Solving the *"Colomerus vitis* conundrum": genetic evidence reveals a complex of highly diverged groups with little morphological differentiation

by

Palesa Mbali Maboeei

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in the Faculty of Sciences (Department of Genetics) at Stellenbosch University

Supervisor: Dr Barbara van Asch (Department of Genetics)

Co-supervisors: Dr Davina Saccaggi (Citrus Research International) and Dr James Wesley-Smith (Sefako Makgatho Health Sciences University)

April 2022

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

April 2022

ABSTRACT

Colomerus vitis (Acari: Trombidiformes: Eriophyidae) includes bud, leaf curl, and leaf blister eriophyoid mites that cause significant economic damage to grapevines worldwide. It has been hypothesised that different genetic or morphological strains of *C. vitis* cause the three different plant symptoms. Eriophyoid mites are morphologically simple, and adaptation to different living conditions and hosts generally results in subtle body plan changes which may be difficult to detect using traditional morphological methods. An integrative taxonomic approach including DNA-based (mitochondrial cytochrome oxidase 1 gene variation and Next Generation Sequencing) and morphological analyses (Low temperature Scanning Electron Microscopy imaging) methods was used to investigate the diversity of grapevine eriophyoid mites presumed to be *C. vitis*. Sanger sequencing of the COI gene in samples of pooled mites collected from grapevine buds and leaf blisters revealed the presence of mixtures of four distinct genetic groups. Subsequent Next Generation Sequencing of the complete mitogenomes of the grapevine mite groups identified by Sanger sequencing was not totally successful but it revealed the presence of a fifth genetic group. The COI sequences had an average intragroup p-distance of 0.82% and an average intergroup p-distance of 20.48%. Based on these results, a multiplex PCR comprising specific primers for each of the five genetic groups was designed to screen samples by capillary electrophoresis with automatic detection of fragment size. Multiplex PCR screenings of bud and leaf blisters consistently confirmed the overall presence of three of the genetic groups in different combinations and proportions. The genetic groups were often found in mixed populations within the same symptomatic grapevine tissue. Bud and blister mites were significantly associated with different genetic groups. The samples used for the analyses were primarily collected in South Africa. Additional samples from the USA, Spain, Israel, and Egypt contributed to insights into the worldwide genetic diversity of *C. vitis*. Additionally, publicly available *C. vitis* COI sequences from Iran revealed two other genetic groups, suggesting that the global diversity within *C. vitis* may exceed what is reported here. For morphological analyses, a total of 67 individual mites collected from grapevines at Nietvoorbij, Western Cape, South Africa from both leaf blisters (n = 45) and buds (n = 22) were analysed using Cryo-SEM imaging. To investigate whether there were any distinct mite morphotypes, specimens were differentiated according to the shape of microtubercles, number of empodial rays, and prodorsal shield patterning. Variations in the morphological characters analysed were observed within the almost indistinguishable species complex. In conclusion, these results indicate that "*C. vitis"* includes several genetic groups of sufficient diversity to justify the proposal of separate species, rather than strains of a single species each associated with different plant symptoms. These putative species occur in mixed populations within the

different plant symptoms, and do not appear to be morphologically unique enough to allow morphological separation of all five species detected here.

OPSOMMING

Colomerus vitis (Trombidiformes: Eriophyidae) behels wingerdknop, blaasvormende en blaar-krul eriophyid-myte. Eriophyid-myte veroorsaak geweldigge ekonomiese skade aan druiwe wingerde wêreldwyd. Daar word hipotetiseer dat verskillende stamme van *C. vitis* die drie verskillende simptome in plante veroorsaak. Eriophyid-myte is morfologies eenvoudig. Aanpassing tot verskillende lewens omstandighede en gashere lei tot subtiele veranderinge in die myte se liggaam plan wat moontlik moeilik is om op te tel deur tradisionele morfologiese metodes. Ons het ’n geïntegreerde taksonomiese benadering gevolg wat beide morfologiese ontleding (Lae temperatuur skandeer-elektronmikroskoop beelding) en DNA gebaseerde (mitochondriese cytochrome oksidase 1 geen variasie en volgende generasie opeenvolging) metodes gebruik het om die diversiteit van wingerdstok eriophyid-myte te ondersoek, wat vermoedelik *C. vitis* is. In monsters van eriophyid-myte wat van wingerd knoppe en blaar blase versamel is het aanvanklike Sanger opeenvolging van die COI geen die teenwoordigheid van vier afsonderlike geneties groepe opgelewer. Die volgende generasie opeenvolging vir die volle mitogenoom van die wingerdstok myte geïdentifiseer deur die Sanger opeenvolging was onsuksesvol, maar dit het die teenwoordigheid van die vyfde genetiese groep geopenbaar deur verwysings na karterings van vorige gegenereerde *C. vitis* COI opeenvolgings. Die COI opeenvolgings het ’n gemiddelde intragroep p-afstand van 0.82% en ‘n gemiddelde intergroep p-afstand van 20.48%. Gebaseer op hierdie resultate het ons ‘n multipleks PCR ontwerp bestaande uit spesifieke primers vir elke een van die vyf genetiese groepe. Hierdie multipleks PCR was gebruik om monsters te screen volgens kapillêre elektroforese met outomatiese bespeuring van fragment grootte. Multipleks PCR screenings van knoppe en blaar blase het konsekwent die teenwoordigheid van drie genetiese groepe bevestig in verskillende kombinasies en proporsies. Die geneties groepe was dikwels gevind in gemengde populasies binne dieselfde simptomatiese wingerdstok weefsel. Knop en blaas myte was aansienlik geassosieer met verskillende genetiese groepe. Vir morfologiese ontleding was ‘n totaal van 67 individuele myte versamel af van blaar blase (n=45) en knoppe (n=22) en ontleed met Cryo-SEM beelding. Om vas te stel of daar onderskeie myt morfotipes was, was mosters onderskei volgens die vorm van mikrotuberkels, hoeveelheid empodiale strale en prodorsale skildpatroon. Die monsters vir die ontleding was hoofsaaklik versamel in Suid-Afrika, alhoewel daar ‘n paar monster van die VSA, Spanje, Israel en Egipte was wat bygedra het tot die genetiese diversiteit. Addisioneel

is daar publiek beskikbare *C. vitis* COI opeenvolgings van Iran twee ander genetiese groepe bygevoeg. Dit stel voor dat die wêreld wye diversiteit binne *C. vitis* moontlik meer is as wat hier geraporteer word. Variasies in die morfologiese karakters wat ontleed is, is waargeneem binne die byna ononderskeibare spesiekompleks. Ter afsluiting, hierdie resultate dui daarop aan dat *C. vitis* nie ‘n complex van naverwante spesies (stamme) is wat met verskillende plant simptome geassosieer word nie. En ons stel voor dat wingerdstok myte bestaan uit verskeie genetiese en morfologiese uiteenlopende spesies.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr Barbara van Asch for her kindness, support and guidance throughout the project and for her infectious excitement and confidence in the project's success. Most importantly, I would like to thank her for giving me the freedom to work at my own pace.

To my co-supervisors Dr Davina Saccaggi and Dr James Wesley-Smith thank you for motivating, sharing your expertise and assisting me throughout the writing process. You both made the often-dreadful experience a delight.

To Mrs Nompumelelo Ndhlovu, thank you for sharing your acarology expertise and for supporting me and attending to all my questions regarding grapevine mites.

To Dr Charnie Craemer whom I am yet to meet, thank you for your contributions to the realisation of this project.

To Dr Elleunorah Allsopp and Ms Muriel Knipe for assisting me with sample collection, sharing their knowledge about grapevine pests and opening their workspace for me.

To Mr Stephan Van der Westhuizen thank you for assisting with all the statistics work done during the project.

Thank you to vineyards and farm managers who gave me full access for sample collection throughout the project.

To my family and friends, without your love and support, this would have not been possible. I love you all and thank you so much.

To WineTech and Stellenbosch University Postgraduate Funding, thank you for funding me and the project.

MBB lab team, thank you for your contribution to my development. It has been a pleasure knowing and learning from you all.

TABLE OF CONTENTS

ABSTRACT iii

OPSOMMING iv

ACKNOWLEDGEMENTS vi

LIST OF FIGURES viii

ABBREVIATIONS x

CHAPTER 1: LITERATURE REVIEW

GRAPEVINE 1

GRAPEVINE BUD OR BLISTER MITE 3

PEST MANAGEMENT 6

TAXONOMY OF *COLOMERUS VITIS* 7

INTEGRATIVE TAXONOMY 8

MOLECULAR APPROACHES 9

AIM OF THIS STUDY 11

CHAPTER 2: GENETIC DIVERSITY OF *COLOMERUS VITIS*

INTRODUCTION 12

MATERIALS AND METHODS 13

RESULTS 28

DISCUSSION 37

CONCLUSION 39

CHAPTER 3: MORPHOLOGICAL DIVERSITY OF GRAPEVINE MITES

INTRODUCTION 41

MATERIALS AND METHOD 42

RESULTS AND DISCUSSION 44

CONCLUSION 57

CHAPTER 4: GENERAL CONCLUSION

STUDY OUTCOMES 59

LIMITATIONS AND PROSPECTS 60

REFERENCES 62

LIST OF FIGURES

Figure 1.1. *Colomerus vitis* worldwide distribution (CABI, 2021). 3
Figure 1.2. (A) Bud that is infested by bud mite (Craemer *et al*., 2012). (B) Leaf blisters on leaves infested with the erineum strain. (C) Underside of grapevine leaves infested with the erineum strain. (D) Leaf curl mite infestation symptom. 6
Figure 2.1. Western Cape map illustrating sampling sites visited during sample collection. Samples were collected at a total of five farms, two of the farms were in Stellenbosch and one vineyard in Simondium, Cape Town, and Upington. 15
Figure 2.2 A. Bud damage indicating bud mite infestation and bud mites captured through a stereomicroscope (Craemer *et al.*, 2012).....15
Figure 2.2 B. Damage on the underside of the leaf indicating blister mite infestation and blister mites captured through a stereomicroscope (Craemer *et al*., 2012). ……..15
Figure 2.3. Grapevine eriophyoid mites initial COI sequence alignment using universal barcoding primers. Estimate of nucleotide diversity was depicted by highlighted disagreements.....26
Figure 2.4. Expected amplicon sizes from amplification by group specific primers (G1, G2, G3). 27
Figure 2.5. Maximum likelihood unrooted tree showing genetic clustering based on the cytochrome c oxidase subunit I (COI) barcoding region of 64 eriophyoid mite sequences (584 bp) presumed to be Colomerus vitis. Nodal support was based on 1000 bootstrap replicates. 30
Figure 2.7. Genetic group distribution of grapevine mites collected from buds and blisters that were screened by multiplex PCR consisting of G1, G2, G3, G4, and G5 primer pairs. ..36
Figure 2.8. Genetic group distribution of summer and winter bud samples screened by multiplex PCR. 37
Figure 3.1. Empodial ray number and complexity in grapevine *Colomerus* specimens. (A) 6-rayed empodial claw with 2-3 sub-rays. (B) 6-rayed empodial claw, that is simple and without subrays. (C) 5-rayed empodial claw with 4 sub-rays. (D) 5 or 6-rayed empodial claw with complex rays. (E) 5-rayed empodial claw with simple arrangement of rays. 49
Figure 3.2. Shape of microtubercles observed in grapevine *Colomerus* specimens. (A) round; (B) pointy, narrow, and elongated; (C) oval; (D) elongated. 52
Figure 3.3. Prodorsal shield ornamentation in *Colomerus* mites. (A) Median and admedian lines are complete. (B) Broken median and median lines. (C) Smooth prodorsal shield towards the frontal lobe and few ridges between setae (sc). 55

Figure 3.4. Female and male genitalia of *Colomerus* specimens. (A) Female genital coverflap: two rows of ridges. (B) Male genitalia: one row of ridges.....56

LIST OF TABLES

Table 2.1. List of primers used for singleplex PCR, Sanger sequencing (LC01490 and HC02198, Folmer et al., 1994), and multiplex PCR of the COI region in eriophyoid grapevine mites presumed to be *Colomerus vitis*.....17
Table 2.2. List of COI sequence samples used to construct the ML trees. The sample list includes samples generated at DAFF, samples generated in the present study, and samples retrieved from GenBank. The list also includes information about the symptom, plant part, collection site, date, and primers used for PCR and sequencing reactions. 21
Table 2.3. Genetic divergence within different groups of eriophyoid grapevine mite sequences presumed to be *Colomerus vitis* (% of maximum intragroup p-distances; K2P) based on a 584 bp alignment of 64 standard COI barcoding sequences. 31
Table 2.4. Genetic divergence between different groups of eriophyoid grapevine mite sequences presumed to be Colomerus vitis (% of intergroup p-distances; K2P) based on a 584 bp alignment of 64 standard COI barcoding sequences. The table also consists standard errors shown in bold. 31
Table 2.5. List of South African grapevine mite samples screened by multiplex PCR using G1, G2, G3, G4, and G5 primer pairs. The list also includes the symptoms present on plant, the plant part where it was collected, the origin/location of the farm and the date the mites were collected. 32
Table 3.1. Morphological characters on *Colomerus* sp. observed with SEM. 44

ABBREVIATIONS

%: Percentage

®: Registered trademark

°C: Degree Celsius

bp: Basepair

C.: *Colomerus*

CA: California

CAF: Central Analytical Facilities

COI: Cytochrome c oxidase subunit I

Covid-19: Coronavirus disease

d.f.: Degrees of freedom

DAFF: Department of Agriculture, Forestry and Fisheries

DALRRD: Department of Agriculture, Land Reform and Rural Development

DNA: Deoxyribonucleic acid

et al.: And others

Fwd: Forward

G: Genetic group

GPGV: Grapevine Pinot gris virus

H_2O: Water

i.e.: That is

ITSI: Internal Transcribed Spacer one

K2P: Kimura Two-Parameter model

LTC: Linked Target Capture

M – Master mix

MA: Massachusetts

MAFFT: Multiple Alignment using Fast Fourier Transform

MAN: Manufacturer

MEGA-X: Molecular Evolutionary Genetics Analysis

Min: Minute

ML: Maximum likelihood

n: number

ng/µl: Nanogram per micro litre

NGS: Next Generation Sequencing

NJ: Neighbor-Joining

nM: Nanomolar

OIV: International Organisation of Vine and Wine

P: P-value

PCR: Polymerase Chain Reaction

p-distances: Proportion distances

pM: Picomolar

Rev: Reverse

S.E.: Standard error

s: Second

SA: South Africa

SAWIS: South African Wine Industry Information and Systems

SEM: Scanning Electron Microscopy

Seq: Sequence

™: trademark

USA: United States of America

V: Valenzano

v: Version

WOSA: Wines of South Africa

X^2: Chi-square value

µl: Micro litre

µM: Micromolar

□X: Average

CHAPTER 1: LITERATURE REVIEW

GRAPEVINE

Cultivation

Vitis vinifera (grapevine, Vitaceae) is a hardy deciduous plant native to Europe, West Asia, and North America (Gerrath *et al*., 2015; Terral *et al*., 2010). The genus *Vitis* occurs primarily in the Northern Hemisphere (Terral *et al*., 2010; Wen, 2007; Chen *et al*., 2007; Moore, 1991). Commercially grown grapevine originates from *Vitis vinifera* subsp. *sylvestris,* the only *Vitis* species native to Europe (Gerrath *et al*., 2015). Domesticated grapevine (*Vitis vinifera* subsp. *vinifera*) cultivation began between 6000 and 8000 years ago in the Near East (Myles *et al*., 2011; McGovern, 2003), from where it expanded to Southern Europe approximately 5000 years ago and then to Western Europe and Egypt approximately 2800 years ago (Myles *et al*., 2010).

Today, grapevines are widely cultivated in temperate regions and are economically important crops worldwide (Myles, 2010). Viticulture has significantly developed globally, primarily through European colonisation (Gerrath *et al.*, 2015). According to the OIV (2019), five countries comprise over 50% of the world's vineyard: Spain (13%), China (12%), France (11%), Italy (9%), and Turkey (6%).

The first record of grapevines in South Africa dates to 1652, when the Dutch East India Company established a refreshment station in Cape Town (WOSA, 2021). Soon after, Jan van Riebeeck planted the first vineyard in 1655, and wine production followed in 1659 (WOSA, 2021). The South African industry has since grown to have over 10000 viticulturists, most of whom are also wine producers. South Africa has the 15th largest area grown under vineyards (OIV, 2019), is the 13th largest table grape producer, 5th largest dried grape producer, 9th largest wine producer, and 6th largest wine exporter globally (OIV, 2019). The country contributed 4% of the world's wine production in 2020 (WOSA, 2021), exporting 319 million litres of wine (SAWIS, 2020) (a significant decrease from previous years due to the effect of Covid-19 on global trade). In South Africa, grapevines are cultivated mostly in the Western Cape, Northern Cape, Free State, and Limpopo provinces (DAFF, 2012). The Western Cape grows more than 80% of the country's table grapes and contributes more than

half to the country's table grape exports (DAFF, 2012). The wine industry in South Africa employed over 269 096 people directly and indirectly and contributed over R 55 billion to the country's GDP in 2020 (SAWIS, 2021).

Pests and diseases

A considerable number of pests and diseases affect grapevines worldwide, including insects, fungi, mites, nematodes, bacteria, and viruses, which are often a cause of phytosanitary concerns and trade limitations (Halawa *et al*., 2015). In South Africa, grapevines are affected by powdery and downy mildew, bacterial leaf spot and black rot (DAFF, 2012). Control of these diseases is usually by selecting resistant cultivars, pruning, fungicide application and vineyard sanitation (DAFF, 2012; Burger and Deist, 1981; Veikondis *et al.*, 2018). A number of insect and mite pests affect grapevines, with banded fruit weevil (*Phlyctinus callosus*, Coleoptera: Curculionidae) and carnation tortrix (*Epichoristodes acerbella,* Lepidoptera: Tortricidae) considered the most critical phytosanitary insect pests (Pryke and Samways, 2007). The most important mite pest on grapevine worldwide is the grapevine bud or erineum mite, *Colomerus vitis* (Trombidiformes: Eriophyidae). If left untreated, bud mite infestation can cause crop losses of up to 56% in Europe, Russia, South Africa, and Australia (Dennill, 1991). *Colomerus vitis* is the focus of the current study.

Eriophyoids on grapevine

According to Amrine and Stasny (1994), six eriophyoid mite species occur on grapevines. The damage caused by three of the grapevine eriophyoid mites (*C. vitis*, *Colomerus oculivitis*, and *Calepitrimerus vitis)* has been widely reported and the mites are considered economically important grapevine mite pests (Smith Meyer, 1981; Craemer and Saccaggi, 2013). *Colomerus vitis* has a worldwide distribution (Figure 1.1), whereas *C. oculivitis* has only been reported in Egypt and Saudi Arabia (Craemer *et al*., 2012). Until 2004, *C. oculivitis* was thought restricted to Egypt and Saudi Arabia. However, at that time, South Africa started intercepting this species on table grape and grapevine budwood imports from various countries (Saccaggi *et al*., 2012, Craemer and Saccaggi, 2013). These interceptions raised concerns about the knowledge of the distribution of *C. oculivitis* and its potential quarantine status and/or occurrence in South Africa (Craemer *et al*., 2012).

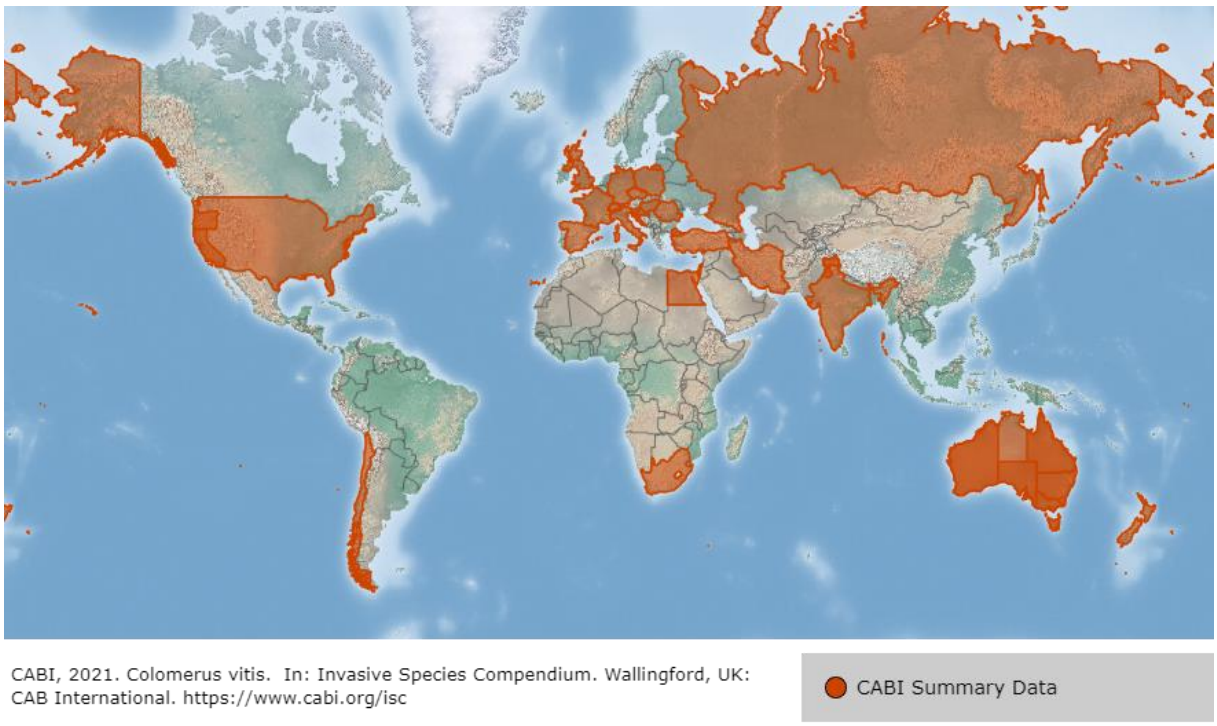


Figure 1.1. *Colomerus vitis* worldwide distribution (CABI, 2021).

GRAPEVINE BUD OR BLISTER MITE

Colomerus vitis is known to possess three “strains” that are characterised by the symptoms they cause on grapevines (Smith and Stafford, 1948). The first “strain” is the bud mite, which attacks the buds, causing the deformation of primordial clusters, distortion of the basal leaves, stunting of the main growth point, and often the death of the overwintering buds, resulting in yield losses and destruction of vineyards (Castillo,1990; Smith Meyer, 1981). The second form is the blister mite which feeds on leaves and causes patches of felt erineum on the lower surface and blister-like swellings on the upper surface, which (arguably) has little economic importance (Meyer and Ueckermann, 1990). The blister mite biology has been intensively studied in the USA and Switzerland (Duso and De Lillo, 1996). The third form is the leaf curl mite, which causes leaf curling and abnormal plant hairs at colony sites. Based on observed plant symptoms, all three strains are present in South Africa (Meyer and Ueckermann, 1990; Meyer and Craemer, 1999; Craemer and Saccaggi, 2013).

As with most eriophyoid mites, *C. vitis* is host-specific and is only able to survive on grapevines, *V. vinifera,* and all its wild subspecies (Smith Meyer, 1981). The mites reproduce by arrhenotokous parthenogenesis, meaning that haploid males are produced from unfertilised eggs, while diploid females develop from fertilised eggs. Thus, a single, unfertilised female can start a new colony. This mode of reproduction also results in a high

degree of genetic inbreeding that can result in genetically indistinct populations (Navia et al., 2010).

The lifecycle consists of larva, nymph and adult stage, with females additionally occurring in two forms: a winter, dormant form (deutogyne) and a summer, reproductive form (protogyne) (Baker, 1996; Lindquist *et al*., 1996). Females produce one to ten eggs per day, and development from egg to adult takes between 10 and 14 days. Immature stages hatch between 5-10 days after eggs are laid and mature around 14 days after hatching. All the strains are distributed through wind, insects, and birds. Bud mites are also carried across through rootstocks and budwood (Smith Meyer, 1981). The lifecycle and symptoms caused by the three strains are discussed separately below.

The bud mite strain (Figure 1.2 A) of *C. vitis* occurs in California, Chile, Spain, Crimea, Egypt, Israel, South Africa and Australia (Duso and De Lillo, 1996). This strain induces hypertrophied cells in the epidermis that subdivide to form “polyps” and elicits repeated division of meristematic cells giving rise to scar tissue (Smith and Schuster, 1963). Bud mite infestation causes scarification of green bark, short basal internodes, flattened shoots, death of terminal and dormant buds, stunting or death of main growth points of the bud along with the development of lateral shoots (witches’ broom growth or zig-zagged shoots), abnormal and crinkled basal leaves with coalescence of venation and cut leaf margins, premature dropping of flower clusters and poor berry set (Smith and Stafford, 1948; Kido and Stafford, 1955; Smith and Schuster, 1963; Gartel, 1970; Castillo, 1990; Smith Meyer, 1981; Baggiolini *et al*., 1969). It is important to note that other physiological factors such as boron deficiency; deficient viticultural practices such as incorrect pruning; or pests such as vineyard phylloxera, plant-parasitic nematodes and some viral diseases may cause similar symptoms. Therefore, grapevines showing these symptoms should be microscopically examined to determine if bud mites are present.

