Diversity of fruit flies (Diptera: Tephritidae) from the South African Highveld and Lowveld: a preliminary assessment of *Wolbachia* infection

By

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Summary

Food security, as far as horticultural production is concerned, requires research into pest control methods at all stages of production. Tephritid fruit flies are among the most serious agricultural pests worldwide, owing to damage caused to vegetables and fruit through female oviposition, and larval development in the fruit, stems and seeds of the host plant. As a result of the large cost of pest management programmes, research into efficient and environmentally benign pest control strategies are always required. One such pest management strategy is the implementation of the incompatible insect technique (IIT) which involves the control of pest populations through bacterium-mediated reproductive suppression.

A potential IIT strategy involves *Wolbachia*-mediated population control. *Wolbachia* is a widespread arthropod endosymbiont that is capable of host reproduction manipulations such as cytoplasmic incompatibility (CI), male killing, and female parthenogenesis, to name a few phenotypic effects. The presence of *Wolbachia* has been confirmed in ~66% of the ~87 tephritid species screened through PCR and sequencing approaches to date, often occurring at a low prevalence which would be advantageous when implementing *Wolbachia*-driven IIT strategies. This project aims to assess the prevalence of *Wolbachia* in fruit flies from the South African highveld and lowveld, as a first step in establishing the viability of a *Wolbachia*-driven IIT control method. To satisfy this aim, this project ascertained the diversity of tephritid fruit flies in the two regions through morphological and molecular identification techniques, screened the collected fruit flies for the native *Wolbachia* endosymbiont, and determined the supergroup to which the *Wolbachia* endosymbiont detected belongs.

A total of 2989 fruit flies were collected during sampling. These fruit flies were identified using morphological keys and found to belong to nine species and three genera. Three of these species, *Bactrocera dorsalis*, *Ceratitis capitata* and *C. cosyra*, were collected across both regions. Species unique to the highveld were *C. pedestris*, *C. quilicii*, *C. simi* and *Dacus ciliatus*, while those unique to the lowveld were *C. rosa* and *C. rubivora*. The structure of these fruit fly assemblages was found to be influenced by the region of collection. The Simpson diversity index indicated that certain species were numerically dominant in the highveld whereas abundance of each species was more even in the lowveld. However, neither the species richness nor the Shannon-Wiener diversity index was significantly different between the two regions.

A subset of 211 specimens were selected for molecular identification using *COI* barcoding primers. Similar to prior studies, we found that *COI* barcoding was incapable of discerning between members of the *Ceratitis* 'FARQ' species complex. The *Ceratitis* FARQ species complex (formerly *Ceratitis* FAR complex) is a grouping of four frugivorous tephritids, *Ceratitis fasciventris*, *C. anonae*, *C. rosa* and *C. quilicii*. The remaining seven species were, however, readily identified through *COI* barcoding. Furthermore, *COI* barcoding successfully revealed the haplotypes that were present in each species. For *B. dorsalis*, a total of five haplotypes were recovered from 47 specimens indicating low haplotype diversity in both regions. Higher levels of haplotype diversity were observed in *C. capitata* (with 30 haplotypes identified from 40 specimens) and *C. cosyra* (35 haplotypes from 53 specimens). For *C. pedestris* (11 haplotypes from 47 specimens) and the two FARQ complex members 17 haplotypes were identified from 52 specimens. The differences in haplotype diversity are likely attributable to the origins of the fruit flies as *B. dorsalis* is an invasive fruit fly species while the other species are endemic to Africa. Furthermore, when performing a Mantel Test with *COI* barcode data, no pattern of correlation between genetic and geographic distances was discernible, indicating an absence of isolation by distance.

Initial *Wolbachia* screening was performed on 246 fruit flies from all the sampling locations. A positive result was obtained from the Mooinooi sampling site, thus locality-specific screening was performed. An additional 331 specimens sampled from Mooinooi were screened bringing the total number of fruit flies screened for the presence of *Wolbachia* to 616. Screening was performed using a two-gene approach in which the *16S rRNA* and *wsp* gene regions were targeted for amplification. There was only one confirmed detection in a male *C. cosyra* fruit fly from the Mooinooi sampling site in the highveld. This corresponds to an overall *C. cosyra* prevalence of 0.005 while for the remaining species, namely *B. dorsalis*, *C. quilicii*, *C. capitata* and *C. pedestris* there was no evidence for *Wolbachia* infection. Genetic characterization of the *Wolbachia* detected in *C. cosyra* revealed that the strain belongs to *Wolbachia* supergroup B, a supergroup associated with multiple arthropods. This supergroup assignment was confirmed through individual gene (*wsp*; *Wolbachia* surface protein) and concatenated multi-locus (*gatB, ftsZ, hcpA, coxA, fbpA* and *wsp*) phylogeny, with sequence data generated through a combination of PCR-Sanger sequencing and PacBio next generation sequencing (NGS). Based on this low *Wolbachia* prevalence, the implementation of *Wolbachia*-driven IIT appears to be plausible if the phenotypic effects of the strain detected do not include the curing of other *Wolbachia* strains. Thus, following a successful trans-infection with a cytoplasmic

incompatibility causing strain of *Wolbachia*, either uni-directional or bi-directional CI may be used as a form of pest control.

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Declaration

I, Onkgopotse Seabi, declare that the dissertation, which I hereby submit towards the degree Magister Scientiae (Entomology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: ……

DATE: 12/12/2022

Disclaimer

This dissertation contains three research chapters that have been prepared as stand-alone manuscripts for publication purposes. Therefore, some unavoidable repetition may be observed between chapters.

