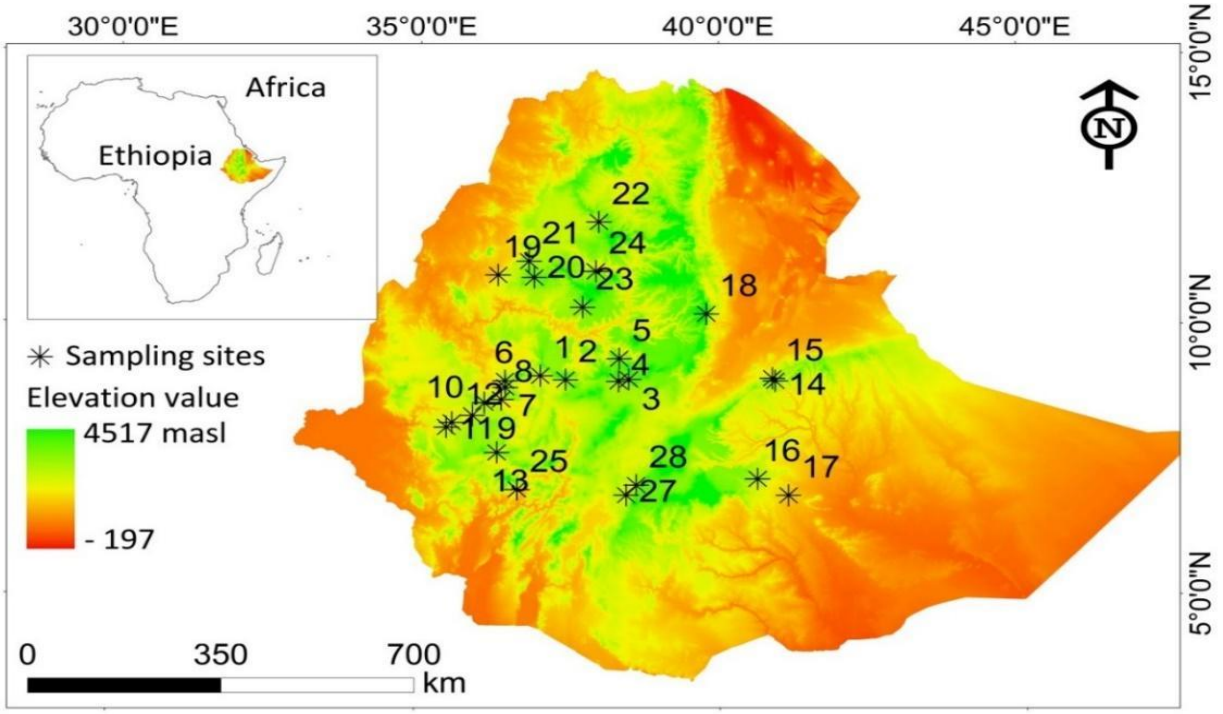

Figure 3.1 Geographic location of surveyed apiaries for varroa sample collection in three regions of Ethiopia (numbers with *indicate specific sampling sites in which some were appeared as overlapping scales**)**

1. Bakko Kejo	6. Gemechisa	11. Witate	16. Ginnir	21. Dangila	26. Shebedino
2. Gedo	7. Jirata	12. Gore /Sagi	17. Rayitu	22. Debre Elias	27. Wondo Genet
3. Holeta	8. Gudina	13. Gera/Afallo	18. Guassa	23. Gozamin	28. Alata Wondo
4. Kimoye	9. Dedessa	14. Gemechis	19. Guangua	24. Mota	
5. Inchini	10.Sanga Fato	15. Chiro	20. Banja	25. Konta	

3.2.2 Sample collection

In this study, random sampling technique was employed to collecte bee colonies for sampling the adult and brood bees for varroa diagnosis. Samples were collected during the major nectar flow seasons of the year, typically between September to November and April to June (Bareke & Addi, 2019). A total of 360 bee samples, comprising of 180 adult bees and 180 brood samples, were collected (Figure 3.1). Both the adult and brood bee samples were collected from different colonies in the same respective apiaries. The bee samples were collected from various sources including beekeepers' backyards, farmer training centers, research stations, university stations, and agricultural college sites, with the technical guidance and support of local experts. The standard procedure of Dietemann et al. (2013) was followed for each sampling technique. In this regard, a sample of adult bees (consisting of 250-300 workers) was collected for each colony by brushing off the middle brood combs directly into wide mouth plastic jars with 8 mm-mesh lids. Similarly, a section of 5 cm × 5 cm sealed brood cells containing about 100-200 broods was collected either from drone or worker broods of the same apiary. Due to the difficulty of accessing traditional hives, which are often hung up on tall trees, only 36 samples were collected from nine locations in traditional hives (Table 3.2). All collected samples were labeled with a permanent marker pen and stored at room temperature until diagnosis.

3.2.3 Sample diagnosis

The collected samples were diagnosed in the respective regional Animal Health Laboratories using the established standard method of varroa research (Dietemann et al., 2013). To dislodge the varroa mites from the adult bee samples, a solution of 1% detergent water (10 ml liquid soap in 1000 ml water) was used and mixed with each sample and vigorously shaken. The mites were then collected by filtering the solution through a double sieved ladle i.e., the contents of the container were discharged through a first sieve (mesh size: 2000 μm) to catch all the bees and let the mites out with the solutions. Then, the solution was poured through the second sieve (mesh size: about 0.5 mm) to collect the mites back from a solution. This washing procedure was

repeated until there were no more mites found on the adult bees. The collected mites were turned down on absorbent papers to let them dry, and thoroughly counted to determine the infestation rate of the mite in adult bees.

Consecutively, varroa mite was examined in brood samples by opening capped brood cells using fine forceps or tooth sticks. An average of 150 pupae (brood) cells were opened from a section of brood sample, and checked for the presence of mites. All the mites found on the pupae, in the cells, on the cell walls were carefully collected, and counted to determine the infestation rate in brood sample.

3.2.4 Determining varroa prevalence and infestation rates

In this study, the prevalence rate (%) of varroa mite was evaluated and determined for each administrative region (Oromia, Amhara and SNNPR) using two steps. Firstly, by dividing the number of apiaries that were tested varroa positive to the total number of assessed apiaries, then multiplied by 100. Secondly, by calculating the proportion of varroa positive colonies in the total sampled colonies with the same procedure. Likewise, the varroa infestation rates (%) were calculated as the number of mites recovered per 100 bee samples (both for adult and brood samples) as described by Dietemann et al. (2013). Briefly, varroa infestation rates (%) in adult bees were expressed as the number of mites per 100 adult bees and infestation rates (%) in brood bees were determined as the number of counted mites per 100 opened brood cells.

Accordingly, the mite infestation level was classified as low (< 3%), medium (3-5%), high (5-10%) and very high (>10%) as described by Szczurek et al. (2020), with little modification for local colonies.

3.2.5 Statistical analysis

The collected data was recorded and organized in Microsoft excel sheets and analyzed using R software version 4.2.3 (R Core Team, 2021). The statistical analysis used in the study varied depending on the type of variable and information obtained. Shapiro-Wilk test was applied to test the data normality and Levene's test was used to test homogeneity of the response variables. Kruskal-Wallis test was performed to compare the varroa mite infestation levels (both in adult and in brood bees) between the three regions (Oromia, Amhara and SNNP). The Mann-Whitney-Wilcoxon test was used to compare the varroa infestation levels between the local hives *versus* framed hives considering the mite infestation in adult bees and in brood bees as response

variables. On the other hand, pairwise t-test was used to compare the mean varroa infestation levels between the adult bees *versus* the brood bees. Data were expressed with significance level set at $P < 0.05$.

3.3 Results

3.3.1 Varroa mite prevalence rate

Varroa mites were present in all three geographic regions of Ethiopia during the study seasons. The overall prevalence of the mite was 89.7% and 89.4% at the apiary level and the colony level, respectively (Table 3.1). The results were varied both at the apiary and colony levels across the sampled regions, with the highest prevalence recorded in the Oromia region. The average prevalence rates at the apiary level for each region were 96.3% in the Oromia region, 85.7% in the Amhara region, and 60.0% in the SNNP region (Table 3.1). Similar trends were observed for the prevalence rates of the mite at the colony level in the three regions, which comprised 95.8 % in Oromia, 85.2 % in Amhara and 71.9 % in SNNPR (Table 3.1).

Table 3 1 Average prevalence rate of varroa mite in apiaries and sampled colonies in Oromia, Amhara and SNNP regions

Regions	Number of apiaries	Varroa +ve apiaries	Prevalence rate (%)	Number of colonies	Varroa +ve colonies	Prevalence rate (%)
Oromia	27	26	96.3	242	232	95.8
Amhara	7	6	85.7	54	46	85.2
SNNP	5	3	60.0	64	46	71.9
Total	**39**	**35**	**89.7**	**360**	**324**	**89.4**

3.3.2 Varroa mite infestation rate

Our study revealed that the infestation rates of varroa mite were significantly varied ($P < 0.001$) among the apiary locations, ranging from 21.4±8.6% (Mean and SD) to 0% (Figure 3.2). Specifically, the varroa infestation rate in Batambe (21.4±8.6), Abadira (20.7±6.2), and Semen Degera (19.9±4.8) locations were considerably higher than the other study locations (Figure 3. 2). Only 10% of the sampled locations were found to be free of the mites, which includes; the Akasha, Guassa, Butuma, and Bullessa locations.

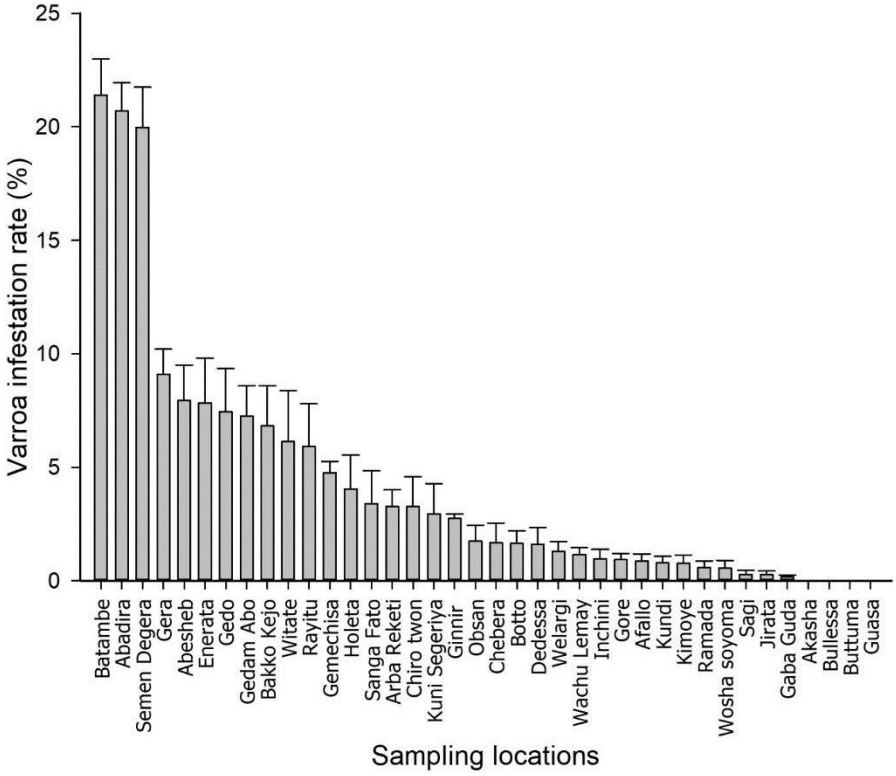

Figure 3. 2 Infestation rates (%) (mean ± SD) of *V. destructor* per 100 bees in *A. mellifera* colonies in Ethiopia. Each study location was indicated for sampled apiaries.

When comparing the two ontogenetic phases (adult *vs* brood phase), the infestation rate of varroa mites in brood bees (5.58±0.76%) was significantly higher when compared to the infestation rate in adult bees (1.93±0.17%) ($t = -6.47$, $df = 179$, $P < 0.0001$, Figure 3. 3c), which highlights that varroa mite infestation is more pronounced in the brood phase of honeybees than in the adult phase.

Likewise, the mean varroa mite infestation level in framed hives (3.8±1.6%) (mean ± SD) was higher than in local hives (0.8±2.9%) (Table 3.2, Figure 3.3b). When considering the bees' ontogenetic phases as response variables, the result of Mann-Whitney-Wilcoxon test revealed that the mite infestation rate in brood bees was significantly higher in modern hives than in the traditional (local) hives ($W=1662.5$, $P = 0.0007$). Similarly, the infestation rates of varroa mite in adult bees were also significantly higher for modern hives when compared to traditional hives ($W=1843$, $P < 0.001$) (Table 3. 2).

Table 3 2 Infestation rate of varroa mite in frame hives and traditional hives as compared for both adult and brood bees. The study locations were limited to where equal samples were collected both for local and frame hives

Study apiaries	IR in Frame (Modern) hives (%)		IR in Local (Traditional) hives (%)		Average IR in modern hives	Average IR in Traditional hives
	Adult bees	Brood bees	Adult bees	Brood bees		
Afallo	0.5±0.4	1.2±1.2	0.0±0.0	0.0±0.00	0.9±0.8	0.0±0.0
Bako Kejo	2.7±1.4	10.9±15.9	0.8±0.8	5.6±5.5	6.8±8.7	3.2±3.1
Chebera	2.5±3.9	4.0±5.5	0.0±0.0	0.2±0.4	3.2±4.7	0.1±0.2
Gedo	2.7±1.7	12.2±9.2	1.5±1.6	3.4±3.8	7.5±5.4	2.4±2.7
Gera	4.7±1.9	13.5±11.1	0.0±0.0	0.0±0.0	9.1±6.5	0.0±0.0
Guassa	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Holeta	1.3±0.5	6.8±7.8	1.5±1.6	0.7±25.6	4.0±4.2	1.1±13.6
Inchini	1.8±1.2	0.1±0.2	0.5±0.4	0.3 ±0.4	0.9±0.7	0.4±0.4
Kimoye	1.2±1.5	1.7±2.4	0.0±0.0	0.2±0.4	1.4±1.9	0.1±0.2
Total mean	**1.9±3.1**	**5.6±2.3**	**0.5±1.2**	**1.2±12.2**	**3.8±1.6**	**0.8±2.9**

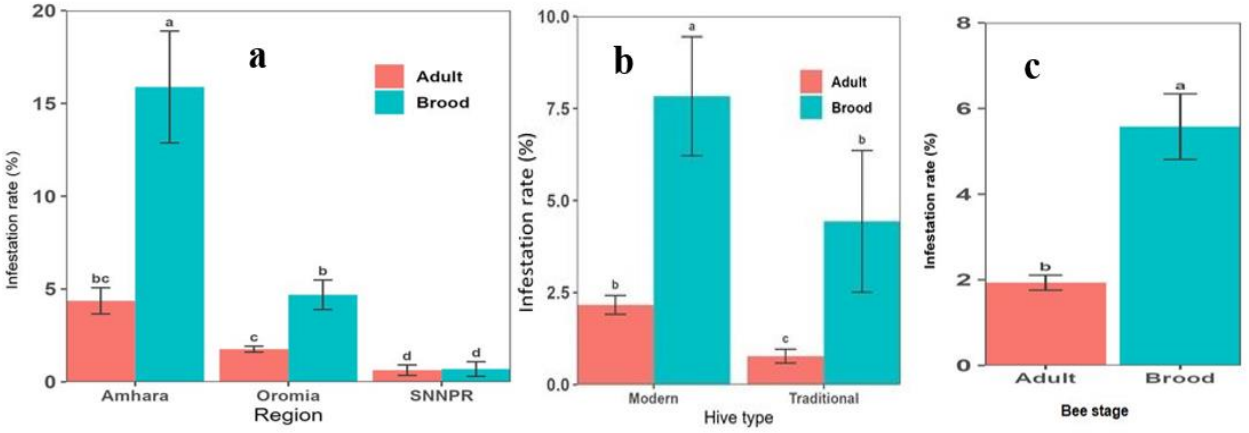

Figure 3.3 Mean varroa infestation rate (mean ± SD) in different categories: **a.** Amhara Vs Oromia Vs SNNPR regions **b.** Traditional Vs Modern beehives **c.** Adult Vs Capped brood bees

The overall varroa mite infestation level among the three regional states was found to be varied, as illustrated in Figure 3. 4, and highlighted with different colored spots. The mean varroa infestation rates were 15.9±3.0 and 4.37±0.7% in the Amhara, 4.7±0.8 and 1.8 ±0.2% in the Oromia and 0.7±0.4 % and 0.6 ±0.3 in the SNNP regions in brood and adult bees, respectively (Figure 3. 3a). Based on the Kruskal-Wallis test at $\alpha = 0.05$, the varroa mite infestation level in brood bees was significantly different among the three regions ($H = 17.54$, $df = 87$, $P < 0.001$). However, for the mite infestations in the adult bees, a significant difference was observed

between Amhara and SNNP ($P < 0.001$) and Oromia and SNNP ($P < 0.001$), while there was no significant difference between Oromia and Amhara ($P = 0.07$). Overall, the highest level of varroa infestation was observed in the northern part (Amhara region) when compared to the centeral (Oromia region) and the SNNP region (Figure 3. 3a). In this study, the lowest varroa mean mite infestation rate was observed in the SNNP region.