All developmental stages of grapevine *Colomerus* occur in the buds throughout the year (Smith Meyer, 1981). They feed on the primordia as the buds swell and lay eggs (Duso and DeLillo, 1996; Smith Meyer, 1981). Mites are carried up axils of young leaves as the shoots grow, and they can also crawl from bud to bud as the season progresses and new buds are formed (Duso and DeLillo, 1996; Smith Meyer, 1981). Grapevine mites start at the stipular bud scales, and as soon as there is an increase in density they penetrate deeper into newly formed buds and gradually reach the primordia. Cluster primordia may be infested from mid-summer onward. The number of infested buds is at its peak in late summer to early autumn. Basal and median buds are usually more infested than apical buds (Bernard *et al*., 2005). It takes approximately 20 days for a generation to be complete.

The leaf blister (erineum) mite strain (Figure 1.2 B, C) feeds on the underside of the leaves leading to gall formation on the top surface of the leaves (Smith Meyer, 1981). Damaged leaf cells multiply and form fine white or yellow hairs, causing felt-like patches called trichomogenic erinea on the under surface of infested leaves (Slepyan, 1969). Patches form in 10 to 30 days from when the mites first start feeding, depending on the leaf development rate. The patches initially appear white, then turn yellow and finally reddish-brown. Patches arise mainly on the apical leaf part, above the upper parts of the incisions. Leaf erinea decrease the photosynthesis ability of the leaf, but usually not to a physiologically damaging point. Thus, the leaf blister strain of *C. vitis* is usually not considered a serious economic pest.

Females of the leaf blister strain overwinters under outer scales of dormant buds (Duso and DeLillo, 1996; Smith Meyer, 1981) and sometimes under bark crevices (Duso and De Lillo, 1996). The females start moving to unfolding leaves as the bud swells in spring, where they start inducing erinea and reproducing (Duso and DeLillo, 1996; Smith Meyer, 1981). Development mostly occurs in the erinea. The first generation requires approximately 25 days, but the subsequent six generations in the season can develop much faster (Mathez, 1965). In late spring and early summer, the mites migrate to apical leaves and induce new erinea. In late summer and the beginning of autumn, the females start migrating to their overwintering sites in the buds. Since these mites are very sensitive to desiccation, they only migrate in the evening to survive (Smith Meyer, 1981).

The leaf curl mite strain (Figure 1.2 D) has a similar life cycle as the erineum mite strain but shows different symptoms (Smith Meyer, 1981). This strain prefers hairy and young leaves and does not induce erinea. Infested leaves become curled or rolled downward in summer, developing a few abnormal trichomes. Stunted growth and scarring of shoots, as well as necrosis and hypoplasia of the leaf underside, can be observed (Smith and Stafford, 1948; Gartel, 1972; Kido, 1981).

Figure 1.2. (A) Bud that is infested by bud mite (Craemer *et al*., 2012). (B) Leaf blisters on leaves infested with the erineum strain. (C) Underside of grapevine leaves infested with the erineum strain. (D) Leaf curl mite infestation symptom.

PEST MANAGEMENT

Eriophyoid mites, including *C. vitis*, are fairly susceptible to available acaricides, but the challenge is getting the compounds in contact with mites, as they are protected within the buds and blisters (van Leeuwen *et al.*, 2010). Timing of application is therefore imperative, usually early in spring when the mites are moving to newly available niches and are therefore exposed (Smith Meyer, 1981; Dennill, 1986). Mites are also susceptible to sulphur, which is routinely used to control powdery and downy mildew (Smith Meyer, 1981). Additionally, pruning of winter buds effectively limits the population. In some cases, a separate acaricide application may not be necessary, and investigation of pest threshold limits are necessary to determine if targeted *C. vitis* acaricide application is required.

There have been studies that have explored biological control through the use or conservation of predatory mites (van Leeuwen *et al.*, 2010). Several predatory mites have been reported as natural enemies of *C. vitis* (Duso and De Lillo, 1996). These include those from the families Phyotoseiidae (Mesostigmata), Tydeidae (Trombidiformes) and Stigmaeidae (Trombidiformes) (Ferragut *et al*., 2008; Bernard *et al.*, 2010; Klock *et al.*, 2011; Vermaak, 2021). The best time for this type of control strategy is in spring, when the eriophyoids are exposed on stipular scales and new bud axils (Smith and Stafford, 1948; Dennil, 1991). Duso and De Lillo (1996) highlighted that *C. vitis* may become an important alternative for prey for some predatory species. Other measures that have been suggested include control using entomopathogenic fungi and mycoacaricides (de Faria and Wraight, 2007).

In the past, only the bud strain of *C. vitis* was considered economically damaging in South Africa, and therefore only this strain was monitored and controlled. Monitoring consisted of in-vineyard inspection for symptoms of bud mite infestation during the growing season and microscopic inspection of winter buds for the presence of *C. vitis.* If either was found to be present, then a preventative acaricidal spray was applied soon after bud burst in the following season. In recent years, however, the leaf blister form is also controlled through acaricidal sprays as it may be damaging on young or susceptible vines (pers. comm. with Dr. E. Allsopp, ARC, 28 Sept. 2021). Monitoring for the leaf blister form is visual while monitoring for the bud form is analysis of dormant buds, as bud mite symptoms are not conclusive and under Integrated Production of Wine (IPW), farmers are penalised if they spray for bud mite without bud analysis (pers. comm. with Dr. E. Allsopp, ARC, 28 Sept. 2021). There are other factors that can cause similar symptoms to bud mite, hence the requirement for bud analysis.

TAXONOMY OF *COLOMERUS VITIS*

Colomerus vitis belongs to the family Eriophyidae within the Eriophyoidea superfamily. Eriophyoidea comprises a large and diverse group of mites that are obligate plant parasites and feed on various plant organs (Petanović and Kielkiewicz, 2010). Most species in the superfamily are host-specific, feeding and completing their lifecycle on a single plant species or, in some cases, a few species within the same plant genus (Meyer and Craemer, 1999). Eriophyoids are microscopic, approximately 200 µm in length, and are often found within sheltered plant structures such as buds or galls (Smith Meyer, 1981; Lindquist *et al*., 1996). Identification is based on the protogyne female (Baker *et al.*, 1996).

Partially due to their cryptic lifestyle, eriophyoids have a reduced body plan, retaining only two pairs of legs and fewer setae as compared to the putative ancestral mite state. This limits the number of characters that can be used to distinguish species in the superfamily (de Lillo *et al*., 2010). It is worth noting that many of the characters used to distinguish eriophyoids have since been shown to be shared between different groups and are therefore uninformative in generic or species classification (Boczek and Shevchenko, 1996). Additionally, Lindquist and Amrine (1996) argued that the challenge was that most authors had used these characters to define genera based on phenetics rather than cladistical.

The importance of morphological characters weighs differently at the different taxonomic levels. At the family level, the development and form of the gnathosoma and the number of prodorsal shield setae are the diagnostic characters used. At the generic and species level, the shape of the opisthosoma (body), the position and orientation of the prodorsal setae, the position, shape and size of the genital coverflap and the presence and arrangement of leg segments and setae are important diagnostic characters (Lindquist *et al*., 1996).

The genus *Colomerus* includes 27 species worldwide (Chandrapatya and Konvipasruang, 2014), 11 of which have been described in Africa (Meyer and Ueckermann, 1990). The genus is defined by having forwardly directed prodorsal setae, protruding genitalia, female genital coverflap with 2 rows of ridges, foretibial setae, and a thick shortened internal genital apodeme (Meyer and Ueckermann, 1990). Two species of *Colomerus* are known on the grapevine, namely *C. vitis* (as discussed above) and *C. oculivitis* (Attiah, 1967). After study of intercepted *C. vitis* and *C. oculivitis* in South Africa, it became clear that the taxonomic distinction between the two was not always clear and that further taxonomic work was needed (Craemer and Saccaggi, 2013).

The most important characters to distinguish *C. vitis* and *C. oculivitis* are the number of empodial rays and the shape of the microtubercles. *Colomerus vitis* has 5-rayed empodia

(Attiah, 1967; Keifer, 1944; Ryke and Meyer, 1960; 1981; Keifer, 1975; Mathez, 1965; Bagdasarian, 1981; Manson, 1984), while those of *C. oculivitis* are 6-rayed (Attiah, 1967). According to Craemer and Saccaggi (2013), the number of empodial rays is the most unambiguous distinguishing morphological character for these species. The shape of microtubercles is variable, ranging between round, oval, and elongate (Attiah, 1967; Mathez, 1985; Bagdasarian, 1981; Manson, 1984; Meyer and Ueckermann, 1990). Other characters which have been described on these species have varied considerably between published descriptions. For instance, the number of ridges on the female genital coverflap has been reported as 12 ridges in *C. oculivitis* (Attiah, 1967; Zaher, 1984), and 13 (Halawa *et al.,* 2015) and 14 (Zaher, 1984) or more than 16 in *C. vitis* (Keifer, 1944; Craemer and Saccaggi, 2013). Similarly, the prodorsal shield ornamentation is variable, with the length and clarity of median and admedian lines varying between descriptions (Smith Meyer, 1981; Manson 1984; Meyer and Ueckermann, 1990; Craemer and Saccaggi, 2013).

INTEGRATIVE TAXONOMY

Delineation of species and classification into larger taxonomic units is challenging when the organisms in question are tiny, have similar morphology and are character poor (Carew *et al.*, 2009). Species complexes often exist within such groups without clear morphological delineation (Skoracka and Magalha, 2015). Due to their reduced body plan and associated lack of informative morphological characteristics, eriophyoid mites often fall into this category. Many species of eriophyoids, particularly those with a broad host range, have been later found to be complexes of morphologically similar or indistinguishable species (Skoracka and Maglha, 2015). Traditional morphological taxonomy becomes restricted in such instances as the number of characters for morphological identifications becomes limited (Matsuda *et al.*, 2013). This is where integrative taxonomy comes into play (Pentanovic, 2016). Integrative taxonomy is the use of multiple approaches for species delimitation (Schlick-Steiner *et al*., 2010). Integrative taxonomy decreases the failure rate in the delimitation of species (Schlick-Steiner *et al.*, 2010). For example, Heethoff *et al.* (2011) reported that the error rate in species delimitation was 28% when using only nuclear DNA, 33% using mitochondrial DNA, 23% using morphological approaches, and 22% using chemical data. However, using two or three approaches together decreased the error rate significantly to 9% and less than 5%, respectively.

There have been considerable amounts of cryptic eriophyid species discovered through integrative taxonomy. Cryptic species are a group of species that cannot be distinguished morphologically but are too genetically diverged to be considered a single species (Bickford *et al.*, 2007). Many eriophyoid mites have economic importance as pests, biological control

agents and pathogen vectors, and thus it is vital to resolve cryptic species within the group. For example, when cryptic species exhibit different epidemiological, pathogenic or host-specific traits, different control and management strategies should be applied (Bickford *et al.*, 2007; Skoracka and Magalha, 2015). If the cryptic species cannot be discriminated, these species-specific strategies cannot be followed, leading to inadequate control. Cryptic species may occur because of recent speciation, high host specialisation or stabilising selection because of habitat homogeneity (Lefébure *et al.*, 2006; Skoracka and Magalha, 2015). Therefore, distinctions and identification of such species benefit from using multiple taxonomic approaches, such as additional morphological methods, molecular methods and biological or physiological studies. The combination of morphological, molecular and biological traits has successfully revealed species complexes in wheat curl mite, *Aceria tosichella* (Trombidiformes: Eriophyidae) (Miller *et al*., 2013), cereal rust mite *Abacarus hystrix* (Trombidiformes: Eriophyidae) (Skoracka and Dabert, 2010) and *Tetra pinnatifidae* (Trombidiformes: Eriophyidae) (Li, Xue and Hong, 2014), to name but a few.

Confirming species assignments is of particular importance when a genus contains a substantial number of pests because distinguishing between a single species and a complex of species affects pest monitoring and biological control programs (Matsuda *et al.*, 2013; Skoracka and Dabert, 2010; Clarke and Walter, 1995; Armstrong and Ball, 2005 ; Xue *et al.,* 2015).

In this study I employ a similar integrative approach to investigate the putative strains of *C. vitis*.

MOLECULAR APPROACHES

Species with limited morphological diversity but which are genetically diverse would benefit from the use and development of molecular methods for their study and accurate identification (Matsuda *et al*., 2013; Xue *et al*., 2015). A good example of this is the study by Skoracka (2009) where traditional taxonomy based on key traits recognised *Abacarus hystrix* (Trombidiformes: Eriophyidae) as a single species with a wide range of hosts, whereas the DNA sequence analysis of the COI region revealed that two host-specific (quackgrass and brome associated) strains were divided into two well-supported clades (Skoracka, 2009). There has been a considerable number of other studies that have been successful in using similar approaches (Caterino *et al*., 2000; Navia *et al*., 2005; Mahani *et al*., 2009).

There are different kinds of molecular species delimitation, including aggregating populations based on a lack of discrete differences; grouping based on fixed nucleotide differences unique to a population; and the tree-based approach based on branch length (Pons *et al*.,

2006). Other recommendations include the use of DNA based taxonomic system, i.e., using the sequence information as the initial information to establish group membership and define species boundaries (Pons *et al*., 2006).

When molecular approaches are used to supplement morphological taxonomic investigations, the nuclear ribosomal Internal Transcribed Spacer (ITS) and mitochondrial cytochrome oxidase 1 gene variation (COI) regions are often used (Wagener *et al*., 2006; Coleman, 2009; Wang and Qiao, 2009; Navajas and Navia, 2010; Pentanovic, 2016).

Mitochondrial analysis

For study of closely related species, mitochondrial genes provide excellent markers for study (Chan *et al*., 2021). This is because mitochondria lack introns, have limited exposure to recombination, have a haploid mode of inheritance, and there are robust primers to enable routine recovery of specific segments (Folmer *et al.*, 1994; Zhang and Hewitt, 1997). Moreover, the 13 protein-coding genes in the mitochondrial genomes are rarely susceptible to indels that might lead to frameshifts (Hebert *et al.*, 2003, Matsuda *et al.*, 2013).

In this study, the mitochondrial cytochrome oxidase I (COI) gene was chosen as a marker. COI possesses a greater range of phylogenetic signal than any other mitochondrial gene (Saccone *et al*., 2009). Its 3rd position nucleotides show a high incidence of base substitution, leading to a rate of molecular evolution that is about three times greater than that of 12S and 16S rDNA. Its evolution is rapid enough to allow the discrimination of closely allied species and phylogenetic groups within a single species (Cox and Hebert, 2001; Wares and Cunningham, 2001), making it more likely to provide deeper phylogenetic insights than alternatives such as cytochrome b. COI markers generally generate a greater resolution of relationships between closely related genetic groups. For instance, this was evident in the phylogenetic analysis of the tick *Rhipicephalis microplus* (Ixodida: Ixodidae) complex, where COI showed stronger support for monophyly of the complex than ITS2, 12S rRNA, and 16S rRNA (Burger *et al*., 2014). Moreover, the COI results confirmed all 13 of the known species and further revealed that two of the known species could contain cryptic species as they did not cluster into monophyletic clades.

DNA-based eriophyoid species identification

The boundaries between what constitutes intraspecific and interspecific distances depend on the taxonomic group studied. In animal studies, the reported intraspecific p-distances are rarely over 2%, and the majority are less than 1% (Avise, 2000). COI divergence in arthropod

studies was estimated to be between 0.1% and 3.6% intraspecific p-distances and between 2.3% and 29.9% interspecific p-distances (Skoracka, 2009). The first eriophyoid mite study (*Abacarus hystrix,* Trombiformes: Eriophyidae*)* that examined COI p-distances reported an average intergroup p-distance of 22.6%, while p-distances within each group (including the newly identified cryptic species) were on average 0.32 % (Skoracka, 2009). There have been several other studies that investigated the genetic diversity by estimating COI p-distances in eriophyoid mites. For instance, Lewandowski *et al*. (2014) reported intraspecific p-distances between 0.1% and 11.9% and interspecific p-distances between 17.3% and 28.6% in all *Trisetacus* species (Trombidiformes: Phytophtidae) sequence pairs analysed. A study on *Metaculus* (Trombidiformes: Eriophyidae) species by Vidovic *et al.* (2015) reported intraspecific p-distances between 0.0% to 0.7%, and interspecific p-distances between 12.1% and 22.6%. Lastly, in a more recent study on the COI divergence of *Diptilomiopus* mites (Trombidiformes: Diptilomiopidae), intraspecific p-distances between 0.2% and 13%, and interspecific p-distances between 25.3% and 41.3% were reported (Liu *et al*., 2019). The mentioned studies give an indication of established intra- and interspecific p-distance boundaries that can guide future eriophyoid mite studies.

AIM OF THIS STUDY

This study aimed to gain insights into the diversity of grapevine *Colomerus* mites using an integrative taxonomic approach. This approach comprised:

1. Molecular taxonomy:
    1. Next Generation Sequencing of the mitogenome
    2. Sanger sequencing
    3. Multiplex PCR screening of the COI region
2. Morphological taxonomy:
    1. Cryo-Scanning Electron Microscopy

The study was divided into two main chapters: the first chapter explored the COI gene diversity of the grapevine *Colomerus* species, while the second chapter looked at the morphological differences within grapevine *Colomerus* species.

CHAPTER 2: GENETIC DIVERSITY OF *C. VITIS*

INTRODUCTION

Grapevines are frequently moved between countries, with pests often being a cause of phytosanitary concern and trade limitations. A considerable number of pests affect grapevines. *Calepimitrimerus vitis*, *Colomerus vitis,* and *Colomerus oculivitis* are eriophyoid mites that affect grapevines. Eriophyoids are usually highly host-specific, with the majority being described from only one host species (Oldfield, 1996). Eriophyoid mites presumed to be *C. vitis* and *C. oculivitis* cause frequent damage in vineyards worldwide (Halawa *et al.*, 2015). Of importance to this study is *C. vitis* because of its geographic distribution and economically damaging effects.

Colomerus vitis is currently classified into three strains according to the symptoms that they cause: bud mite, erineum mite, and leaf curl mite (Smith and Stafford, 1948). The damages caused include adverse effects on plant growth, yield and quality loss and transmission of plant pathogens (Valenzano *et al.*, 2020). Grapevine bud and blister mites have been demonstrated to transmit Grapevine Pinot Gris Virus (GPGV) between grapevines (Pearce and Australia, 2019). Grapevine mites are minute and often hide in the host plant (e.g., buds and sheaths) which in turn makes them hard to detect (Lindquist *et al.*, 1996). Their resistance to pesticides increases their economic importance as they become more challenging to control (Navajas and Navia, 2010).

Grapevine mites have limited characters used for their morphological distinction. The strains are hardly distinguishable by morphological characters (Craemer and Saccaggi, 2013). Consequently, previous studies that looked at the morphology of *C. vitis* found in different plant tissues and symptoms (bud, curl, and blister mites) categorised them as conspecific (e.g. Smith and Stafford, 1948). However, Carew *et al*. (2004) analysed the ribosomal Internal Transcribed Spacer 1 (ITS1) and microsatellites and reported that bud and blister mites were potentially different species that were closely related. Contrary to the findings of Carew *et al.* (2004), a recent study reported no differences in ITS1 sequences between *C. vitis* individuals collected from the buds and blisters (Valenzano *et al.*, 2020).

The disaccord about the species status of *C. vitis* shows the need for extensive taxonomic studies that integrate multiple taxonomic approaches. Supplementing traditional taxonomy with a DNA-based systems may counteract some of the problems identified with the former. Traditionally, taxonomy of a group is based on morphology and DNA information is fitted into the system of already defined taxonomic groups (Pons *et al.*, 2006). The traditional taxonomic approach is usually limited as it does not account for the presence of cryptic species and gene histories that contribute to species recognition (Pons *et al*., 2006). Additionally, the traditional taxonomic approach disregards that genetic differentiation is not always accompanied by morphological differences (Hu *et al.*, 2019). In contrast, the DNA based system may consider the uncertainty of species limits by permitting confidence intervals when allocating species-defined nodes (Hebert *et al*., 2003), and branch length methods do not require the definition of populations before a test of aggregation can be performed (Davis and Nixon, 1992).

The current study developed a DNA-based identification system for *C. vitis* for four main reasons. Firstly, *C. vitis* individual mites are microscopic and have limited morphological characters to study diversity and consequently present challenges for morphological identification. Secondly, because of the challenges posed by the lack of consensus regarding the diversity and species status of *C. vitis*. Thirdly, efficient and accurate identification is vital for its control because of the economic importance of grapevines. Lastly, a robust barcoding pipeline for *C. vitis* could be adapted for commercial use in routine diagnostics and optimise control and management.

This chapter aimed to show the genetic diversity of grapevine *Colomerus* species presumed to be *C. vitis* using a marker-assisted approach and analysis of mitogenome sequences. Sanger sequencing and multiplex PCR of the COI barcoding region and NGS were used to investigate the genetic variations and associations of the genetic groups of the grapevine *Colomerus vitis* ‘strains’.

MATERIALS AND METHODS

This study builds on an initial dataset of mitochondrial cytochrome c oxidase subunit I (~710 bp) sequences (DNA barcodes) generated at the Department of Agriculture, Forestry and Fisheries (DAFF) and supplied by Dr D. Saccaggi. The sequence dataset consisted of grapevine eriophyoid mites collected from the Agricultural Research Council Nietvoorbij farm (-33.915, 18.861), the DAFF plant quarantine station in Stellenbosch (-33.946, 18.827), and from grape and grapevine imports from various countries between 2011 and 2015.

Eriophyoid mites were manually collected from fresh grapes, leaves, dormant grapevine cuttings and bud sticks, locally grown or imported from Egypt, Spain, Israel, and the United States of America (Saccaggi *et al*., 2012; Craemer and Saccaggi, 2013). Additional samples were taken during the course of this study. Full details on sample collection, Sanger sequencing, and multiplex PCR procedures are provided below.

Grapevine plant material collection from the field

Grapevine leaves and buds showing symptoms of mite infestation (leaf galls, leaf curl, and stunted growth or bud death) were collected at four farms in the Western Cape province and one farm in the Northern Cape province of South Africa on several dates between 2011 and 2013, as well as between 2018 and 2021 (Figure 2.1; Figure 2.2. A, B). Plant material collected from different grapevine blocks was stored in separate Ziploc bags to avoid cross-contamination. In addition, different plant tissues (leaves and buds) from the same grapevine were also collected in separate Ziploc bags. The plant samples were kept in a cooled Styrofoam box during fieldwork and transferred to a 4°C refrigerator upon arrival at the laboratory until subsequent analyses.

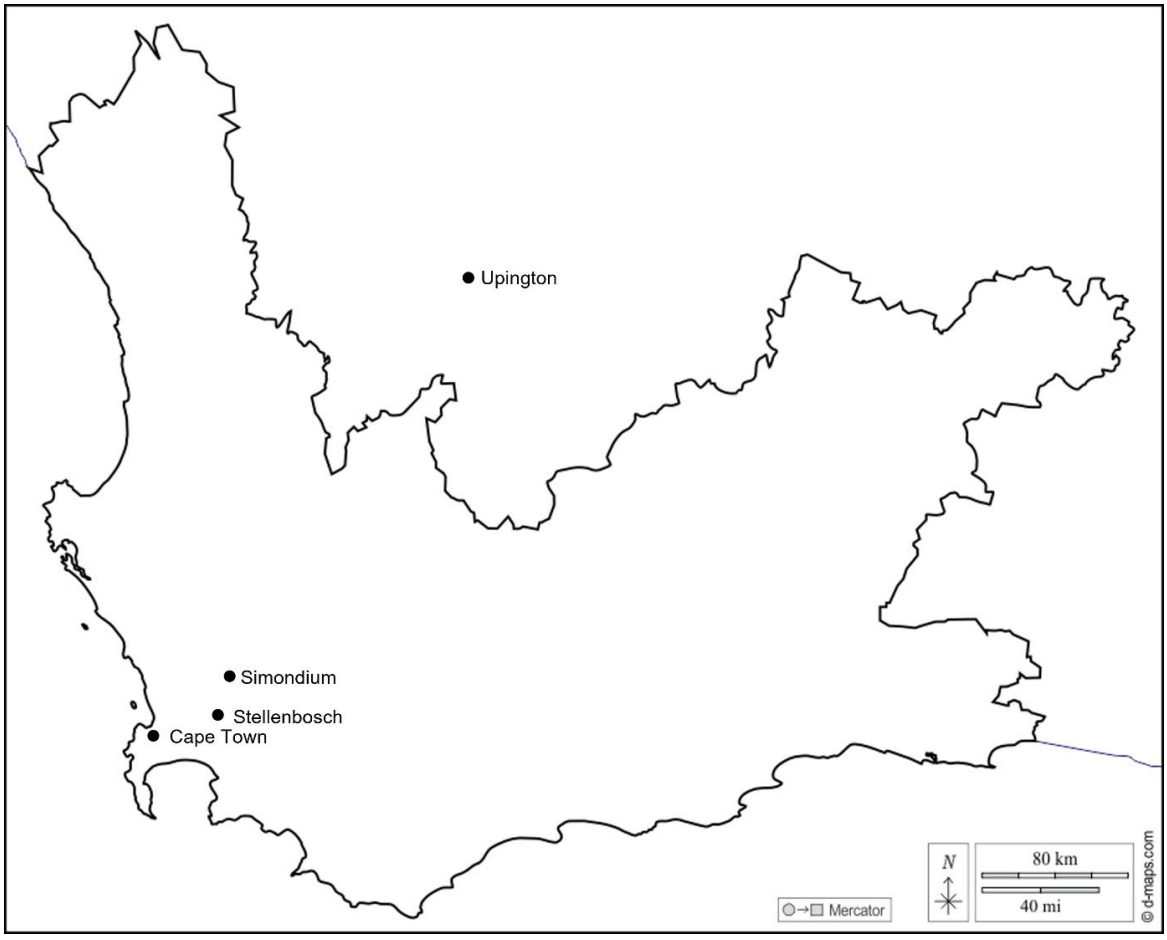


Figure 2.1. Western Cape map illustrating sampling sites visited during sample collection. Samples were collected at a total of five farms, two of the farms were in Stellenbosch and one vineyard in Simondium, Cape Town, and Upington.