Chapter 1. General Background

1.1.General background

1.1.1. The need for food security

The United Nations has defined global food security as a scenario in which "all people have physical and economic access to sufficient, safe and nutritious food that meets their food preferences and dietary needs for an active and healthy life" (FAO 1996). Threats to food security include an increasing human population and climate change, thus scientists have been looking for ways to protect and increase food crop yields on farms (Faisal and Parveen 2004, Wheeler and Von Braun 2013). Food security is particularly important in sub-Saharan Africa where nearly a quarter of the human population is undernourished (FAO 2013) and undernourishment is projected to increase due to a proportionally higher increase in the human population on this continent (Rahman and Westley 2001, Bongaarts 2009, Ekesi et al. 2009b).

An increase in agricultural production is vital to mitigate the effects of population growth and climate change. Scientists and policymakers recognise that agroecology has the potential to address environmental and social issues within food production (Bezner Kerr et al. 2021). Agroecology refers to the holistic approach that incorporates ecological, health, social, and economic considerations into agricultural and food systems design and implementation (Bezner Kerr et al. 2021). Agroecological practices aim to optimize ecological processes and minimize social-ecological costs from agriculture such as soil degradation, water contamination, greenhouse gas emissions, exhaustion of non-renewable resources, and inequitable social structures (Wezel et al. 2014, Agroecological 2019).

1.1.2. Agroecosystems and pests

Agroecosystems refer to human-modified ecological systems used for the production of food, fibre or other agricultural products (Conway 1987). Agroecosystems are created through the transformation of the great diversity of original natural systems to a simpler assemblage of crops, pests, weeds, beneficial, and non-invasive species. These systems are characterized by high net productivity, simple linear trophic chains, low species and genetic diversity, open mineral cycles, low resilience (stability), high entropy, simple habitat heterogeneity and synchronized phenology (Odum 1969, Altieri et al. 1983). Monocultures are favoured as they align with agricultural objectives, which are generally to achieve maximum profit, minimize year-to-year instability in production, and prevent long-term degradation of the productive capacity of the agroecosystem.

South Africa reported production losses in the agricultural sector of 2.3% for the 2017/18 period, of which 0.5% was due to a decrease in horticultural production (DAFF 2019b). This is despite the horticulture sector being a major economic value generator as it provides opportunities for exports as well as other economy stimulating activities (DAFF 2019b, a). South African agricultural production is conducted on farms, which are an example of an agroecosystem. These environments have a dense concentration of basic food resources for arthropod pests; thus, control methods need to be dispatched to compensate for the lack of self-regulating mechanisms in monocultures (Root 1973, Nicholls and Altieri 2004). Furthermore, as South Africa engages in trade to interregional and global markets, arthropod pests must be monitored, and control solutions acceptable to consumers must be developed.

To counter losses caused by insects and other pests, a vast array of control methods have been developed. Suggested methods mostly focus on the manipulation of biotic factors as abiotic factors can only be studied and manipulated on a limited scale (Smith 2000). The manipulation of biotic factors includes management techniques such as biological control of pests (Ekesi et al. 2009b). In such approaches, one organism is used to reduce the population density of another organism (including animals, weeds, and diseases). Biological control reduces rather than eradicates pests, such that the pest and natural enemy remain in the agroecosystem at low densities (Bale et al. 2008). DeBach (1964) defined arthropod biological control as 'the study and uses of parasites, predators, and pathogens for the regulation of host (pest) densities. Many biological control schemes targeted against insect and mite pests use predatory insects and mites, insects that parasitize other insects (parasitoids) or nematodes; these are referred to as 'macrobial agents' (Bale et al. 2008). In contrast, the term 'microbial agents' refers to bacteria, viruses and fungi that are developed and applied for arthropod biological control.

Efficient pest control methods could lead to the alleviation of undernourishment, improved food quality, as well as an increase in contribution to international markets (Ekesi et al. 2009b). However, before pest control can be considered the pest needs to be identified and studied. This is very important for tailoring tactics to effectively control pest populations. A group of arthropod pests that is monitored throughout global and South African horticultural ecosystems are the true fruit flies (Diptera: Tephritidae). Tephritid flies are insects that cause damage to crops through female oviposition and larval development in the fruit, stems, and seeds of host plants (Aluja 1994). Female oviposition results in the introduction of spoilage microbes, which lead to fruit breakdown. Larval development is dependent on ingestion of the decaying flesh

of the fruit. This damage may reduce the yield by up to 80% depending on the plant variety and season (Ekesi et al. 2014).

Reduction in yield, along with expensive control measures, the potential imposition of quarantine restrictions or a complete block on market access, support the need for further research into control methods for these pests (Verghese et al. 2004). Several International Standards for Phytosanitary Measures have been published by the International Plant Protection Convention to address invasion by fruit flies (Devorshak 2007, Lefebvre et al. 2015). These measures include the development of legislation or action plans to decrease the spread and damage caused by invasive species. In order to align themselves with the requirements of different legislative bodies, agricultural practitioners implement different control practices. A common prerequisite of these control practices is the correct identification of the insect pest (Nyeko et al. 2002, Lefebvre et al. 2015). White and Elson-Harris (1992) reported on the morphology and distribution of fruit flies, the information from this publication is regularly updated with further morphological identifications, molecular and behavioural studies. This is essential because of the high levels of diversity, invasion potential and associated changes in geographical range/distribution. The geographical range/distribution of fruit flies is constantly changing owing to the human-mediated transportation of infected fruits and vegetables (Saccaggi et al. 2016). Thus, it is important to ensure accurate identifications for accurate compilation of geographical distribution and range. It is therefore clear that novel sustainable control methods can only be developed with an understanding of this family of fruit flies.