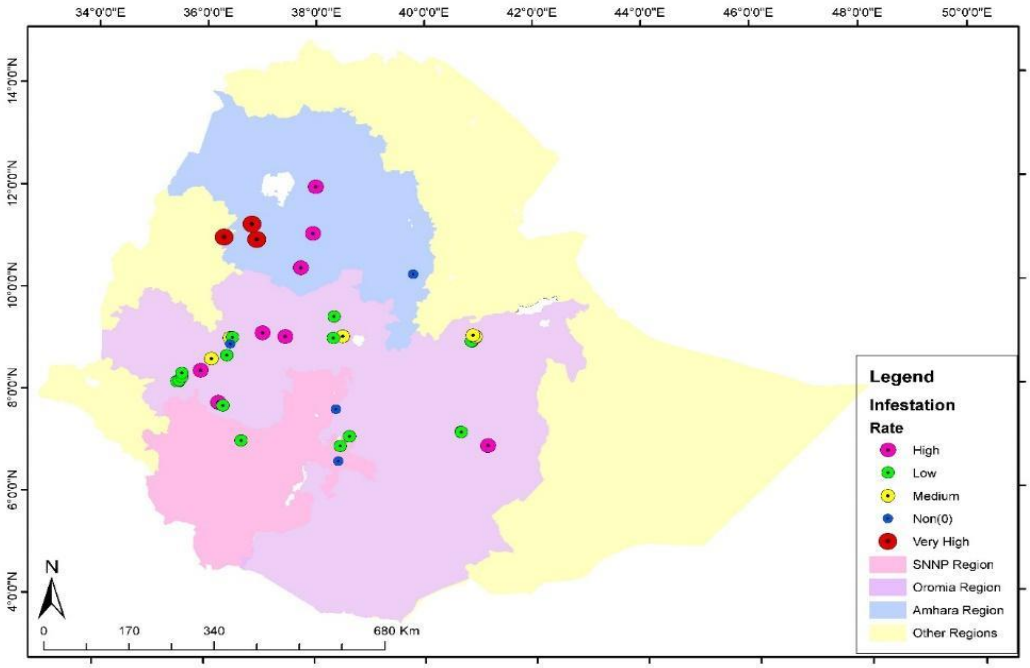

Figure 3. 4 Distribution and infestation rate of varroa mite in three regions of Ethiopia (Dots with different colors indicate apiaries with varied infestation rates as indicated in the listed legend)

3.4 Discussions

This study provides an insight into the distribution and infestation level of the *Varroa destructor* mite in Ethiopia, covering a wide geographic area. A comprehensive assessment was conducted involving 360 local honeybee colonies, *A. mellifera* in 39 apiaries. The study focused on determining the prevalence and infestation levels of the parasitic mite with regard to administrative regions, hive types and the bees' ontogeny phases. The findings of this study revealed that the mite was conspicuously distributed across all the studied administrative regions including, Oromia, Amhara and SNNPR regions. However, significant variations were observed

in the prevalence and infestation levels of the mite among the regions, the sampled colonies, as well as among the hive types. Our result revealed an overall of 89% mite prevalence in the country, ranging from 71.9% to 95.8%. This result was higher than previous findings reported 82% in the Tigray region of Ethiopia (Begna 2014), 83% in Kenya (Muli et al., 2014), 59% in Uganda (Chemurot et al., 2016), and 48% in Tanzania (Mumbi et al., 2014).

The differences in the prevalence of varroa mites observed across the country may be attributed to various regional factors, such as changes in environmental conditions, seasonal flora of the area, beekeeping management practices, and variations in local honey bee subspecies (Giacobino et al., 2017; Hillayová et al., 2022). Although climatic data was lacking in this study, Ethiopia is known for its highly diverse topography, followed by variation in climatic factors (Cheung et al., 2008; Etana et al., 2020; Ware et al., 2023). Such spatial variability in climatic factors, between regions and even between seasons, can create opportunities for the spread of different pests and pathogens beyond their ecological niches if not regularly monitored. Earlier studies showed that changes in climate patterns and geographical variation have significantly impacted the distribution of *V. destructor* for the past ten years in honey bee colonies of East Africa (Muli et al., 2014; Chemurot et al., 2016; Giliba et al., 2020). Particularly, Giliba et al. (2020) demonstrated that changing climate patterns posed a high risk to the spread of the *V. destructor* mite in Tanzania. Likewise, the varied prevalence and infestation levels of varroa in Ethiopia appears to be associated with ecological variations among geographic regions, while further research is required to identify suitable and non-suitable climatic factors to predict the potential risk of the parasite in the country.

In this study, the higher the prevalence rate of varroa mite in the Oromia region may be associated with the higher the sample size and also the honeybee population in the region. As described by Addi and Bareke (2019), the abundance and diversity of bee flora resources in the Oromia region appear to support a larger colony population as the result of consistent brood rearing and colony swarming than other regions in Ethiopia. This feature likely contributed to the widespread distribution of varroa mites in the potential beekeeping areas of the Oromia region as compared to the Amhara and SNNP regions. This is in agreement with previous reports that demonstrated the strong correlation between varroa mite distribution and the dynamics of its host

population (Chemurot et al., 2016; Muhoro, 2017). In addition, Messan et al. (2021) suggested that the seasonal availability of broods and the high tendency of colony swarming can promote the extensive distribution of *V. destructor* within specific geographic areas. Likewise, previous studies conducted in Africa have observed similar patterns of varroa distribution in areas with strong honeybee colonies, but they have the ability to maintain the mite population below the infestation threshold (Muli et al., 2014; Strauss et al., 2015; Chemurot et al., 2016).

On the other hand, the significantly higher varroa infestation levels per 100 bees observed in certain apiaries in the Amhara region, for example, 21.4±8.6% in Batambe, 20.7±6.2% in Abadira, and 19.9±4.8 % in Semen Degera highlights the possible impact of the varroa mite on local honeybees. Thus, further investigation is required to better understand the parasite's potential effect on local honeybees and identify associated risk factors specific to these areas, then to take immediate monitoring actions. In previous studies, factors such as drought, scarcity of bee forage, intensive agricultural practices, and specific colony trading practices have been identified as potential constraints of beekeeping sector in the Amhara region (Kerealem et al., 2009; Kalayu et al., 2018; Bihonegn and Begna, 2021). These constraints and their synergistic effect would likely weaken the local honeybee colonies and reduce their defense behaviors (such as hygienic and grooming behavior), against the varroa mite infestation. Previous researches also identified that different factors such as nutritional stress, climate change, and human activities can adversely affect the health of honeybees and impede their ability to defend against pests and pathogens (Vandame and Palacio, 2010; Giacobino et al., 2017). Particularly, the common practice of honeybee colony movement and trading in the northern regions (Amhara and Tigray regions) (Kerealem et al., 2009; Gebretinsae & Tesfay, 2014; Bihonegn & Begna, 2021) may have contributed to the higher levels of varroa infestation in the above particular apiary locations. Because local beekeepers in Ethiopia generally lack awareness about contagious pathogens in their colony and there is no established pathogen monitoring strategy in the country. In line with this, Owen (2017) suggested that global colony trade and migratory beekeeping practices might contribute to the rapid spread of bee parasites and pathogens, especially in the absence of monitoring.

The current study also revealed a significantly lower level of varroa infestation in colonies in local hives than in movable frame hives. Similar findings were also reported by Gebremedhn et

al. (2019) demonstrating the lower level of varroa mite infestation in traditional hives when compared to movable frame hives in the Tigray region, particularly during the season of May-June. However, Chemurot et al. (2016) did not find a significant difference in varroa infestation level among different hive types in Uganda. Rather, the same study identified that bee hives that were frequently inspected had significantly higher mite infestation levels than those that were not inspected. These observations indicate that local beekeeping apiaries are less likely to be influenced by human activities and less exposed to bee parasites and pathogens than modern beekeeping apiaries (Lounsberry et al., 2010; Owen, 2017), although the underlying reasons were not well explained. In the Ethiopian context, several traditional beekeeping practices and the honeybees' behavior may have contributed to the lower levels of varroa infestation in local hives than in modern hives. For example, beekeepers typically use a total comb removal during honey harvesting in local hives (Kebede & Adgaba, 2011), which promotes *de novo* comb building and this condition can disrupt the mites' reproduction life cycle and reduce the mite population growth in honey bee colonies. Moreover, the higher tendency of colony absconding and seasonal migration in the traditional hives (Ayinalem T & Zeleke M, 2017) would support the evidence that lower varroa mite infestation levels were recorded in these hives. In Ethiopia, beekeepers use some specific swarm attractants in traditional hives, which are often derived from specific plant species known for their natural medicinal properties (e.g., *Ocimum bacilicum and Lippia adoensis)* (Tewodros et al., 2017; El-Bolok & Mahfouz, 2021; Kebebe et al., 2022) but there is no evidence to suggest whether this condition helps bees avoid exposure to pathogens and pests. On the other hand, Taric et al. (2019) indicated that non-treating colonies with acaricides could provide better conditions for local honeybees to develop population level immunity and resistance against pests and pathogens.

On the other hand, the higher mite infestation levels recorded in framed hives may be linked with human activities. Modern beekeeping involves frequent activities such as colony inspections, artificial feeding, queen rearing, colony splitting, and other management practices that could increase the likelihood of pathogen transmission from infected colonies to healthy ones (Navajas et al., 2010). For example, Chemoret et al. (2016) have explained the potential impact of frequent colony inspection in increasing the level of varroa mite infestation. As there are no established regulations or guidelines for preventing the spread of bee pathogens and parasites in Ethiopia, many local beekeepers do not take any precautions during beekeeping

management practices. Thus, the increased level of varroa mite infestation in modern beehives observed in this study could be linked to such human beekeeping practices conducted without adequate precautions.

In this study, the higher the varroa infestation rate observed in the brood bees (5.6±0.8%) than in the adult bees (1.9±0.2%) suggests that varroa mites prefer to infest brood larvae (pupae) for feeding on the soft body tissues of the larvae and to complete their life cycle in the brood cells (Rosenkranz et al., 2010; Yang et al., 2021). Thus, the presence of honey bee broods, particularly drone broods, is very essential for the existence and reproduction of varroa mites in a colony. According to previous studies, the prolonged development period of drone pupae creates a conducive environment for female varroa mite to reproduce her offspring and complete the reproductive cycle within the same cells (Boot, 1995; Boot et al., 1999). Boot (1995) also highlighted that varroa mites invade drone cells approximately 12 times more frequently than worker cells within a honey bee colony, implying that invasion into drone cells occurs much faster than invasion into worker cells. On the other hand, adult worker bees exhibit regular grooming behavior which can minimize the mite infestation rates by removing and damaging adult mites from their bodies (Pritchard, 2016). Interestingly, African honeybees have been recognized evolving strong grooming behaviors identified as a key factor in reducing varroa infestation at colony level (Bąk & Wilde, 2015; Pritchard, 2016; Nganso et al., 2017).

In general, the present study provides valuable insights into the current prevalence and infestation of varroa mite at a wide range of locations in Ethiopia. It is important to note that various factors, such as changes in climate patterns and global human activities, have the potential impact to shift the current varroa infestation levels to the level of devastating effect on local honeybee populations and the beekeeping industry. For instance, the higher varroa infestation levels observed in this study in some specific apiary sites in the Amhara region highlight an alarming condition regarding the possible impact of the mite on the local honeybees. Therefore, regular monitoring and assessment on the mite infestation and prevalence rates are highly recommended to mitigate the rapid spread of the mite in the country and to establish sustainable and long-term varroa management strategies. The current governmental expansion of modern beekeeping in Ethiopia should incorporate precautions and preventive strategies to

control the spread of varroa mites across the country. Furthermore, conducting comprehensive research on the behavioral and genetic adaptations of local honeybee populations in response to varroa mite infestation is indispensable to understand the host-parasite dynamics, thereby used for selection breeding of resilient colonies.

CHAPTER FOUR

Genetic diversity and population structure of *Varroa destructor* mite in honeybee (*Apis mellifera)* colonies of Ethiopia

4.1 Introduction

The varroa mite (Acari: Varroidae) is one of the invasive species affecting both managed and wild honeybees and recognized as a serious biotic threat to the global apiculture industry. The mite was initially described as *Varroa jacobsoni* (Oudemans) in Java, on its native host of the Asian honeybees (*A. cerana*) (Oudemans, 1904). During the mid-nineteenth century, the Western honeybees (*A. mellifera*) were introduced to Asia for the beekeeping purpose and the parasite encountered a transformative host shift (Chantawannakul & Ramsey, 2018). Since then, the lineage *V. destructor* is identified as the most widespread and devastating pest of the Western honeybees (*A. mellifera*) (Anderson & Trueman, 2000). Currently, the genus varroa comprises four distinct species: *V. destructor. V. jacobsoni, V. rindereri, V. underwoodi* and among these, *V. destructor* and *V. jacobsoni* are the common parasites having gene introgression (Anderson & Trueman, 2000; Genersch & Aubert, 2010). Notably, *V. destructor* is a detrimental parasite of honeybees and has successfully established and crippling in its new host, *A. mellifera* populations over the world (Traynor et al., 2020).

Genomic studies using mitochondrial DNA sequencing provided that the linage *V. destructor* comprises about six haplotypes and only two of them (Korea and Japan haplotypes) were recognized to infest populations of the new host, *A. mellifera* (Anderson and Trueman 2000). The Japanese haplotype is relatively non-virulent, whereas the Korean haplotype is known to be extremely virulent and destroying millions of commercial bee colonies in the northern hemisphere (Anderson & Trueman, 2000; Bai et al., 2021). But various studies indicate that there is a potential lineage admixture as well as significant levels of genetic differentiation within and between the varroa mite populations infesting *A. mellifera* colonies (Beaurepaire et al., 2019; Dietemann et al., 2019; Beaurepaire et al., 2022; Zheng et al., 2023). These evidences have provided scientists to advance marker-assisted host selection techniques to enrich naturally occurring varroa resistance honeybee traits in commercial beekeeping (Sainsbury et al., 2022). Because, the mite could exhibit potential changes in its population and genetic structure for the

successful adaptation and co-evolution with its host (Thompson, 2019; Moro et al., 2021; Schmid-Hempel, 2021). However, the selection of specific adaptations in parasites is not uniform across different host populations, as various selective forces may influence the phenomenon (Gandon & Van Zandt, 1998). These forces may comprise diverse environmental factors and unique local host responses, contribute to the genetic diversification of parasite populations at different geographic scales, which gives rise to geographic mosaics of coevolution (Thompson, 2005). Hence, studies on genetic diversity evidence would help to identify how the mite population can affect the specific host across regional differences. In fact, the recently updated varroa genome (Techer et al., 2019) is becoming a powerful tool to help understand varroa evolution in response to dynamic host defenses, and its successful invasion in various bee populations. Consequently, previous studies performed mitochondrial DNA sequences and microsatellite marker analysis to determine the genetic diversity of varroa populations in different honeybee populations in different Western countries (Beaurepaire et al., 2019; Lin et al., 2021a; Beaurepaire et al., 2022). However, the low genetic diversity and an admixture of *V. destructor* lineage have been largely reported within and between mite populations.

In Africa, *V. destructor* was recently reported and information on its genetic diversity and population structure is very limited. On the other hand, the diverse tolerance/resistance behaviors of African honeybees (Fazier et al., 2010; Nganso et al., 2017) might enhance genetic differentiation in the *V. destructor* populations parasitizing African honeybee populations. This can be assumed from the potential adaptations and rapid spread of the mite across the continent despite substantial defensive response and natural selection of African honeybees. However, recent mitochondrial DNA analyses have revealed the occurrence of only the Korean haplotype (K1 type) among varroa populations in Uganda and Kenya (Chemurot et al., 2016; Nganso et al., 2017), but there is no information on varroa species identification in Ethiopia. Apart from this, the genetic variations among regional varroa populations and mechanisms used behind the mite co-adaptation in local honeybees remain unknown. Thus, assessing the genetic profile of this parasite within populations is very vital to get a better understanding of the mite evolutionary adaptations, and it is a prerequisite to design sustainable and effective control programs. In this study, we performed mtDNA sequencing at *Cytb* and *COX1* genes and SNP markers analysis for the first time to understand the specific varroa genetic structure in Ethiopian honeybees. Diversity array technology (DArT) uses single nucleotide markers and is the most effective way

to cover large portions of the genome information at high throughput, particularly in species with low genetic variations (Wenzl et al., 2004; Wittenberg et al., 2005). Therefore, understanding the genetic traits among mite populations using such molecular analysis will benefit in marker-assisted selection of varroa resistant honeybee colonies and further pave the way for long-term and sustainable control of the parasite.

4.2 Material and methods

4.2.1 Varroa mite sampling

In this study, a multistage purposive sampling procedure was used to select study areas and apiry locations. But, bee colonies were randomly selected from each apiry loction for sample collection. Adult worker honeybee samples (consisting of 200-300 bees each) were collected from 28 different apiary locations in the Eastern, Western, Central, and Southern parts of Ethiopia (Appendix 2). The detergent wash method was used to isolate adult varroa mites from the worker honeybees, following the standard procedure of varroa research (Dietemann et al., 2013). Accordingly, a total of 76 varroa samples were collected and grouped into four hypothetical populations (Eastern, Western, Central, and Southern) based on their origins. The collected mite samples were then preserved in a mite-tight kit containing 97% ethanol and stored at -20°C until DNA extraction.

4.2.2 Mitochondrial DNA extraction and sequencing

The mtDNA extraction was performed using NucleoSpin DNA kit according to the standard manufacturer procedures. Cytb was amplified using the primers provided by Navajas et al., 2010 (5'-GCAGCTTTAGTGGATTTACCTAC-3',and 5'-CTACAGGACACGATCCCAAG-3') while COX1 gene was amplified by using primers (5'-GGAGTAGGTACAGGTTGAACGG-3' and 5'-ACAACCCCAGCAATAATAGCAA-3') (Lin et al., 2021). The total PCR volume was 58 µL, containing 3 µL of 10 × PCR buffer, 0.6 µL of DNTP (2.5 mmol/L), 1µL of each primer (10 mmol/L), 0.3µL of Taq DNA polymerase, 29 µL of template DNA, and 23.1 µL ddH2O. For the COX1 gene, PCR amplification was started by 95^{o}C for 2 min, followed by 40 cycles with 95^{o}C for 1 min, 55^{o}C for 1 min and 72^{o}C for 1 min. The step of final elongation was performed at 72 °C for 5 min. For the Cytb PCR amplification was started by 95^{o}C for 2 min, followed by 40 cycles with 95^{o}C for 1 min, 52^{o}C for 1 min and 72^{o}C for 1 min. The step of final elongation

was performed at 72 °C for 5 min. In addition to the samples sequenced in this study, we incorporated 169 sequences of Cytb and 104 sequences of COX1 from Gene bank.