Figure 2.2 A. Bud damage indicating bud mite infestation and bud mites captured through a stereomicroscope (Craemer *et al*., 2012).

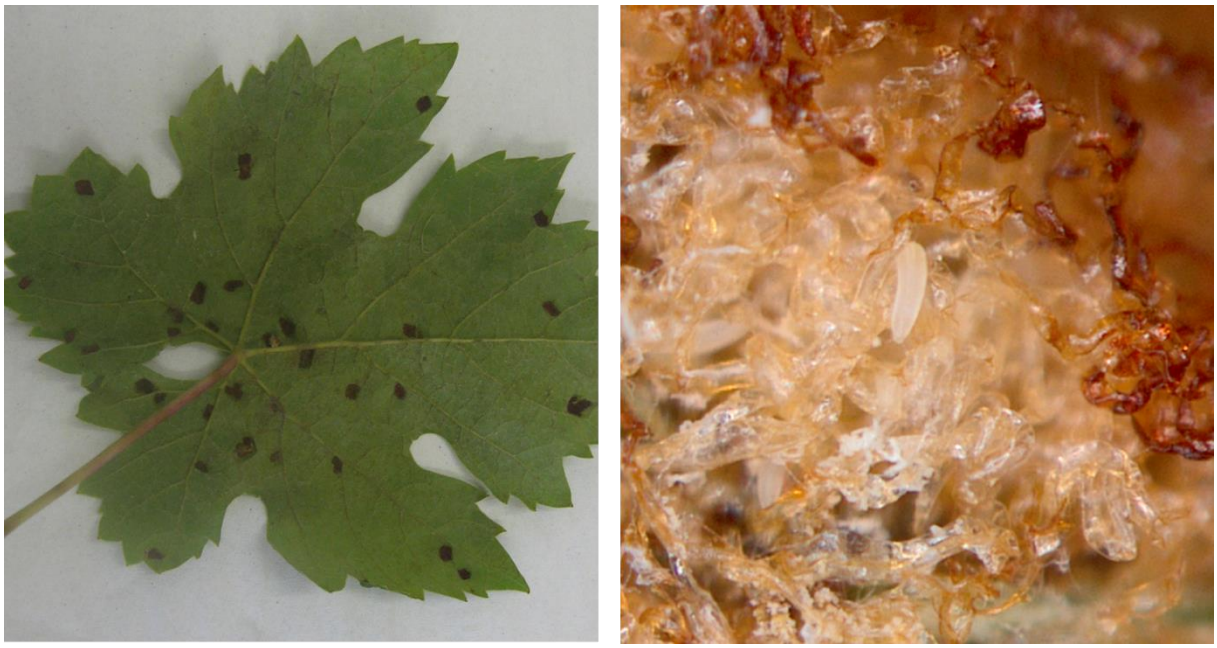


Figure 2.2 B. Damage on the underside of the leaf indicating blister mite infestation and blister mites captured through a stereomicroscope (Craemer *et al*., 2012).

Eriophyoid mite retrieval from plant material for multiplex PCR analyses

The presence of eriophyoid mites was confirmed on plant material through a NexiusZoom 1902-PG stereomicroscope at 30 X magnification (SMM, Cape Town). Following positive detection, the region of the plant material with mite infestation was excised using a sterile blade and transferred directly to a PCR Eppendorf tube (0.2 ml) with 100 µl absolute ethanol and stored at -20 °C until further processing. Next, mites were separated from plant material by a tailored method that uses centrifugation and filtration through a 9.5 mm steel screen. The steel screen filters the ethanol and mites while retaining the plant material. The filtered ethanol solutions containing the mites were then evaporated at 45 °C on a heat block until the mite pellet was dry.

DNA extraction

DNA was extracted from the dry mite pellet using the Qiagen QIAamp® DNA Micro Kit (QIAGEN, Stanford, CA, USA), following the Isolation of Genomic DNA from Tissues protocol. The protocol was adapted to half volumes of all reagents because of the microscopic size of eriophyoid mites. DNA extract purity (260/230 nm and 260/280 nm ratios) and concentration (ng/µl) were estimated using a NanoDrop™ ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

Polymerase Chain Reaction (PCR) and Sanger sequencing

Initially, the standard cytochrome c oxidase subunit I (COI) barcoding region (~710 bp of the 5' end of the gene) was amplified using the universal primer pair (LCO1490 and HC02198) (Folmer *et al*., 1994) (Table 2.1). PCR amplifications were conducted in total volumes of 5 µl, comprising of 2.5 µl of Qiagen Multiplex PCR kit (QIAGEN), 1 µl of DNA template, 0.5 µM of each primer, and 1.2 µl MilliQ H_2O. Thermocycling conditions consisted of initial denaturation at 95 °C for 15 min; 35 cycles at 94 °C for 30 s, 54 °C for 90 s, and 72 °C for 90 s; and a final extension at 72 °C for 10 min. PCR products were sequenced in the forward direction or bidirectional when the forward sequence resulted in poor quality. For all sequencing reactions, the BigDye Terminator v3.1 Cycle Sequencing Kit, following the manufacturer's recommendations, was used (Applied Biosystems) at the Central Analytical Facilities of the University of Stellenbosch, South Africa. Subsequently, group-specific primers (G1, G2, G3, G4, and G5 were designed

using sequences generated by the mentioned universal primers (Table 2.1). Subsequent PCR amplifications were carried out using the group-specific primers. DNA extracts of confirmed genetic groups were sequenced bidirectionally, using the group-specific primers to verify that the primers were amplifying the intended region. Subsequently, potential primer interactions (self-dimers and cross primer-dimers) were analysed on the Thermo Fisher multiple primer analyser. From the primer interactions assessment, it was evident that there was a need to separate the multiplex PCR into two separate sets of primers. The first set of primers consisted of G1, G2, and G3 primer pairs, and the second set consisted of G4 and G5 primer pairs. The multiplexes were divided into two reactions comprising species-specific primers to screen for the genetic groups' presence. All sequences were curated by excluding low-quality sequences and manually resolving ambiguities in good quality sequences.

Table 2.1. List of primers used for singleplex PCR, Sanger sequencing (LC01490 and HC02198, Folmer *et al*., 1994), and multiplex PCR of the COI region in eriophyoid grapevine mites presumed to be *Colomerus vitis*.

Primer name	**Nucleotide sequence**	**Sequence length (bp)**	**Type of primers**
LCO1490	GGTCAACAAATCATAAAGATATTGG	710	Universal (singleplex)
HC02198	TAAACTTCAGGGTGACCAAAAAATCA		
G1-F	GAATAGAATTATCGCAGACAGG	433	Species specific (multiplex)
G1-R	CTAACACGATCTATACAAGAC		
G2-F	GCGTCTAGAACTATCTCAAAC	162	
G2-R	TCATTACAACCAAGCATCAATG		
G3-F	ACGAATGGAATTGTCTCATAC	252	
G3-R	CCAGAAGCTAACAAAGAGGA		
G4-F	GAGGATTTGGGAATTGATTAATC	241	
G4-R	GAAACACCTCCTAAATGAAGAG		

G5-F	GAAATTGATTAATTCCATTGATGC	306	
G5-R	CACGATCCATAATAGCTCCTC		

Genetic divergence and genetic clustering

Estimation of intragroup and intergroup p-distances and clustering analyses were performed using a final dataset that included four types of sequences (1) sequences recovered using the universal invertebrate barcoding primers (LCO1490 and HCO2198, Folmer *et al*., 1994), (2) sequences obtained with the group-specific primers used in the multiplex, (3) sequences recovered by mapping of new NGS data to COI sequences (see the section below), and (4) COI sequences of *Colomerus vitis* retrieved from GenBank (Table 2.2). Multiple sequence alignments were performed using the MAFFT v7.450 algorithm (Katoh *et al.*, 2002; Katoh and Standley, 2013) in Geneious Prime® 2020.1.2 (Kearse *et al.*, 2012; Prime, 2012). A maximum likelihood tree was constructed in IQ-Tree (Minh *et al*., 2020). The best partitioning scheme was based on the edge-linked greedy strategy (Lanfear *et al*., 2012) making use of automatic model selection (Chernomor *et al*., 2016; Kalyaanamoorthy *et al*., 2017). Nodal support was investigated using 1000 replicates for ultrafast bootstraps and SH-aLRT branch tests (Guindon *et al*., 2010; Hoang *et al*., 2018). Intragroup and intergroup pairwise distances (p-distances) were determined on MEGAX (Kumar *et al.*, 2018) using the Kimura-2-parameter (K2P) model (Kimura, 1980) with pairwise deletion of sites and bootstrap support test of 1000 replicates.

Multiplex PCR, capillary electrophoresis, and fragment analyses

Multiplex PCR uses multiple primer pairs to simultaneously amplify different DNA regions (Elnifro *et al.*, 2000). The technique is beneficial when the sample is likely to contain DNA from different sources. This is the case for grapevine material infested with eriophyoid mites, as observed in the initial Sanger sequencing using universal barcoding primers (LCO1490 and HCO2198, Folmer *et al.,* 1994) (Figure. 2.3). The initial COI sequence data allowed for the design of primers specific to the five identified genetic groups of *C. vitis* (G1, G2, G3, G4, and G5). The primers could amplify fragment sizes between 162 bp and 433 bp (Figure. 2.4). The short fragments increase the PCR efficiency since grapevine eriophyoid mites are microscopic, and DNA concentrations recovered tend to be low (X = 8.8 ng/µl) (Powell *et al.*, 2020). Additionally, shorter target sequences also increase the chances of amplifying multiple fragments simultaneously (Powell *et al.*, 2020). Moreover, target fragment sizes were spaced out in terms of size by at least 40 bp to avoid uncertainty in cases of co-amplification. Importantly, primers were optimised to work at the same annealing temperature (54 °C) during multiplex PCR. Fragments obtained from group-specific primers were bi-directionally sequenced to validate that intended sequences had been amplified.

Following that, grapevine mite DNA samples were screened for the presence of the identified genetic groups using the multiplex PCR protocol. The forward primers were labelled at the 5'-end using the 6-FAM™†† dye (Inqaba Biotech™, Pretoria, South Africa) followed by automatic fragment size detection by capillary electrophoresis. Since genetic groups' fragments were well separated in size, one dye was adequate to distinguish between the four groups.

Table 2.2. List of COI sequence samples used to construct the ML trees. The sample list includes samples generated at DAFF, samples generated in the present study, and samples retrieved from GenBank. The list also includes information about the symptom, plant part, collection site, date, and primers used for PCR and sequencing reactions.

Sequence name / GenBank reference number	Symptom	Plant part	Origin	Collection date	PCR primers	Sequencing primers	Sample source
06-05A_Folmer-F_E11_14	Leaf galls	Leaf	DAFF, Stellenbosch, SA	2011/07/21	Folmer	LCO1490	DAFF
07-05C_Folmer-F_G11_20	Leaf galls	Leaf	DAFF, Stellenbosch, SA	2011/07/29	Folmer	LCO1490	
07-05D_Folmer-F_H11_23	Leaf galls	Leaf	DAFF, Stellenbosch, SA	2011/09/11	Folmer	LCO1490	
10_04 B_Folmer REV_H03_24	-	-	-	2011/09/14	Folmer	HC02198	
192_Fol Fwd_C11_08	-	Fresh grapes	Egypt	2011/09/15	Folmer	LCO1490	
2_Grape-Israel_2_Folmer-F_E05_14	-	Fresh grapes	Israel	2011/09/15	Folmer	LCO1490	
204-LTC	Bud mite	Shoot	Nietvoorbij, Stellenbosch, SA	2011/09/21	Linked Target Capture	Linked Target Capture	
236_Fol Fwd_D11_11	-	Fresh grapes	Egypt	2011/09/26	Folmer	LCO1490	
237_Fol Fwd_E11_14	-	Fresh grapes	Egypt	2011/09/27	Folmer	LCO1490	
3 21_4_C_vitis_Folmer fwd_G05_20	Leaf galls	Leaf	DAFF, Stellenbosch, SA	2011/10/01	Folmer	LCO1490	
36-LTC	Bud mite	Shoot	Nietvoorbij, Stellenbosch, SA	2011/10/17	Linked Target Capture	Linked Target Capture	
4_Grape-Egypt_2_Folmer-F_G05_20	-	Fresh grapes	Egypt	2011/10/17	Folmer	LCO1490	

Sequence name / GenBank reference number	Symptom	Plant part	Origin	Collection date	PCR primers	Sequencing primers	Sample source
444-LTC	Leaf galls	Leaf	Nietvoorbij, Stellenbosch, SA	2012/02/15	Linked Target Capture	Linked Target Capture	
451_Fol Fwd_G11_20	-	Fresh red seeded grapes	Spain	2012/02/16	Folmer	LCO1490	
465_Fol Fwd_H11_23	-	Fresh red grapes	Egypt	2012/02/16	Folmer	LCO1490	
5_Grape-Spain_1_Folmer-F_H05_23	-	Fresh grapes	Spain	2012/02/17	Folmer	LCO1490	
526_Fol Fwd_B12_06	-	Fresh grapes	Spain	2012/03/01	Folmer	LCO1490	
543_Fol Fwd_D12_12	-	Fresh grapes	Spain	2012/07/27	Folmer	LCO1490	
6_Grape-Spain_2_Folmer-F_A06_03	-	Fresh grapes	Spain	2012/08/28	Folmer	LCO1490	
7_Grape-USA_1_Folmer-F_B06_06		Budstick/canes	USA	2012/08/31	Folmer	LCO1490	
747_Fol Fwd_E12_15	leaf curl	Leaf	Bon Valleh farm, Robertson, SA	2013/01/07	Folmer	LCO1490	
8_Grape-USA_2_Folmer-F_C06_09	-	Budstick/canes	USA	2013/01/31	Folmer	LCO1490	
880_Fol Fwd_F12_18	-	Fresh grapes (red globe)	SA	2013/01/31	Folmer	LCO1490	
894_Fol Fwd_G12_21	-	Budstick imports	USA	2013/01/31	Folmer	LCO1490	
896_Fol Fwd_H12_24	-	Budstick imports	USA	2013/01/31	Folmer	LCO1490	
917_Fol Fwd_A01_01	-	Grapes- big red berry	Egypt	2013/02/05	Folmer	LCO1490	

Sequence name / GenBank reference number	Symptom	Plant part	Origin	Collection date	PCR primers	Sequencing primers	Sample source
93-LTC	Leaf galls	Leaf	Nietvoorbij, Stellenbosch, SA	2013/02/08	Linked Target Capture	Linked Target Capture	
D2_Folmer-F_B09_06	-	-	-	2013/03/05	Folmer	LCO1490	
D3_Folmer-F_C09_09	-	-	-	2013/03/05	Folmer	LCO1490	
MN164416	Blister	Leaf	Iran	2013/03/05	Folmer	LCO1490	GenBank
MN164417	Blister	Leaf	Iran	2013/03/05	Folmer	LCO1490	
MN164418	Blister	Leaf	Iran	2013/03/05	Folmer	LCO1490	
MN164419	Blister	Leaf	Iran	2013/03/05	Folmer	LCO1490	
MN164420	Blister	Leaf	Iran	2013/03/12	Folmer	LCO1490	
MN164421	Blister	Leaf	Iran	2013/03/12	Folmer	LCO1490	
MN164422	Blister	Leaf	Iran	2013/03/12	Folmer	LCO1491	
MN164423	Blister	Leaf	Iran	2013/03/12	Folmer	LCO1492	
MN164424	Blister	Leaf	Iran	2013/05/09	Folmer	LCO1493	
MN164425	Blister	Leaf	Iran	2013/05/09	Folmer	LCO1490	
MN164426	Blister	Leaf	Iran	2015/05/01	Folmer	LCO1490	
MN164427	Blister	Leaf	Iran	2015/05/01	Folmer	LCO1490	

Sequence name / GenBank reference number	Symptom	Plant part	Origin	Collection date	PCR primers	Sequencing primers	Sample source
NGS_106	Leaf curl	Leaf	DAFF, Stellenbosch, SA	2015/05/01	-	-	Current Study
NGS_224	Bud mite	Bud	DAFF, Stellenbosch, SA	2020/02/10	-	-	
NGS_Nietvoorbij01-02	Bud mite and leaf blister	Leaf and bud	Nietvoorbij, Stellenbosch, SA	2020/02/10	-	-	
PC1_Fol_R_29jul2011	Leaf galls	Dormant grapevine cuttings	-	2020/02/10	Folmer	HC02198	
PC3_Fol_R_29jul2011	Leaf galls	Dormant grapevine cuttings	DAFF, Stellenbosch, SA	2020/02/10	Folmer	HC02199	
T20_Fol_R_08mar2012	Leaf galls	Leaf	DAFF, Stellenbosch, SA	2020/02/10	Folmer	HC02198	
G1 ref - 224 assembly consensus sequence	Bud mite	Bud	DAFF, Stellenbosch, SA	2020/02/10	-	-	
G2 ref - 106 assembly consensus sequence (reversed)	Leaf curl	Leaf	DAFF, Stellenbosch, SA	2020/02/10	-	-	
G2 ref - 204 assembly consensus sequence (reversed)	Bud mite	Bud	Nietvoorbij, Stellenbosch, SA	2020/02/10	-	-	
G2 ref - Nietvoorbij01-02 assembly consensus sequence (reversed)	Bud mite and leaf blister	Bud and leaf	Nietvoorbij, Stellenbosch, SA	2020/09/30	-	-	
G3 ref - 204 assembly consensus sequence	Bud mite	Bud	Nietvoorbij, Stellenbosch, SA	2020/09/30	-	-	
G3 ref - Nietvoorbij01-02 assembly consensus sequence	Bud mite and leaf blister	Bud and leaf	Nietvoorbij, Stellenbosch, SA	2020/09/30	-	-	
G4 896 ref - 224 assembly consensus sequence	Bud mite	Bud	DAFF, Stellenbosch, SA	2020/09/30	-	-	

Sequence name / GenBank reference number	Symptom	Plant part	Origin	Collection date	PCR primers	Sequencing primers	Sample source
G4 896 ref - Nietvoorbij01-02 assembly consensus sequence (reversed)	Bud mite and leaf blister	Bud and leaf	Nietvoorbij, Stellenbosch, SA	2020/09/30	-	-	
G4 USA ref - Nietvoorbij01-02 assembly consensus sequence	Bud mite and leaf blister	Bud and leaf	Nietvoorbij, Stellenbosch, SA	2020/09/30	-	-	
G5 ref - 106 assembly consensus sequence	Leaf curl	Leaf	DAFF, Stellenbosch, SA	2020/09/30	-	-	
G5 ref - 204 assembly consensus sequence	Bud mite	Bud	Nietvoorbij, Stellenbosch, SA	2020/09/30	-	-	
G5 ref - Nietvoorbij01-02 assembly consensus sequence	Bud mite and leaf blister	Bud and leaf	Nietvoorbij, Stellenbosch, SA	2020/09/30	-	-	
V2 ref - 106 assembly consensus sequence	Leaf curl	Leaf	DAFF, Stellenbosch, SA	2020/09/30	-	-	
V2 ref - 204 assembly consensus sequence	Bud mite	Bud	Nietvoorbij, Stellenbosch, SA	2020/09/30	-	-	
V2 ref - Nietvoorbij01-02 assembly consensus sequence	Bud mite and leaf Blister	Bud and leaf	Nietvoorbij, Stellenbosch, SA	2020/09/30	-	-	
V3 ref - 204 assembly consensus sequence	Bud mite	Bud	Nietvoorbij, Stellenbosch, SA	-	-	-	
V3 ref - 224 assembly consensus sequence	Bud mite	Bud	DAFF, Stellenbosch, SA	-	-	-	
V3 ref - Nietvoorbij01-02 assembly consensus sequence	Bud mite and leaf blister	Bud and leaf	Nietvoorbij, Stellenbosch, SA	-	-	-	

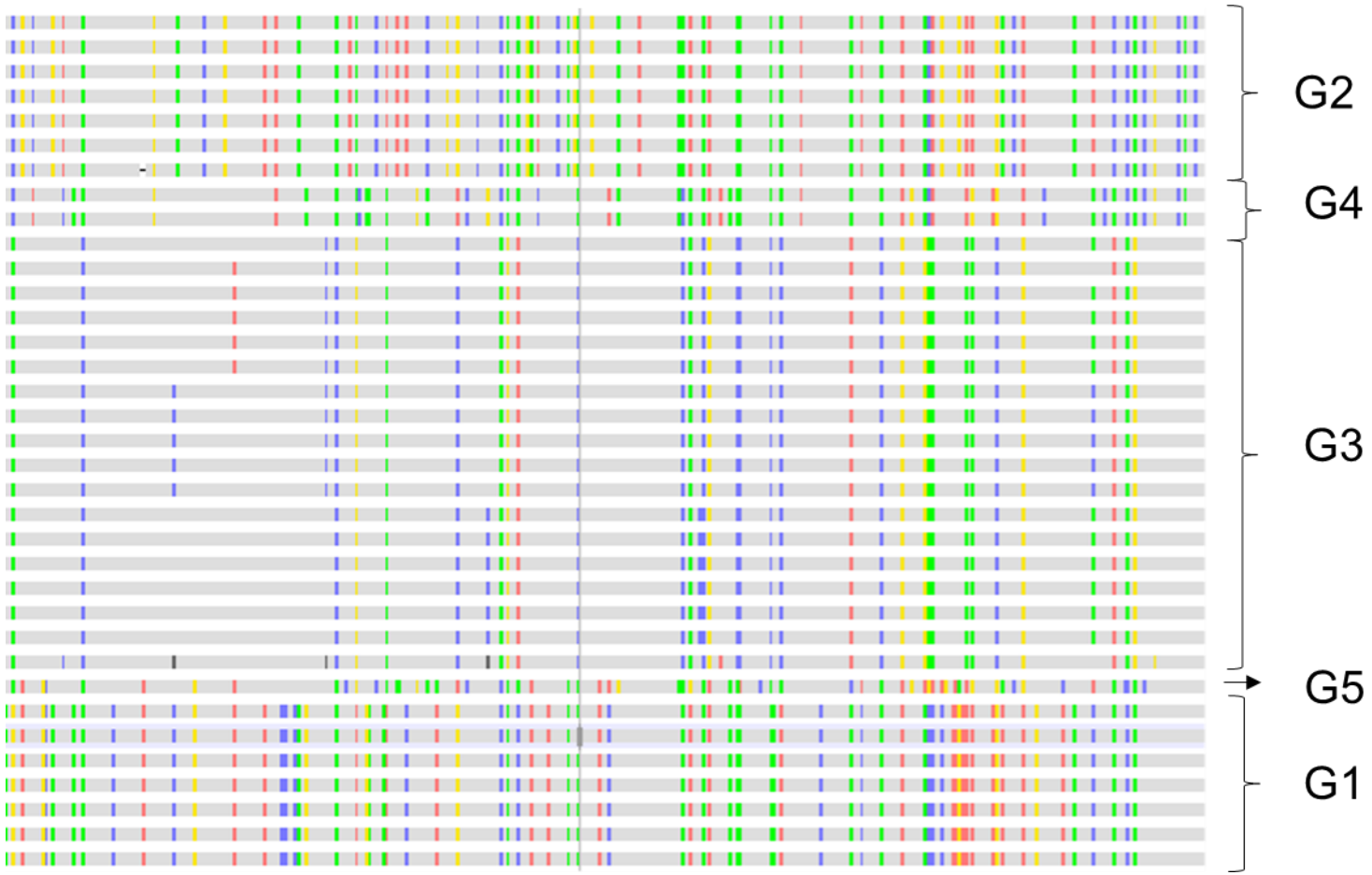


Figure 2.3. Grapevine eriophyoid mites' initial COI sequence alignment using universal barcoding primers. Estimate of nucleotide diversity was depicted by highlighted disagreements.