1.1.3. Tephritid fruit flies

Tephritidae represents one of the largest dipteran families, comprising 4,200 species within 500 genera, worldwide (White and Elson-Harris 1992, Aluja and Norrbom 1999). Of these, approximately 250 species are of economic importance due to their association with commercial fruit and vegetable crops (White and Elson-Harris 1992). The recorded African tephritids of economic importance span 150 species within more than 50 genera (Grové and de Beer 2012). Globally, the most relevant horticultural pest genera include *Anastrepha* (Schiner, 1868), *Bactrocera* (Macquart, 1846), *Ceratitis* (Macleay, 1829), *Dacus* (Fabricius, 1805), *Rhagoletis* (Loew, 1862), *Trirhithrum* (Bezzi, 1918), and *Zeugodacus* (Hendel, 1927) (White and Elson-Harris 1992, Grové and de Beer 2012, Birke et al. 2013). These genera each have their own native distribution and host-plant interactions.

Ceratitis is a predominantly Afrotropical genus and comprises an estimated 95 described species. The larvae of this genus develop in fruit. *Ceratitis* is hypothesised to have evolved in East Africa (Baliraine et al. 2004). For the cosmopolitan *C. capitata* (Weidemann, 1824; Figure 1.1.a), South Africa is considered part of its native range owing to higher genetic variation than other countries around the world and the lack of genetic difference between individuals found in South Africa and elsewhere in Africa (Karsten et al. 2015). It is widely distributed throughout agroecosystems in South Africa. In contrast, *C. rosa* (Karsch, 1887; Figure 1. 1.b) only occurs in warmer, wetter and more coastal parts of the country (De Meyer et al. 2008). Early documentation on *C. rosa s.l.* indicated that there was an "R2", "cool" or "highland" morphotype (Virgilio et al. 2013, Tanga et al. 2018). Subsequent investigations that derived behavioural (Juarez et al. 2015), genetic (Virgilio et al. 2018), morphological and physiological evidence (De Meyer et al. 2016) ultimately led to the "R2" morphotype being elevated to species status, viz. *Ceratitis quilicii* (De Meyer, Mwatawala and Virgilio, 2018; Figure 1.1.c). This species prefers cool higher altitudes whereas *C. rosa s.s* prefers warm, lower altitudes (Mwatawala et al. 2015). However, in the northern parts of South Africa, *C. quilicii* and *C. rosa* occur sympatrically in some regions (Virgilio et al. 2013).

The marula fruit fly, *Ceratitis cosyra* (Walker, 1849; Figure 1.1.d), infests mango and guava, but its distribution in southern Africa is limited to the subtropical regions of the North East and East Coast and follows the distribution of the marula tree, *Sclerocarya birrea*, which is an important wild host (De Villiers et al. 2013). In South Africa, *Ceratitis pedestris* (Bezzi, 1924; Figure 1.1.g) is associated with stone fruits and thus is a pest found in litchi orchards (Grové and de Beer 2012). Furthermore, this species has strong associations with the plant genus *Strychnos* (Loganiaceae; Linnaeus, 1753), several species of which occur naturally in southern Africa (Adebowale 2014), and tomato (Solanaceae; Linnaeus) (De Meyer and Freidberg 2005, Grové and de Beer 2012). Another member of the *Ceratitis* genus found in South Africa is *C. rubivora* (Coquillett, 1901;Figure 1.1.h), the distribution of this fruit fly is linked to regions used for the production of blackberry (*Rubus fruticosus*; Linnaeus), loganberry (*Rubus loganobaccus*; James Harvey Logan,1881) and raspberry (*Rubus idaeus*; Linnaeus) and the regions with indigenous berries (Karsten et al. 2018, Sochor et al. 2018).

Another indigenous fruit fly genus is *Dacus*, which contains 177 described species most of which are endemic to Africa. *Dacus ciliatus* (Loew,1862; Figure 1.1.e) is reported throughout Africa and infests at least 16 host plants belonging to the Cucurbitaceae, with research efforts

to control this pest focussing on melon, pumpkin, and squash (Vayssières et al. 2008, Kamali et al. 2013).

In addition to these indigenous fruit flies, *Bactrocera* represents a genus of Indo-Australian origin, with only 11 species present in Africa (Grové and de Beer 2012). This includes the highly invasive *Bactrocera dorsalis* (Hendel, 1912; Figure 1.1.f) which, following first detection in 2003, has spread throughout sub-Saharan Africa (Lux et al. 2003). The spread of *B. dorsalis* is suggested to be due to its broad host range, wide climate tolerance and high dispersal capacity that is coupled to global trade routes (Peterson and Denno 1998, Wan et al. 2012). In 2007 and 2008, a single male specimen was intercepted on South Africa's northern border and the incursion was confirmed in 2010 when several individuals were detected in three provinces; Limpopo, North-West and Gauteng (Manrakhan et al. 2015). Ecological niche and climatic models based on the global distribution of *B. dorsalis* have predicted the potential for further range expansion southwards, in South Africa (Stephens et al. 2007, Hill and Terblanche 2014).

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

Figure 1.1 Images of (a) *Ceratitis capitata* photographed by Andre Coetzer, (b) *Ceratitis rosa* male photographed by Nina Parry, (c) Male and female *Ceratitis quilicii* photographed by Nina Parry, (d) *Ceratitis cosyra* photographed by Andre Coetzer, (e) *Dacus ciliatus* extracted from Qatar eNature, (f) *Bactrocera dorsalis* photographed by Nina Parry, (g) *Ceratitis pedestris* photographed by Nina Parry and (h) *Ceratitis rubivora* photographed by Nina Parry.