Both *Cytb* and COX1 sequences were preliminarily aligned using Clustal W (Thompson et al 1994) as implemented in the Mega software v 7.0.26 (Kumar et al 2015) and edited using Bio edit. DnaSP 5.0 was used to calculate the number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (Hd) and nucleotide diversity (Pi). The phylogeographer network was constructed using the median-joining method available in the software Network 10.2.0.0 (Fluxus Technology Ltd, Colchester, England).

4.2.3 DNA extraction and genotyping for SNP markers

The varroa samples were sent to the Biosciences eastern and central Africa (BecA) hub of the International Livestock Research Institute (ILRI) located at Nairobi, Kenya. DNA extraction was done using a nucleomag tissue extraction kit following the DArT extraction protocol (Kilian et al., 2012). The DNA concentrations were adjusted in the range of 50-100 ng/µl. The quality and quantity of extracted DNA were checked on 0.8% agarose and verified using the Nanodrop 2000c spectrophotometer (NanoDrop Lite, LT2878, Thermo Scientific, USA). Libraries were constructed for genotyping the varroa genomic DNA following the procedure by Kilian et al. (2012). DArTSeq complexity reduction method (Wenzl et al., 2004) was applied through digestion of genomic DNA using a combination of *PstI* and HPaII enzymes. Polymerase chain reaction (PCR) adapters were ligated to the *Pstl* fragment ends followed by PCR amplification of adapter-ligated fragments. Genotype by sequencing was conducted using single read sequencing runs for 77 bases as described by Kilian et al. (2012). The genomic DNA was genotyped by next generation sequencing using the Illuminia Hiseq2500. The sequenced data were analyzed using DarTsoft v.7.4.7 and scored as binary data "1" for presence or "0" for absence of marker in the genomic representation of each sample. The markers were then aligned to the common varroa reference genome (GCA_002443255.1) at National Center for Biotechnology Information (NCBI) in Genebank to identify the Varroa haplotype.

4.2.4 Markers quality analysis

All SNP marker obtained from DArT Pty Ltd were tested for their call rate (%), polymorphism information content (PIC), reproducibility (%) and One ratio. PIC shows marker diversity within the population and indicates the importance of the marker for linkage analysis. Reproducibility

measures the proportion of technical replicate assay pairs for which the marker score exhibited consistency (Alam et al., 2018). The call rate determines the success of reading the marker sequence across the samples. Hence, SNPs with reproducibility $\geq$ 0.95, call rate $\geq$ 75% were retained for further analysis. Markers with more than 10% missing data and minor allele frequency less than 5% were eliminated from analysis.

4.2.5 Analysis of genetic diversity and population structure

Genetic diversity analysis was conducted using DArTseq-based SNP markers for each varroa sample collected from the four cross sectional locations. After filtering markers with less than 10% missing data and eliminating the DArT loci with unknown chromosome positions, a total of 1414 SNP markers were distributed across 9 chromosomes and maintained for analysis. Genomic diversity related parameters including overall genetic diversity, availability, major allele frequency (MAF), and polymorphic information content (PIC) were calculated using R packages diveRsity, function divBasic (Keenan et al., 2013). The genetic distance among populations was assessed based on the proportion of shared alleles derived from SNP marker datasets, employing the Jaccard similarity index and transformed using the R-package vegan (Oksanen, 2010). Neighbor-joining trees were constructed based on pair-wise genetic distances among varroa samples using the R-package ape (Paradis et al., 2004). Analysis of molecular variance (AMOVA) was also performed using Arlequin v3.5.2.2 software (Excoffier & Lischer, 2010). For a more in-depth understanding of population structure, a Bayesian clustering approach was employed using STRUCTURE v.2.3.4 (Pritchard et al., 2000), and the number of hypothetical subpopulations (K) was estimated through grouping genetically similar samples. Hence, ten individual Markov Chain Monte Carlo (MCMC) simulations were run for each K-value (from 1 to 10), with a burn-in length of 50,000-100,000 iterations. The optimal K value was detected by ΔK (delta K) using web-based "Structure Harvester" software (Earl & VonHoldt, 2012).

4.3 Results

4.3.1 Identification of Varroa mite strain

The result of mtDNA sequencing both for *Cytb* and *COX1* gene showed only a single haplotype belonged to the Korean strain (K1-haplotype). This haplotype was identified in all the varroa samples collected from *A. mellifera* colonies in Ethiopia. For *Cytb* gene analysis, a total of 19

haplotypes were found within 177 sequences of 850 bp. including the sequences derived from the gene bank. Haplotype 11 (K1-haplotype that includes varroa in Ethiopia) (Figure 4.1) contributed for 83.6% of all the sequences (n =177). In this analysis, H11 (K1-haplotype) was reported from South Korea, Japan, Serbia, France, Turkey and Ethiopia as indicated in Figure 4. 1. Turkey has the largest haplotype number with 8 haplotypes while all the rest nations are represented only by single haplotype. Overall, the genetic diversity from *Cytb* gene analysis showed a value of nucleotide diversity (π) of 0.00498 and haplotype diversity (Hd) of 0.8821.

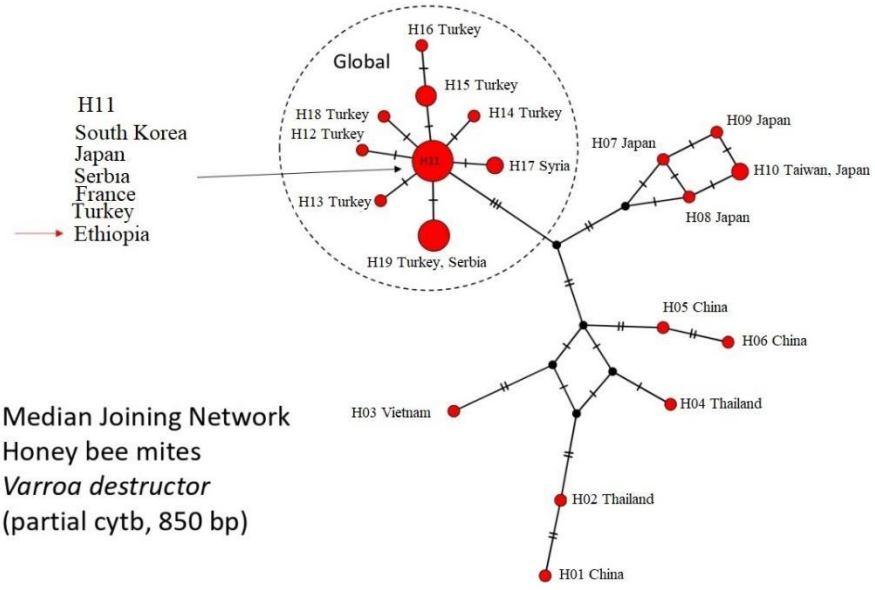

Figure 4.1 Median joining network analysis for Ctyb gene

For the COX1 gene, a total of 13 haplotypes were found including the haplotype found in Ethiopia (Haplotype 13). This haplotype contributed for 50% of all the other haplotypes revealing extremely low genetic diversity among varroa mite populations. The COX1 haplotype in Ethiopia is more prevalent and reported also in 12 nations including China, Vietnam, Thailand, South Korea, Philippines, Japan, New Zealand, Turkey, Serbia, France, USA, and Argentina (Figure 4. 2). The overall nucleotide and haplotype diversity (π) from COX1 gene analysis indicates the value of 0.00817 and 0.7356 respectively.

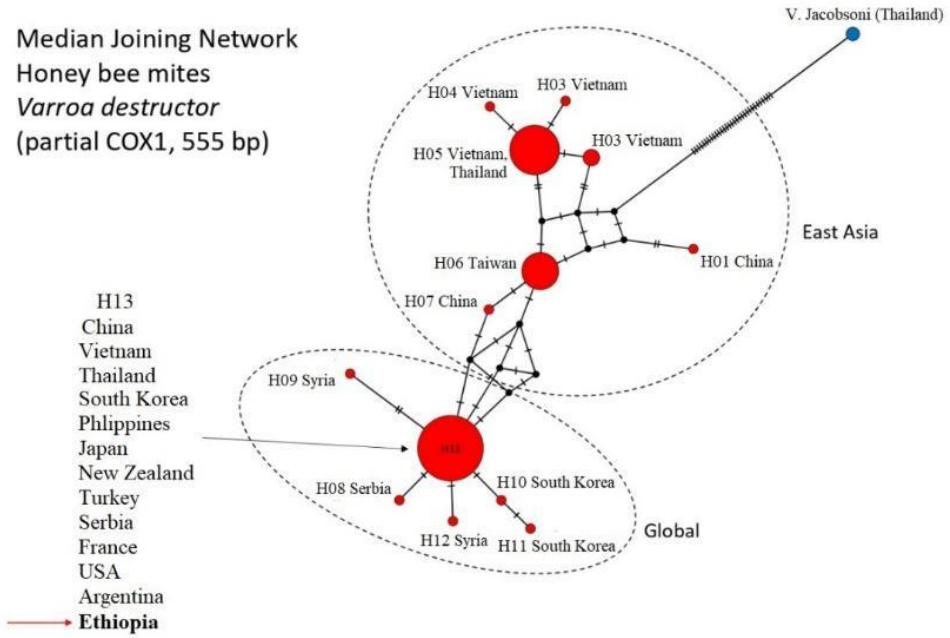

Figure 4.2 Median joining network analysis for COX1 gene

4.3.2 SNP markers quality

After filtering SNP markers with call rate < 75% and One Ratio < 0.5%, a total of 1414 SNP markers were generated for 76 varroa samples and used for further analysis. The majority of the SNP markers (36%) have shown > 95% call rate, of which all were found to be 100% reproducible (Figure 4. 3). Likewise, about 19% markers also displayed > 90 % call rate, however, 454 (32%) SNP markers with a low call rate (< 75%) (Figure 4. 3) were eliminated and not considered for further analysis.

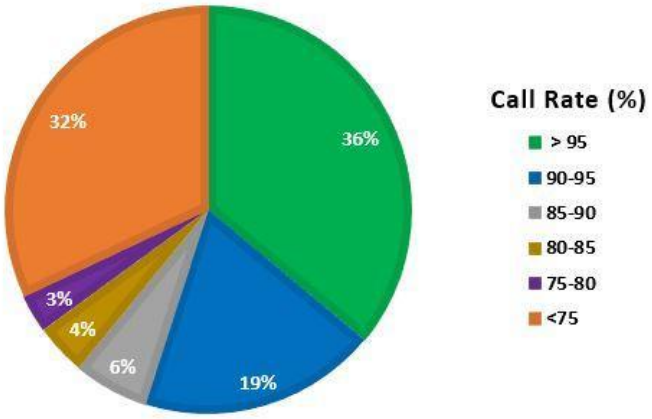

Figure 4. 3 Distribution of SNP markers data for quality parameter analysis

Polymorphic information content (PIC) distribution among SNP markers indicates that the largest proportion (65.48%) were categorized in the lowest range of PIC class (0.05- 0.15) and only 3.67% of SNP markers were observed in the highest PIC class 0.45-0.50 (Figure 4. 4). The remaining percentages of markers vary and distributed unequally across the rest PIC values. The median (0.25) was located far from the average PIC value and the data exhibited approximately unequitable distribution on either side of the median.

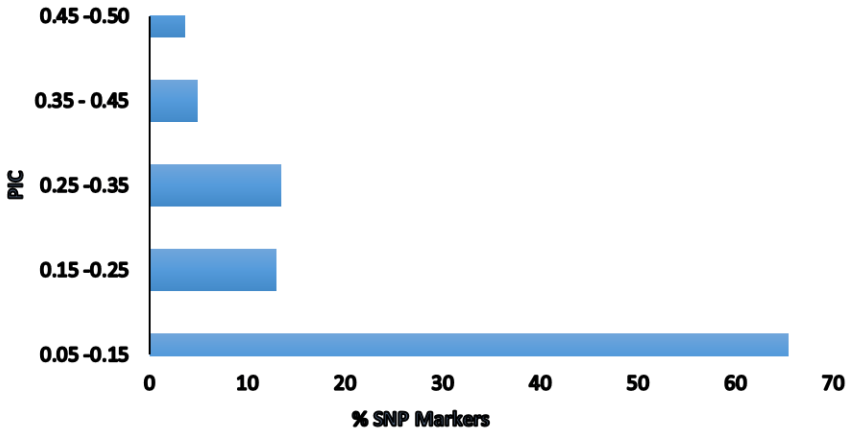

Figure 4. 4 Frequency distribution of PIC values for SNP markers used in Varroa mite genotyping

4.3.3 Genetic relationships and population structure among Varroa samples

Genetic relationships were estimated through their genetic distances among varroa samples collected from different area codes (Appendix-2). The weighted neighbor-joining cluster analysis of SNP markers provided three hypothetical clusters, C-I, C-II and C-III (Figure 4. 5A). The majority of the parental lines (77.7%) were predominantly grouped in C-III (blue), while the others (11.8%) were grouped in C-I (red) and the remaining 10.5% were grouped into C-II (green) (Figure 4. 5A). Briefly, Cluster-I consists of samples WSB1, SSH1, WHC1, BBD1, IAA2, WSG3, WSS2, IAY3 and BD1 and Cluster-II includes sample codes SSH2, BBb1, WSK1, WSS3, WSB2, BBD4, EWG4 and BGN1, while all the rest of the samples were grouped under Cluster-III. In this result, each cluster was represented at least by more than three samples from four geographic locations (Eastern, Western, Central and Southern), indicating all the three clusters contained a mixture of samples.

The population structure analysis of the total 76 samples shows the overall proportion of membership of the sample locations in each of the three clusters as illustrated in the bar plot for K = 3 (Figure 4. 5B). The hypothetical sub-populations were classified into four categories;

Eastern, Western, Central and Southern according to the sample's origin and consisted of 39.47% and 22.95%, 25.0% and 6.58% members, respectively (Table 4. 3). The hypothetical founder population seen in C-II (red) is represented mainly by samples from Central and Eastern parts, while that of sub populations C-III (green) consisted mainly for the Western samples and sub-pops in C-I (blue) was represented for samples collected from Southern and Central parts (Figure 4. 5B).

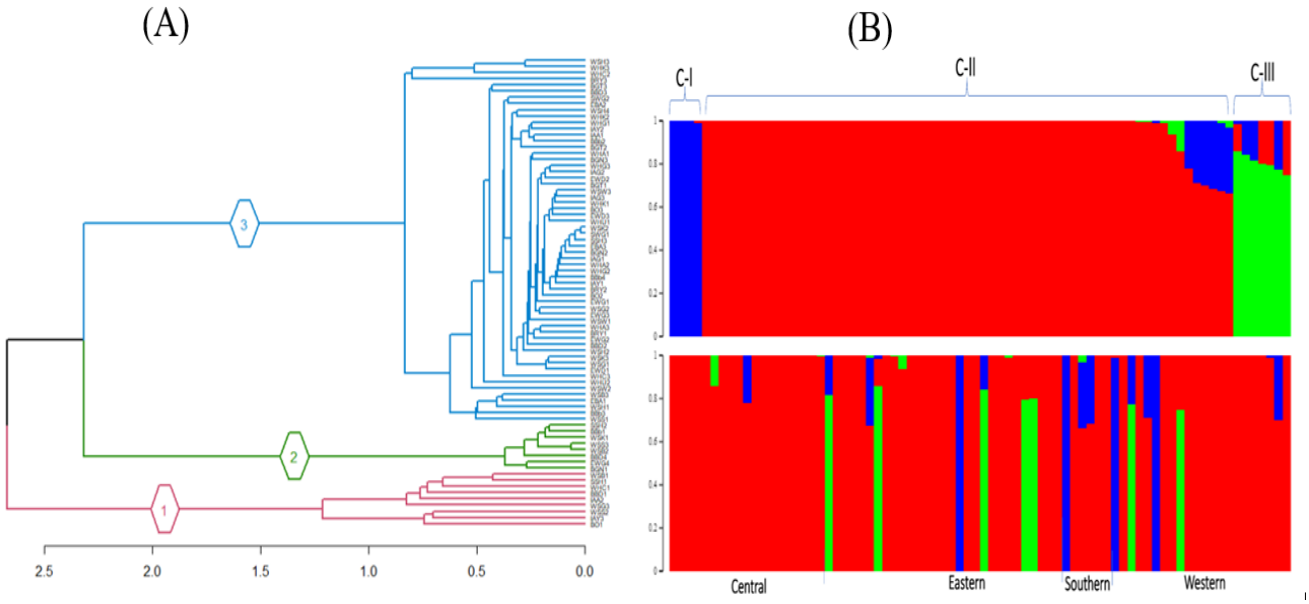

Figure 4.5 Genetic relationship among *V. destructor* genotypes collected from different regional locations (Central, Eastern, Western and Sothern) of Ethiopia. (A) Weighted neighbor-joining dendrogram, (B) estimated population structure at optimum value k = 3.

The population structure analysis showed the overall proportion of membership of the samples in each of the three clusters. The simulations (logarithm probability relative to standard deviation, ΔK) estimated from the SNP markers indicated a sharp peak at K = 3 (Figure 4. 6) which is the real K similar to what was observed in the phylogenetic cluster analysis (Figure 4. 5A). This indicates the overall proportion of membership of the samples were allocated in each of the three clusters.