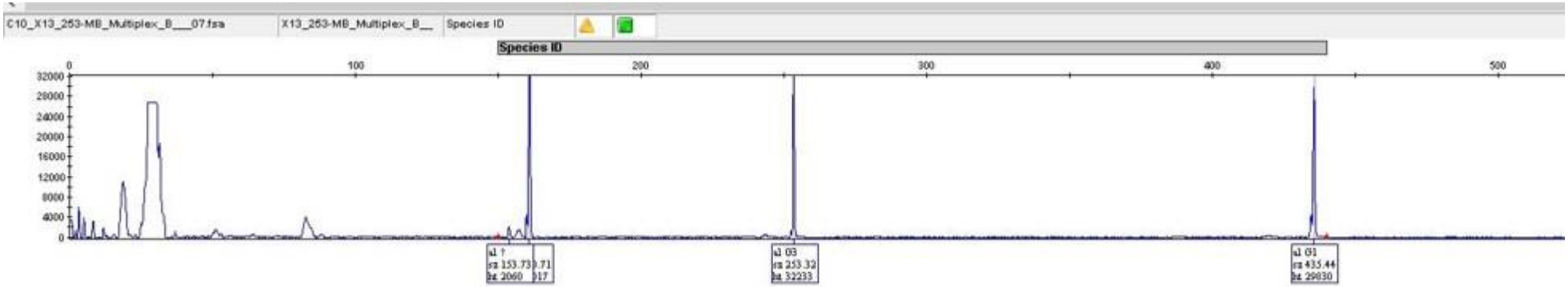


Figure 2.4. Expected amplicon sizes from amplification by group specific primers (G1, G2, G3).

Multiplex PCR amplifications were performed in total volumes of 5 µl comprising 2.5 µl (1X) Qiagen kit, 1 µM primer mix, 0.5 µl DNA template, and 1 µl MilliQ H_2O. The thermocycling conditions were as follows: initial denaturation of 95 °C for 15 min; 27 cycles at 94 °C for 30 s, 54 °C for 90 s, 72 °C for 90 s; and final denaturation at 72 °C for 10 min. Capillary electrophoresis made use of LIZ600 as an internal size standard at the Central Analytical

Facilities of Stellenbosch University. The data were analysed using GeneMapper Software Version 4.0 (Applied Biosystems). A project folder was created for routine screening of the presence of genetic groups on GeneMapper. The folder had defined bins based on the expected fragment size of each genetic group. The bins acted as a fragment size guide for easier identification of the specific genetic groups detected.

Next-Generation Sequencing (NGS)

Four eriophyoid mite DNA samples were sequenced (two from bud mites, one mix of blister and bud mites, and one from leaf curl mites: Table 2.2) using the Ion Torrent™ S5™ platform. Firstly, the DNA was assessed using a NanoDrop™ ND-1000 spectrophotometer, and low Tris-EDTA buffer (ThermoFisher Scientific) to assess DNA quality and purity, respectively. Secondly, library preparation was performed using Ion Xpress™ gDNA Fragment Library Kit (ThermoFisher Scientific) according to the manufacturer's protocol (MAN0009847, REV J.0). Subsequently, DNA was purified and end-repaired using Agencourt™ AMPure™ XP reagent (Beckman Coulter, Brea, CA, USA) and ligated on the blunt-end of IonCode™ Barcode Adapters using Agencourt™ AMPure™ XP reagent (Beckman Coulter). The fragments in the library were then quantified using Library TaqMan™ Quantitation Kit according to the manufacturer's protocol (MAN0015802, Rev. B.0). The libraries were diluted to 60 pM and combined in equal concentrations for sequencing template preparation using 540™ Chef Kit (ThermoFisher Scientific). Massively parallel sequencing was performed on Ion Torrent S5™ as described in the MAN0010851 REVF.0. protocol. The Torrent Suite Version 5.12 Software was used for flow space calibration and base caller analysis using standard analysis parameters.

RESULTS

The initial COI dataset included highly diverged sequences indicating three distinct genetic groups of eriophyoid mites. Additionally, the sequences showed the simultaneous presence of two or more genetic groups, i.e., DNA mixtures. DNA mixtures are difficult to separate using Sanger sequencing because of the resulting superimposed peaks; however, the initial sequences allowed for the development of group-specific primers for use in multiplex PCR. The multiplex PCR test simultaneously detected the different genetic groups to overcome the difficulty and limitations of Sanger sequencing in analyses of DNA mixtures.

Genetic clustering and divergence of COI sequences

Sanger sequencing using universal primers resulted in a high number of ambiguous nucleotides caused by superimposed peaks. The superimposed peaks suggested the presence of multiple genetic groups in the samples. Consequently, group-specific primers were designed and optimised to produce sequences of single nucleotide peaks in each variable position representative of the genetic groups. Sanger sequencing using group-specific primers resulted in 58 COI sequences, and after manual curation involving exclusion of sub-optimal sequences, 31 remained. The sequence sizes were between 560 – 711 bp. The NJ tree further alluded to the presence of at least 3 genetic groups within the *C. vitis* COI sequences analysed: G1 (n = 7), G2 (n = 7), G3 (n = 18). The maximum pairwise distance of the 34 sequences was 23.97%. The high genetic divergence of the sequences suggests the presence of more than one species within the presumed *C. vitis* COI sequences. Following that, intragroup p-distances of each genetic group were estimated. The maximum pairwise distance within each genetic group were all less than 2%, suggesting that each of the groups were conspecific. Finally, the intergroup p-distances between each group were estimated to be 21.98 % (G1, G2), 22.46 % (G1, G3), and 22.60 % (G2, G3).

A Maximum likelihood (ML) tree was built using COI sequences recovered by Sanger sequencing (n = 34), GenBank (n = 12), and by mapping NGS reads to reference genetic group sequences (n = 18) (Figure 2.5). The ML tree was constructed from an alignment of 584 bp sequences (n = 64). The sequences in the alignment had 350 identical sites (59.9%). The publicly available COI sequences directly submitted on GenBank were from *C. vitis* samples collected in Iran (Jinbo *et al*., 2011). Some of these sequences clustered with G1 (n = 4), some formed a new cluster V2 (n=7), and one was completely different from all other sequences V1 (n =1).

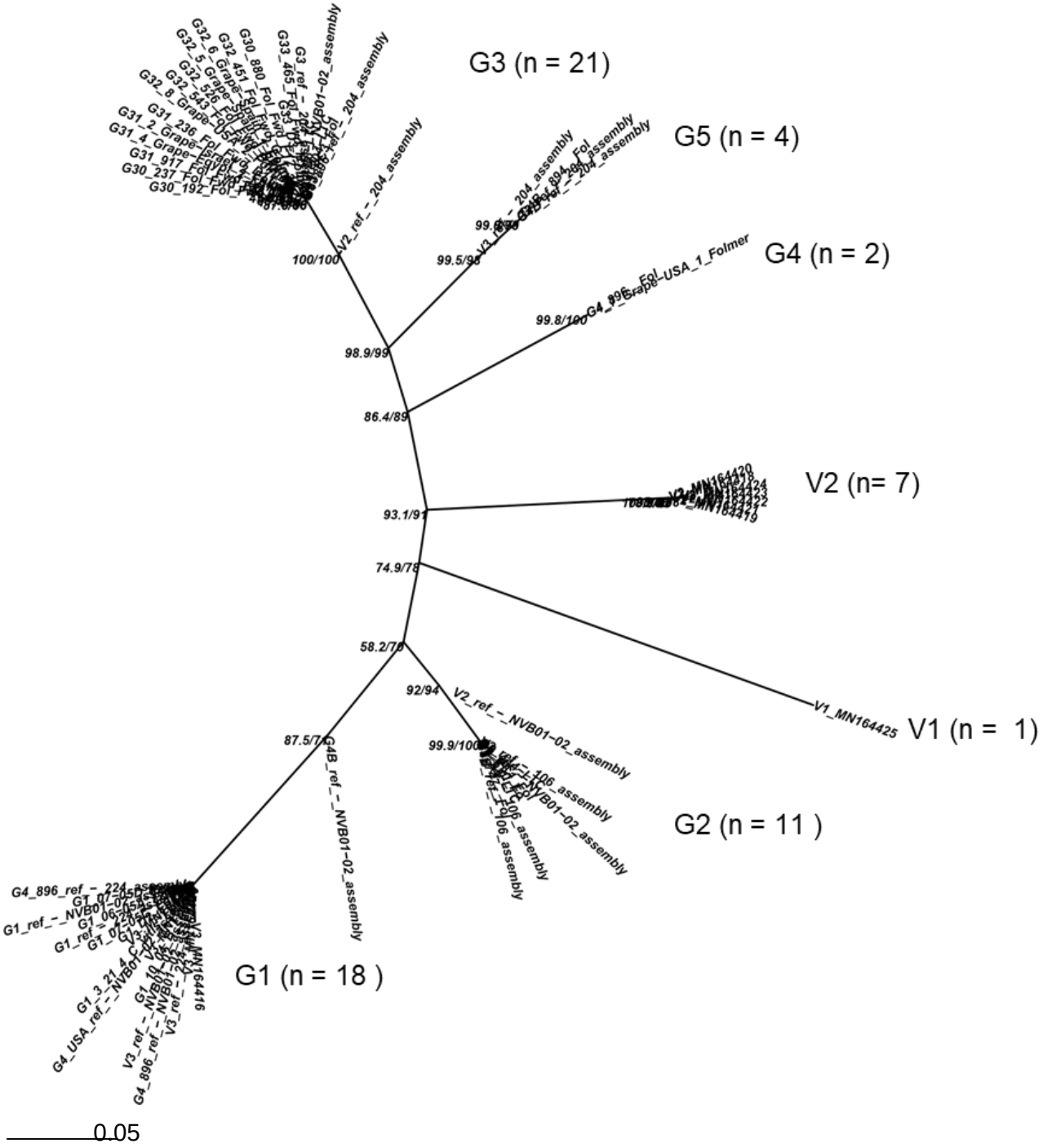


Figure 2.5. Maximum likelihood unrooted tree showing genetic clustering based on the cytochrome c oxidase subunit I (COI) barcoding region of 64 eriophyoid mite sequences (584 bp) presumed to be *Colomerus vitis*. Nodal support was based on 1000 bootstrap replicates.

The G1 cluster consisted of samples from Iran (n = 4) and South Africa (n = 14). While the G2 cluster only had sequences from South Africa (n = 11). The G3 (n = 21) cluster was the most geographically diverse as it consisted of sequences from South Africa (n = 14), Egypt (n = 1), Israel (n = 1), Iran (n = 3), and Spain (n = 2). G4 (n = 2) and G5 (n = 4) sequences were all from USA bud stick imports.

Based on the ML tree sequence clustering, estimates of intraspecific and interspecific p-distances were calculated. The maximum intragroup p-distances ranged between 0% (G2) and 3.51% (G5) with an average of 1.48% (Table. 2.3), indicating that sequences within each cluster were conspecific with the possible exception of G5. In contrast, the intergroup p-distances ranged between 17.2% (G4 – G5) and 25.03% (G5 – V1), indicating highly diverged groups possibly representing different species (Table 2.4).

Table 2.3. Genetic divergence within different groups of eriophyoid grapevine mite sequences presumed to be *Colomerus vitis* (% of maximum intragroup p-distances; K2P) based on a 584 bp alignment of 64 standard COI barcoding sequences.

Grapevine mite groups	**n**	**Max intragroup p-distance (%)**	**S.E.**
Group 1	18	2.44	0.0007
Group 2	11	0.00	0.0027
Group 3	21	1.92	0.0026
Group 4	2	0.00	0
Group 5	4	3.51	0.0037
Group V1	1	na	na
Group V2	7	1.03	0.0019
Group All sequences	64		

Table 2.4. Genetic divergence between different groups of eriophyoid grapevine mite sequences presumed to be *Colomerus vitis* (% of intergroup p-distances; K2P) based on a

584 bp alignment of 64 standard COI barcoding sequences. The table also consists standard errors shown in bold.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group V1	Group V2
Group 1		**0.0221**	**0.0212**	**0.0194**	**0.0205**	**0.0206**	**0.0204**
Group 2	22.41		**0.0226**	**0.0223**	**0.0205**	**0.0237**	**0.0201**
Group 3	23.31	23.5157		**0.0195**	**0.0186**	**0.0224**	**0.0204**
Group 4	18.45	21.92	17.82		**0.0185**	**0.0214**	**0.0196**
Group 5	20.85	21.11	18.29	17.21		**0.0225**	**0.0199**
Group V1	21.56	24.84	23.93	21.95	25.03		**0.0218**
Group V2	20.10	19.48	20.86	19.44	20.70	22.38	

Multiplex PCR of *C. vitis* DNA samples using genetic group-specific primers

The objective of the multiplex PCR was to screen *C. vitis* samples for the presence of the five identified genetic groups at vineyards in the Western Cape of South Africa. The species-specific primers used for the two multiplexes (multiplex 1: G1, G2, and G3; multiplex 2: G4 and G5) amplified fragment sizes between 162 bp – 433 bp (Figure 2.4.).

Multiplex PCR screening for the presence of the five genetic groups was performed on 274 DNA samples (Table 2.5). From the total screened, 188 samples (114 from buds and 74 from leaf blisters) were positive for the presence of at least one of the genetic groups, excluding G4 and G5 which were not successfully amplified (Figure 2.6). Additionally, no amplification was obtained in 86 of the samples, although it is unknown whether this was due to no mites being present in the sample, failure of the PCR, or whether mites were present but did not fall into one of the five targeted genetic groups. The identified genetic groups were often in mixed populations within the same symptomatic tissue.

Table 2.5. List of South African grapevine mite samples screened by multiplex PCR using G1, G2, G3, G4, and G5 primer pairs. The list also includes the symptoms present on plant,

the plant part where it was collected, the origin/location of the farm and the date the mites were collected.

Sample/Job no. (X- number)	**Symptoms**	**Plant part / niche**	**Origin**	**Date**
X13/204	Bud mite	Shoot	Nietvoorbij - Stellenbosch	05/03/2013
X13/226	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	12/03/2013
X13/106	Leaf curl	Shoot	DAFF - Stellenbosch	31/01/2013
X13/163	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	05/02/2013
X13/167	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	06/02/2013
X13/217	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	07/03/2013
X13/224	Bud mite	Shoot	DAFF - Stellenbosch	12/03/2013
X13/226	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	12/03/2013
X13/235	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	14/03/2013
X13/237	Bud mite	Shoot	Nietvoorbij - Stellenbosch	15/03/2013
X13/243 - X13/247 (n = 4)	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	19/03/2013
X13/251	Bud mite	Shoot	Nietvoorbij - Stellenbosch	19/03/2013
X13/253	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	20/03/2013
X13/254	Bud mite	Shoot	Nietvoorbij - Stellenbosch	20/03/2013
X13/255	Bud mite	Shoot	Nietvoorbij - Stellenbosch	20/03/2013
X13/270	Leaf galls	Leaf	DAFF - Stellenbosch	27/03/2013
X13/382 -- X13/387 (n = 5)	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	15/04/2013
X13/403	Leaf galls	Leaf	DAFF - Stellenbosch	22/04/2013
X13/411	Leaf curl	Buds	DAFF - Stellenbosch	17/04/2013
X13/418	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	26/04/2013
X13/420	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	30/04/2013
X13/421	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	30/04/2013
X13/422	Leaf galls	Shoot	Nietvoorbij - Stellenbosch	23/04/2013
X13/427	Bud mite	Leaf	Nietvoorbij - Stellenbosch	23/04/2013
444-204 artificial mix		-	-	-

Sample/Job no. (X- number)	Symptoms	Plant part / niche	Origin	Date
X13/444	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	09/05/2013
X13/452	Leaf galls	Leaf	DAFF - Stellenbosch	15/02/2013
X13/91	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	06/02/2013
BD025 – BD048 (n = 23)	Bud mite	Winter buds	Groot Phesantekraal - Durbanville	2018/09/03
BD154 – BD165	Bud mite	Winter buds	Unknown farm - Upington	2018/09/03
BD177	Bud mite	Winter buds	Unknown farm - Upington	2018/09/03
BD178	Bud mite	Winter buds	Unknown farm - Upington	2018/09/03
LB001 – LB026 (n = 26)	Leaf blister	Leaf	Nietvoorbij - Stellenbosch	2018/03/06
LB028 – LB034 (n = 4)	Leaf blister	Leaf	Plasir de Merle - Simondium	2018/04/24
WBNV001 – WBNV022 (n = 22)	Bud mite	Winter buds	Nietvoorbij - Stellenbosch	2020/08/25
WBNV045 – WBNV064 (n = 19)	Bud mite	Winter buds	Nietvoorbij - Stellenbosch	2020/08/25
NVB009 – NVB032 (n = 23)	Leaf galls	Leaf	Nietvoorbij - Stellenbosch	2020/02/10
LPDM001 – LPDM005 (n = 5)	Leaf curl	Leaf	Plasir de merle - Simondium	22/03/2021
SBSB001 – SBSB002 (n = 10)	Bud mite	Summer bud	Somerbosch - Simondium	22/03/2021
WBPD001 – WBPD005 (n = 5)	Bud mite	Winter buds	Plasir de merle - Simondium	2020/08/25
WBPD031 – WBPD 040 (n = 10)	Bud mite	Winter buds	Plasir de merle - Simondium	2020/08/25
SBB001 – SBB010 (n= 10)	Leaf galls	Leaf	Somerbosch - Stellenbosch	22/03/2021

Sample/Job no. (X- number)	Symptoms	Plant part / niche	Origin	Date
SBB024 – SBB033 (n = 9)	Leaf galls	Leaf	Somerbosch - Stellenbosch	22/03/2021
WBSB001 – WBSB010 (n = 10)	Bud mite	Winter buds	Somerbosch - Stellenbosch	2020/08/25
NVBB011 – NVBB039 (n = 28)	Bud mite	Summer bud	Nietvoorbij - Stellenbosch	27/11/2020
SBSB021 – SBSB036 (n = 15)	Bud mite	Summer bud	Somerbosch - Stellenbosch	25/02/2021

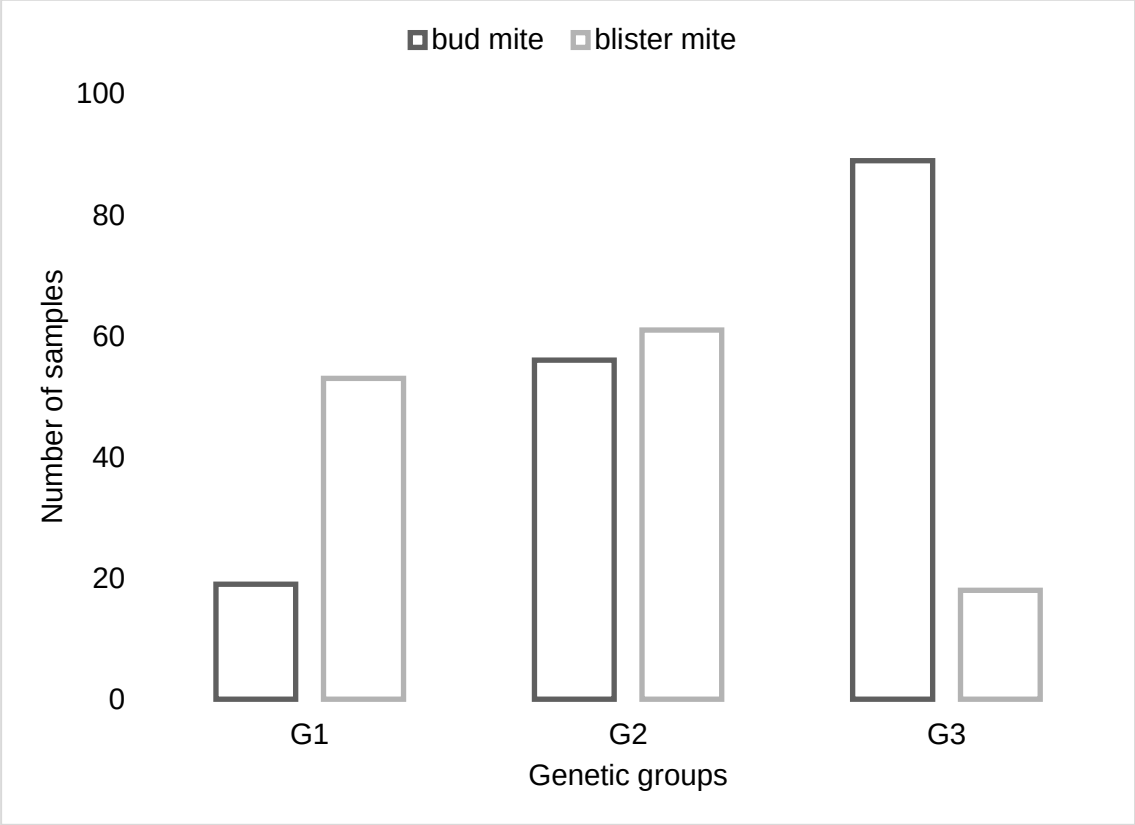


Figure 2.6. Genetic group distribution of all bud and blister mite samples analysed through multiplex PCR regardless of whether it was identified in a mixture or exclusively.

A total of 114 bud mite samples were screened, the majority were identified as G3 (41.23 %), followed by the co-presence of G2 and G3 (27.9%), with the least prevalent mixture being the co-presence of G1 and G2 (2.63%) (Figure 2.7). Of the blister mite samples screened, the majority showed the co-presence of G1 and G2 (44.59%), and the least prevalent grouping being the co-presence of G1 and G3 (1.35%) (Figure 2.7).

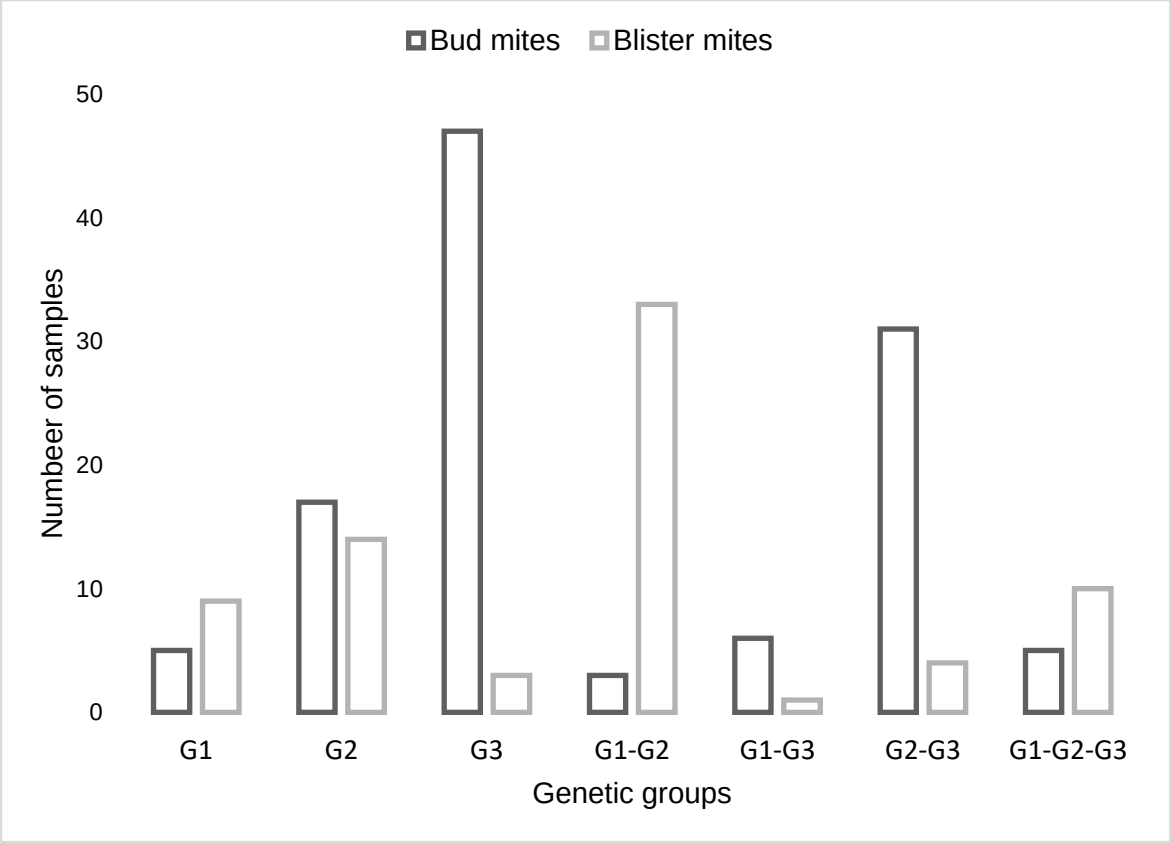


Figure 2.7. Genetic group distribution of grapevine mites collected from buds and blisters that were screened by multiplex PCR consisting of G1, G2, G3, G4, and G5 primer pairs.

Significant associations of G3 (X^2= 27.359; d.f.= 6; *P* = <0.0001) and co-presence of G2-G3 (X^2= 13.349; d.f.= 6; *P* = <0.0001) were observed in buds, whereas the co-presence of G1-G2 (X^2= 40.830; d.f.= 6; *P* = <0.0001) was significantly prevalent in leaf blisters. Moreover, there were differences in genetic groups associations between winter and summer buds (Figure 2.8). G3 (X^2=17.597; d.f.= 6; *P* = <0.0001) was significantly associated with winter buds and the co-presence of G2-G3 (X^2=15.051; d.f.= 6; *P* = <0.0001) was associated with summer buds.