1.1.4. Molecular identification of tephritid fruit flies

The development of fruit fly control protocols is dependent on their correct identification. Currently, this identification is mostly ascertained through the use of morphological characteristics (White and Elson-Harris 1992, Aluja and Norrbom 1999). However, agricultural practitioners, dipterists and policymakers have found that morphological characteristics yield poor resolution of higher relationships within the Tephritidae family (Aluja and Norrbom 1999). Furthermore, the use of molecular makers can provide molecular identification or diagnostics for fruit fly species at their various developmental stages (Dhami et al. 2016). This has resulted in research into alternative species identification methods, including the use of molecular techniques (Armstrong and Ball 2005).

Oligonucleotide arrays using molecular markers derived from intron sequence polymorphisms were used for the identification of *B. dorsalis* (Naeole and Haymer 2003). Although this method together with species-specific PCR methods allows the discrimination between some of the closely related species in the *B. dorsalis* complex, it cannot delineate species which share alleles (Naeole and Haymer 2003). Early literature demonstrates cytochrome oxidase I (*COI*) barcoding as reliable for the discrimination of a diverse range of taxa to species level, with this method being utilised to identify insects belonging to the Ephemeroptera (Ball et al. 2005) and Phthiraptera (Whiteman et al. 2004). However, other studies using *COI* barcoding found that variation in this gene was insufficient for separating members in the *Ceratitis* 'FARQ" complex (Jiang et al. 2014), which includes *C. rosa* and *C. quilicii*.

Unfortunately, while whole mitochondrial or nuclear genome sequencing (single nucleotide polymorphism or "SNPs") may be one of the more effective host identification methods, it is very expensive and would not be accessible to all countries for pest identification (Bennette et al. 2015, Papanicolaou et al. 2016). As a result, an accurate, cost-effective molecular identification method is required to allow for the development of effective control methods through the confirmation of pest identity. It has been suggested that a combination of *COI* barcoding (mitochondrial genome) and microsatellite profiling (nuclear genome) would allow for separation of members within species complexes (Nardi et al. 2003). Moreover, haplotype networks allow for intraspecific/population-level analyses that permit inferences of genealogical relationships, biogeography, and the relatedness of populations (Leigh and Bryant 2015). Once the identity of the pest has been clarified, tailored control methods can be considered for the identified pest.

1.1.5. Tephritid control methods

Pest identification may assist with the implementation and the design of pest control methods aimed at various fruit flies. Research into the development of more effective control methods is supported by the limited success observed with fruit fly trapping, insecticidal control, and biological control (Antonidakj et al. 1991, Carey 1991, Daane and Johnson 2010). An integrated programme utilising these established techniques seems most promising, however insecticidal control of tephritid populations has been challenged by the development of resistance in fruit flies (Vargas et al. 2008, Vontas et al. 2011).

Control of pests with insecticides is fraught with problems such as resistance, fruit contamination and the elimination of beneficial insects. Research into alternative approaches, including genetic control methods is therefore supported (Vargas et al. 2008). The sterile insect technique (SIT) is a cost-effective and environmentally friendly genetic control method that involves the release of sterilised males to induce unsuccessful mating (Enkerlin 2005). The species-specific manner in which SIT works as well as the fact that resistance is less likely to develop makes this a desirable control method. However, SIT is hampered by problems that include below standard fruit fly rearing, refugia harbouring fertile breeding fruit flies, and funding (Barnes et al. 2002). These limitations have led to other control protocols being considered. One of these is the Incompatible Insect Technique (IIT), which is an approach that uses microbe interactions, mainly *Wolbachia*-driven, to render insects incapable of producing viable offspring (Pagendam et al. 2020).

1.1.6. Effects of microbial interaction on fruit flies

Microbial interactions are known to benefit insects through nutritional supplementation, tolerance to environmental perturbations and maintenance or the development of the host immune system (Bahrndorff et al. 2016). A study by Behar et al. (2008) investigating the nutritional role of gut microbes revealed that the intestinal bacteria found in *C. capitata* provide the host with significantly higher amounts of lipids and proteins when compared to antibiotic-treated flies. The gut bacteria in *B. dorsalis* might produce enzymes that help in the digestion of polysaccharides, such as xylan, pectin, cellulose and starch (Saha and Ray 2015). The contribution of various microbes to the nutrition of their hosts includes nitrogen fixation as seen in *B. tryoni* fed dinitrogen-fixing strains of *Klebsiella oxytoca* and *Enterobacter* spp., which have nitrogenase activity (Murphy et al. 1988). The natural diet of wild flies is poor in organic nitrogen but does contain amino acids, uric acids, and proteins. Thus, microbial

contributions in digestion, nitrogen fixing, and amino acid breakdown aid in nutrient acquisition for fruit flies (Raza et al. 2020). Beneficial microbial interactions with tephritids extend to behavioural changes as Damodaram et al. (2016) found that *B. dorsalis* males were attracted to females that were positive for infection with *K. oxytoca*. Cheng et al. (2017) found that a gut microbe belonging to the genus *Citrobacter* plays a role in the degradation of trichlorphon, which is an organophosphate insecticide leading the authors to conclude that symbiont-mediated insecticide resistance can be developed in *B. dorsalis*.