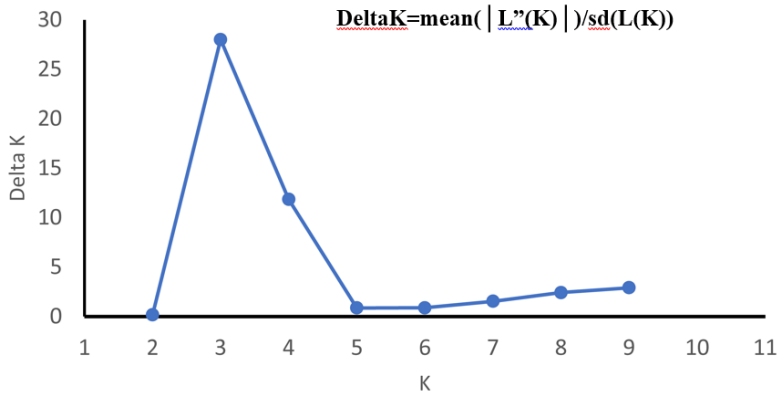

Figure 4. 6 Delta K for different numbers of hypothetical subpopulations (K) of *V. destructor* mite

4.3.4 Genetic diversity

The overall mean genetic diversity of varroa populations in this study was 0.14 which ranged from 0.08 to 0.27 (Table 4. 1). PIC varied from 0.26 to 0.33 with an average of 0.29 and average allelic frequency was 0.94. Based on these, the result indicates that samples collected from the Eastern region (which includes West Hararge and Bale zones) appears to have the highest relative genetic diversity (0.27), with allelic number of 2.0 and PIC value of 0.33. While, the lowest genetic diversity was recorded for varroa samples collected from southern and western regions (0.08 each) although there was no significance difference compared to the average gene diversity for samples collected from the central region (0.11) (Table 4. 1).

Table 4. 1 Estimation of gene diversity, allelic frequency and PIC based on DArT SNP marker

Sample location	Sample Size	No. of observations	Allele No.	Allele frequency	Gene Diversity	PIC
Central	19	17.6	1.60	0.90	0.11	0.29
Eastern	30	28.6	2.00	0.95	0.27	0.33
Southern	5	4.8	1.30	0.96	0.08	0.26
Western	22	20.5	1.50	0.93	0.08	0.27
Mean	19	17.9	1.53	0.94	0.14	0.29

4.3.5 Genetic distance among populations

Genetic distance quantifies the extent of genetic variations among individuals, populations within a species, and typically described by allelic variation (Beaumont et al., 1998). In this result, the varroa populations exhibited the lowest genetic distance (< 0.083) and correspondingly the highest genetic identity (> 0.91) among sample origins (Table 4. 2).

Table 4. 2 Genetic distances (below diagonal) and genetic identity (above diagonal) among 76 *V. destructor* samples based on SNPs analysis

Sample location	Central	Eastern	Southern	Western
Central		0.926	0.938	0.932
Eastern	0.083		0.918	0.940
Southern	0.062	0.082		0.940
Western	0.068	0.059	0.061	

4.3.6 Analysis of molecular variance (AMOVA)

Analysis of molecular variance (AMOVA) showed highly significant differences ($P < 0.0001$) among varroa populations and within individual samples. But a low level of variation (2%) was observed among mite populations collected from different regional locations, while the higher genetic variation was found among sample individuals (72%) followed by within individuals (26%) (Table 4. 3).

Table 4. 3 Analysis of molecular variance (AMOVA) for *V. destructor* genotypes based on SNP markers

Source of Variation	d.f	MS	SS	Est. Var.	Prop. of variance	P-Value
Among populations	3	114.04	342.11	0.806	2 %	< 0.0001
Among individuals	72	85.72	6171.67	36.13	72 %	< 0.0001
Within individuals	76	13.45	1022.00	13.45	26 %	< 0.0001
Total	151	180.96	7535.78	50.39	100 %	

*Note: d.f = the degrees of freedom, MS = mean square, SS = sum square, Est. Var.= estimated variance.

4.4 Discussions

This study provides the first genetic diversity analysis of the varroa mite in Ethiopia. On the bases of both mtDNA sequencing and SNP marker analysis, the results revealed that only a single haplotype of *V. destructor*, belonging to the Korean strain (K1-haplotype) was found in Ethiopia. This is consistent with previous studies reported for the prevalence of the same

haplotype in East Africa, including Kenya (Nganso et al., 2017), Tanzania (Fazier et al., 2010) and Uganda (Chemurot et al., 2016). This implies that *V. destructor* mites introduced to Africa could potentially originate from genetically homogeneous populations, which may exhibit continuous inbreeding characteristics. In agreement with this, Traynor et al. (2020) explained that *V. destructor* colonizing *A. mellifera* honeybees in Africa and African-derived colonies lacks complete genetic diversity. In addition, the high swarming and migration tendency of African honeybee colonies (Nuru et al., 2002; Pirk et al., 2016) may have contributed for the high gene flow and homozygosity among the varroa populations infesting colonies in different geographic areas. However, it is difficult to conclude the source of this invasive parasitic mite due to the fact that this haplotype is the most virulent and prevalently distributed lineage at global scale (Traynor et al., 2020). Although its parasitism impact depends on the survival and susceptibility of honeybee subspecies (Moro et al., 2021), the presence of this haplotype in Africa could be a potential threat to the beekeeping sector and should be monitored.

Interestingly, the results of both the SNP and mitochondrial DNA sequence analysis in this study were consistent revealing the varroa species found in Ethiopia have extremely low genetic diversity. The SNP markers analysis indicated that the majority of SNPs were distributed in the lowest range of PIC class (0.05-0.15) (Figure 4. 4) suggesting the low genetic differentiation among varroa populations collected from different locations. This is supported by the finding of a small genetic distance (< 0.083) and low average gene diversity (0.14) among varroa populations. These results indicate that varroa mites introduced into Ethiopia likely originated from a single haplotype with a closely related genotype. This indicates that the varroa parasite has been spreading from genetically homozygous lineages in the country. In agreement with this, previous studies also reported the low genetic diversity of *V. destructor* in different countries. For example, Rasolofoarivao et al. (2017) employed nuclear microsatellite analysis for 344 mite samples and found a low genetic diversity, with only 3 polymorphic alleles in Madagascar. Likewise, Ogihara et al. (2020) conducted mtDNA sequences for the *V. destructor* (K-haplotype) samples and found no regional variation within all populations in Japan and Navajas et al. (2010) also couldn't found any single nucleotide polymorphisms in Cytb genes within the same haplotype of *V. destructor* in Asia. Kelomey et al. (2017) also reported the Korean (K) haplotype of varroa strain in Benin using mitochondrial and microsatellite markers analysis. Such a very low genetic diversity in most of the varroa mite haplotypes suggests the minimum adaptation of the parasite

against the host defensive traits, particularly in resistant honeybee colonies in Africa (Beaurepaire et al., 2019). It was explained that the defensive responses of resistant honeybees exhibit the potential to influence the adaptive capacity and genetic variability of *V. destructor* mites in survival colonies of *A. mellifera* (Kence et al., 2013). In this regard, it is expected that the strong defensive responses of local honeybees, *A. m. bandasii* colonies against the *V. destructor* observed in our study (Chapter 5) could be linked with the limited genetic diversity among varroa populations. However, this might be influenced by several other factors including bottleneck effects and further investigations are required to understand the potential role of host resistance traits and their possible impact on varroa mite population genetics.

On the other hand, the high genetic diversity in varroa mites found in countries like Turkey, Vietnam, Thailand and China (Figure 4.1 & 4.2), based on gene sequences of 169 Cytb and 104 COX1 derived from Gene bank strongly suggests that *V. destructor* has already established at its origin in Asia (Oudemans, 1904). This genetic divergence between varroa populations suggests the successful adaptations of the parasite to changing environmental conditions, thereby affecting susceptible host colonies (Lin et al., 2021a). It was explained that a higher genetic diversity generally indicates that species are more adaptable to the environment, while low genetic diversity among species is associated with decreased fitness and adaptability to varying environmental conditions (Frankham et al., 2017). For example, Markert et al. (2010) elucidated the strong correlation between gene diversity and the ability of *Americamysis bahia* populations to adapt to environmental changes. Concomitantly, the relatively varied varroa linage genetic diversity found in some European countries (Traynor et al., 2020), might also be associated with the adaptation of the parasite causing devastating effect on the survival of susceptible *A. mellifera* colonies (Moro et al., 2021). This implies that the genetic diversity of the *V. destructor* might undergo adaptive evolution in response to its host susceptibility (Giliba et al., 2020).

In this study, the analysis of pair-wise genetic distance in SNP markers revealed the presence of three distinct population clusters, irrespective of the geographic locations. Consequently, Varroa samples collected from different apiary locations were grouped in the same clusters, while samples from the same locations were also grouped in different clusters (Figure 4.5A). Similarly, analysis of population structure revealed that varroa genotypes did not exhibit a distinct correlation with specific geographic locations, as each cluster encompassed at least one sample

from all four hypothetical populations (Figure 4. 5B). Consequently, the assignment of test genotypes appears independent of their geographical origins, suggesting the limited heterozygosity nature of Varroa populations, likely attributed to the effects of mite inbreeding (Dietemann et al., 2019; Techer et al., 2022). These findings align with previous observations indicating a dynamic population structure in *V. destructor* mites, likely driven by the rapid fixation of rare mutations due to inbreeding, followed by subsequent gene admixture when foundress mites co-infest host cells (Beaurepaire et al., 2017). Although varroa mite reproduces sexually, mating takes place between close relatives that enhances inbreeding among populations. This reproduction system (adelphogamy), coupled with the haplo-diploid and pseudo-arrhenotoky sex determination systems, are responsible for the observed extreme low genetic diversity (Cornuet et al. 2006). Additionally, the dispersal nature of the parasite, for example, through colony migration and movement could contribute to unidirectional gene flow among mite populations, potentially resulting in the homozygosity of parasite populations across diverse geographic locations (Boulinier et al., 2016; DeGrandi-Hoffman et al., 2017). In line with this, the result of AMOVA also further confirmed a very low proportion of genetic variation (2%) (Table 4.3), indicating a distinctive lack of genetic diversity among varroa populations in Ethiopia.

In general, our study revealed a low genetic diversity among *V. destructor* populations collected from different apiary locations in *A. mellifera* colonies in Ethiopia. This explains the genetic composition of *V. destructor* is not predominantly influenced by sampling locations, indicating its spread could be from genetically homogeneous lineages. Such a lack of genetic differentiation among varroa populations in Ethiopia may have influenced the mites' adaptation to overcome the host defenses and this could have contributed to the ability of local honeybees to defend against the parasitism of the mite. Thus, understanding the genetic profile of this invasive parasite provides valuable insights into the complex host-parasite relationships between varroa mite and local honeybees and lays the foundation for the development of mite control strategies.

CHAPTER FIVE

Defensive behaviors of the central highland honeybees, *Apis mellifera bandasii* against *Varroa destructor* in Ethiopia

5.1 Introduction

The ecto-parasitic mite, *Varroa destructor*, and the honeybee are the two interacting host-parasite arthropods that have become a subject of growing interest for many scientists around the world. Honeybees are the only sources of food for the varroa mite, and the mite's life cycle coincides with the development of honey bee pupae inside the brood cells (Rosenkranz et al., 2010; Yang et al., 2021). This parasitic mite primarily feeds on the fat bodies of the bees (Ramsey et al., 2019) while also spreading other bee pathogens like bee viruses, which ultimately lead to the highest rate of honeybee mortality (Le Conte et al., 2010; Hristov et al., 2020; Traynor et al., 2020). This feeding activity of the mite and its associated secondary infections can cause huge losses of the honeybee population, and become a threat to colony survival and beekeeping industry around the world (Noël et al., 2020; Flores et al., 2021; O'Shea-Wheller et al., 2022). In addition to reducing the potential beekeeping production, Varroa also has negative effects on the pollination capacity of a colony, which in turn significantly affects crop production for food security (Abrol & Sharma, 2013; Malfroy, 2015). The approach so far taken to control the mite, particularly using chemical treatments over the past 30 years did not completely solve the problem due to the spread of acaricide resistant mites and the risk factors associated with acaricide residues (Plettner et al., 2017; Kablau et al., 2020). As a result, *V. destructor* remains a complex invasive parasite, crippling the Western honeybee, *A. mellifera*, in the world for many generations (Nazzi & Le Conte, 2016; Traynor et al., 2020). In contrast, the mite has been considered a harmless pest to its native host, the Eastern honeybee *(A. cerana)* due to their naturally evolved defensive traits developed over a long evolutionary period (Peng et al., 1987; Boecking & M Spivak, 1999; Rath, 1999). Thus, a balanced host-parasite relationship has been established between *A. cerana* and the *V. destructor* mite, revealing the potential resistance of *A. cerana* honeybees to the mite infestation (Grindrod & Martin, 2021, 2023).

Likewise, some *A. mellifera* colonies in Africa and African-derived populations have shown varying degrees of tolerance or resistance towards the infestation of Varroa mite without the beekeepers' interventions (Büchler et al., 2010; Locke, 2016b; Castilhos et al., 2023). Particularly, African honeybee populations are likely to be less threatened by the impact of *V. destructor* compared to European honeybees (Muli et al., 2014; Nganso et al., 2017; Tibatá et al., 2021). Although the mite has continued its rapid spread across many African countries over the past 15 years, there has been no report on the visible colony losses linked to Varroa mite infestation in African continent (Allsopp et al., 1997; Dietemann et al., 2009; Fazier et al., 2010; Begna, 2014; Chemurot et al., 2016; Muli et al., 2014). The long co-existence of honeybee subspecies with the mite in the absence of chemical treatment is suggestive of the potential resistance or tolerance of the honeybee populations evolving natural adaptations against the Varroa mite. Behavioral defenses such as hygienic and grooming behaviors are important natural traits that enable resistant honeybee populations to survive and co-exist with the varroa mites, particularly in Africa (Fazier et al., 2010; Muli et al., 2014).

Hygienic behavior involves targeting, opening and removal of diseased, injured, parasitized, or dead broods by the worker honeybees (Peng et al., 1987; Otto Boecking & Marla Spivak, 1999; Spivak & Reuter, 2001). In particular, when hygienic behavior targets the detection and removal of varroa infested brood cells, it is termed as "Varroa Sensitive Hygiene Behavior (VSH)" (Mondet et al., 2015). On the other hand, grooming behavior involves the potential dislodging and damage of ecto-parasites from the bodies of adult bees either by themselves or by their nest mates, where they reduce the population of parasites below the danger threshold level (Aumeier, 2001; Mondet et al., 2020; Russo et al., 2020). In fact, previous studies demonstrated that African honeybees displayed stronger hygienic and grooming behaviors than their European honeybee counterparts (Muli et al., 2014; Nganso et al., 2017). Nevertheless, it is anticipated that the diverse bee species in Africa are likely to exhibit different responses tailored to combat the negative impact of the Varroa mite (Mondet et al., 2020).

Furthermore, local honeybee populations in Ethiopia have assumed to evolve some sort of resistance or tolerance traits to maintain a stable host-parasite relationship against the aggressive infestation behaviors of varroa parasitism. In fact, Pirk et al. (2016) reviewed how the treatment-free beekeeping approach in Africa has allowed honeybees to develop some natural resistance

traits or behavioral adaptations to combat the negative effects of different pests and pathogens. However, the specific resistance or tolerance mechanisms employed by these honeybee populations against the mite remain unclear. Therefore, this study was designed to determine whether the hygienic and grooming behaviors of *A. m. bandasii* could contribute for the defensive mechanisms against the *V. destructor* mite infestation. Understanding such natural defense behaviors will provide relevant insights into future selective breeding and enhance resistance traits in the local honeybee stocks.

5.2 Materials and methods

5.2.1 Study location

The study was conducted from September 2021 to June 2022 in the laboratory and at the apiary site of Holeta Bee Research Center, located at 09^{o}03'.24" N and 038^{o}30'.72" E, altitude 2400 m a.s.l. about 33 kilometers in the west direction of the capital city, Addis Ababa, Ethiopia. The climate of the study area is characterized by temperate to humid weather conditions with an average temperature of 14.15°C (rangies from 6.2-22.1°C), annual rainfall of 1091.51 mm (ranges from, 800 -1500 mm) per year and mean relative humidity of 60.6%. The main vegetation types in the study area include *Guzotia s*pp., *Acacia* spp., *Eucalyptus globulus, Vernonia amygdalina, Trifolium* spp., *Plantago lanceolata, Brassica carinata* and *Isoglossa laxa* (Fichtl & Adi, 1994).

5.2.2 Experimental set up and honeybees

A total of 15 queen-right colonies (headed by naturally mated queens) of *A. m. bandasii*, the local honeybee race, were established in standard Langstroth frame hives, a year prior to the commencement of the experiment. All experimental colonies originated from locally caught swarms, and established in the uniform apiry conditions, and they were checked for the presence of naturally infested varroa mites. Then, adult bee samples were taken from all colonies and the varroa mites were separated from each bee samples using the standard method of detergent wash (Dietemann et al., 2013). Thereafter, the samples were diagnosised in the laboratory to count the number of recovered mites from total sampled bees. Finally, the mite infestation rate (%) for

each colony was determined and epressed as the number of mites counted per 100 adult worker bees as described in section 3.2.4.

5.2.3 Evaluation of hygienic behavior (HB)

Hygienic behavior was assessed in the established colonies (N = 15) using the standard pin-killed brood assay method as described in Büchler et al. (2013). After selecting the section of bee comb containing caped young pupae cells (white-to purple-eyed stage), this section was punctured with a circular (5cm, ID) polyvinyl chloride (PVC) pipe to demark the entire row of cells that surrounds approximately 164 cells (Figure 5. 1). The number of empty cells within each circular comb section was counted and recorded. Then, every capped pupa within the marked section of the comb was pin-killed with a fine insect pin (entomological pin No -2) and the combs were placed back into the test colonies.

After 24 and 48 hrs, the frame with the comb sections was taken out from the respective colonies to count and record the number of removed cells and the remaining dead broods at both consecutive periods. Moreover, the marked section of the comb was photographed for later count and confirmation, as indicated below (Figure 5. 1). Then, the number of fully removed pin-killed pupae cells from each test frame was recorded after 24 and 48 hours and expressed as the percentage of brood removal rate (%). Lastly, the total percentage of dead brood removal rate (%) was calculated according to (Kebede, 2006) as follow (Eq-1):

$$R = \frac{K-E-C}{T-E} x\ 100 \quad \ldots\ldots 1$$

Where; R = Percentage of dead brood removal rate (%) in time interval

K = Number of removed dead broods in time interval

C = No. of empty cells in the section before the test

E = No. of non-removed brood cells after the test

T = Total number of cells in the demarcated brood section

Finally, the experimental colonies were classified into three groups: high, medium, and low hygienic colonies based on brood removal rates after 24 hrs. Consecutively, colonies uncapped and removed dead broods of more than 90%, 60-90%, and less than 60% were classified as high, medium, and low hygienic behaviors, respectively (Medina-Flores et al., 2014).