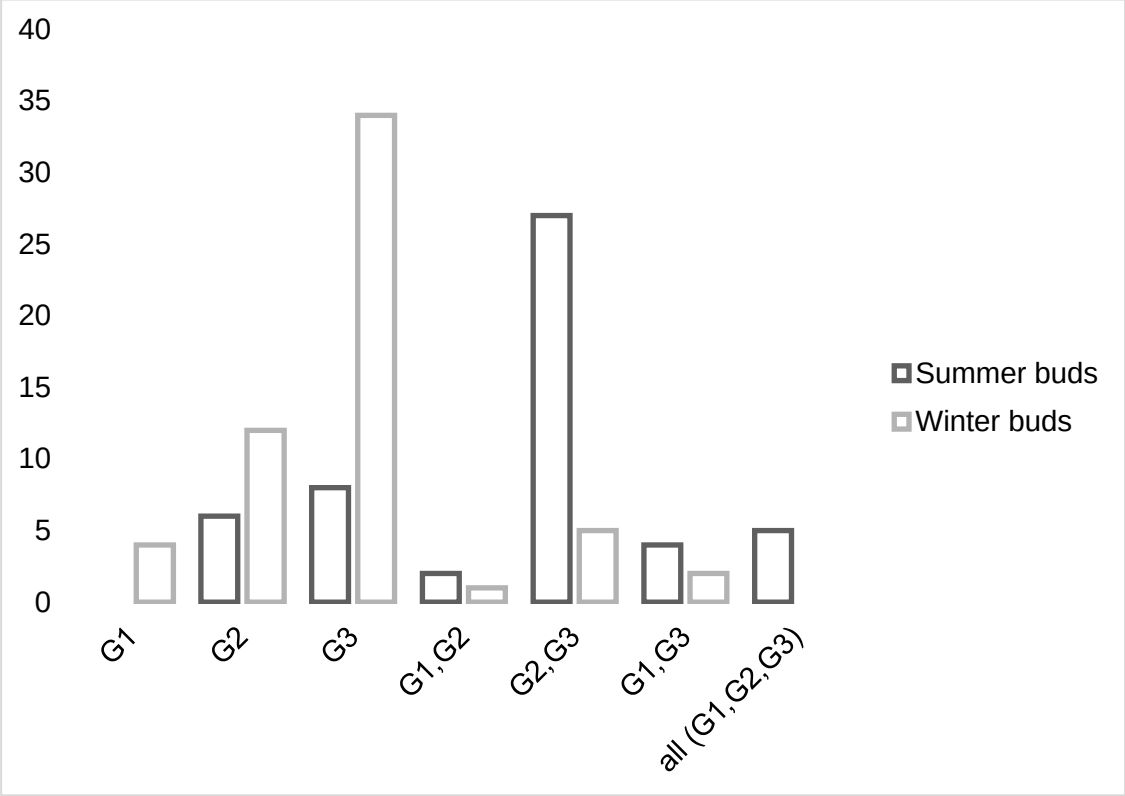


Figure 2.8. Genetic group distribution of summer and winter bud samples screened by multiplex PCR.

Next-Generation Sequencing

NGS was performed to recover the full mitogenome and achieve a better resolution of the genetic diversity of grapevine *Colomerus vitis*. However, attempts to recover the full mitogenome(s) of *C. vitis* were unsuccessful because DNA concentration achieved after extractions were extremely low (average, 8.8 ng/µl), mainly due to the small size (and hence limited DNA availability) of eriophyoid mites. Nevertheless, reads generated by NGS could be used to recover the COI region, and this was subsequently added to the data analysed for genetic clustering and divergence.

DISCUSSION

According to current literature, it is unclear whether *C. vitis* is a complex of multiple species or a single eriophyoid mite species consisting of multiple strains. In cases such as this, traditional taxonomy can benefit by supplementation with molecular tools to study organisms with limited available morphological characters (Navia *et al.,* 2005; Caterino *et al*., 2000).

Maximum likelihood tree and genetic diversity estimates

The clustering of the sequences on the ML tree that consisted of sequences recovered by Sanger sequencing, GenBank, and NGS showed multiple genetic groups within *C. vitis.* The additional diversity introduced by sequences recovered from GenBank suggests that *C. vitis* might be more diverse than is reported in this study. Moreover, the results suggest genetic differentiation according to the geography of these mite populations. However, to confirm this hypothesis, a study of the geographic genetic distribution would require a worldwide sample collection of these mites.

The intergroup p-distances were between 17.21% and 25.03%, indicating that the genetic groups diverged highly. The maximum intragroup p-distances within each genetic group in this study were between 0% and 3.51%. The within p-distances were less than the widely used barcoding threshold of 2 - 3% typically used to separate species (Hebert *et al.*, 2004; Meyer and Paulay, 2005) in each genetic group except for G5 (3.51 %). These results suggest conspecificity within the genetic groups (G1 (n = 18), G2 (n = 11), G3 (n = 21), G4 (n = 2), and V2 (n = 7). The high genetic divergence in G5 (n = 4) suggests that there may be more than one genetic group within the cluster, but more specimens of this group would be needed to confirm. Previous eriophyoid mite studies have found intraspecific p-distances between 0% and 13%, and interspecific p-distances range were between 2% and 41.3% (Skoracka, 2009; Lewandowski *et al*., 2014; Vidovic *et al*., 2015; Liu *et al*., 2019). These ranges are far wider than that usually considered conspecific in barcoding studies (Hebert *et al.*, 2004; Meyer and Paulay, 2005), implying either that eriophyoids typically show greater genetic diversity within species, or that many currently recognised species are in fact complexes of morphologically cryptic species. Thus, this study's intra- and interspecific p-distances are consistent with the reported range in eriophyoid mite studies. Moreover, the genetic diversity estimates support the classification of bud and blister mites as separate species, consistent with the findings of Carew *et al.* (2004). However, the high intergroup p-distances between the groups associated with buds and blister mites dispute that these species are closely related, as reported by Carew *et al.* (2004). More eriophyoid mite studies would increase confidence in intra- and interspecific boundaries. Lastly, the COI region gave excellent resolution of the genetic diversity in the current study as there was no overlap in the intra- and interspecific p-distances' ranges.

Co-presence of highly diverged groups in the same sample is common in *C. vitis*

Statistical analysis of the multiplex screening results revealed that G3 and the co-presence of G2-G3 were significantly more prevalent in buds than leaf blisters. In contrast, the co-presence of G1-G2 was more prevalent in leaf blisters. These results suggest some genetic differentiation according to the plant tissue the mites were collected from, although overlap is also common. There have been reports of such genetic differentiation in taxa due to their feeding habits in Eriophyoidea (e.g. Skoracka, 2009).

Winter buds had a significantly higher presence of G3, whereas co-presence of G2-G3 was more prevalent in summer buds. The results contrasted with what is expected of winter and summer bud mites. In winter there is an expectation that there would be more diversity of genetic groups as all mites from all groups hibernate in the buds and therefore there is a high chance to have more genetic groups. In summer blister mites and leaf curl mites migrate to the leaves, leaving only bud mites in the buds. As such, less genetic groups are expected to be seen in the summer buds. The significant change in prevalence of specific genetic groups between summer and winter suggested genetic differentiation according to the season the grapevine mites were collected.

Failure to detect any genetic groups in 86 samples may be due to a few reasons. Firstly, and most likely, it may be because the centrifugation and filtration process failed to capture any mites before DNA extraction. Secondly, it could be because the mites present belonged to other genetic groups not accounted for by the primers used for the multiplex PCR. Finally, it could also be that those samples belong to the G4 and G5 groups, of which I had not been successful in confirming they were optimal for detection of the groups.

Blisters had a greater diversity and mixture of genetic groups than was detected in buds. This might be because bud mites are restricted to the bud and complete their lifecycle there (Smith Meyer, 1981). As such, the genetic variation would be limited compared to that of blister mites. Blister mites overwinter in buds but reproduce and cause damage in leaves and return to the buds at the end of summer.

CONCLUSION

Grapevine *Colomerus* species represent a group of highly diverged cryptic species. The grouping patterns of the limited number of sequences from other countries point to the genetic diversity of *C. vitis* potentially being greater than what is reported in this study. Moreover, grapevine *Colomerus* species are minute with a reduction in characters, making it hard to distinguish them morphologically. This highlights the need to complement traditional taxonomy with molecular approaches for identification.

Multiplex PCR analyses revealed three genetic groups that often coexist in plant tissues. Depending on the plant tissue and season, one group or more may be more prevalent. These results highlight the importance of studying the diversity to optimise identification procedures of these organisms for accuracy. Accurate identification improves the ability to apply the most appropriate control strategies for that type of mite. Furthermore, the COI genetic groups could be beneficial for routine identification and confirmation of species presence in vineyards and interception of the mites in grapevine imports and exports, particularly in countries where grapevine mites are a quarantine concern.

Studies on grapevine *Colomerus* species would benefit from using the same specimens for both the molecular and morphological analyses. Using the same specimens for both approaches would increase the confidence when assessing associations of specific morphotypes to genetic groups and symptoms caused on plants. However, this is difficult due to different collection and handling techniques and the destructive methods sometimes necessary for morphological and molecular work. Additionally, sequencing the complete mitogenome(s) or genome of grapevine *Colomerus* species would also allow for estimating phylogenetic relationships among the genetic groups detected in the study, and between these and other eriophyoid mites.

CHAPTER 3: MORPHOLOGICAL DIVERSITY OF GRAPEVINE MITES

INTRODUCTION

The superfamily Eriophyoidea comprises tiny, highly specialised plant-feeding mites (Meyer and Craemer, 1999). Eriophyoid mites are small and wormlike, with the average female adult body approximately 200 µm in length (Smith Meyer, 1981; Lindquist *et al.*, 1996). Life stages consist of larvae, nymphs, adult males and at least one form of an adult female, with females being most prevalent (Manson and Oldfield, 1996). Males have a similar body form to females but are usually smaller and have distinctly different genitalia (Lindquist *et al.*, 1996). In some populations, two forms of females may be present: the protogyne, or the summer form, and the deutogyne, which is the secondary or overwintering form (Baker *et al*., 1996). Deutogynes are not known in all species, and in species where a deutogyne is known, it may be present only during adverse conditions and therefore may not always be present in a particular population. For this reason, taxonomic descriptions are based on the protogyne (Baker *et al*., 1996).

Scanning Electron Microscopy (SEM)

SEM can be utilised to visualise features that enhance the detail of the morphological descriptions. Its large depth of field makes 3D-like images to be perceived and with higher resolution than is possible with light microscopy. Briefly, an electron beam is scanned in a raster pattern over the surfaces of organisms, generating secondary electrons which are used to render detailed surface views of samples in the images captured. The images characteristically have high resolution, and a wide range of magnifications are possible, revealing structural detail, which is crucial in taxonomy, especially for descriptions of microscopic organisms. The use of SEM in acarology successfully began in the 1960s (Sabatini *et al.*, 1963), although there are limitations to this approach. Specimens need to be immobilised on adhesive carbon supports (e.g. dorsally or ventrally) making the glued side unavailable for imaging, and almost impossible to retrieve the organisms after imaging without damage (Fisher and Dowling, 2010). A further limitation is that the high vacuum environment in conventional SEM instruments demands that organisms are dried prior to

observation. Unfortunately, even the gentlest protocols often lead to structural collapse that distorts fine detail in the samples. However, since the advent of low-temperature (cryo) SEM methods in the 1970s, it has been possible to observe fully-hydrated specimens at low temperature (<-100 °C), thus precluding the need for chemical intervention and drying, making it an ideal strategy to study the natural state of the eriophyoid mite (Echlin *et al.*, 1971; Echlin, 1978; Achor *et al.*, 2001). Although analysing specimens using SEM is expensive and time-consuming, the additional information and detail it contributes to taxonomical descriptions make it crucial in integrative taxonomy.

This chapter aimed to find unambiguous differentiating characters in grapevine *Colomerus* through the use of cryo-SEM images. These images were used to assess morphological diversity and subsequently assign specimens to morphological groups.

MATERIALS AND METHOD

Sample collection

Grapevine leaves and buds showing presumed *Colomerus vitis* infestation symptoms were collected at the Agricultural Research Council Nietvoorbij farm (-33.914, 18.861) in Stellenbosch in March 2021. The collected plant material was stored at 4 °C, and bud and leaf material were kept in separate Ziploc bags until SEM imaging. Then, live mites were individually picked from the leaf blisters and buds and placed on double-sided carbon tape using minute pins with the aid of a stereomicroscope.

Cryo-SEM

A cryo-fixation technique adapted from Rahbani *et al.* (2013) was used to prepare specimens and study them using a Zeiss Supra 55VP SEM equipped with a Gatan Alto cryo-stage (Gatan, USA). A double-sided tape containing the mites was attached to a specimen carrier and rapidly (ms) plunge-frozen in sub-cooled (-210 °C) liquid nitrogen to avoid the formation of ice crystals. The specimen carrier was then transferred under vacuum and without warming to the pre-cooled stage of the cryo-preparation chamber (-140 °C). Samples were then warmed to -100 °C and held at that temperature for 3-5 minutes to sublime any water contamination that may have condensed on the surface of the mites, before cooling again to -140 °C and sputter-coating with platinum for 60 s. Samples were subsequently transferred

to the cryo-SEM stage at -140 °C and imaged at between 2-5 kV accelerating voltages. Images of the whole body, prodorsal shield, microtubercles, genital cover flap and empodium were captured in high resolution to assess variation within the specimens collected. Images were focussed on the characters listed by Amrine and Manson (1996) as essential for eriophyoid species descriptions, namely the prodorsal shield, coxi-genital region, legs, empodia and lateral, dorsal and ventral views of the whole mite, including the microtubercle details. These are also characters considered important for differentiation between *C. vitis* and *C. oculivitis* (Attiah, 1967; Keifer, 1944; Keifer, 1975; Mathez, 1965; Bagdasarian, 1981; Manson, 1984; Halawa *et al.*, 2015). As mentioned previously, SEM is limited by the orientation of the specimen on the stub and therefore it was not possible to observe all the characters on each specimen.

RESULTS AND DISCUSSION

Morphology was assessed for a total of 67 *Colomerus* specimens (45 mites from leaf blisters and 22 from buds). The characters used were the number of rays on the empodial featherclaws, the shape of microtubercles, the prodorsal shield ornamentation, and the female genital coverflap (Table 3.1).

Table 3.1. Morphological characters on *Colomerus* sp. observed with SEM.

Morphological characters		**Blister mites**		**Bud mites**	
Number of empodial rays	**Specimens on which this character could be seen**	**29**		**4**	
	4 rays (most likely nymphs or larvae)	2	7 %	0	0 %
	5 rays	18	62 %	0	0 %
	6 rays	9	31 %	4	100 %
Shape of microtubercles	**Specimens on which this character could be seen**	**47**		**21**	
	Pointy, narrow and elongate	10	21 %	5	24 %
	Oval	7	15 %	2	10 %
	Round	22	47 %	13	62 %
	Intermediate (oval/round)	8	17 %	1	5 %
Prodorsal shield ornamentation	**Specimens on which this character could be seen**	**13**		**10**	
	Median and admedian lines complete or almost complete	6	46 %	0	0 %
	Median and admedian lines broken	5	38 %	0	0 %
	Smooth (no median or admedian lines visible)	2	15 %	10	100 %
Genital coverflap	**Specimens on which this character could be seen**	**16 (Females), 1 (Male)**		**11 (Females), 1 (Male)**	
	Females: two rows, top row 11-14 ridges; bottom row 9-14 ridges	16	100 %	11	100 %
	Males: one row, 11 and 13 Ridges	1	100 %	1	100 %

Spikes on distal margin of femur and genu	**Specimens on which this character could be seen**	**47**		**22**	
	Spikes present	8	17 %	4	18 %
	Spikes absent	39	83 %	18	82 %

Empodial rays

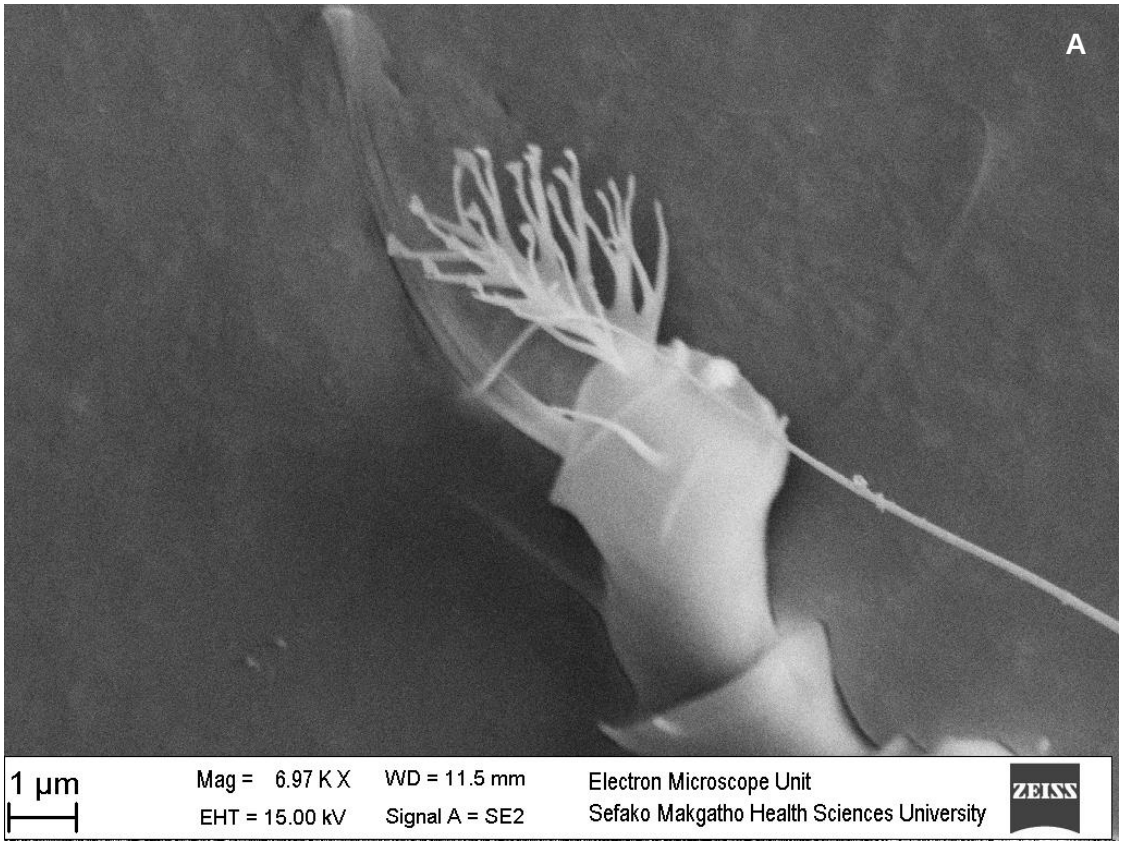
The number of empodial rays has been considered the only unambiguous distinguishing character between *C. vitis* and *C. oculivitis* (Craemer and Saccaggi, 2013b; Halawa *et al.*, 2015). In the current study, specimens were observed with 4 rays, 5 rays and 6 rays (Figure 3.1; Table 3.1). It is likely that the 4-rayed specimens were nymphs or larvae but I was unable to confirm this as the other necessary features were not visible. I did not consider them in further analysis or discussion. Moreover, in some specimens, rays were relatively simple, easy to observe and count, whereas for others, the empodium shape was more complex (Figure 3.1). The complex empodia did not prohibit counting the number of rays in the SEM images, but it may present challenges in the case of traditional slide-mounted specimens.

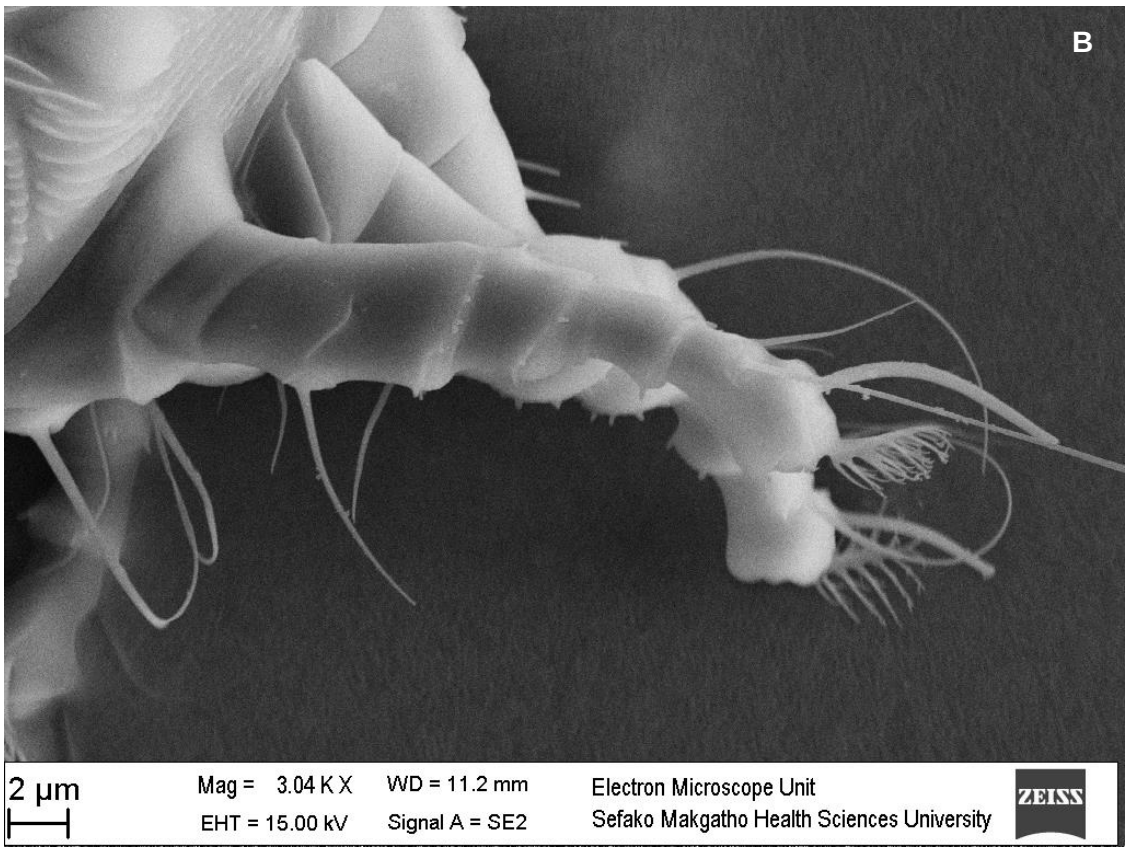
Of the blister mite specimens assessed, the number of empodial rays was between 4 and 6, consistent with the description of African *Colomerus* species (Meyer and Ueckermann, 1990). About 62% of specimens had 5-rayed empodia, consistent with descriptions of *C. vitis* worldwide (Attiah, 1967; Keifer, 1944; Ryke and Meyer, 1960; Smith Meyer, 1981; Keifer, 1975; Mathez, 1965; Bagdasarian, 1981; Manson, 1984). However, 31% had 6-rayed empodia, as described for *C. oculivitis.*

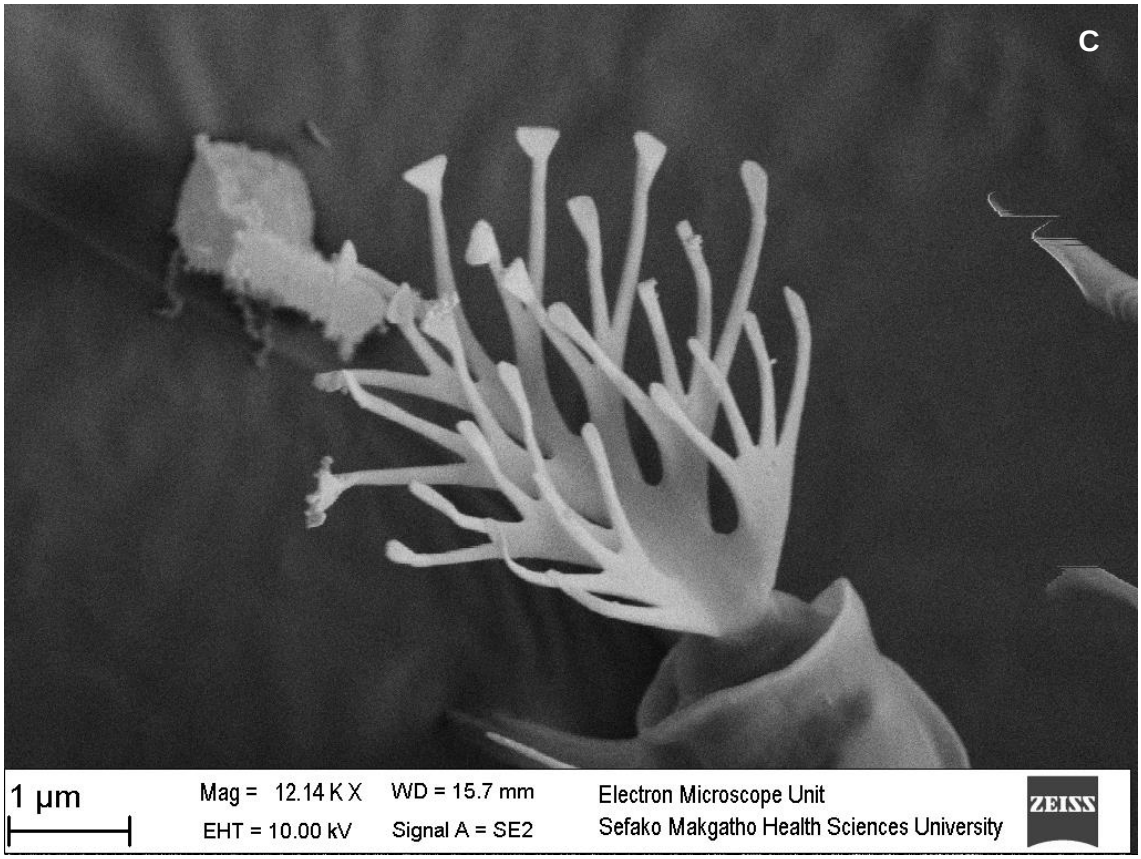
The empodium could only be observed in 4 bud mite specimens, and in these, it was 6-rayed. The 6-rayed empodia observed are consistent with descriptions of *C. oculivitis* (Attiah, 1967). This is not surprising as there has been speculation *C. oculivitis* might be the bud strain of *C. vitis* (Craemer *et al.,* 2012).