Microbes have also been reported to have detrimental effects on tephritid fitness. Sarakatsanou et al. (2011) reported that an artificial *Wolbachia pipientis* (Hertig) infection in *C. capitata* resulted in reduced fitness through shortening of adult lifespan, reduction of female fecundity, and in some lines, increasing egg-to-adult mortality. Bacteria from the genus *Wolbachia* have also been mentioned as a possible cause for speciation mediated through interference with host reproductive systems. This is evident in a Brazilian study done on the South American fruit fly, *Anastrepha fraterculus* (Wiedemann), which suggests that cytoplasmic incompatibility may give rise to pre-zygotic isolation and speciation (Dias et al. 2016). Although an impressive find, further research is needed to ascertain whether this is species-level or population-level speciation (Dias et al. 2016). As research has found that *Wolbachia* infection can lead to a wide range of outcomes, *Wolbachia*-driven manipulation has been considered as a genetic control method for arthropods, however further research into *Wolbachia*-tephritid interactions is needed.

1.1.7. *Wolbachia*

The genus *Wolbachia* (Hertig, 1936) comprises widespread arthropod endosymbionts that occur in an estimated 20-50% of invertebrates worldwide (Haine et al. 2005, Hilgenboecker et al. 2008). *Wolbachia* belongs to the family Anaplasmataceae, which is closely related to the tick-borne genera *Ehrlichia* and *Anaplasma*. The current consensus is that *Wolbachia* shares a recent common ancestor with *Ehrlichia*, with their divergence associated with a derived ability in *Wolbachia* to manipulate host reproduction (Anderson and Karr 2001). *Wolbachia* classification is based on the delineation of 16 supergroups annotated alphabetically from A-F and H-Q (Bleidorn and Gerth 2017). The term supergroup is employed to avoid confusion with designation of more closely related groups (Zhou et al. 1998b). The separation into the various supergroups is based on their phylogenetic relationships and investigations into these groups have also revealed similar symbiotic relationships and host specificity in certain supergroups

(Bleidorn and Gerth 2017). Whereas supergroups A and B occur in a broad range of arthropods and F occurs in diverse nematodes and arthropods, supergroups M, N, O, P are highly specific (Table 1.1; Gerth et al. 2014). *Wolbachia* are present in most arthropod tissues, including ovaries and testes (Dobson et al. 1999, Cheng et al. 2000).

Table 1.1: Summary of the hosts and the lifestyle of 13 of the 16 *Wolbachia* supergroups described (adapted from Lo *et* al. (2002), Werren *et* al. (1995) and Wang *et* al. (2016)). The lifestyles for supergroups H-Q have not been reported.

Wolbachia Supergroup	Host/s	Lifestyles
A and B	Arthropods	Parasitism, facultative, proximate & obligate mutualism
C and D	Filarial nematodes	Obligate mutualism
E	Springtails and mites	Parasitism/evolved dependencies
F	Termites and nematodes	Parasitism/obligate mutualism
H	Termites	
K	Mite (*Bryobia*)	
M	Aphid (*Cinara* and *Toxoptera*)	
N	Aphid (*Toxoptera*)	
O	Silverleaf whitefly (*Bermisi tabaci*)	
P	Mites (*Syringophilopsis* and *Torotrogla*)	
Q	Mites	

Wolbachia, like other vertically transmitted bacteria, are dependent on the host for survival and transmission. The occurrence of a *Wolbachia* infection has been linked to the provision of important nutrients, vitamins, and proteins to the host (Zug and Hammerstein 2015, Kamtchum-Tatuene et al. 2017). This is evident as *Wolbachia* has been implicated in the mediation of host protection against arboviruses, which stems from competition for resources, the pre-activation of the host immune system, or the induction of microRNA (miRNA) (Zug and Hammerstein 2015, Kamtchum-Tatuene et al. 2017).

The aforementioned occurrences appear to benefit the host. However, *Wolbachia* infection can also cause detrimental effects to the host. These are mainly due to *Wolbachia* infection resulting in changes to the reproductive system of the host (Kamtchum-Tatuene et al. 2017). These

changes to the reproductive system are referred to as reproductive manipulations and can include the skewing of the sex ratio in favour of more females, male-killing, parthenogenesis induction, and feminisation of individuals that are genetically male (Anderson and Karr 2001, Haine et al. 2005). Additionally, the most common reproductive manipulation is cytoplasmic incompatibility, which occurs when *Wolbachia*-infected males fertilize eggs from uninfected females leading to embryonic mortality. Initially, cytoplasmic incompatibility was understood to be a pattern of modification and rescue (*mod*/*resc*) genes. The *Wolbachia mod* (modification) factors were suggested to act as a toxin or imprint of the male germline, while the *resc* (rescue) factors acted as an antidote (Presgraves 2000). Further research into cytoplasmic incompatibility by LePage et al. (2017), found that cytoplasmic incompatibility occurs due to the occurrence of gene regions encoding cytoplasmic incompatibility factor A and B (*cif*A and *cif*B). These authors found that the dually expressed gene products of *cif*A and *cif*B are factors that result in unsuccessful fertilisation. Cytoplasmic incompatibility has the effect of increasing the likelihood of *Wolbachia* infection spreading throughout a population and represents a natural example of 'gene drive' (Macias et al. 2017, Wedell et al. 2019).

The discovery of these *Wolbachia*-mediated reproductive manipulations has led to these bacteria being regarded as potential biological and genetic control agents. Frentiu et al. (2014) reported that *Aedes aegypti* (Linnaeus, 1762) infected with the wMel strain of *Wolbachia* showed reduced replication and transmission of the dengue virus (*Flavivirus*, Flaviviridae) in laboratory trials. Similar replication reduction has been reported in other RNA viruses such as yellow fever (van den Hurk et al. 2012), chikungunya (Moreira et al. 2009) and West Nile (Hussain et al. 2013). The "Eliminate Dengue Programme" was initially implemented to investigate the use of *Wolbachia*-infected mosquitoes to reduce dengue transmission (Kolopack et al. 2015).