5.2.4 Evaluation of grooming behavior (GB)

The grooming behaviors were evaluated in ten selected experimental colonies (N=10). The experimental colonies were selected having uniform population size based on the standard method of estimating colony population (Delaplane et al., 2013) in order to minimize the biased effects of varying colony strengths on grooming activity. Then, the original bottom boards of selected experimental colonies were removed and replaced with modified screened bottom boards following the procedure of Pettis and Shimanuki (1999). The screens were designed to allow only the passage of mites through, but not allow to pass the bees. To intercept the falling mites, mite-collecting trays were covered with white cardboard and smeared with sticky, non-toxic petroleum jelly (Vaseline®). The trays were maintained in the hives, and the fallen mites were collected every 24 hrs from the cardboard papers for three consecutive days. On each data collection day, the slide board was removed, and fallen mites were collected, cleaned, and reintroduced into the bottom boards of the hive.

Subsequently, the collected fallen mites were counted and examined for body damage under a Zeiss Primo Star light microscope, Germany (Mg. Power 40X). Each examined mite was assigned as "damaged" or "undamaged" categories for the analysis. The proportion of damaged mites (%) in each colony was expressed by dividing the number of damaged mites over the total number of fallen mites at the end of the collection time (after 48 h). The damaged mites were also further grouped into different damage categories following previously established classifications parameters (Corrêa-Marques et al., 2000).

5.2.5 Statistical analyses

All statistical analyses were carried out using R-Software version 4.1.3 (R Core Team 2021). Data were checked for normality using the Shapiro-Wilk test, and they were normally distributed. A pairwise sample t-test was used to compare brood removal rates after 24 hrs and 48 hrs, as well to compare the percentage of damaged and undamaged mites. The Pearson correlation test and linear regression model were used to estimate the varroa infestation rates in relation to brood removal rates both at 24 and 48 hours and similarly to compare the infestation rate in relation to the percentage of damaged and undamaged fallen mites separately. The average daily fallen mites per colony was determined by dividing the total number of fallen mites to the number of collection days following Strauss et al. (2015).

5.3 Results

5.3.1 Evaluation of hygienic behavior

In this result, the average removal rates of pin-killed broods from the cell combs (Figure 5.1) were used to determine the level of hygienic behavior in experimental colonies. The results revealed that there were variations in removing the pin-killed broods among the colonies. The mean percentage removal of pin-killed brood (Mean ±SD) were 83.98 ±14.3% (ranging from 57.6% to 98.1%) after 24 hrs and 97.6±3.4% (ranging from 89.4% to 100%) after 48 hrs (Table 5.1 and Figure 5. 2A). The mean varroa infestation rate was 4.06±3.78 (ranging from 0.16 to 13.43 %) for the experimental colonies (N=15) (Table 5.1).

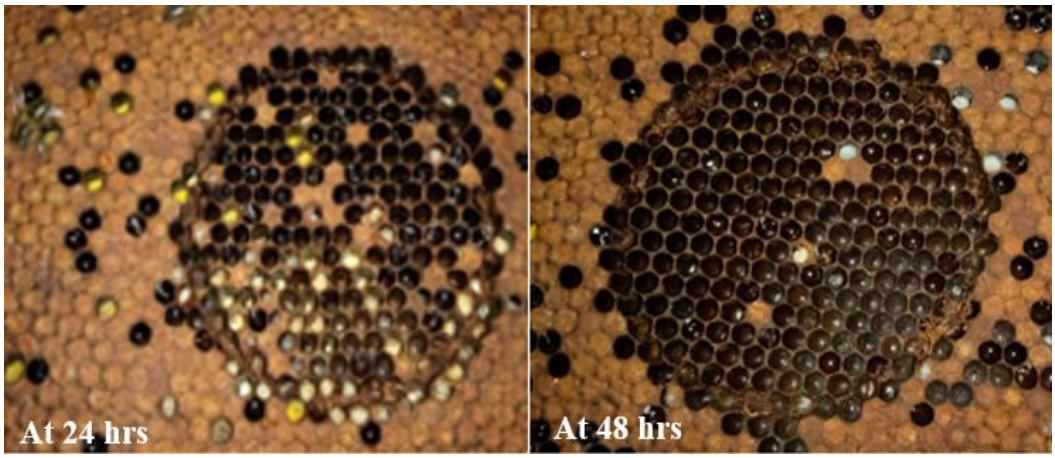

Figure 5. 1. Sections of bee comb indicating the removal of pin-killed broods after 24 and 48 hours from *A. m. bandasii* colonies

Table 5. 1 Description of pin-killed brood removal rates after 24 and 48 hours and the varroa infestation rates, in A. m. bandasii colonies

Description	N	Min	Max	Mean	Std. D
After 24 h	15	57.62	98.10	83.98	14.26
After 48 h	15	89.40	100.00	97.60	3.4
IR	15	0.16	13.43	4.06	3.78

The classification of hygienic behaviors of colonies indicated that 53.5% (8 of 15) of the colonies removed more than 90% pin-killed broods after 24 hrs, showing higher hygienic performances. While the other 40.0% (6 of 15) colonies removed 60-90% of the dead broods,

and were classified as medium HB. Yet, 6.5% of the colonies were removed, less than 60% of the pin-killed broods, and classified as low HB (Figure 5. 2B).

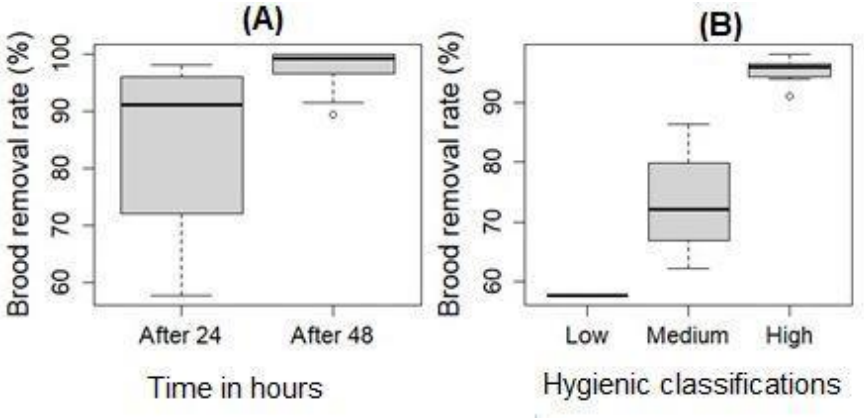

Figure 5. 2 The mean percentage of pin-killed brood removal rates in time periods **(A)** and the classification of hygienic behavior for central highland honeybee, *A. m. bandasii* colonies **(B)**

There was negative correlation between the Varroa mite infestation rate (IR) and the proportion of pin-killed brood removal rate in honeybee colonies, both after 24 and 48 hrs (Pearson correlation: r = -0.81, P < = 0.001 and r = -0.483, P = 0.039, respectively) (Figure 5. 3A).

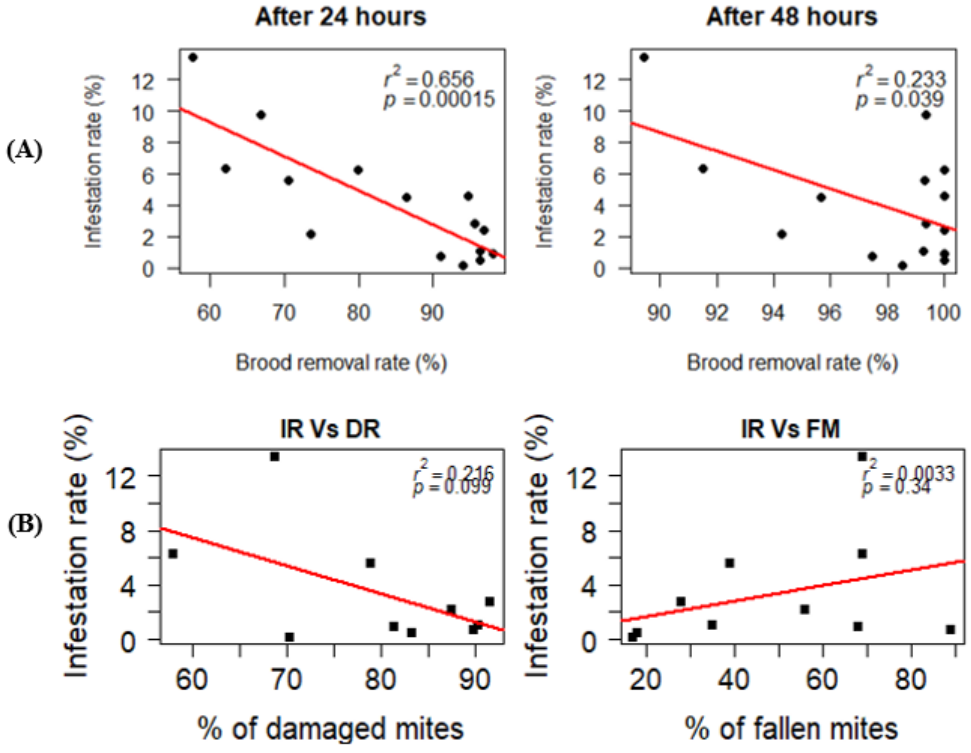

Figure 5. 3 Correlations between varroa mite infestation rate and brood removal rates across time periods (A), as well as the correlations between varroa mite infestation rate and the percentages of damaged mites and fallen mites (B) in the experimental colonies

5.3.2 Evaluation of grooming behavior

The results of grooming behavior exhibited by the experimental colonies (N=10) in terms of fallen and damaged mites are depicted in Table 5.2 and Figure 5. 4B. Of the total 488 fallen mites collected from the bottom boards, 80.0±16.3% (Mean ± SD) were damaged, while 20±13.0% were undamaged, and these values were significantly different (t (df)= value of t; $P < 0.001$) (Figure 5. 4B). The average daily count of fallen mites (Mean ± SD) in experimental colonies was 16.3±10.2, ranging from 5.7±3.5 to 29.7±14.2. The higher number of daily fallen mites was recorded for colonies 6, 4 and 2, while colonies 3, 7 and 9 showed a lower number of daily fallen mite counts (Table 5.2).

Table 5. 2 The mean daily count of fallen mites (Mean ± SD) and the percentage of damaged and undamaged mites (Mean± SD) within experimental colonies of *A. m. bandasii*

Colony code (N=10)	No. of daily fallen mites	% damaged mites	% undamaged mites
C-1	13.0±11.4	78.9±9.2	21.1±1.8
C-2	23.0±8.2	57.9±22.0	42.1±2.1
C-3	5.7±3.5	70.4±17.9	29.6±0.4
C-4	23.0±7.8	68.8±18.6	31.2±4.1
C-5	22.7±8.7	81.4±10.2	18.6±11.3
C-6	29.7±14.2	89.9±3.8	10.1±6.2
C-7	6.0±3.6	83.3±20.8	16.7±1.7
C-8	11.7±3.2	90.3±10.9	9.7±6.4
C-9	9.3±4.2	91.7±14.4	8.3±0.7
C-10	18.7±18.7	87.5±8.5	12.5±9.1
Average	**16.3±10.23**	**80.0±16.3**	**20±13.0**
P-Value	$P < 0.001$	$P < 0.001$	

The results indicate that there was no significant correlation between the percentage of damaged mites and infestation rates ($R = r^2=0.216$, $P = 0.099$). Similarly, the association between the

percentage of fallen mites and the mite infestation rate was not statistically significant (r^2 = 0.0033, P = 0.34; Figure 5. 3B).

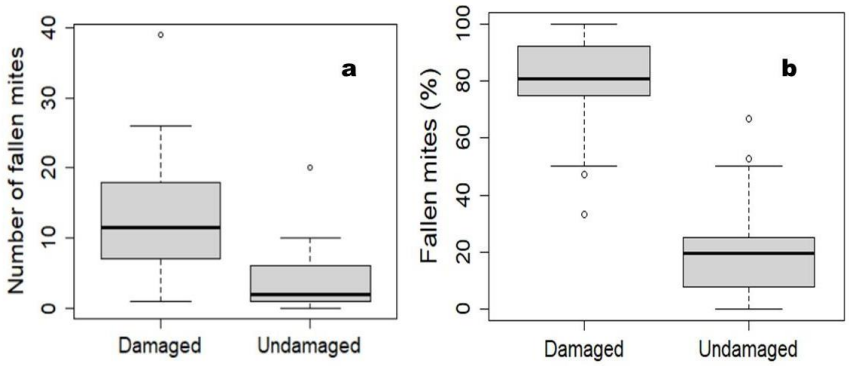

Figure 5. 4 The average number of daily fallen mites (a) and the percentage distribution between damaged and undamaged mites (b) in local honeybees, *A. m. bandasii*

In this study, about 10 categories of damages were observed on the bodies of the mites (Figure 5. 5), and all injuries could be inflicted from the grooming activities of worker bees. Notably, three distinct new damage categories were observed in this study which include: carcass-empty dorsal shield + empty ventral shield; damaged legs + damaged gnathosoma + damaged shield; and hollow in the dorsal shield + damaged legs (Figure 5. 5 D, H and J). The damaged legs (the complete or partial loss of one or more legs) (Figure 5. 5C) and damaged dorsal shields (Figure 5. 5J) were the most frequently observed (presented more than five times when examined under the microscope) body damages, with the proportion of mite injuries at 38.2% and 29.2%, respectively. Other damages represented in this assessment included combined injuries in the mite bodies and legs.

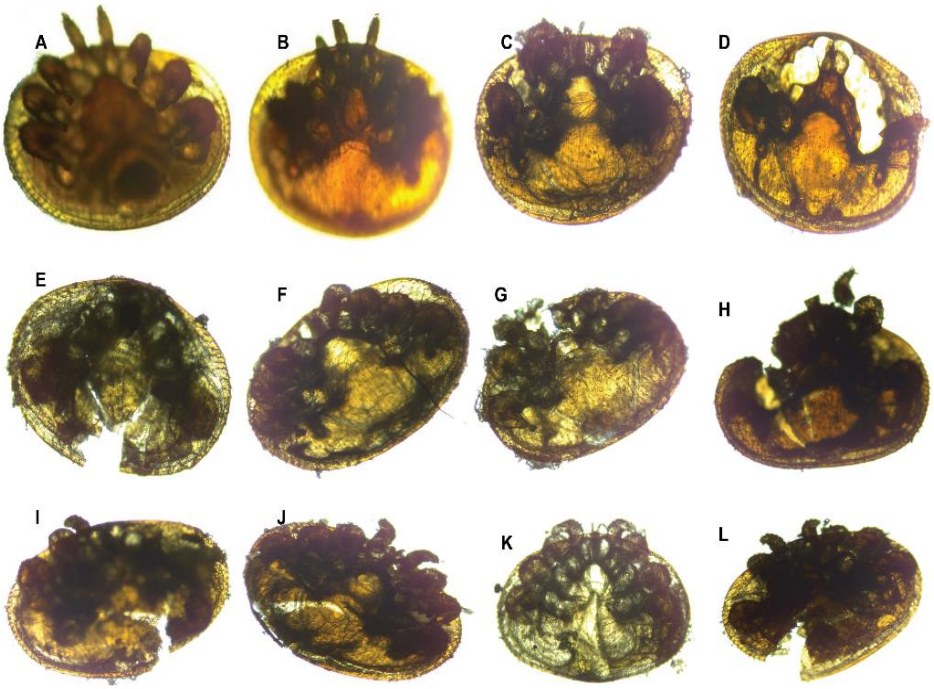

Figure 5. 5 Morphological views of damaged *V. destructor* mites under examination using Primo Star (Zeiss) microscope (40x Mg) during the evaluation of *A.m. bandasii* grooming behavior. The figure illustrates ventral and dorsal view of undamaged mite (A-B) and damage categories inflicted to legs (C), carcass-empty dorsal + empty ventral shield (D), damaged empty dorsal shield + missing legs (E), damaged legs + empty dorsal shield (F), damaged legs + damaged gnathosoma (G), damaged legs + damaged gnathosoma + damaged shield (H), hollow in the dorsal shield + damaged legs (I), damaged dorsal shields (J), hollow in the ventral shield + damaged legs (K) and hollow in the dorsal shield (L).

5.4 Discussions

Honeybees exhibit a wide range of natural defensive behaviors against the infestation of pathogens, pests and parasites in order to survive. Particularly, resistant honeybees display various physiological and behavioral defenses to limit the spread of pathogen and parasite infections within a colony (Mondet et al., 2020). However, such natural traits considerably vary based on bees' genetic factors and environmental conditions, as well as prevailing pathogens and parasites (Meixner et al., 2015). Several studies suggested that hygienic and grooming behaviors play key roles as defense mechanisms and enable honeybee populations to survive the effects of brood diseases and parasitic mite (Otto Boecking & Marla Spivak, 1999; Nganso et al., 2017; Morfin, Espinosa-Montaño, et al., 2020; Khan & Ghramh, 2021). In the present study, we

assessed the potential contribution of hygienic and grooming behaviors in the central highland honeybee, *A. m. bandasii* in Ethiopia as defense strategies against the infestation of *V. destructor* mite.

Our results revealed that a higher level of hygienic behavior (96.7%) expressed in the tested colonies of the *A. m. bandasii* honeybee race after 48 hrs. This demonstrates the central highland honeybees have greater ability to detect and remove the infested broods from the comb cells. As explained by Medina-Flores et al. (2014), colonies with a HB of more than 95% within 48 hrs are genetically resistant to different infectious pathogens, and are categorized as strong hygienic colonies. However, the hygienic behaviors expressed in our study varied among experimental colonies (Table 5.1). These variations among colonies of the same subfamilies might be influenced by factors such as the age of the worker bees performing the hygienic tasks (Panasiuk et al., 2010), or the heritability of their genetic traits (Arathi & Spivak, 2001). In fact, individual worker honeybees within the same subfamilies may also differently respond to different stressors including pest and pathogens (Roberts and Hughes, 2014; Dalmon et al., 2019). In agreement with our finding, a recent investigation by Hunde and Hora (2022) has also showed the performance of *A. m. bandasii* colonies in terms of high level of hygienic behavior (96.42%), good brood rearing, nectar production, and high aggressive behavior. Similarly, higher percentages of HBs have been also reported in different subspecies of Ethiopian local honeybees, including *A. m. scutellata* colonies (95.7%) (Shitaneh et al., 2022) and *A. m. weyi gambella* subspecies (92.16%) (Aleme et al., 2017).