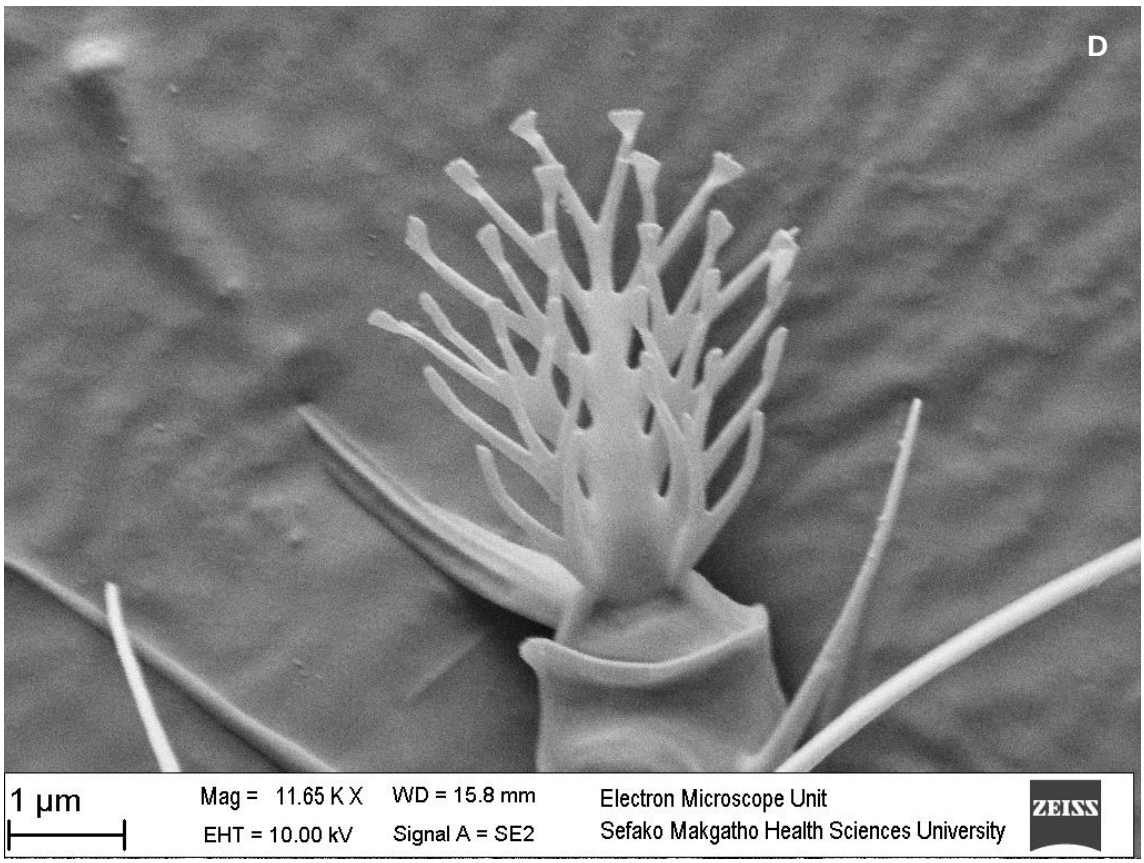
Intraspecific variation of the number of empodial rays has previously been reported in Eriophyoidea (Meyer and Ueckermann, 1990) but has never been noted in *C. vitis* or *C. oculivitis* (Craemer and Saccaggi, 2013). Thus, it is more likely that the difference in empodial rays observed here is an interspecific character. This would indicate that *C. oculivitis* (6-rayed empodia) and *C. vitis* (5-rayed empodia) are both present in South Africa, but that *C. vitis* only occurs in the blisters, while *C. oculivitis* occurs in both blisters and buds.

However, due to the limited number of bud mite specimens in which empodial rays could be observed, more specimens would be needed to confirm that only the 6-rayed species (putatively *C. oculivitis*) occurs in the buds.









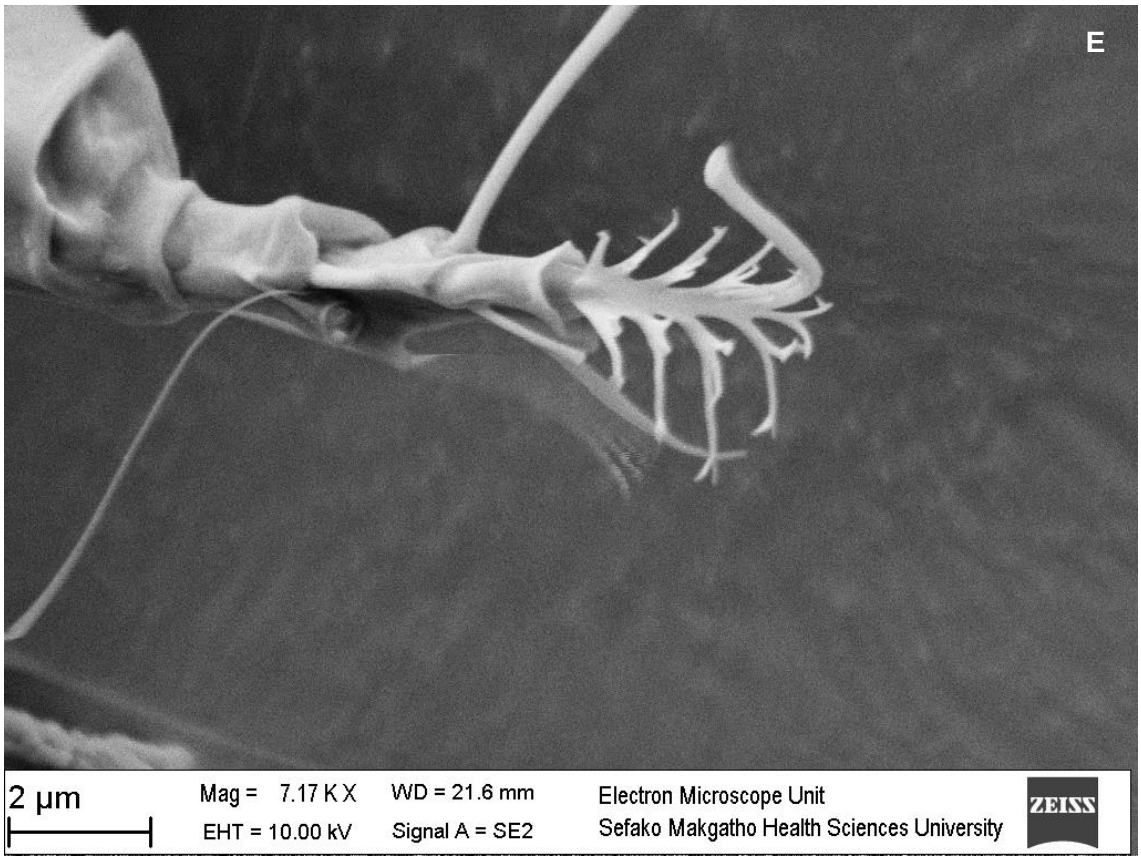
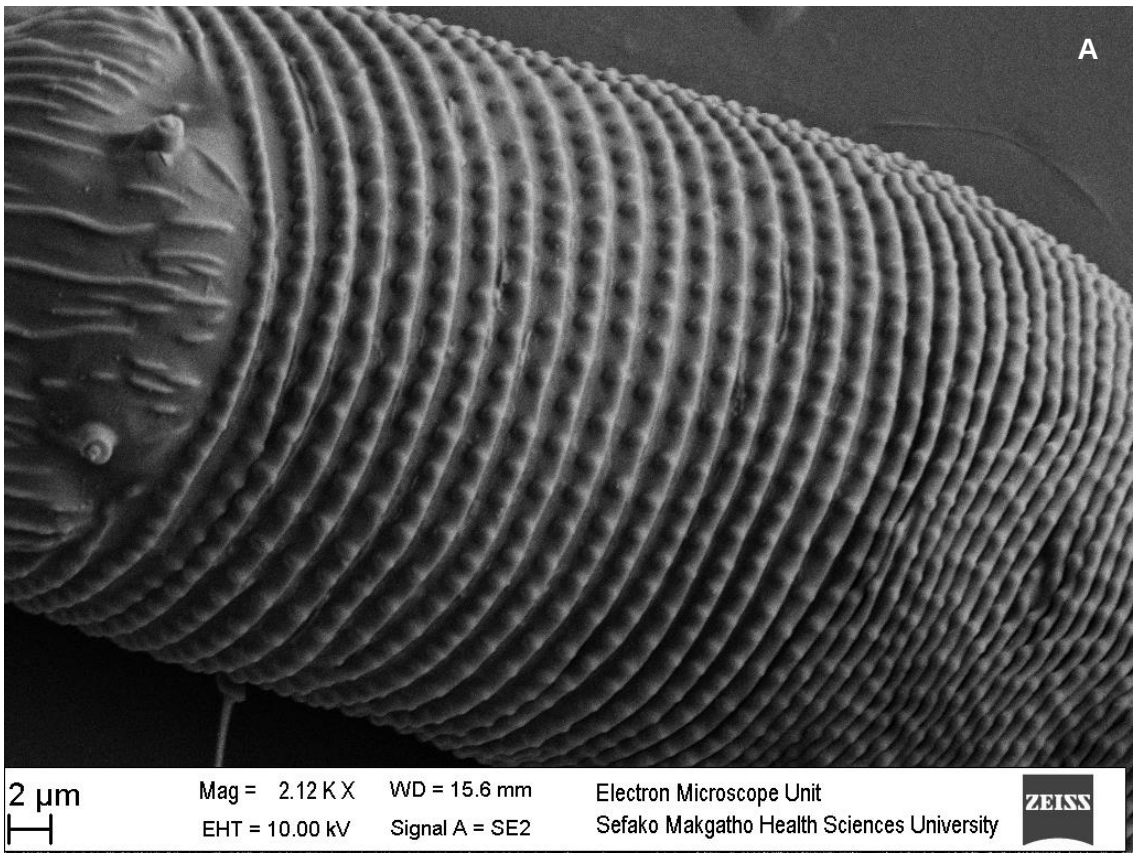


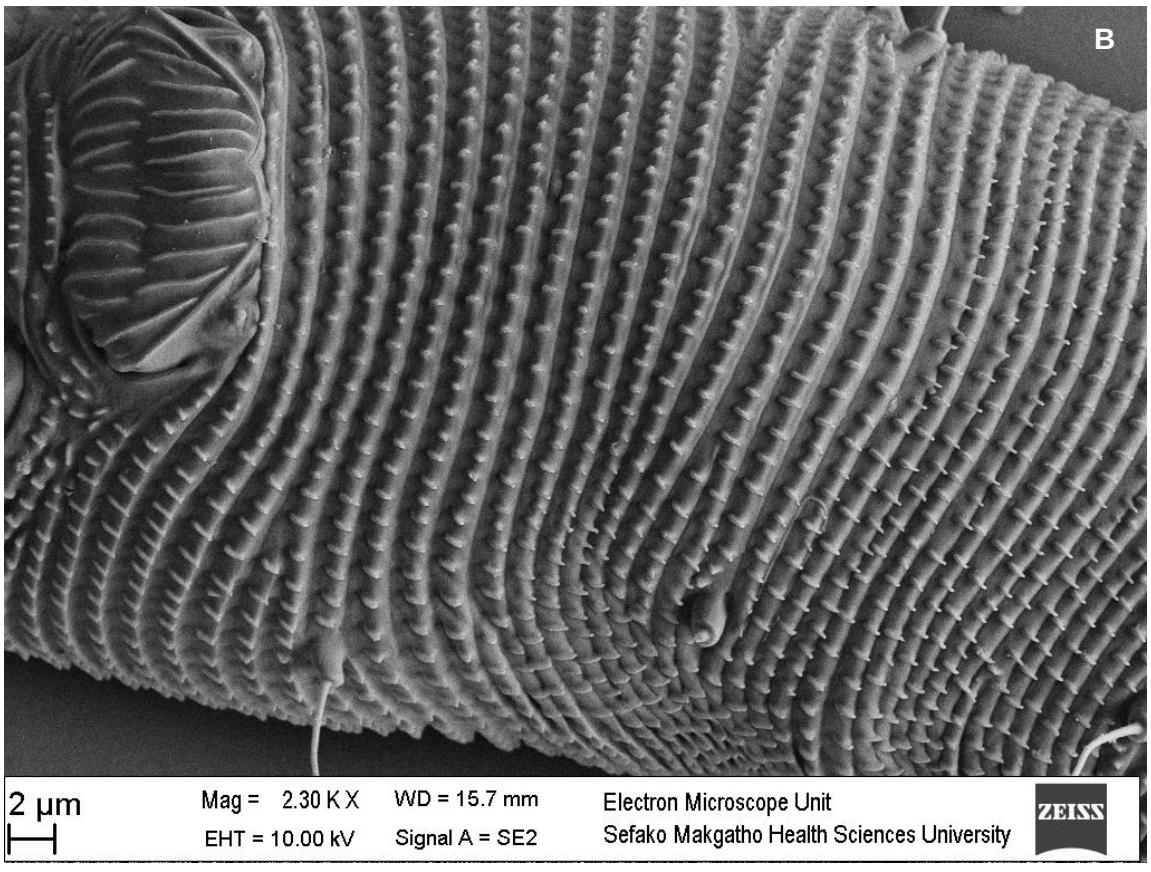
Figure 3.1. Empodial ray number and complexity in grapevine *Colomerus* specimens. (A) 6-rayed empodial claw with 2-3 sub-rays. (B) 6-rayed empodial claw, that is simple and without sub-rays. (C) 5-rayed empodial claw with 4 sub-rays. (D) 6-rayed empodial claw with complex rays. (E) 5-rayed empodial claw with simple arrangement of rays.

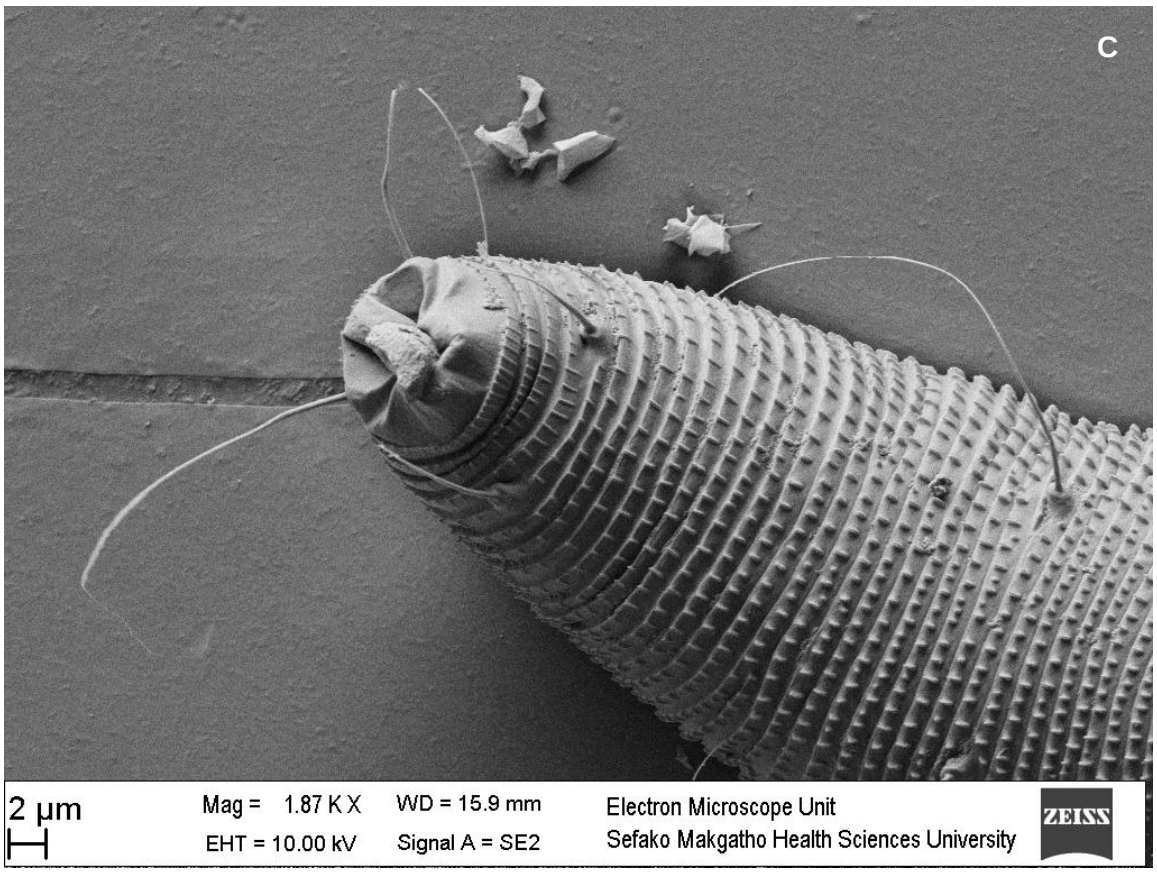
Shape of microtubercles

Craemer and Saccaggi (2013) noted variation in the shape of the microtubercles and recommended the inclusion of this character in studying the grapevine *Colomerus* species. In this study, the shape of microtubercles varied from pointy, narrow, and elongated through oval to round (Figure 3.2). The elongate microtubercles were always clearly distinguishable, but the differentiation between oval and round was sometimes unclear, and we categorised species as intermediate between these two.

Rounded microtubercles were most common (51% overall), consistent with descriptions of *C. vitis* by Mathez (1965) and Attiah (1967). The next most common microtubercle shape was pointy, narrow and elongate (22% overall), similar to the original description of *C. oculivitis* by Attiah (1967). Lastly, 13% of specimens had oval microtubercles, consistent with Bagdasarian (1981) and Manson (1984) descriptions of *C. vitis.* The shape of microtubercles could not be linked to any other character and could not be associated with either bud or blister mite specimens. However, this may be due to the limitation of SEM for observing all characters on each specimen. Another technique that can observe all characters on a single specimen may well find correlations. Thus, although observations in this study agree with that of Halawa *et al.* (2015), in that the shape of the microtubercles cannot be used to distinguish between species or strains in this complex, the study can not discount their importance in morphological studies.







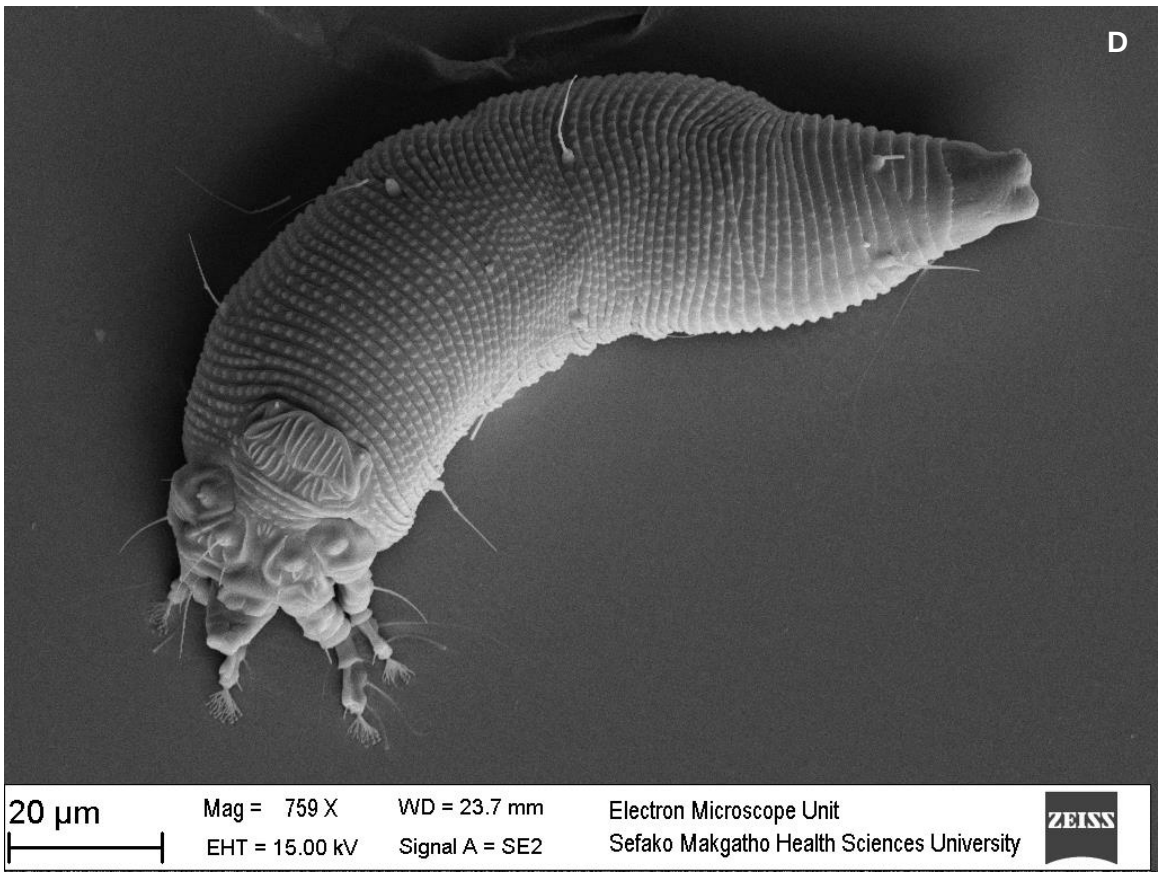


Figure 3.2. Shape of microtubercles observed in grapevine *Colomerus* specimens. (A) round; (B) pointy, narrow, and elongated; (C) oval; (D) elongated.