The programme was subsequently expanded to develop *Wolbachia* as an intervention to control mosquito-transmitted viruses such as Zika and chikungunya, with the expanded programme renamed the World Mosquito Programme (WMP) (O'Neill 2018). The WMP reported that the effect of cytoplasmic incompatibility-inducing *Wolbachia* to reduce dengue virus outbreaks occurred in areas where high levels of *Wolbachia* (surpassing an unstable equilibrium point estimated to be less than 0.3 for the wMel strain of *Wolbachia*) were present (O'Neill 2018,https://www.worldmosquitoprogram.org). The WMP website boasts similar results in communities in Asia, Latin America, and Oceania. However, O'Neill (2018) reported that while

Wolbachia maintains itself at a very high frequency in the wild mosquito population it is rarely at complete fixation.

Wolbachia infection can be confirmed through the use of multilocus sequence typing (MLST). Baldo et al. (2006) developed a *Wolbachia* MLST which uses five conserved genes namely *gatB, coxA, hcpA, ftsZ* and *fbpA*. These genes are housekeeping genes that a broadly distributed across the genome and subjected to purifying selection (Baldo and Werren 2007). The aforementioned housekeeping genes have been shown to be consistent in supergroup classification of a strain (Baldo et al. 2006), thus allowing for support for the initial *Wolbachia* surface protein (*wsp*) supergroup assignment. The *wsp* gene has been used routinely for infection confirmation and supergroup designation (Baldo et al. 2005). However, *wsp* sequences have been shown to be highly recombinant thus making *wsp*-based supergroup designation unreliable (Baldo et al. 2005).

Although MLST was developed as an answer to the *wsp* shortfall, false positives and negatives have been reported for the *fts*Z and *glt*A housekeeping genes assays (Casiraghi et al. 2005). The 16S rRNA gene region has been reported as informative about infection status, gene transfer and supergroup designation especially when combined with the *wsp* gene region assay (Morrow et al. 2015). To evaluate the suitability and effectiveness of pest control in programmes that rely on *Wolbachia* infection, the occurrence of infection in the pest population before and during the programme needs to be accurately measured. Recording of both the proportion of infected individuals within the population (prevalence) and the change in levels of infection over time are important parameters describing the infection frequency of *Wolbachia*. Mateos et al. (2020) reviewed the potential for *Wolbachia pipientis* driven IIT and concluded that PCR screening is the most utilized infection confirmation method and that host-encoded genes should also be amplified to remove concerns about DNA integrity and the possibility of false negatives.

1.1.8. *Wolbachia* in the Tephritidae

In tephritid fruit flies, PCR and sequencing approaches have found that ~66% of ~87 screened tephritid species have at least one record of positive *Wolbachia* screening, and that these positive *Wolbachia* detected belong to the A and B Supergroup (Mateos et al. 2020). Twelve *Anastrepha* species in South America were shown to be infected with *Wolbachia wMel* strain belonging to supergroup A, thus showing that *Wolbachia* is occurrence is high (100% of the twelve species sampled) in members of this genus (Coscrato et al. 2009). In *B. dorsalis*,

Wolbachia has been reported at low prevalence in populations from China (1.27%) and Thailand (1.15%) (Kittayapong et al. 2000, Sun et al. 2007). Gichuhi et al. (2019) reported a prevalence of 3.6% in the period between 2005 and 2009, and 1.1% in 2017 when 357 *B. dorsalis* individuals, collected in Tanzania, Uganda, and Sudan, were tested for infection and 10 were found to be positive for *Wolbachia*.

Wolbachia prevalence in specimens of eight *Bactrocera* species and *Dacus axanus* (Hering) collected in tropical far north Queensland, Australia during summer ranged from 0% to 100% (Morrow et al. 2014); *B. bryoniae* (7.85%), *B. decurtans* (16.7%), *B. frauenfeldi* (14.7%), *B. neohumeralis* (9.8%), *B. peninsularis* (0%), *B. perkinsi* (100%, only five flies tested), *B. strigifinis* (13.5%), *B. tryoni* (4.1%) and, *D. axanus* (10%). A single individual of *Bactrocera ascita* (Hardy) was reported to be infected with up to five *Wolbachia* strains (Jamnongluk et al. 2002). Thus, it is important to be considerate of possible multiple infections and varying prevalence among different species when screening for the presence of *Wolbachia*.

The association between *Wolbachia* and tephritid flies is important due to the potential for it to be manipulated for pest control. The use of *Wolbachia*-mediated control has a few requirements, the first of which is that *Wolbachia* strains are transferable between diverse host systems and insect genera and the expected phenotype is expressed (Schneider et al. 2013). Thus far multiple researchers have reported the successful transfer of *Wolbachia* by micro-injection from donor to recipient hosts within the same insect order, followed by the confirmation of their phenotype in the novel host (Poinsot et al. 1998, Zabalou et al. 2004, McMeniman et al. 2009). The second requirement is that the transferred infection and expected phenotypes persist stably in the novel host. Schneider et al. (2013) demonstrated that infections by multiple *Wolbachia* strains can shift in prevalence after artificial host transfer. They suggest that this may be due to either stochastic or selective processes.

Laboratory trials have demonstrated that *Wolbachia* can be used for economic pests like tephritid fruit flies. Zabalou et al. (2004) demonstrated that *Wolbachia* could be successfully transferred across species in a laboratory setting to induce cytoplasmic incompatibility in *C. capitata*. *Wolbachia* has been shown to infect a range of polyphagous fruit flies (Kittayapong et al. 2000) that include the important pest genera *Bactrocera, Ceratitis, Dacus* and *Rhagoletis* (Coscrato et al. 2009). This suggests the potential for *Wolbachia* strains to control pest fruit flies and reduce their impact on food production. This aforementioned research has aided in the development and investigations into an environmentally friendly fruit fly control method.