Compared to reports from other countries, the average hygienic behavior displayed in Ethiopian honeybee races was higher than those reported in Kenya (81.0%) (Nganso et al., 2017), Egypt (72.5%) (Kamel et al., 2003), Ecuador (80 %) (Masaquiza et al., 2021), and Chile (20-80%) (Araneda et al., 2008). Such differences could be attributed to several factors, including the bees' genetic factors, geographic locations, climatic conditions, and seasonal variations. Although the level of hygienic behavior varies among different bee species, several studies suggested that African honeybees in general, display strong hygienic behavior that suppresses the varroa mite reproduction cycle and population growth than the honeybees of western countries (Muli et al., 2014; Mondet et al., 2015; Gebremedhn et al., 2019; Mondet et al., 2020).

Interestingly, our results demonstrate a negative correlation between the HBs and varroa mite infestation rate among the tested colonies both at 24 and 48 hrs. This supports the evidence that honeybees with higher HBs can limit the reproduction cycle of the mite, thereby reducing its infestation level in the colonies (Kim et al., 2018). Similarly, Muli et al. (2014) reported a strong negative relationship between hygienic behavior and the varroa mite infestation rate in *A. m. scutellata* colonies in Kenya, Tanzania and Uganda. This could be linked to specific tasks of worker honeybees in which they exhibit to detect and remove varroa-infested broods from comb cells, which is termed as "Varroa Sensitive hygiene behavior" (VSH) (Harris et al., 2010; Mondet et al., 2020; Spivak & Danka, 2021). Several studies suggest that VSH results in reducing the reproductive potential and population growth of mites, which in turn limits the infestation and spread of mites in colonies (Kim et al., 2018; Spivak & Danka, 2021). During the activities of VSH, the worker bees' antennal physiology can play a key role in detecting odor coming from varroa-infested larvae, which then triggers the cleaning of infested broods from the comb cells (Parker et al., 2012; Mondet et al., 2015). In this study, local honeybee *(A. m. bandasii)* colonies demonstrated active hygienic behavior by removing pin-killed larvae, indicating a potential expression of VSH. This behavior suggests their ability to survive the destructive impact of Varroa mites by reducing mite infestation levels below the threshold for significant damage. However, it is worth noting that the timing of brood removal can influence the apparent resistance of honeybees, determining the overall rates of varroa parasitism in colonies (Spivak & Danka, 2021).

Apart from hygienic behavior, the tested colonies (*A. m. bandasii)* displayed a higher level of grooming behavior in terms of mean daily fallen mites and percentage of damaged mites. The higher percentage of fallen mites and the higher proportion of damaged mites observed in this study reflect the intensive grooming ability of *A. m. bandasii* colonies to fight against the destructive nature of *V. destructor* mite. The percentage of grooming behavior (80.0±16.3%) recorded in this study was considerably higher when compared to the previous report for *A. m. simensis* colonies in the northern region of Ethiopia, displayed 34.78% and 41.89% GB during active and dry seasons, respectively (Gebremedhn et al., 2019). In addition, the percentage of GB for *A. m. scutellata* colonies in Kenya was reported to be 21.3% (Nganso et al., 2017) and significantly lower than the present study. This explains a strong grooming behavior of *A. m.*

bandasii stocks that could likely inflict damage to the mites' bodies and might be contributed for colony survival. In agreement with this, Pritchard (2016) explained that colonies inflicting about 60% body damage to the total fallen mites are capable of limiting the mite infestation level and can survive the varroa infestation without chemical treatment. Consequently, the higher intensity of grooming behavior and injuries that the bees inflict on the body of the mite can significantly influence the mites' reproduction cycle and population growth in varroa-surviving colonies (Dadoun et al., 2020; Russo et al., 2020).

In the present study, about ten body damage categories were examined from the total fallen mites. It appears that all the damages have been inflicted by the grooming activities of worker bees, which is consistent with the previous investigation by Corrêa-Marques et al. (2000). Importantly, a significant overlap of damage categories was observed between our result and the findings of recent studies in Argentina (Russo et al., 2020) and Kenya (Nganso et al., 2017; Russo et al., 2020). Although there were multiple damages examined in our study, the damaged legs (total or partial loss of one or more legs), and damaged dorsal shield were the most frequently recorded damages. This observation suggests that the primary target of worker honeybees is to destroy the legs and external bodies of the fallen adult mites, which then inhibits the mite movement and re-infestation in the hive. Such targeted damages would eventually lead to the death of mites and have potential to reduce varroa population growth and infestation levels in the hives (Corrêa-Marques et al., 2000). However, there were variations in the level of damaged mites across experimental colonies, and this would indicate that grooming behavior is not the only defense mechanism of honeybees against mites. Because, several driving factors can considerably influence the degree of grooming behaviors among honeybee stocks, even for colonies existing in the same geographic region (Boecking et al., 2000; Masaquiza et al., 2021). Therefore, considering driving factors would be important during selection breeding programs to enhance resistant bee stocks.

In general, this study reveals the potential expression of hygienic and grooming behavioral traits of *A. m. bandasii* colonis against the *V. destructor* mite. These findings provide valuable insights into natural defense mechanisms that help local honeybee populations in combating the impacts of *V. destructor* mite without the need for beekeepers' intervention. Understanding such natural adaptations and driving factors is crucial to develop effective selective breeding strategies for local honeybee populations in the country.

CHAPTER SIX

Efficacy of Propolis extract for the control of *Varroa destructor* (Mesostigmata: Varroidae), ectoparasite of the honeybees

6.1 Introduction

The increased mortality and population decline of honeybees (*Apis mellifera*) in recent years have raised significant concerns among beekeepers and the scientific community. Various biotic and abiotic stressors and their synergistic effects have been attributed to the global decline and losses of honeybee populations (Nazzi et al., 2012; Lin et al., 2023). Wide reports indicate that *Varroa destructor* is among the factors causing the most devastating effect to the Western honeybees *(A. mellifera*) and has becoming a serious threat for global apiculture industry (*A. mellifera*) (Smith et al., 2013; Giacobino et al., 2015; Traynor et al., 2016). Apart from the damage it causes by feeding on adults and brood bees, its ability to transmit and spread of multiple viruses throughout colonies has made *V. destructor* a global threat to honeybee populations (Tentcheva et al., 2004; Wilfert et al., 2016). Therefore, most *A. mellifera* colonies require treatment to have a chance of surviving against this parasite infestation, while a few populations of honeybees' exhibit resistance or tolerance traits without treatments.

Different varroa control methods, including biological, physical and chemical approaches, have been employed in the last few decades. In particular, synthetic acaricides have been intensively used to control varroa mite infestation in honeybees for the past many years (Gracia et al., 2017). However, the use of synthetic acaricides has become ineffective due to emergence of acaricide-resistant mite populations, the hazardous effects of acaricides, and the contamination of bee products with acaricides residue (Bogdanov et al., 1998; de Mattos et al., 2017). These challenges have shifted the attention of many scientific studies towards searching for alternatives to combat with the mites' infestation. Hence, the use of natural products, including plant derivatives, has emerged as a promising alternative to the use of synthetic acaricides (Damiani et al., 2014; Tutun et al., 2018). Previous studies have assessed the effectiveness of organic acids (Akyol & Yeninar, 2009), botanical extracts and essential oils (Qayyoum et al., 2013; Lindberg et al., 2000; Damiani et al., 2011) and propolis extracts (Garedew et al., 2002; Pusceddu et al., 2018) for the control of mite infestation in honeybee colonies. The use of these natural products

is highly important for maintaining the safety and health of honeybees while controlling mite infestations below the economic threshold level.

Propolis is among the novel hive products that have been widely used by people in traditional medicine since ancient times (Burdock, 1998). It is a mixture of plant resins collected by worker honeybees from different plant parts, including tree buds, sap flows, and various botanical sources, to which the bees add salivary secretions and enzymes (Marcucci, 1996; Ayad et al., 2024). It is the proactive hive component that plays an important role in enhancing the social immunity of honeybees. Naturally, honeybees use propolis for sealing and sterilizing the internal parts of their hives so as to protect the colony from pest attack and infestation against numerous pathogens and parasites (Simone-Finstrom et al., 2017; Lipiński, 2019). The biological activity and pharmacological properties of propolis have been extensively studied (Banskota et al., 2001; Stojanović et al., 2020), revealing a broad spectrum of antimicrobial (Kujumgiev et al., 1999; Bonvehí & Gutiérrez, 2012), antifungal (Silici et al., 2005), antiviral (Gekker et al., 2005), and immunomodulatory (Dimov et al., 1991) properties. Furthermore, the use of propolis in controlling the honeybee pests and parasites like wax mouths and varroa mite infestations has been explored in different countries (Garedew et al., 2002; Garedew et al., 2004; Ararso & Legesse, 2016; Simone-Finstrom et al., 2017). Because these products naturally occur in bee colonies and possess significant acaricide activities (Dimetry et al., 2005).

However, the bioactive compositions of propolis and its efficacy against pests and pathogens varies according to the geographical and botanical origins, season, bees' genetics, and environmental factors (Bankova, 2005; Rufatto et al., 2018; do Nascimento et al., 2019). Likewise, the quality and quantity of collected propolis depend on plant diversity and availability, source and term of gathering, beekeeping techniques and practices…etc.(Bankova et al., 2006; Souza et al., 2016). Variations in colour, odour and chemical compounds are evident based on the origin of propolis and the season of collection (Souza et al., 2016). Moreover, some bee colonies have more devoted behaviour on propolis collection than others have. Previously, propolis was not incorporated into pest management due to its adhesive properties (Simone-Finstrom et al., 2017). However, in recent years, it has gained significant attention as scientific researches explored the advantageous of propolis over synthetic acaricides in controlling several honeybee pest and pathogens (Silici et al., 2005; Drescher et al., 2017; Pusceddu et al., 2021).

African honeybees are anticipated to produce large amount of crude propolis as self-medication against various microorganisms and hive intruders, particularly during the period of brood rearing to provide a prophylactic defensive barrier (Garcia et al., 2013; Simone-Finstrom et al., 2017; Nganso & Torto, 2021). Nevertheless, the use and application of propolis against honeybee pests and pathogens is very limited in Africa.

The divesrse ecological richness and floral sources in Ethiopia may potentially provide a complex and beneficial hive product with significantly varied compositions. As a result, the biological active compounds in propolis may vary depending on the availability of plant species across different regions and beekeeping management practices. According to Nuru et al. (2002), although propolis can be harvested from every hive type, a significantly higher amount can be produced from traditional hives than from modern hives. Thus, it is important to evaluate the acaricidal effect of propolis collected from different locations against the *V. destructor* mite. So far, there is limited research attempt on the application of this natural hive product in the control of varroa mite. Therefore, the present study was designed to evaluate the efficacy of propolis collected from traditional and modern beehives for the control of *V. destructor* under laboratory conditions.

6.2 Material and methods

6.2.1 Study area

The varroa mite collection and bioassay tests were conducted in the apiary site and in the Holeta Bee Research Centre laboratory. Holeta is found in the West Shoa Zone of Oromia Region, about 33km from Addis Ababa to the Ambo road. It is located at 09°06′32″.94 N, 38°49′02″. 49 E, with an average altitude of 2365 m a.s.l. The central highland honeybee (*A. m. bandasii*) colonies were priorly established in the langstroth beehives at the apiary site and were used for the sample collection. These colonies were naturally infested by *V. destructor* mites, and the mites were sampled during the bee brooding seasons.

6.2.2 Propolis collection

Propolis samples were collected from an individual colony of honeybees (*A. m. bandasii*) from Wayu Tuka and Diga districts of East Wollega Zone, Oromia, Ethiopia. The samples were harvested from traditional and modern hives by scraping the resins from the hive entrances, inside the hive walls and between hive cracks. The collected propolis samples were wrapped by aluminium foil, allowed to dry at room temperature, and weighed. All the collected samples were

pooled together based on the hive types and stored in the refrigerator at -4 degree Celsius until extraction.

6.2.3 Extraction and preparation of propolis

Before sample extraction, the collected propolis samples were frozen in a refrigerator (-20 ^{o}C) for 24 h. The frozen propolis was grounded and homogenized using an ultra-centrifugal coffee grinder (Figure 6. 1b) and the powders were stored in labelled, airtight plastic bags at room temperature until extraction. Crude propolis extraction was undertaken at the research Lab of Organic Chemistry, Department of Chemistry, Addis Ababa University, following the procedure described in Bankova et al. (2019). Before extraction, 96% ethanol was diluted to 70% in distilled water. From each sample, 50g of powder was weighed and added into an extraction flask (500ml volume) containing 70% ethanol (1:30 w: v) following the procedure used by (Garedew et al., 2002). These mixtures were kept for 24 h and then were centrifuged for 10 min at 1644× g. Then, the suspension was filtered using a Whatman grade 1 filter paper (Whatman International Ltd Maidstone England, England) (Figure 6. 1c) at room temperature. The material retained in the filter paper was re-extracted twice as described above under the same conditions. The recovered filtrates were concentrated using a vacuum rotary evaporator (Heidolph, Germany) under reduced pressure at 40^{o}C (Figure 6. 1d). The dried propolis extract obtained was then weighed and the yield of extraction was calculated using the formula: Yield (%) = (Pe/Pm) × 100; where Pe is the mass of the residue (g) after evaporation of propolis from 70% ethanol extract and Pm is the mass of the crude propolis sample (g) (Pujirahayu et al., 2014). Then, propolis extract obtained after evaporation was dissolved in distilled water to reduce the effect of ethanol solution on the experimental mites. Finally, three different concentrations of 5%, 7%, and 10% (w/v) were prepared and utilized in the laboratory bioassay.

Figure 6. 1 Extraction process of crude propolis collected from honeybee colonies **a)** Crude propolis **b)** Grinded propolis **c)** Filtration of extracted propolis **d)** Concentrating using rotary evaporator

6.2.4 Varroa mite collections

Adult female varroa mites were collected from naturally infested honeybee colonies established at the apiary site of HBRC. The standard sugar shake method was used to dislodge the mites from the bees following the procedure of Dietemann et al. (2013). Briefly, 1 teaspoon of powdered sugar (about 7g) was poured into each bee sample and thoroughly rolled in meshed glass jars. The jars were inverted down, and then shacked over a piece of paper to recover the mites. The fallen mites were rinsed with a 1x phosphate-buffered saline solution in order to dissolve and remove the sugar particles. After that, the recovered adult mites were transferred into a petri dish with moist filter paper containing fifth instar bee pupae and incubated in a dark room at 34°C and 55% relative humidity (RH). These pupae were used for bioassay tests within 3h from the time of collection.

6.2.5 Bioassay testing

To assess the effects of ethanolic extract of propolis on mite mortality, six mites per experiment were placed on a petri dish lined with a piece of moist filter paper (3 × 3 cm) following the

procedure described by Damiani et al. (2010). The mites were treated by directly applying 200 µl of each of the prepared concentrations: 5%, 7%, and 10% (w/v) of proplis extract on the dorsal side of the idiosoma of the six mites using micropipette (Figure 6. 2). Similarly, control treatments involved the direct application of 200µl of distilled water on the dorsal side of six mites in the same condition. All the Petri dishes were incubated at 34°C with 55% relative humidity. Mite survival was subsequently observed under a Zeiss Primo-star light microscope (MP 40X), and mortalities were recorded at time intervals: 30 minutes, 1 h, 2 h, 3 h, and 4 h, a total of 5 observation points. Mites were considered alive when they made some movement if touched, while lethal with a condition that the mites cannot be moving at all. Each treatment was replicated three times for all the concentrations. Finally, the percentage of mite mortality rate at each time interval was corrected using the following formula:

$$\% \text{ Mortality} = \frac{Number\ of\ dead\ individual\ mites\ in\ treatment}{Total\ number\ of\ mites\ in\ treatment} \text{ X } 100$$

Figure 6. 2. Different concentration bioassay test of propolis extracts on *V. destructor* mite

6.2.6 Statistical analysis

All collected data were entered into Microsoft excel and statistical analyses was performed using SPSS software (Version 25). Corrected mortality rates of mites at each concentration level of the propolis extracts were analysed using descriptive statistics and analysis of variance (ANOVA). Data were checked for normality using the Shapiro-Wilk test, and they were normally

distributed. Means ± S.D values were compared using Tukey's least significant differences at $P < 0.05$.

6.3 Results

The acaricidal efficacy of ethanol extracts of propolis collected from traditional and modern beehives against *V. destructor* at different concentration levels are shown in Tables 1 and 2. Statistically significant differences between the treatments and the control group (water) were observed ($P < 0.05$) (Table 1). Ethanol extracts of propolis at 10% concentration had the highest toxicity against the varroa mite after 1hr exposure time, causing 100% mite mortality irrespective of the hive types. In addition, the test product collected from both hive types caused 100% mortality of varroa mite at 7% concentration 3 hours post treatment.

Particularly, propolis extracts from traditional hives have caused significantly different mite mortality rates; 11%, 27%, and 50% for treatments of 5%, 7%, and 10% concentrations, respectively, after the first exposure time (Table 6.1). Notably, a 10% propolis extract caused 100% mite mortality within 1hr exposure, whereas the 5% and 7% concentrations resulted in 35% and 66.67% mite mortality, respectively. Although lower concentrations of propolis remain effective to cause mite mortality, they require relatively longer duration to achieve full efficacy compared to the 10% concentration. Specifically, the 5% concentration attained 100% mite mortality 4 hr post-application (Table 6.1).