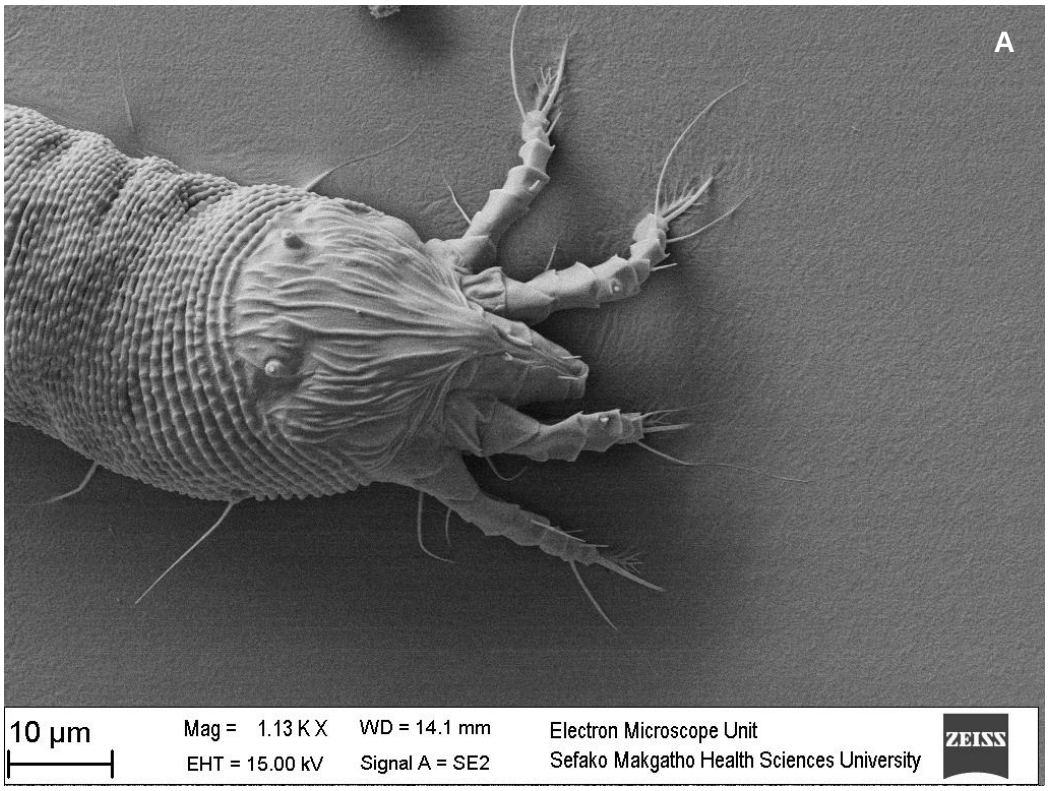
Prodorsal shield

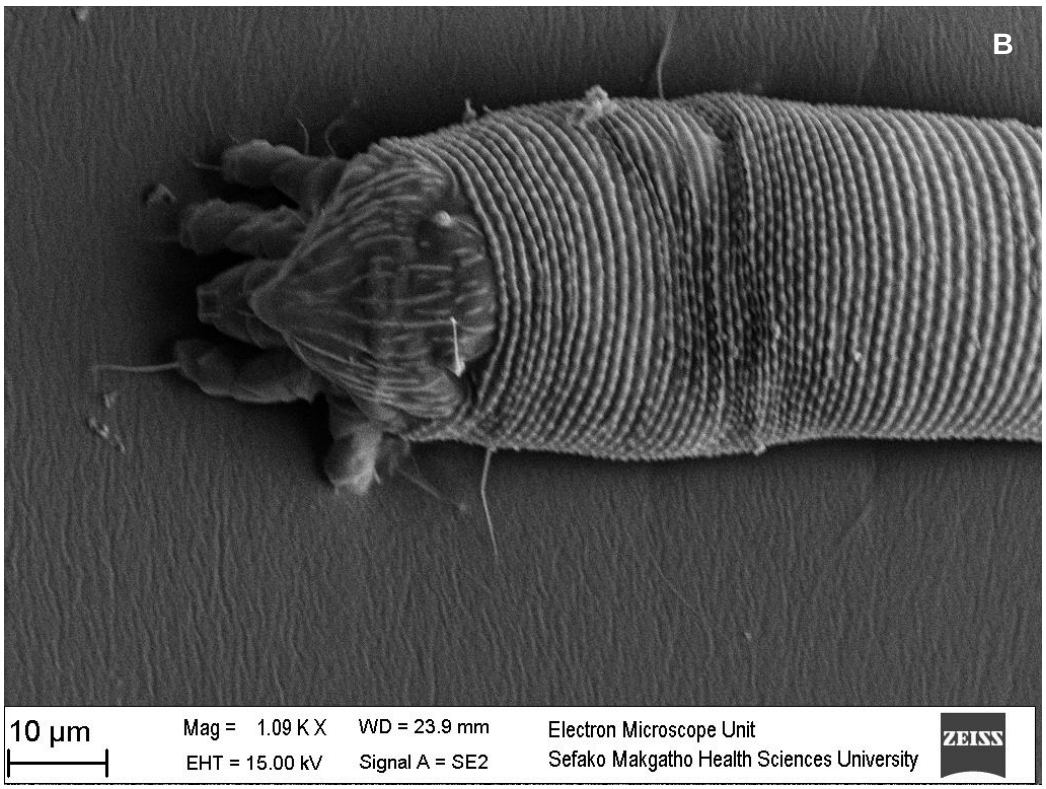
The prodorsal shield ornamentation was divided into three types: complete or almost complete median and admedian lines; median and admedian lines clearly broken; and smooth prodorsal shield with no median or admedian lines visible. In some samples, there was ornamentation, but the median and admedian lines could not be determined (n = 6) (Figure 3.3). This variability is consistent with different *C. vitis* descriptions (Keifer, 1944; Keifer, 1975; Mathez, 1965; Bagdasarian, 1981) where both incomplete (i.e. broken) or almost complete median and admedian lines were reported. Meyer and Ueckermann (1990) also reported a generally not well defined prodorsal shield.

However, the observation of a smooth prodorsal shield in all bud mite specimens that were dorsally captured and in 15% of blister mite specimens is inconsistent with published literature (Attiah, 1967; Keifer, 1944; 1975; Mathez, 1965; Bagdasarian, 1981; Manson, 1984; Meyer and Ueckermann, 1990). In some of these specimens, a few small ridges could

be observed between setae *sc.* With traditional slide-mounting techniques, there is a risk of over-clearing the specimen, resulting in less visible prodorsal shield ornamentation. It may be that in the past, when specimens with reduced prodorsal shield ornamentation were observed, it was assumed that this was due to over-clearing and thus was not considered diagnostic. However, cryo-SEM precludes over-clearing of the mite, and thus this method can confirm that a large percentage of grapevine *Colomerus* specimens do, in fact, have smooth prodorsal shields. Moreover, this prodorsal shield type was consistent throughout all bud mite specimens captured dorsally and could be used to differentiate between bud and blister mites.

It must be noted that a smooth prodorsal shield might indicate the presence of deutogynes, as they have been reported to have a reduced prodorsal shield ornamentation compared to protogynes (Lindquist, 1996). However, considering that these samples were taken in March (late summer, and still very hot at the sampling site), if deutogynes were present in the population, one would expect to see a mixture of both protogynes and deutogynes in the sample, with protogynes likely being more common. Thus, it is unlikely that these types represent a deutogyne form.





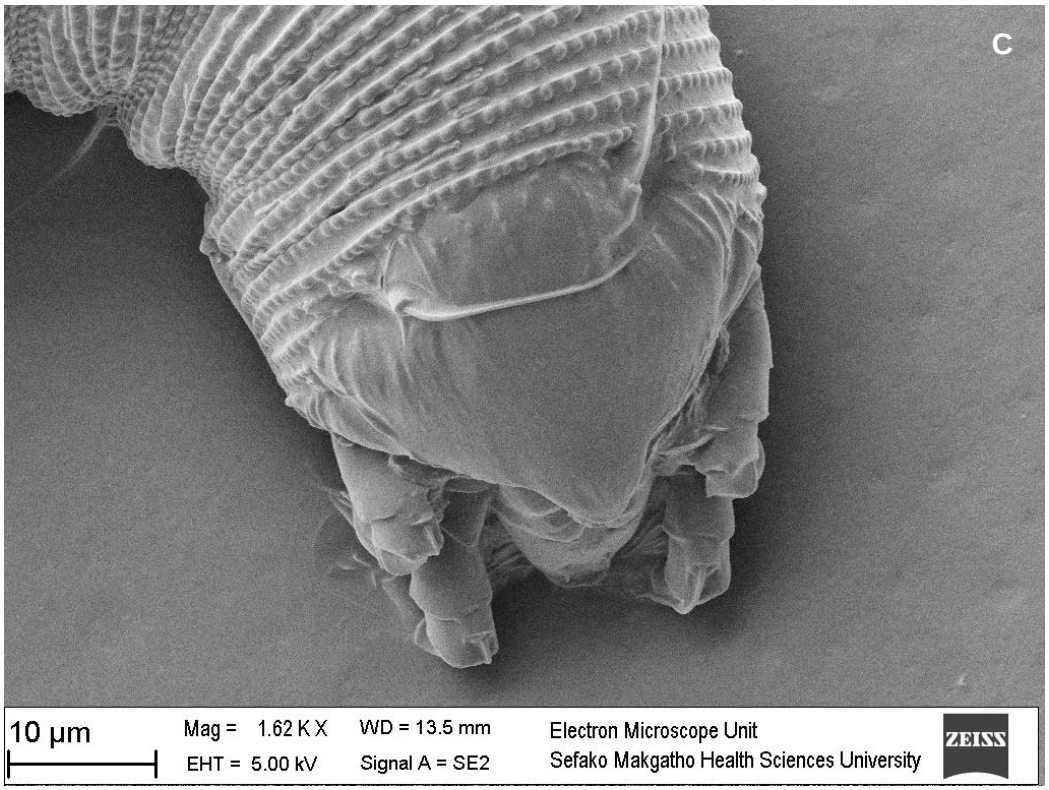
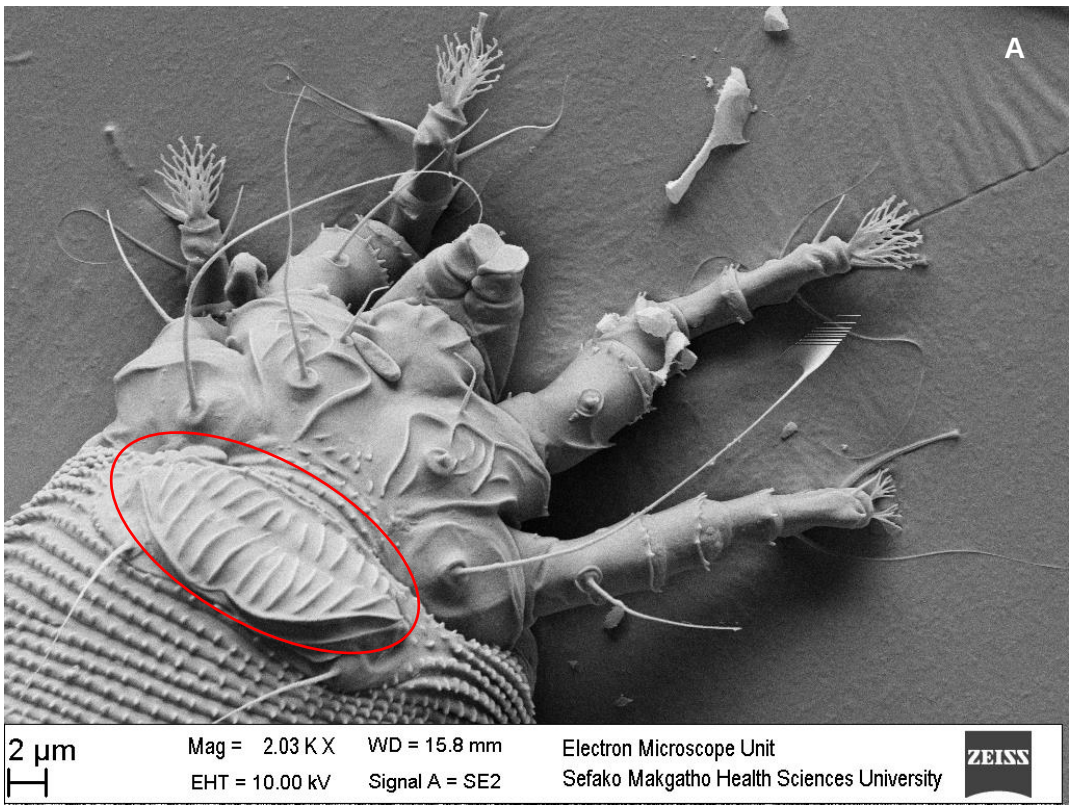


Figure 3.3. Prodorsal shield ornamentation in *Colomerus* mites. (A) Median and admedian lines are complete. (B) Broken median and admedian lines. (C) Smooth prodorsal shield towards the frontal lobe and few ridges between setae (sc).

Female genital coverflap

The female genital coverflap had two rows of ridges (Figure 3.4 A), consistent with this diagnostic feature of the tribe. The top row had 11-14 ridges, while the bottom row had 9-14 ridges. The top row generally had more ridges than the bottom row. Observations in this study were contrary to reports of greater than 16 ridges in *C. vitis* (Keifer, 1944; Craemer and Saccaggi, 2013), but in line with other studies observing 10, 13 or 14 ridges in *C. vitis* (Halawa *et al.*, 2015; Zaher, 1984) and 12 ridges in *C. oculivitis* (Attiah, 1967; Zaher, 1984). It should be noted that the *Colomerus* type in Halawa *et al.* (2015) described with only one row of ridges on the genital coverflap is instead a description of the male, as can be seen from the figures accompanying the description (N. Ndlovu and D. Saccaggi, personal communication). In this study, males were also observed with a single row of ridges in the genital region (Figure 3.4 B).



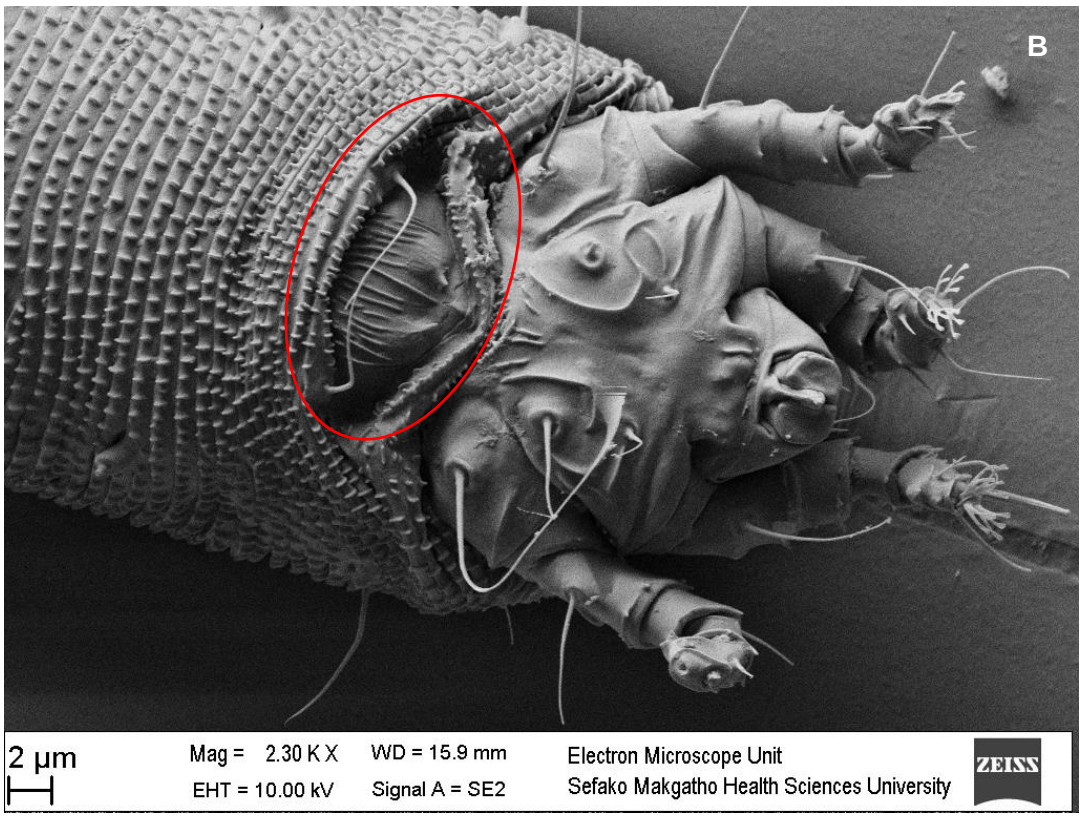


Figure 3.4. Female and male genitalia of *Colomerus* specimens. (A) Female genital coverflap: two rows of ridges. (B) Male genitalia: one row of ridges.

Other characters

In addition to the above widely used morphological characters, other characters were observed which may be of use in distinguishing between mites within this complex. This included the number of annuli without microtubercles on the posterior of the dorsum, and the presence of spikes on leg segments. In both bud and blister mites, microtubercles extended to the last annuli ventrally but were not present from between the 10^{th}-8^{th} annuli on the dorsum posteriorly. Thus, the observations in this study are contrary to reports by (Halawa *et al.*, 2015), in which the absence of microtubercles on the posterior annuli was observed only in specimens with rounded microtubercles.

This study observed another morphological character that may be of importance. Some specimens had spikes or sharp protuberances along the distal margin of the femur and genu segments on both legs. This was observed in all bud mite specimens that had oval or elongated microtubercles, but randomly in some blister mite specimen. It could not consistently be associated with any other morphological characteristic.

Bud and blister mites

The number of rays observed in blister mite specimens (4-, 5-, and 6-rays) was more diverse than that observed in bud mite specimens (6-rays). Additionally, the blister mite's prodorsal shields had more diversity than the prodorsal shield of specimens collected from the buds. The diversity within the grapevine *Colomerus* specimens characters in South Africa might be partly due to the long history of grapevine cultivation and transportation around the world (Garnas *et al.*, 2016). The introduction of grapevine from multiple countries might have contributed to the diversity observed in the grapevine *Colomerus* species analysed. Furthermore, when describing *Colomerus* species morphologically, there have been recommendations to describe them as a complex rather than on a species level (Craemer and Saccaggi, 2013b; Halawa *et al.*, 2015). This is because of potential intraspecific variations within grapevine *Colomerus* species, making it challenging to find characters that can be used to identify grapevine *Colomerus* specimens to a species level unambiguously. These suggestions highlight the need to complement traditional taxonomy with molecular methods.

CONCLUSION

The number of rays and prodorsal shield patterns observed in blister mite specimens was more diverse than that observed in bud mite specimens. This suggests that the mite population within blisters may be more diverse than within buds and may possibly represent more than one species in the blisters.

It is worth mentioning that there was a lack of resolution in differentiating *C. vitis* through morphology compared to COI divergence analyses. These mites are small, have limited distinct morphological characters, and are highly host specialised. Consequently, this leads to a limited diversity in the body structure. The results further highlight the need to supplement traditional taxonomy with molecular approaches. The challenge with using morphology methods to investigate species is that speciation is not always complemented by morphological differentiation between taxa (Bickford *et al.*, 2007), hence the need to supplement traditional taxonomy with other approaches.

The specimens analysed in this study show variation consistent with that reported by Craemer and Saccaggi (2013). At the two extremes, morphotypes consistent with *C. vitis*

descriptions (five rays and round microtubercles) and *C. oculivitis* (six rays and pointy, narrow, and elongate microtubercles) could be observed. However, many specimens displayed characteristics intermediate between these two extremes, suggesting the presence of a morphologically indistinguishable species complex with a graduation in morphological characteristics. The study did not necessarily disprove existing descriptions of the grapevine *Colomerus* complex but revealed the existing diversity within *Colomerus vitis*. I observed specimens consistent with all previously published descriptions, as well as specimens outside the range of published descriptions, pointing towards even more diversity within the complex than previously reported.

The artefact-free, enhanced imagery provided by cryo-SEM gave essential insights into the exact shape of the characters, allowing more detailed observation of the features. This improved resolution will be instrumental in re-examining slide-mounted specimens to determine if distinct morphotypes are clear when all features can be examined together in each specimen.

CHAPTER 4: GENERAL CONCLUSION

The study aimed to investigate the species diversity of *C. vitis* through integrative taxonomic approaches. The taxonomic approaches consist of morphological analysis (cryo-SEM imaging) and DNA based methods (mitochondrial cytochrome oxidase 1 gene variation and Next Generation Sequencing).

The study consisted of three main chapters. The first chapter was the literature review, highlighting the history, relevance, and economic contributions of grapevine cultivation in South Africa. Moreover, the chapter reviewed state of the field in *C. vitis* research and introduced main concepts explored within the present study. The second chapter explored the genetic diversity of grapevine *Colomerus* species presumed *C. vitis* through a marker-assisted approach (Sanger sequencing and multiplex PCR of the COI barcoding region) and mitogenome sequences (NGS) analysis. Lastly, in the third chapter, the morphological diversity of *C. vitis* was assessed through cryo-SEM imaging.

STUDY OUTCOMES

In chapter two, two novel methods were developed. The first method separates grapevine mites from the plant material. The method makes use of centrifugation and filtration by a 9.5 mm steel screen. The second method was a species-specific multiplex PCR that allows simultaneous detection of different genetic groups within *C. vitis.* Additionally, I showed that the COI barcoding region of *C. vitis* sequences analysed were too diverged to be considered one species. Seven genetic groups were identified from *C. vitis* COI sequences analysed, five genetic groups were identified in the present study (G1, G2, G3, G4, and G5) and an additional two were identified from *C. vitis* COI sequences deposited in GenBank (V1 and V2). The study also revealed that the genetic groups often co-exist in plant tissues. Furthermore, there was evidence of genetic differentiation according to the geography of the mite COI sequences studied. Statistical analysis of the multiplex results gave strong support

for genetic group associations. In summer buds there was a higher prevalence of the co-presence of G2-G3, whereas in winter buds there was a higher prevalence of G3, while in leaf blisters the co-presence of G1-G2 was more prevalent.

Finally, in the third chapter, high-resolution images of *C. vitis* were produced, which allowed for the observation of morphological characters that are otherwise unclear on slide-mounted specimens. From the images I saw variations in the characters analysed and considered useful for differentiating eriophyoid mites. The number of empodial rays observed were between 4 – 6, and either simple or complex. However, most of the samples were 5-rayed as expected for *C. vitis* and a considerable number were 6-rayed. Microtubercles were mostly round as expected for *C. vitis*. However, there was also a considerable amount of pointy, narrow and elongate microtubercles previously associated with *C. oculivitis.* The presence of 6-rayed empodia and pointy, narrow, and elongate microtubercles which are character descriptions of *C. oculivitis* suggested the coexistence of *C. vitis* and *C. oculivitis* in South Africa. Overall, there was more diversity in blister mites compared to bud mites. Three types of prodorsal shields were observed. (1) Complete or almost complete median and admedian lines, (2) clearly broken median and admedian lines, and (3) smooth prodorsal shield with no median or admedian lines visible. All three were observed in blister mites, whereas only the 3rd type was observed in buds. Additionally, in blister mites I observed 4-, 5-, and 6-rayed empodia whereas only 6-rayed empodia were observed in bud mites.

LIMITATIONS AND PROSPECTS

The multiplex PCR developed in this study requires PCR and a capillary electrophoresis step using a sequencer, which is time-consuming. Additionally, mites are minuscule; consequently, not enough DNA was attained to recover the complete mitogenome(s) of *C. vitis.* Moreover, the same *C. vitis* specimen could not be used for both genetic and morphological analyses as the methods used were destructive to the specimens. Furthermore, cryo-SEM imaging could not allow for the analysis of all features together in one specimen. Lastly, genetic groups could not be identified to a species level.

Further studies into the grapevine *Colomerus* species complex may benefit from devising a protocol for specimens' concurrent morphological, molecular and symptom analysis. Such a study would facilitate associations of morphotypes to molecular genetic groups identified and

symptoms caused on the grapevine. Additionally, DNA extraction of grapevine mites may be optimised to allow for recovery of sufficient DNA concentrations for sequencing the complete mitogenome(s), or genome of grapevine *Colomerus* species. The complete genome would also increase the resolution and reveal the extent of genetic diversity within the complex. Lastly, multiplex PCR method may be developed for commercial application to detect the presence/absence of grapevine mites in plant material. It may further be adapted to real-time PCR for grapevine mite diagnostics.

REFERENCES

Achor, D. S. *et al.* (2001). Relative advantages of low temperature versus ambient temperature scanning electron microscopy in the study of mite morphology. *International Journal of Acarology*, 27(1), pp. 3–12. doi: 10.1080/01647950108684218.

Amrine Jr, J.W. and Manson, D.C.M. (1996). 1.6. 3 Preparation, mounting and descriptive study of eriophyoid mites. *World crop pests*, *6*, pp.383-396.

Amrine, J.W. and Stasny, T.A. (1994). *Catalog of the Eriophyoidea (Acarina: Prostigmata) of the world*. Indira Publishing House.

Armstrong, K.F. and Ball, S.L. (2005). DNA barcodes for biosecurity: invasive species identification. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *360*(1462), pp.1813-1823.

Avise, J.C. (2000). *Phylogeography: the history and formation of species*. Harvard university press.

Attiah H. H. (1967). Eriophyes oculivitis n. sp., A new bud mite infesting grapes in the U.A.R. *Pakistan Journal of Biological Sciences*, 14(2), pp. 112–117.

Bagdasarian (1981). Eriophyoid mites of the fruit trees and bushes from Armenia. *Armenian SSR Academy of Sciences Press, Erevan*.

Baggiolini, M., Guignard, E., Hugi, H. and Epard, S. (1969). Contribution à la connaissance de la biologie de lérinose de la vigne et nouvelles possibilités de lutte. *Revue Suisse de Viticulture Arboriculture Horticulture*, 1: 50-52

Baker, E.W. (1996). *Eriophyoid mites of the United States*. Indira Publishing House.

Bernard, M. B., Horne, P. A., and Hoffmann, A. A. (2005). Eriophyid mite damage in *Vitis vinifera* (grapevine) in Australia: *Calepitrimerus vitis* and *Colomerus vitis* (Acari: Eriophyidae) as the common cause of the widespread «Restricted Spring Growth» syndrome. *Experimental and Applied Acarology*, 35: 83-109.

Bernard, M.B., Cole, P., Kobelt, A., Horne, P.A., Altmann, J., Wratten, S.D. and Yen, A.L. (2010). Reducing the impact of pesticides on biological control in Australian vineyards: pesticide mortality and fecundity effects on an indicator species, the predatory mite Euseius victoriensis (Acari: Phytoseiidae). *Journal of Economic Entomology*, *103*(6), pp.2061-2071.

Bickford, D., Lohman, D.J., Sodhi, N.S. and Ng, P.K.L., others (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*, 22(3), pp. 148–155. doi: 10.1016/j.tree.2006.11.004.

Boczek, J. and Shevtchenko, V.G. (1996). Ancient associations: eriophyoid mites on gymnosperms. In: E.E. Lindquist, M.W. Sabelis and J. Bruin (Editors), Eriophyoid Mites. Their biology, natural enemies and control. *Elsevier Science Publ*., Amsterdam, The Netherlands, pp. 217-225.

Burger, J. and Deist, J. (1981). Wingerdbou in Suid-Afrika. *Trio-Rand/SA Litho*.

CABI. (2021). Centre for Agriculture and Biosciences International. Wallingford, UK: CAB International. Last accessed 9/30/2021. CABI.org.