These pest control approaches include SIT, which has been discussed previously, and incompatible insect technique (IIT), which involves the use of *Wolbachia*'s cytoplasmic incompatibility (CI) to control a pest. With the prevalence of *Wolbachia* ranging from low to 100% infection in different species and populations, it is important to identify fruit flies that are naturally infected with *Wolbachia* as well as the phenotypic effects of *Wolbachia* strains to support development of IIT.

1.2. Project objectives

To address the challenge that fruit flies present to farmers in the South African agricultural industry, this project aimed to assess the viability of a *Wolbachia*- driven insect incompatibility technique control method. To accomplish this an understanding of the prevalence of *Wolbachia* in fruit fly populations and the diversity of tephritid hosts is necessary. The successful undertaking of this would involve the morphological and molecular identification of fruit fly hosts and detection and characterisation of *Wolbachia* in wild-caught fruit flies.

To achieve these aims, my objectives were to:

1. Establish the species diversity of tephritid flies in two fruit producing regions of South Africa, the highveld and lowveld, based on morphological identification.
2. Assess the intraspecific genetic diversity of tephritid flies from the highveld and lowveld using a mitochondrial cytochrome oxidase I gene barcoding approach.
3. Determine the prevalence and diversity of *Wolbachia* in naturally infected tephritid species from the highveld and lowveld using PCR-sequencing and next generation sequencing.

Chapter 2. Diversity of fruit flies (Diptera: Tephritidae) in the South African Highveld and Lowveld

2.1 Introduction

Agroecosystems are ecological systems modified by human beings to produce food, fibre or other agricultural products (Conway 1987). These ecological systems are created through the transformation of the original natural systems, which are known for high levels of diversity, to a manicured assemblage of crops, pests and weeds. These transformed agroecosystems have high net productivity, simple linear trophic chains, low species and genetic diversity, open mineral cycles, low resilience (stability), high entropy, simple habitat heterogeneity and synchronized phenology (Odum 1969, Altieri et al. 1983). Owing to a preference for monocultures in these systems, they are regarded as immature due to short temporal permanence, human control and low resilience (stability) (Turnbull 1969, Altieri et al. 1983). Monocultures are preferred in these instances as they align with agricultural objectives, which are mainly to achieve maximum profit, minimize year-to-year instability in production, and prevent the degradation of their agroecosystem. Basic ecological processes *viz*. competition, herbivory and predation still occur but these are now overlaid and regulated by agricultural processes of cultivation, subsidy, control, harvesting and marketing (Conway 1987).

These high production systems serve as an environment with a dense concentration of basic food resources for potential arthropod pests; therefore, monocultures need pest control, which was usually achieved through the application of pesticides. The application of pesticides was used to subsidize the lack of self-regulation. Currently, pest control through pesticide application does not align with consumer concerns regarding pesticide residue on produce thus, consumers argue for more sustainable agricultural systems characterized by pesticide use reduction or avoidance (Nitzko et al. 2022). Agricultural practices that meet all or most of the abovementioned targets allow for access to interregional and global markets, this unfortunately increases the chances for the introduction of invasive arthropod pests (Suckling et al. 2019).

True fruit flies (Diptera: Tephritidae) are frugivorous insects that are a feature of horticultural ecosystems, globally. They can cause direct damage to crops when females oviposit and larvae develop in the fruit, stems and seeds of host plants (Aluja 1994). Female oviposition allows for the introduction of spoilage microbes, which lead to fruit breakdown and rot. Larval development in frugivorous fruit flies is dependent on the ingestion of the decaying flesh of the fruit. This damage may reduce yield by up to 80% depending on the plant variety and season (Ekesi et al. 2014). Two species of tephritid fruit flies - the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann, 1824) and the Natal fruit fly, *C. rosa s.l.* (Karsch, 1887) - cause

economic losses in the South African deciduous fruit industry to the value of US $3 million per annum (Barnes and Venter 2006). In 1997, a study conducted in Israel, the Palestinian Territories and Jordan reported that the direct damage (yield loss and control costs) and indirect damage (environmental impact and market loss) caused by *C. capitata* amounted to US$192 million per year (Enkerlin and Mumford 1997). To mitigate fruit fly-mediated economic loss in 2007, the horticulture industry of Florida, USA spent over US$4.8 million in exclusion and detection activities while Mediterranean countries spent approximately US$13.6 million on various control programmes (Enkerlin and Mumford 1997, Pierre 2007). In Ghana, depending on the mode of spraying used, chemical treatment an acre of mango plantation for pest fruit fly control ranges from US$688 to US$915.20 for the two harvesting seasons per year (Banson and Egyir-Yawson 2014). When Suckling et al. (2016) analysed 211 eradication programmes against 17 fruit fly species, they deduced that the cost of eradication ranged from US$0.1 million to US$240 million and averaged about US$12 million. These high control costs have led to multiple fruit fly species being considered quarantine pests with the imposition of quarantine restrictions on exporting countries (Verghese et al. 2004) and strict regulation of fruit transported in airline passenger baggage due to their role as major invasion pathways (Liebhold et al. 2006).