Table 6. 1 Mean percent mortality of varroa mites after exposure for 4 hrs to different concentration of propolis extracts collected from traditional hives

Concentrations	% Mean ± Std. mortality of Varroa mite in time intervals				
	30 m	**1 h**	**2 h**	**3 h**	**4 h**
5%	11.1 ± 1.4^{c}	35.00 ± 19.2^{b}	77.8 ± 18.8^{b}	88.9 ± 7.7^{b}	100 ± 0.0^{a}
7%	27.8 ± 6.6^{b}	66.7 ± 22.2^{ab}	88.9 ± 6.7^{ab}	100 ± 0.0^{a}	100 ± 0.0^{a}
10 %	50.0 ± 8.7^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}
H_20	0.00 ± 0.0^{d}	0.00 ± 0.0^{d}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{b}

- *Means with different letters (a, b, c) indicate statistically significant differences ($P < 0.05$) between treatment groups.*

Likewise, ethanol extracts of propolis from modern beehives had significant mortality effects on varroa mites at different treatment doses and exposure times ($P < 0.05$) (Table 6.2). At higher concentrations (10%), ethanol extracts of propolis resulted in more than 83% mortality of varroa mite after 1 hr and beyond post-applications (Table 6.2). Even at the 7% concentration, there was a notable increase in mite mortality across all exposure times. At the concentration of 5%, the mite mortality rates increased steadily with longer times, reaching 94.4% after 4 hr post exposure.

Table 6. 2 Mortality rates of varroa mites after 4-hr post exposure to different concentrations of ethanol extracts of propolis collected from modern (Langstroth) hives

Concentrations	% Mean ± Std. mortality of Varroa mite in time intervals				
	30 m	**1 h**	**2 h**	**3 h**	**4 h**
5%	5.6 ± 6.11^{c}	22.2 ± 4.4^{c}	55.6 ± 20.3^{b}	77.8 ± 14.6^{b}	94.4 ± 0.0^{a}
7%	16.7 ± 11.3^{b}	55.6 ± 6.3^{b}	83.3 ± 15.3^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}
10%	50.0 ± 7.6^{a}	83.3 ± 16.4^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}	100 ± 0.0^{a}
H_20 (control)	0.00 ± 0.0^{d}	0.00 ± 0.0^{d}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{b}

- *Means with different letters (a, b, c) indicate statistically significant differences ($P < 0.05$), between treatment groups*

6.4 Discussions

For the sustainable management of *V. destructor* in honeybee colonies, it is imperative to investigat alternatives to the application of synthetic acaricides. Recently, natural products like plant derivatives are becoming common alternatives for the safer methods to counteract the mite infestations and to minimize the potential negative impacts of synthetic acaricides on honeybees and their hive products (Bava et al., 2023). This finding unveiled demonstrated the efficacy of propolis extracts in controlling *V. destructor* in laboratory bioassays, consistent with previous research findings (Garedew et al., 2004; Nganso and Torto, 2021). Ethanolic extracts of propolis harvested from both traditional and modern hives gave promising results in reducing mite survival. This efficacy may be attributed to the chemical composition of propolis, including phenolic acids and flavonoids, which are released upon extraction and exert significant acaricidal effects on various pests and parasites (Bankova, 2005; Dimetry et al., 2005). Previous studies also pointed out that acaridical effects of propolis against varroa mites linked to its chemical constituents, which comprise various volatile components found within the propolis (Asgharpour

et al., 2020; Ghallab et al., 2021). These components may disrupt the normal biological and physiological activities of varroa mites (Drescher et al., 2017). The authors suggested that propolis play a potential role as an immune defense mechanism against various pests and pathogens within the colony. Other studies also showed that honey bees' resin collection was positively associated with the levels of parasites and pathogens, indicating an adaptive mechanism of honeybees for self-medication using propolis (Garcia et al., 2013; Simone-Finstrom et al., 2017). Thus, the presence of crude propolis within the hives enhances honeybee fitness by enhancing their brood viability, worker lifespan, hygienic behaviour, honey production, and pollen reserves as suggested by previous studies (Nicodemo et al., 2013; Nicodemo et al., 2014). More importantly, Garedew et al. (2002) highlighted that when propolis is extracted particularly using ethanol, it exhibits a potential lethal and narcotic effect on varroa mites. This was recently confirmed by Nganso and Torto (2021) that ethanolic extract of propolis is more effective in affecting the survival of varroa mite compared to crude propolis in African honeybees.

In addition to its lethal effect, propolis treatment was observed to induce a narcotic effect on *V. destructor* mites. This was observed when the mites remained inactive during the first hour post treatment initiation, while it was absent in the control groups (water treatment). In line with this, previous studies have also shown that ethanolic extract of propolis exhibit significant narcotic and lethal effects on *V. destructor* mites. However, the durationa of narcosis and mortality rates are influenced by the concentration of propolis, the solvent used for extraction, and the length of contact time (Garedew et al., 2002; Pusceddu et al., 2018). Garedew et al. (2003) suggested that mite narcosis typically induced by volatile compounds in propolis that disrupt neuronal transmission, leading to decreased heat production and oxygen consumption, eventually cause mite death.

In this study, effects of different propolis concentrations on mite mortality were also assessed across different exposure times. The results revealed that mite mortality increased as the concentration and exposure periods increased. At higher concentration (10% w/v), propolis extract killed 100% of exposed varroa mites within 1 hr exposure time. This finding is consistent with a previous report that indicated ethanolic extracts from German propolis caused 100% mite mortality due to contact with 10% propolis extract within a shorter exposure time (Garedew et

al., 2002). Similarly, Ararso and Legesse (2016) found that higher propolis concentrations at 8% and 10% w/v had higher toxicity effects, causing 80% and 90% mortality of wax moth larvae, respectively. Accordingly, it is necessary to apply propolis extracts at higher doses for the control of honeybee pests and parasites in topical applications. However, the varroacidal effect of propolis is similar for all concentrations and not statistically different ($P > 0.05$) after 4 h of exposure. This suggests that lower concentrations of propolis extract exhibit effectiveness as well but require more time to achieve comparable mortality rates.

The relatively higher mortality of mites induced by propolis extracts from traditional hives compared to those from modern hives over each observation period may be attributed to differences in their chemical composition. This may be further related with the larger amount of propolis stored for longer time can be produced from traditional hives than from modern hives as observed by Nuru et al. (2002). As there is no routine beekeeping management inspection inside traditional basket hives, propolis obtained from these hives tends to remain for longer periods, leading to aging. Whereas propolis from modern hives is often harvested fresh due to more frequent hive inspections (Nuru et al., 2002). As a result, aged propolis in traditional hives probably accumulates more chemical and biological components over time than fresh propolis. In support of this, Schmidt et al. (2014) evaluated that aged propolis retains significant radical scavenging and antimicrobial properties compared to fresh propolis harvested from the same geographic areas.

Overall, the findings of this study revealed concentration based and time-dependent efficacy of propolis extract in control of varroa mites. The laboratory assay results indicated that directly exposing mites in Petri dishes to different concentrations of ethanolic extracts of propolis caused high varroa mortality, but with varying mean mortality rate across different concentrations and exposure times. The higher concentration and longer exposure time were highly effective causing higher mortality rates of varroa mites. Therefore, propolis extracts can be viable alternatives to synthetic acaricides for the control of *V. destructor* mite. Identification and characterization of biologically active molecules in propolis harvested from various geographical regions of Ethiopia is an important area that has to be focused on optimizing its application under real in-hive conditions.

CHAPTER SEVEN

General Conclusions and Recommendations

7.1 Conclusions

Honeybees play a crucial role in both the economy and ecology, providing essential pollination services vital for agricultural production. However, their populations are currently facing a severe decline, and dramatic colony losses worldwide. Several biotic and abiotic factors and their synergetic effects are attributed to the global colony losses.

Among these factors, the varroa mite *(V. destructor)* is recognized as the most significant threat to honeybees, affecting both wild and managed colonies globally. Despite its presence being reported in Ethiopia for about 14 years, research on the distribution of this parasite and its impact on local honeybee populations remains extremely limited. This thesis provides comprehensive insight into *V. destructor* mite infestation, genetic diversity, host defensive behavior, and mite management in local honeybee populations in Ethiopia. Based on the main findings of the study, the following concluding remarks were drawn:

- The current study identified a widespread distribution of the varroa mite across the studied areas of Ethiopia with varied prevalence and infestation level.
- The prevalence and infestation rates of varroa mite were varied across the regional states, between the bee developmental stages, and among beekeeping management practices
- Human beekeeping practices were considered as the main factors contributed for varroa mite widespread in Ethiopia.
- The current study revealed that varroa infestation rate was significantly higher in brood bees than in adult bees, indicating that the mites strongly prefer brood larvae so that the mites feed on the soft tissue of the brood and reproduce within the brood cells.
- In addition, higher varroa mite infestations were found in modern hives compared to traditional local hives, indicating that intensive beekeeping practices in modern beekeeping, such as frequent hive inspections, may contribute to the increased spread of parasites among modern beekeeping.
- The study on varroa genetic diversity represents the first comprehensive attempt to describe *V. destructor* populations in Ethiopia. Analysis mtDNA sequencing and SNP

markers revealed a single haplotype (K1-hplotype) of *V. destructor* with the very low genetic diversity among varroa populations in Ethiopia.

- The introduction and subsequent spread of varroa mite into Ethiopia could be likely from genetically homozygous lineages and there may be high gene flow and inbreeding among the varroa populations.
- The study indicates that local honeybee *(A.m. bandasii)* colonies demonstrated strong defensive behaviors against varroa mite, expressing higher hygienic and grooming behaviors.
- Laboratory bioassay tests demonstrated that ethanolic extracts of propolis have a potential varroacidal effect against *V. destructor* mites, suggesting propolis could be effective in controlling this parasitic mite.

Overall, this thesis has provided new insights and expanded knowledge on the distribution, genetic profile, host behavioural response and non-chemical control of *V. destructor* mite in Ethiopia. The findings of this study are essential for developing effective and sustainable control strategies against the *V. destructor* mite and and to ensure the survival of honeybee population, thereby enhancing their productivity in the country.

7.2 Recommendations

Although this study represents a significant step forward in understanding the invasive pattern of *V. destructor* mite in Ethiopia, several knowledge gaps still need to be addressed. Thus, the following recommendations are forwarded to fully understand the parasitism of *V. destructor* in Ethiopia and to develop sustainable control strategies:

- Regular monitoring and continuous surveillance of honeybee colonies are essential to understand the dynamics of mite infestation and mitigate its impact on honeybee health and the beekeeping industry.
- Increase awareness among beekeepers and the whole community about the presence and adverse impacts of this parasitic mite on honeybee health and their production.
- Strengthen the skills and knowledge of beekeepers and extension workers to enhance the survival of local honeybee populations.

- Promote the adoption of non-chemical alternatives instead of synthetic acaricides for controlling *V. destructor* mites, thereby enhancing organic beekeeping practices in Ethiopia.
- There must be a national strategy that regulate the introduction and transmission of honeybee pathogens and parasites in the expansion of modern beekeeping in Ethiopia to ensure the long-term health and productivity of honeybee colonies.
- Further studies are recommended to evaluate the dynamics of mite infestation and prevalence, and to determine the economic thresholds beyond which *V. destructor* mites pose a significant threat to local honeybee populations.
- Understand the in-depth host-parasite interactions between the varroa mite and different honeybee races in the country is required to determine the resilience of local honeybees against the parasitism of varroa mite.
- Identifying the genetic sources and population structure of varroa mite populations in local honeybees using highly polymorphic markers and at large scale.
- Explore the natural product-based methods for mite control practices to ensure sustainable and long-term mite management strategies.
- Further studies are required to assess the potential side effects of propolis extract on bee broods and practical feasibility of using this product in beekeeping practices at field conditions.

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APPENDICES

Appendix 1

Table. S1 Sampling locations, altitudes and coordinates for varroa mite infestation in Ethiopia

No.	Sampling Site	Altitude (mt)	Coordinates	districts	Zones	Region
1.	Bakko Kejo	1678 m	9°07’08.24” N, 37°02’35.28” E	Bakko	West Shoa	Oromiya
2.	Gedo	2490 m	9°02’50.38” N, 37°27’25.31” E	Chalya		
3.	Holeta	2498 m	9°03’24.48” N, 38°30’05.72” E	Wolmerra		
4.	Kimoye	2219 m	9° 01'02.01” N, 38°19'59.98” E	Ejere		
5.	Inchini	2450 m	9°26'33.57” N, 38°20'34.32” E	Ada Berga		
6.	Gemechisa	2161 m	9°01’16.46” N, 36°27’38.01” E	Diga	East Wollega	
7.	Jirata	2256 m	9°01’21.43” N, 36°30’03.47” E			
8.	Gudina	2162 m	8°53’56.11” N, 36°27’56.37” E	Leka Dulecha		
9.	Dedessa	1395 m	8°40’16.25” N, 36°24’04.16” E	Dabo Hanna	Buno Bedele	
10.	Sanga Fato	2041 m	8°36’32.18” N, 36°07’28.88” E	Degga		
11.	Witate	1602 m	8°22’19.65” N, 35°55’37.46” E	Yayo	Illu A/bora	
12.	Gore	2036 m	8°09’03.35”N, 35°32’08.63” E	Alle		
13.	Sagi	1680 m	8°09’07.14” N, 35°30’05.62” E			
14.	Kundi	1789 m	8°10’15.80” N,35°32’51.63” E			
15.	Gaba Guda	1722 m	8°14’14.95” N, 35°35’27.26” E	Mettu		
16.	Botto	1644 m	8°18’58.25” N, 35°35’21.23” E			
17.	Gera	2098 m	7°44'37.73” N, 36°14'59.39” E	Gera	Jimma	
18.	Afallo	1923 m	7°40'57.64” N, 36°19'59.02” E			
19.	Kuni Segeriya	2232 m	9°01’24.49” N, 40°54’52.18” E	Gemechis	West Hararge	
20.	Welargi	1895 m	8°56’52.78”N, 40°50’33.17” E			
21.	Wachu Lemay	2040 m	8°57’06.51” N, 40°50’03.04” E	Chiro		
22.	Arba Reketi	2269 m	9°03’02.38” N, 40°54’40.67” E			
23.	Chiro town	1788 m	9°04’23.28” N, 40°52’03.17” E			
24.	Akasha	2073 m	7°11’34.83” N, 40°37’01.63” E	Ginnir	East Bale	
25.	Obsan	1987 m	7°09’51.04” N, 40°38’39.61” E			
26.	Ginnir 01	1127 m	6°52’57.33” N, 41°07’50.77” E			
27.	Rayitu	1109 m	6°53’04.70” N, 41°07’23.13” E	Rayitu		
28.	Guassa	3366 m	10°16'53.74”N, 39°46'67.21” E	Menz Gera	North Shoa	Amhara
29.	Semen Degera	1320 m	10°59’50.19”N, 36°20’04.31” E	Guangua	Awi Zone	
30.	Batambe	2560 m	10°57’19.95”N, 36°55’57.39” E	Banja		
31.	Abadira	2120 m	11°15'31.28”N, 36°50'38.61” E	Dangila		
32.	Abesheb	2271 m	11°59’59.93”N, 37°59’59.97” E	Debre Elias	East Gojjam	
33.	Enerata	2485 m	10°24'14.99" N, 37°44'03.84" E	Gozamin		
34.	Gedam Abo	2493 m	11°04’37.06”N, 37°57’07.10” E	Mota		
35.	Chebera Churchura	1245 m	6°59'28.82” N, 36°40'06.40” E	Konta	Dawuro	SNNP
36.	Ramada	1917 m	6°53’29.43” N, 38°27’33.70” E	Shebedino	Sidama	

37.	Wosha Soyoma	1779 m	7°04’42.14” N, 38°37’34.03” E	Wondo Genet	Sidama	
38.	Bullessa	1858 m	7°37’12.82” N, 38°22’49.72” E	Alata Wondo	Sidama	
39.	Buttuma	1936 m	6°35’47.72” N, 38°25’27.85” E	Alata Wondo	Sidama	

Appendix 2

Table S1 Sample Code for Varroa mite genotyping by SNP markers

S-No	S-Code	Zone	District	Population group
V-1	BA1	East Bale	Akasha 1	Eastern Ethiopia
V-2	BA2	East Bale	Akasha 2	Eastern Ethiopia
V-3	BA3	East Bale	Akasha 3	Eastern Ethiopia
V-4	BBD1	Buno Bedele	Degga 1	Western Ethiopia
V-5	BBD2	Buno Bedele	Degga 2	Western Ethiopia
V-6	BBD3	Buno Bedele	Degga 3	Western Ethiopia
V-7	BBD4	Buno Bedele	Degga 4	Western Ethiopia
V-8	BBDb1	Buno Bedele	Dabo 1	Western Ethiopia
V-9	BBDb2	Buno Bedele	Dabo 2	Western Ethiopia
V-10	BBDb3	Buno Bedele	Dabo 3	Western Ethiopia
V-11	BBDb4	Buno Bedele	Dabo 4	Western Ethiopia
V-12	BGN1	East Bale	Ginnir 1	Eastern Ethiopia
V-13	BGN2	East Bale	Ginnir 2	Eastern Ethiopia
V-14	BGN3	East Bale	Ginnir 3	Eastern Ethiopia
V-15	BGNT1	East Bale	Ginnir Town 1	Eastern Ethiopia
V-16	BGNT2	East Bale	GinnirTown 2	Eastern Ethiopia
V-17	BGTN3	East Bale	Ginnir Town 3	Eastern Ethiopia
V-18	BO1	East Bale	Obsan 1	Eastern Ethiopia
V-19	BO2	East Bale	Obsan 2	Eastern Ethiopia
V-20	BO3	East Bale	Obsan 3	Eastern Ethiopia
V-21	BRY1	East Bale	Rayitu 1	Eastern Ethiopia
V-22	BRY2	East Bale	Rayitu 2	Eastern Ethiopia
V-23	BRY3	East Bale	Rayitu 3	Eastern Ethiopia
V-24	EWD1	East Wollega	Diga 1	Western Ethiopia
V-25	EWD2	East Wollega	Diga 2	Western Ethiopia
V-26	EWD3	East Wollega	Diga 3	Western Ethiopia
V-27	EWG1	East Wollega	Gobbu 1	Eastern Ethiopia
V-28	EWG2	East Wollega	Gobbu 1	Western Ethiopia
V-29	EWG3	East Wollega	Gobbu 2	Western Ethiopia
V-30	EWG4	East Wollega	Gobbu 3	Western Ethiopia
V-31	IAA1	I/Aba bora	Alle 1	Western Ethiopia
V-32	IAA2	I/Aba bora	Alle 2	Western Ethiopia