Carew, M. E., Goodisman, M. A. D. and Hoffmann, A. A. (2004). Species status and population genetic structure of grapevine eriophyoid mites. *Entomologia Experimentalis et Applicata*, 111(2), pp. 87–96. doi: 10.1111/j.0013-8703.2004.00149.x.

Carew, M., Schiffer, M., Umina, P., Weeks, A. and Hoffmann, A. (2009). Molecular markers indicate that the wheat curl mite, *Aceria tosichella* Keifer, may represent a species complex in Australia. *Bulletin of Entomological Research: Cambridge University Press*, 99, pp. 479-486. doi:10.1017/S0007485308006512.

Castillo, R (1990). Damage of Colomerus vitis Pgst. on buds in the area of Jerez de la Frontera. *OILB/SROP Bull*., 13(7): 150-153.

Caterino, M.S., Cho, S. and Sperling, F.A. (2000). The current state of insect molecular systematics: a thriving Tower of Babel. *Annual review of entomology*, *45*(1), pp.1-54.

Chan, A. H. E., Chaisiri, K., Saralamba, S., Morand, S. and Thaenkham, U. (2021). Assessing the suitability of mitochondrial and nuclear DNA genetic markers for molecular systematics and species identification of helminths. *Parasites and vectors*, *14*(1), pp.1-13.

Chandrapatya, A. and Konvipasruang, P. (2014). Complementary description of *Colomerus novahebridensis* Keifer (Acari , Eriophyidae), with a discussion about the constitution of the genus and its economic importance , and a tentative key to *Colomerus* Newkirk and Keifer species. *ZooKeys* 434, pp. 17–35. doi: 10.3897/zookeys.434.7308.

Chen, F., Guo, Y.B., Wang, J.H., Li, J.Y. and Wang, H.M. (2007). Biological control of grape crown gall by Rahnella aquatilis HX2. *Plant disease*, *91*(8), pp.957-963.

Chernomor, O., Von Haeseler, A. and Minh, B.Q. (2016). Terrace aware data structure for phylogenomic inference from supermatrices. *Systematic biology*, *65*(6), pp.997-1008.

Clarke, A.R. and Walter, G.H. (1995). " Strains" and the classical biological control of insect pests. *Canadian Journal of Zoology*, *73*(10), pp.1777-1790.

Coleman, A. W. (2009). Is there a molecular key to the level of “biological species” in eukaryotes? A DNA guide. *Mol. Phylogenet. Evol*. 50: 197Ð203.

Cox, A.J. and Hebert, P.D. (2001). Colonization, extinction, and phylogeographic patterning in a freshwater crustacean. *Molecular ecology*, *10*(2), pp.371-386.

Craemer, C., Saccaggi, D. L. and Makutoana, M. M. C. (2012). *Colomerus* spp. (Prostigmata: Eriophyoidae) on grapevine: yet another confusing eriophyoid sibling species complex? *European Acarology Assocuiation Congress (EURAAC)*. Vienna, Austria.

Craemer, C. and Saccaggi, D. L. (2013). Frequent quarantine interception in South Africa of grapevine *Colomerus* species (Trombidiformes: Prostigmata: Eriophyidae): taxonomic and distributional ambiguities. *International Journal of Acarology*, 39(3), pp. 239–243. doi: 10.1080/01647954.2013.767856.

Davis, J.I. and Nixon, K.C. (1992). Populations, genetic variation, and the delimitation of phylogenetic species. *Systematic Biology*, *41*(4), pp.421-435.

Dennill, G.B. (1986). An ecological basis for timing control measures against the grape vine bud mite *Eriophyes vitis* Pgst. *Crop Protection*, *5*(1), pp.12-14.

Dennill, G.B. (1991). A pruning technique for saving vineyards severely infested by the grape vine bud mite *Colomerus vitis* (Pagenstecher)(Eriophyidae). *Crop protection*, *10*(4), pp.310-314.

Department of Agriculture, Forestry and Fisheries (2012). Grapes: Production Guideline. Available at: https://www.nda.agric.za/docs/brochures/grapesprod.pdf.

Duso, C. and De Lillo, E. (1996). 3.2. 5 Grape. In *World Crop Pests* (Vol. 6, pp. 571-582). Elsevier.

Echlin, P. (1971). The application of scanning electron microscopy to biological research. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *261*(837), pp.51-59.

Echlin, P. (1978). Low temperature scanning electron microscopy: a review. *Journal of Microscopy*, 112(1), pp. 47–61. doi: 10.1111/j.1365-2818.1978.tb01153.x.

Elnifro, E. M., Ashshi, A. M., Cooper, R. B. and Klapper P. E. (2000). Multiplex PCR: Optimization and application in diagnostic virology. *Clinical Microbiology Reviews,* 13 (4), pp. 559-570. doi: 10.1128/CMR.13.4.559-570.2000.

de Faria, M.R. and Wraight, S.P. (2007). Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. *Biological control*, *43*(3), pp.237-256.

Ferragut, F., Gallardo, A., Ocete, R. and López, M.A. (2008). Natural predatory enemies of the erineum strain of *Colomerus vitis* (Pagenstecher) (Acari, Eriophyidae) found on wild grapevine populations from southern Spain (Andalusia). *Vitis-Geilweilerhof-*, 47(1), pp. 51–54.

Fisher, J. R. and Dowling, A. P. G. (2010). Modern methods and technology for doing classical taxonomy. *Acarologia*, 50(3), pp. 395–409. doi: 10.1051/acarologia/20101981.

Folmer, O., Black, M., Hoeh, W., Lutz R. and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology*, 3(5), pp. 294–299. doi: 10.1071/ZO9660275.

Garnas, J.R., Auger-Rozenberg, M.A., Roques, A., Bertelsmeier, C., Wingfield, M.J., Saccaggi, D.L., Roy, H.E. and Slippers, B. (2016).Complex patterns of global spread in invasive insects: Eco-evolutionary and management consequences. *Biological Invasions*. *Springer International Publishing,* 18(4), pp. 935–952. doi: 10.1007/s10530-016-1082-9.

Gärtel, W. (1970). Shoot damage and stunted growth as a result of the simultaneous occurrence of boron deficiency and vine leaf gall mites, *Eriophyes vitis*, in the un-irrigated southern vine-growing regions of Chile. *Weinberg und Keller*, *17*, pp.159-200.

Gerrath, J., Posluszny, U. and Melville, L. (2015). Taming the wild grape: Botany and horticulture in the Vitaceae. *Springer.*

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic biology*, *59*(3), pp.307-321.

Halawa, A. M., Ebrahim, A. A., Abdallah, A. A. M. and Azza, A. M. (2015). Taxonomical Revision of the Genus *Colomerus* Newkirk and Keifer (Acari : Eriophyidae) in Egypt. *Middle East Journal of Agriculture Research*, 4(1), pp. 67–76.

Hebert, P. D. N., Ratnasingham, S. and Jeremy, R. (2003). Barcoding animal life : cytochrome c oxidase subunit 1 divergences among closely related species. pp. 96–99. doi: 10.1098/rsbl.2003.0025.

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. and Hallwachs, W. (2004). Ten species in one : DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*.

Heethoff, M., Laumann, M., Weigmann, G. and Raspotnig, G. (2011). Integrative taxonomy: combining morphological, molecular and chemical data for species delineation in the parthenogenetic *Trhypochthonius tectorum* complex (Acari, Oribatida, Trhypochthoniidae). *Frontiers in Zoology*, *8*(1), pp.1-10.

Hoang, D.T., Chernomor, O., Von Haeseler, A., Minh, B.Q. and Vinh, L.S. (2018). UFBoot2: improving the ultrafast bootstrap approximation. *Molecular biology and evolution*, *35*(2), pp.518-522.

Hu, C. C., Wu, Y.Q., Ma, L., Chen, Y.J. and Ji, X. (2019). Genetic and morphological divergence among three closely related Phrynocephalus species (Agamidae). pp. 1–15.

International Organisation of Vine and Wine (2019). Statistical Report on World Vitiviniculture. Available at: https://www.oiv.int/public/medias/6782/oiv-2019-statistical-report-on-world-vitiviniculture.pdf.

Jinbo, U., Kato, T. and Ito, M. (2011). Current progress in DNA barcoding and future implications for entomology. *Entomological Science,* 14, pp. 107-124. doi:10.1111/j.1479-8

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K., Von Haeseler, A. and Jermiin, L.S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature methods*, *14*(6), pp.587-589.298.2011.00449.x.

Katoh, K., Misawa, K., Kuma, K. and Miyata, T. (2002). MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Research*, 30(14), pp. 3059–3066. doi: 10.1093/nar/gkf436.

Katoh, K. and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, 30(4), pp. 772–780. doi: 10.1093/molbev/mst010.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C. and Thierer, T. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12), pp. 1647–1649. doi: 10.1093/bioinformatics/bts199.

Keifer, H. H. (1944). Eriophyid studies XIV. *Bulletin of the California Department of Agriculture*, *33*(1), pp.18-38.

Keifer, H.H. (1975). The Eriophyoidea Nalepa, injurious eriophyoid mites. *Mites injurious to economic plants*, pp.327-591.

Kido, H. and Stafford, E.M. (1955). The biology of the grape bud mite *Eriophyes vitis* (Pgst.). *Hilgardia*, *24*(6).

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of molecular evolution*, *16*(2), pp.111-120.

Klock, C. L., Botton, M. and Ferla, N. J. (2011). Mitefauna (Arachnida: Acari) associated to grapevine, *Vitis vinifera* L. (Vitaceae), in the municipalities of Bento Gonçalves and Candiota, Rio Grande do Sul, Brazil. *Check List*, 7(4), pp. 522–535.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6), pp. 1547–1549. doi: 10.1093/molbev/msy096.

Lanfear, R., Calcott, B., Ho, S.Y. and Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular biology and evolution*, *29*(6), pp.1695-1701.

van Leeuwen, T., Witters, J., Nauen, R., Duso, C. and Tirry, L. (2010). The control of eriophyoid mites: state of the art and future challenges. *Experimental and Applied Acarology*, 51(1), pp. 205–224. doi: 10.1007/s10493-009-9312-9.

Lefébure, T., Douady, C.J., Gouy, M., Trontelj, P., Briolay, J. and Gibert, J. (2006). Phylogeography of a subterranean amphipod reveals cryptic diversity and dynamic evolution

in extreme environments. *Molecular ecology*, *15*(7), pp.1797-1806. doi: 10.1111/j.1365-294X.2006.02888.x.

Lewandowski, M., Skoracka, A., Szydło, W., Kozak, M., Druciarek, T. and Griffiths, D.A. (2014). Genetic and morphological diversity of *Trisetacus* species (Eriophyoidea: Phytoptidae) associated with coniferous trees in Poland: phylogeny, barcoding, host and habitat specialization. *Experimental and Applied Acarology*, *63*(4), pp.497-520.

Li, H. S., Xue, X. F. and Hong, X.Y. (2014). Cryptic diversity in host-associated populations of Tetra pinnatifidae (Acari: Eriophyoidea): what do morphometric, mitochondrial and nuclear data reveal and conceal? *Bulletin of entomological research*, 104(2), pp. 221–32. doi: 10.1017/S0007485313000746.

de Lillo, E., Craemer C., Amrine, J.W. and Nuzzaci, G. (2010). Recommended procedures and techniques for morphological studies of Eriophyoidea (Acari: Prostigmata). *Experimental and Applied Acarology*, 51(1), pp. 283–307. doi: 10.1007/s10493-009-9311-x.

Lindquist, E.E. and Amrine, J.W. Jr. (1996). Systematics, Diagnoses for Major Taxa, and Keys to Families and Genera with Species on Plants of Economic Importance. pp 33–87 In: Lindquist, E.E.; Sabelis, M.W. & Bruin, J. (Eds). Eriophyoid Mites: Their Biology, Natural Enemies and Control. *World Crop Pests 6. Elsevier Science*, Amsterdam.

Lindquist, E. E., Sabelis, M. W. and Bruin, J. (1996). *Eriophyoid mites: their biology, natural enemies and control.* Elsevier. Edited by J. Lindquist, E.E.; Sabelis, M.W. and Bruin.

Liu, Q., Yuan, Y.M., Lai, Y., Wang, G.Q. and Xue, X.F. (2019). Unravelling the phylogeny, cryptic diversity and morphological evolution of *Diptilomiopus* mites (Acari: Eriophyoidea). *Experimental and Applied Acarology*, *79*(3), pp.323-344.

Mahani, M.K., Inomata, N., Saboori, A., Tabatabaei, B.E.S., Ishiyama, H., Ariana, A. and Szmidt, A.E. (2009). Genetic variation in populations of *Allothrombium pulvinum* (Acari: Trombidiidae) from Northern Iran revealed by mitochondrial cox I and nuclear rDNA ITS 2 sequences. *Experimental and Applied Acarology*, *48*(4), pp.273-289.

Manson, D.C. (1984). Eriophyinae (Arachnida: Acari: Eriophyoidea). *Fauna of New Zealand*, *5*.

Manson, D. C. M. and Oldfield, G. N. (1996). Chapter 1.4 Biology and ecology 1.4.1 Life forms, deuterogyny, diapause and seasonal development. *World Crop Pests*. Elsevier, 6

(Eriophyoid Mites Their Biology, Natural Enemies and Control), pp. 173–183. doi: 10.1016/S1572-4379(96)80009-1.

Mathez, F. (1965). *Contribution a l'étude morphologique et biologique d'Eriophyes vitis Pgst., agent de l'Erinose de la vigne* (Doctoral dissertation, ETH Zurich).

Matsuda, T., Fukumoto, C., Hinomoto, N. and Gotoh, T. (2013). DNA-based identification of spider mites: Molecular evidence for cryptic species of the Genus Tetranychus (Acari: Tetranychidae). *Journal of Economic Entomology*, 106(1), pp. 463–472. doi: 10.1603/EC12328.

McGovern, P.E. (2003). Ancient wine: the search of the origin of the viniculture. *Princeton University Press, New Jersey*.

Meyer, M. (Smith) and Ueckermann, E. A. (1990). African Eriophyoidea: the genus *Colomerus* Newkirk and Keifer, 1971 (Acari: Eriophyidae). *Phytophylactica*, 22, pp. 15–21.

Meyer, M. K. P. (Smith) and Craemer, C. (1999). Mites (Arachnida: Acari) as crop pests in southern Africa: An overview. *African Plant Protection*, 5(1), pp. 37–51.

Meyer, C. P. and Paulay, G. (2005). DNA barcoding: Error rates based on comprehensive sampling. *PLoS Biology*, 3(12), pp. 1–10. doi: 10.1371/journal.pbio.0030422.

Minh, B.Q., Schmidt, H.A., Chernomor, O., Schrempf, D., Woodhams, M.D., Von Haeseler, A. and Lanfear, R. (2020). IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Molecular biology and evolution*, *37*(5), pp.1530-1534.

Miller, A. D., Skoracka, A., Navia, D., de Mendonca, R.S., Szydło, W., Schultz, M.B., Smith, C.M., Truol, G. and Hoffmann, A.A. (2013).Phylogenetic analyses reveal extensive cryptic speciation and host specialization in an economically important mite taxon. *Molecular Phylogenetics and Evolution*, 66(3), pp. 928–940. doi: 10.1016/j.ympev.2012.11.021.

Moore, M.O. (1991). Classification and systematics of eastern North American *Vitis* L.(Vitaceae) north of Mexico. *Sida, contributions to botany*, pp.339-367.

Myles, S., Boyko, A.R., Owens, C.L., Brown, P.J., Grassi, F., Aradhya, M.K., Prins, B., Reynolds, A., Chia, J.M., Ware, D. and Bustamante, C.D. (2011). Genetic structure and domestication history of the grape. *Proceedings of the National Academy of Sciences*, *108*(9), pp.3530-3535.

Navajas, M. and Navia, D. (2010). DNA-based methods for eriophyoid mite studies: Review, critical aspects, prospects and challenges. *Experimental and Applied Acarology*, 51(1), pp. 257–271. doi: 10.1007/s10493-009-9301-z.

Navia, D., De Moraes, G.J., Roderick, G. and Navajas, M. 2005. The invasive coconut mite Aceria guerreronis (Acari: Eriophyidae): origin and invasion sources inferred from mitochondrial (16S) and nuclear (ITS) sequences. *Bulletin of Entomological Research,* 95, pp. 505–516.

Navia, D. and Flechtmann, C.H. (2005). A new eriophyoid mite in the genus *Disella* from Ilex paraguariensis in Brazil. *Zootaxa*, *1037*(1), pp.23-28.

Navia, D., Ochoa, R., Welbourn, C. and Ferragut, F. (2010). Adventive eriophyoid mites: A global review of their impact, pathways, prevention and challenges. *Experimental and Applied Acarology*, 51(1), pp. 225–255. doi: 10.1007/s10493-009-9327-2.

Oldfield, G.N. 1996. Diversity and host plant specificity. pp 199–216 In: Lindquist, E.E.; Sabelis, M.W. & Bruin, J. (Eds). Eriophyoid Mites: Their Biology, Natural Enemies and Control. *World Crop Pests 6. Elsevier Science,* Amsterdam.

Pearce, I. and Australia, V. (2019). A comprehensive review of Grapevine Pinot gris virus (GPGV) FINAL REPORT TO WINE AUSTRALIA Project Number : VHA 1701 Principal Investigator : Inca Pearce Research Organisation : Vinehealth Australia (January).

Pentanovic, R. U. (2016). Towards an integrative approach to taxonomy of Eriophyoidea (Acari , Prostigmata) - an overview. *Ecologica Montenegrina*, 7, pp. 580–599.

Petanović, R. and Kielkiewicz, M. (2010). Plant-eriophyoid mite interactions: Specific and unspecific morphological alterations. Part II. *Experimental and Applied Acarology*, 51(1), pp. 81–91. doi: 10.1007/s10493-009-9328-1.

Pons, J. and Barraclough, T.G., G ómez-Zurita, J., Cardoso, A., Duran, D. P., Hazell, S., Kamoun S., Sumlin W. D. and Vogler A. P. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology*, 55(4), pp. 595–609. doi: 10.1080/10635150600852011.

Powell, C., Caleca, V., Knipe, M., Rhode, C., Allsopp, E. and van Asch, B. (2020). DNA-based identification of larvae offers insights into the elusive lifestyles of native olive seed wasps in South Africa. 28(2), pp. 433–446.

Prime, G. (2012). User Manual Geneious Prime. *Data Base*, 3304(January), pp. 1–148.

Pryke, J. S. and Samways, M. J. (2007). Current control of phytosanitary insect pests in table grape vineyards of the Hex River Valley , South Africa. *African Entomology*, *15*(1), pp.25-36.doi: 10.4001/1021-3589-15.1.25.

Rahbani, J., Behzad, A.R., Khashab, N.M. and Al-Ghoul, M. (2013). Characterization of internal structure of hydrated agar and gelatin matrices by cryo-SEM. *Electrophoresis*, 34(3), pp. 405–408. doi: 10.1002/elps.201200434.

Ryke, P. and Meyer, M. (1960). South african gall mites, rust mites and bud mites (Acarina : Eriophyidae) of economic importance. *South African Journal of Agricultural Science* 3(2), pp. 231–242.

Sabatini, D. D., Bensch, K. and Barrnett, R. J. (1963). Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *The Journal of cell biology*, 17, pp. 19–58. doi: 10.1083/jcb.17.1.19.

Saccaggi, D. L., Craemer, C. and Venter, J. H. (2012). Mites intercepted on grapevine material imported to South Africa. *7th Symposium of the European Association of Acarologists (EURAAC)*. Vienna, Austria, pp. 81–82.

Schlick-Steiner, B.C., Steiner, F.M., Seifert, B., Stauffer, C., Christian, E. and Crozier, R.H. (2010). Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual review of entomology*, *55*, pp.421-438.

Skoracka, A. (2009). Description of *Abacarus lolii* n. sp.(Prostigmata: Eriophyoidea: Eriophyidae), a cryptic species within a grass–feeding *Abacarus* complex. *International Journal of Acarology*, *35*(5), pp.405-417.

Skoracka, A. and Dabert, M. (2010). The cereal rust mite *Abacarus hystrix* (Acari: Eriophyoidea) is a complex of species: evidence from mitochondrial and nuclear DNA sequences. *Bulletin of entomological research*, 100(3), pp. 263–272. doi: 10.1017/S0007485309990216.

Skoracka, A. and Magalha, S. (2015). Cryptic speciation in the Acari : a function of species lifestyles or our ability to separate species ? *Experimental and Applied Acarology, 67(2),* pp.165-182*.* doi: 10.1007/s10493-015-9954-8.

Slepyan, E.I., Landsberg, G.S. and Maltshenkova, N.I. (1969). The gall of the mite Eriophyes vitis Pgst. (Acarina, Eriophyidae) as its ecological niche. *Entomol. Rev*., 48: 67-74.

Smith, L.M. and Stafford, E.M. (1948). The bud mite and the erineum mite of grapes. *Hilgardia*, *18*(7).

Smith, L.M. and Schuster, R.O. (1963). The nature and extent of *Eriophyes vitis* injury to *Vitis vinifera* L. *Acarologia*, *5*(4), pp.530-539.

Smith Meyer, M.K. (1981). *Mite pests of crops in southern Africa*. Department of Agriculture and Fisheries. Science Bulletin 397, 92pp.

South African Wine Industry Information and Systems (2021). Macro-Economic Impact of the Wine Industry on South African Economy. Available at: https://www.sawis.co.za/info/download/Macro-economic_Impact_of_the_Wine_Industry_2019_Final_(2).pdf.

Terral, J.F., Tabard, E., Bouby, L., Ivorra, S., Pastor, T., Figueiral, I., Picq, S., Chevance, J.B., Jung, C., Fabre, L. and Tardy, C. (2010). Evolution and history of grapevine (Vitis vinifera) under domestication: new morphometric perspectives to understand seed domestication syndrome and reveal origins of ancient European cultivars. *Annals of botany*, *105*(3), pp.443-455.

Valenzano, D., Tumminello, M.T., Gualandri, V. and de Lillo, E. (2020). Morphological and molecular characterization of the *Colomerus vitis* erineum strain (Trombidiformes: Eriophyidae) from grapevine erinea and buds. *Experimental and Applied Acarology*, *80*(2), pp.183-201. doi: 10.1007/s10493-020-00470-w.

Veikondis, R., Burger, P., Vermeulen, A., Van Heerden, C.J. and Prins, R. (2018). Confirmation of the effectiveness and genetic positions of disease resistance loci in “Kishmish Vatkana” (Ren1) and “Villard Blanc” (Ren3 and Rpv3). *South African Journal of Enology and Viticulture*, 39(2), pp. 185–195. doi: 10.21548/39-2-2685.

Vermaak, M., Ueckermann, E.A., Veldtman, R. and Addison, P. (2021). An Overview of Mites on Grapevine and the Discovery of a New Phytoseiidae Species: Typhlodromus (Typhlodromus) spiceae. *South African Journal of Enology and Viticulture*, *42*(1), pp.91-99.

Vidović, B., Cvrković, T., Marić, I., Chetverikov, P.E., Cristofaro, M., Rector, B.G. and Petanović, R. (2015). A new *Metaculus* species (Acari: Eriophyoidea) on *Diplotaxis tenuifolia*

(Brassicaceae) from Serbia: a combined description using morphology and DNA barcode data. *Annals of the Entomological Society of America*, *108*(5), pp.922-931.

Wagener, B., Reineke, A., Löhr, B. and Zebitz, C.P. (2006). Phylogenetic study of Diadegma species (Hymenoptera: Ichneumonidae) inferred from analysis of mitochondrial and nuclear DNA sequences. *Biological Control*, *37*(2), pp.131-140.

Wang, J.F. and Qiao, G.X. (2009). DNA barcoding of genus *Toxoptera* Koch (Hemiptera: Aphididae): identification and molecular phylogeny inferred from mitochondrial COI sequences. *Insect Science*, *16*(6), pp.475-484.

Wares, J.P. and Cunningham, C.W. (2001). Phylogeography and historical ecology of the North Atlantic intertidal. *Evolution*, *55*(12), pp.2455-2469.

Wen J. (2007). Vitaceae. In: Kubitzki K ed. *The families and genera of vascular plants*. Berlin: Springer–Verlag. **9**: 467–479.

Wines of South Africa (2021). History: Three Centuries of Cape Wine. Available at: https://www.wosa.co.za/The-Industry/History/Three-Centuries-of-Cape-Wine/.

Xue, X. F., Han, X. and Zhang, Z. Q. (2015). Correct identification and biosecurity decision-making: Two species instead of one in Aceria genistae complex (Acari: Eriophyidae) in New Zealand. *Systematic and Applied Acarology*, 20(1), pp. 71–86. doi: 10.11158/saa.20.1.8.

Zaher, M.A. (1984). Survey and ecological studies on phytophagous, predaceous and soil mites in Egypt. I: Phytophagous mites in Egypt (Nile valley and Delta). *PI 480 Programme. USA Project No. EG. ARS, 30. Grant. No, FG, Eg.,* 81. 228pp

Zhang, D.X. and Hewitt, G.M. (1997). Assessment of the universality and utility of a set of conserved mitochondrial COI primers in insects. *Insect Molecular Biology*, *6*(2), pp.143-150.