V-33	IAG1	I/Aba bora	Gore 1	Western Ethiopia
V-34	IAG2	I/Aba bora	Gore 2	Western Ethiopia
V-35	IAG3	I/Abab bora	Gore 3	Western Ethiopia
V-36	IAY1	I/Aba bora	Yayo 1	Western Ethiopia
V-37	IAY2	I/Aba bora	Yayo 2	Western Ethiopia
V-38	IAY3	I/Aba bora	Yayo 3	Western Ethiopia
V-39	SS1	Sidama	Shebedino 1	Southern Ethiopia
V-40	SS2	Sidama	Shebedino 2	Southern Ethiopia
V-41	SS3	Sidama	Shebedino 3	Southern Ethiopia
V-42	SW1	Sidama	Wondogenet 1	Southern Ethiopia
V-43	SW2	Sidama	Wondogent 2	Southern Ethiopia
V-44	WHA1	West Hararge	Arba Rakate 1	Eastern Ethiopia
V-45	WHAR2	West Hararge	Arba Rakate 2	Eastern Ethiopia
V-46	WHAR3	West Hararge	Arba Rakate 3	Eastern Ethiopia
V-47	WHC1	West Hararge	Chiro 1	Eastern Ethiopia
V-48	WHC2	West Hararge	Chiro 2	Eastern Ethiopia
V-49	WHC3	West Hararge	Chiro 3	Eastern Ethiopia
V-50	WHG1	West Hararge	Gemechisa 1	Eastern Ethiopia
V-51	WHG2	West Hararge	Gemechisa 2	Eastern Ethiopia
V-52	WHG3	West Hararge	Gemechisa 3	Eastern Ethiopia
V-53	WHK1	West Hararge	Kuni sagaria 1	Eastern Ethiopia
V-54	WHK2	West Hararge	Kuni sagaria 2	Eastern Ethiopia
V-55	WHK3	West Hararge	Kuni sagaria 3	Eastern Ethiopia
V-56	WHU1	West Hararge	Univesrity 1	Eastern Ethiopia
V-57	WHU2	West Hararge	Univesrity 2	Eastern Ethiopia
V-58	WSB1	West Shewa	Bakko 1	Central Ethiopia
V-59	WSB2	West Shewa	Bakko 2	Central Ethiopia
V-60	WSB3	West Shewa	Bakko 3	Central Ethiopia
V-61	WSG1	West Shewa	Gedo 1	Central Ethiopia
V-62	WSG2	West Shewa	Gedo 2	Central Ethiopia
V-63	WSG3	West Shewa	Gedo 3	Central Ethiopia
V-64	WSH1	West Shewa	Holeta 1	Central Ethiopia
V-65	WSH2	West Shewa	Holeta 2	Central Ethiopia
V-66	WSH3	West Shewa	Holeta 3	Central Ethiopia
V-67	WSH4	West Shewa	Holeta 4	Central Ethiopia
V-68	WSK1	West Shewa	Kimoye 1	Central Ethiopia
V-69	WSK2	West Shewa	Kimoye 2	Central Ethiopia
V-70	WSK3	West Shewa	kimoye 3	Central Ethiopia
V-71	WSS1	West Shewa	Sokondo 1	Central Ethiopia
V-72	WSS2	West Shewa	Sokondo 2	Central Ethiopia
V-73	WSS3	West Shewa	Sokondo 3	Central Ethiopia
V-74	WSW1	West Shewa	Wolmera 1	Central Ethiopia

V-75	WSW2	West Shewa	Wolmera 2	Central Ethiopia
V-76	WSW3	West Shewa	Wolmera 3	Central Ethiopia

Table S1. Cytb sequences of Varroa mite acquired from Gene bank and the haplotype sequences belongs

NO	**Accation number**	**Sampling country-Site**	**Haplotype**
1	GQ379100	China-Xishuanbanna	1
2	GQ379102	Thailand-BangChangtay	2
3	GQ379099	Vietnam-Hanoi	3
4	GQ379101	Thailand-ChiangMai	4
5	GQ379106	China-Dayao	5
6	GQ379105	China-Kunming	6
7	GQ379110	Japan-Shikoku	7
8	GQ379109	Japan-Machida	8
9	GQ379112	Japan-Tokyo	9
10	GQ379107	Taiwan-Taichung	10
11	GQ379108	Japan-Tokyo-TamagawaUni	10
12	45875985_GUL598_R_f	Ethiopia	11
13	45876005_GUL600_Gin_f	Ethiopia	11
14	45876029_GUL602_Y_f	Ethiopia	11
15	45876043_GUL604_Ba_f	Ethiopia	11
16	45876067_GUL606_G_f	Ethiopia	11
17	45876081_GUL608_Un_f	Ethiopia	11
18	45876104_GUL610_H_f	Ethiopia	11
19	45876128_GUL612_Ge_f	Ethiopia	11
20	AJ493124	France-Avignon	11
21	AP019523	Japan-OnoCity	11
22	GQ379094	Korea-Seoul	11
23	JX970944	Serbia1-Belgrade	11
24	MW553985	Turkey	11
25	MW553986	Turkey	11
26	MW553989	Turkey	11
27	MW553990	Turkey	11
28	MW553991	Turkey	11
29	MW553992	Turkey	11
30	MW553993	Turkey	11
31	MW553994	Turkey	11

32	MW553995	Turkey	11
33	MW553998	Turkey	11
34	MW553999	Turkey	11
35	MW554001	Turkey	11
36	MW554003	Turkey	11
37	MW554005	Turkey	11
38	MW554006	Turkey	11
39	NC004454	France-Avignon	11
40	OM929196	Turkey	11
41	OQ104612	Turkey	11
42	OQ104613	Turkey	11
43	OQ104614	Turkey	11
44	OQ104615	Turkey	11
45	OQ104616	Turkey	11
46	OQ104617	Turkey	11
47	OQ104618	Turkey	11
48	OQ104619	Turkey	11
49	OQ104620	Turkey	11
50	OQ104621	Turkey	11
51	OQ104622	Turkey	11
52	OQ104623	Turkey	11
53	OQ104624	Turkey	11
54	OQ104625	Turkey	11
55	OQ104626	Turkey	11
56	OQ104627	Turkey	11
57	OQ104628	Turkey	11
58	OQ104629	Turkey	11
59	OQ104630	Turkey	11
60	OQ104631	Turkey	11
61	OQ104632	Turkey	11
62	OQ104633	Turkey	11
63	OQ104634	Turkey	11
64	OQ104635	Turkey	11
65	OQ104636	Turkey	11
66	OQ104637	Turkey	11
67	OQ104638	Turkey	11

68	OQ104639	Turkey	11
69	OQ104640	Turkey	11
70	OQ104641	Turkey	11
71	OQ104642	Turkey	11
72	OQ104644	Turkey	11
73	OQ104645	Turkey	11
74	OQ104646	Turkey	11
75	OQ104647	Turkey	11
76	OQ104649	Turkey	11
77	OQ104651	Turkey	11
78	OQ104652	Turkey	11
79	OQ104653	Turkey	11
80	OQ104654	Turkey	11
81	OQ104656	Turkey	11
82	OQ104657	Turkey	11
83	OQ104658	Turkey	11
84	OQ104659	Turkey	11
85	OQ104660	Turkey	11
86	OQ104662	Turkey	11
87	OQ104663	Turkey	11
88	OQ104664	Turkey	11
89	OQ104665	Turkey	11
90	OQ104666	Turkey	11
91	OQ104667	Turkey	11
92	OQ104668	Turkey	11
93	OQ104669	Turkey	11
94	OQ104670	Turkey	11
95	OQ104671	Turkey	11
96	OQ104672	Turkey	11
97	OQ104673	Turkey	11
98	OQ104675	Turkey	11
99	OQ104676	Turkey	11
100	OQ104677	Turkey	11
101	OQ104678	Turkey	11
102	OQ104679	Turkey	11
103	OQ104680	Turkey	11

104	OQ104681	Turkey	11
105	OQ104682	Turkey	11
106	OQ104683	Turkey	11
107	OQ104684	Turkey	11
108	OQ104685	Turkey	11
109	OQ104686	Turkey	11
110	OQ104687	Turkey	11
111	OQ104688	Turkey	11
112	OQ104689	Turkey	11
113	OQ104690	Turkey	11
114	OQ104691	Turkey	11
115	OQ104692	Turkey	11
116	OQ104693	Turkey	11
117	OQ104694	Turkey	11
118	OQ104695	Turkey	11
119	OQ104696	Turkey	11
120	OQ104697	Turkey	11
121	OQ104698	Turkey	11
122	OQ104699	Turkey	11
123	OQ104700	Turkey	11
124	OQ104701	Turkey	11
125	OQ104702	Turkey	11
126	OQ104703	Turkey	11
127	OQ104704	Turkey	11
128	OQ104705	Turkey	11
129	OQ104706	Turkey	11
130	OQ104707	Turkey	11
131	OQ104708	Turkey	11
132	OQ104709	Turkey	11
133	OQ104710	Turkey	11
134	OQ104711	Turkey	11
135	OQ104712	Turkey	11
136	OQ104713	Turkey	11
137	OQ104714	Turkey	11
138	OQ104715	Turkey	11
139	OQ104716	Turkey	11

140	OQ104717	Turkey	11
141	OQ104718	Turkey	11
142	OQ104719	Turkey	11
143	OQ104720	Turkey	11
144	OQ104721	Turkey	11
145	OQ104722	Turkey	11
146	OQ104723	Turkey	11
147	OQ104724	Turkey	11
148	OQ104725	Turkey	11
149	OQ104726	Turkey	11
150	OQ104727	Turkey	11
151	OQ104728	Turkey	11
152	OQ104729	Turkey	11
153	OQ104730	Turkey	11
154	OQ104731	Turkey	11
155	OQ104732	Turkey	11
156	OQ104733	Turkey	11
157	OQ104734	Turkey	11
158	OQ104735	Turkey	11
159	OQ104736	Turkey	11
160	OQ104674	Turkey	12
161	OM929194	Turkey	13
162	MW553996	Turkey	14
163	MW553987	Turkey	15
164	MW553997	Turkey	15
165	MW554002	Turkey	15
166	MW554004	Turkey	15
167	MW554000	Turkey	16
168	LN873225	Syria-Tartous	17
169	LN873229	Syria-AlQunaitra	17
170	MW553988	Turkey	18
171	JX970945	Serbia, Peshter1-SuviDo	19
172	OM929195	Turkey	19
173	OQ104643	Turkey	19
174	OQ104648	Turkey	19
175	OQ104650	Turkey	19

176	OQ104655	Turkey	19
177	OQ104661	Turkey	19

Table S2. COX1sequence acquired from gene bank and haplotype the sequences belong to

No	**AccNum**	**Country/Site**	**haplotype**
1	GQ387678	Thailand Bang Chantay	1
2	GQ387679	Thailand Chiang Mai	1
3	GQ379068	China_Dayao	1
4	MW599142	Vietnam	2
5	MW599143	Vietnam	2
6	MW599144	Vietnam	2
7	MW599126	Vietnam	3
8	KR528380	Vietnam	4
9	GQ379061	Vietnam_Hanoi	5
10	GQ379064	Thailand_Bang Changtay	5
11	KR528379	Vietnam	5
12	KR528382	Vietnam	5
13	KR528383	Vietnam	5
14	MW599120	Vietnam	5
15	MW599121	Vietnam	5
16	MW599122	Vietnam	5
17	MW599123	Vietnam	5
18	MW599124	Vietnam	5
19	MW599125	Vietnam	5
20	MW599127	Vietnam	5
21	MW599131	Vietnam	5
22	MW599132	Vietnam	5
23	MW599133	Vietnam	5
24	MW599135	Vietnam	5
25	MW599136	Vietnam	5
26	MW599137	Vietnam	5
27	MW599148	Vietnam	5
28	MW599149	Vietnam	5
29	MW599150	Vietnam	5
30	MW599151	Vietnam	5
31	MW599152	Vietnam	5

32	MW599153	Vietnam	5
33	MW599154	Vietnam	5
34	MW599155	Vietnam	5
35	MW599156	Vietnam	5
36	AJ784872	Taiwan_Taichung	6
37	GQ379069	Taiwan_Taichung	6
38	MW599107	Taiwan	6
39	MW599108	Taiwan	6
40	MW599109	Taiwan	6
41	MW599110	Taiwan	6
42	MW599111	Taiwan	6
43	MW599112	Taiwan	6
44	MW599113	Taiwan	6
45	MW599114	Taiwan	6
46	MW599115	Taiwan	6
47	MW599116	Taiwan	6
48	MW599117	Taiwan	6
49	MW599118	Taiwan	6
50	MW599119	Taiwan	6
51	GQ379059	China_Hunan	7
52	JX970938	Serbia_Belgrade	8
53	LN873226	Syria_Al-Qunaitra	9
54	MW725316	SKorea	10
55	MW725317	SKorea_BWK	11
56	LN873222	Syria_Tartous	12
57	GQ379056	SKorea_Seoul	13
58	GQ379060	China_Xishuanbanna	13
59	KR528381	Vietnam	13
60	MW599128	Vietnam_HDCQ	13
61	MW599129	Vietnam_HDCQ	13
62	MW599130	Vietnam_HDCQ	13
63	MW599140	Vietnam_LSTT	13
64	MW599145	Vietnam_TLTP	13
65	MW599146	Vietnam_TLTP	13
66	MW599147	Vietnam_TLTP	13
67	MW725307	SKorea_SJC	13

68	MW725308	SKorea_PYJ	13
69	MW725309	SKorea_PSK	13
70	MW725310	SKorea_LJW	13
71	MW725311	SKorea	13
72	MW725312	SKorea_KHJ	13
73	MW725315	SKorea_PH	13
74	MW725318	SKorea_LSS	13
75	MW725319	SKorea_AJH	13
76	MW725320	SKorea_LSH	13
77	MW725321	SKorea_HTJ	13
78	KR528385	Philippines	13
79	AP019523	Japan_Ono City	13
80	AY163547	USA	13
81	**Bed**	Ethiopia_Bedele	13
82	**Chi**	Ethiopia_Chiro	13
83	EZI356_R	Ethiopia_Rayitu	13
84	EZI358_Ba	Ethiopia_Bakko	13
85	**G**	Ethiopia_Gemechisa	13
86	**Ge**	Ethiopia_Gedo	13
87	**Gin**	Ethiopia_Ginnir	13
88	**H**	Ethiopia_Holeta	13
89	JX970939	Serbia_Peshter1-Suvi_Do	13
90	MG793455	New Zealand	13
91	MN179648	Thailand_ChiangMai	13
92	MT462459	Argentina_Castelar	13
93	MT462460	Argentina_Balcarce	13
94	MT462461	Argentina_Rafaela	13
95	MT462462	Argentina_Formosa	13
96	MT462463	Argentina_Leales	13
97	MT462464	Argentina_Ascasubi	13
98	MT462465	Argentina_Castelli	13
99	MT462466	Argentina_Famailla	13
100	MT462467	Argentina_Reconquista	13
101	MT462468	Argentina_SantaFe	13
102	NC004454	France_Avignon	13
103	OK560013	New Zealand_Hamilton	13

104	OK560014	New Zealand_Ashburton	13
105	OK560015	New Zealand_Porirua	13
106	OK560016	New Zealand_Fielding	13
107	OM929188	Turkey	13
108	OM929189	Turkey	13
109	OM929190	Turkey	13
110	**Si**	Ethiopia_Sidam	13
111	**Un**	Ethiopia_Gera	13
112	**Y**	Ethiopia_Yayo	13

Appendix 3

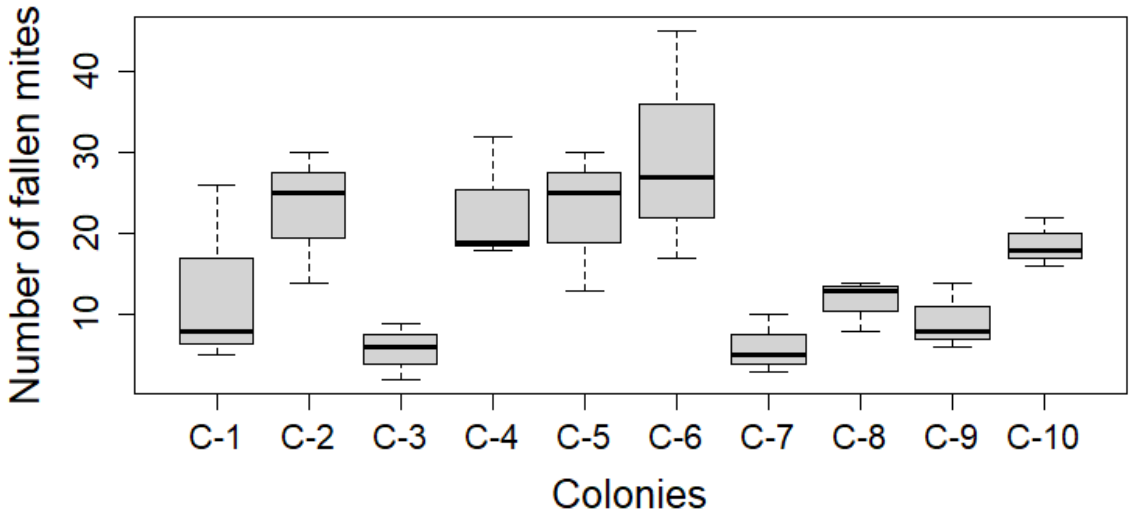

Figure S1- Mean daily fallen mites in each experimental colonies