Tephritidae represents 4,200 described species in 500 genera (White and Elson-Harris 1992) with many species representing sub-economic pests or having no pest status at all. These non-pests or indigenous fruit flies may represent a source of novel/future pest species given that ecological shifts can occur in response to global environmental change. However, further information is needed on the basic biology of these species to determine their pest potential. A well-known example of this threat involves the apple maggot, *Rhagoletis pomonella* (Walsh, 1867), which underwent a sympatric shift from its native host hawthorn (*Crataegus* sp.) to the introduced domesticated apple (*Malus domestica* (Borkhausen,1803)) in the north-eastern United States approximately 150 years ago (Berlocher and Feder 2002, Hood et al. 2013, Authority et al. 2020). The apple maggot fly is now considered a major economic pest of apples in North America owing to this host-race shift (Boller and Prokopy 1976). Tephritid fruit flies are also capable of arthropod-mediated ecosystem services that are not considered pest behaviours in their native ranges. In Asia, fruit flies from the genus *Bactrocera* (Maquart,1846) act as pollinators for *Bulbophyllum* (Orchidaceae: Epidendroideae) flowers (Tan 2009).

In Africa, there are 150 recorded tephritid genera and more than 50 species are considered to be of economic importance (Grové and de Beer 2012). Most species that use agricultural produce as hosts and are thus of economic concern belong to the genera *Bactrocera*, *Dacus* (Fabricius, 1805), *Zeugodacus* (Hendel, 1927), *Anastrepha* (Schiner, 1868), *Trirhithrum* (Bezzi, 1918)and *Ceratitis* (Macleay, 1829) (Virgilio et al. 2009, Grové and de Beer 2012). Tephritid fruit flies that have been detected in South Africa are recorded as falling within nine genera namely *Bactrocera*, *Ceratitis, Coelotrypes* (Bezzi, 1923), *Corpophthoromyia* (Hardy, 1977), *Dacus*, *Elaphromyia* (Bigot, 1859), *Munromyia* (Bezzi, 1922), *Perilampsis* (Bezzi, 1920) and *Trirhithrum* (Manrakhan et al. 2017, Grové and de Beer 2019). These do not represent all the tephritids present in South Africa, the others are far less studied thus there is very little information available. This may be owing to the fact that they breed in non-commercialised plants and plant parts, *viz*. leaves and flowers (Karsten et al. 2018).

Ceratitis is a predominantly Afrotropical group and comprises an estimated 95 described species. This genus is hypothesised to have evolved in East Africa (Baliraine et al. 2004). In the case of the cosmopolitan species *C. capitata*, South Africa is considered part of its native range as Karsten et al. (2015) found no genetic difference between individuals found in South Africa and elsewhere in Africa. Furthermore, *C. capitata* is widely distributed throughout agroecosystems in South Africa. In contrast, *C. rosa* only occurs in wetter and more coastal parts of the country (De Meyer et al. 2008). Early documentation on *C. rosa s.l.* indicated that there was an "R2", "cool" or "highland" morphotype of *C. rosa* (Virgilio et al. 2013, Tanga et al. 2018). Subsequent investigations that derived behavioural (Juarez et al. 2015), genetic (Virgilio et al. 2018), morphological and physiological evidence (De Meyer et al. 2016) led to the "R2" morphotype being awarded species status. *Ceratitis quilicii* (De Meyer, Mwatawala and Virgilio, 2018) prefers cool higher altitudes with *C. rosa s.s* preferring warm, lower altitudes (Mwatawala et al. 2015). However, in the northern parts of South Africa, *C. quilicii* and *C. rosa* occur sympatrically (Virgilio et al. 2013). The marula fruit fly, *Ceratitis cosyra* (Walker,1849), infests mango and guava, but its distribution in southern Africa is limited to the subtropical regions of the North East and East Coast following the distribution of the marula tree, *Sclerocarya birrea*, which is an important wild host (De Villiers et al. 2013).

The invasive fruit fly, *Bactrocera dorsalis* (Hendel, 1912) was recorded on the African continent in 2003 when it was detected in Kenya (Lux et al. 2003). In 2007 and 2008, a single male specimen was intercepted on South Africa's northern border and an incursion was

confirmed in 2010 when several individuals were intercepted in the provinces of Limpopo, North-West and Gauteng (Manrakhan et al. 2015). Ecological niche and climatic models based on the global distribution of *B. dorsalis* have predicted the potential for range expansion further south in South Africa (Stephens et al. 2007, Hill and Terblanche 2014).

South Africa spans the subtropics at the interface between tropical, subtropical, and temperate weather systems, and consequently experiences distinct summer-, winter- and year-round rainfall zones (Roffe et al. 2021). The lowveld region, specifically Mbombela (previously known as Nelspruit), represents the strongest summer rainfall zone with an estimated average rainfall of 783-1200 mm per year (Adam et al. 2013, Roffe et al. 2021). The highveld region of South Africa consists of the high plateaus of South Africa, covering about 30% of the country's land area. The highveld has a semi-arid climate, with a long-term annual average rainfall of 545 mm and a high annual evaporative demand that can be four times more than received rainfall (Zerizghy et al. 2012). Using past observations from the period 1980-2016 and model simulations up to 2050, Jury (2019) extrapolated the rate of warming experienced in South Africa and predicted that it will increase by 0.02 to 0.03°C/yr. While there are some doubts about how these temperature changes will affect tephritid populations (Hill et al. 2016), there may be opportunities for some species to expand their range as temperature and rainfall directly affect their development, reproduction and survival. Global climate change, and variability in temperature have been attributed to the abundance, distribution, and high fruit infestation by fruit flies in different agroecological zones (Tanga et al. 2018).

This study aims to determine the occurrence and diversity of fruit flies in the highveld and lowveld regions of South Africa. I expect to find the highest fruit fly diversity in areas that have a wider variety of plant hosts and low levels of pest management. Furthermore, I expect to find *B. dorsalis* in higher numbers in the lowveld than in the highveld of South Africa, as the point of entry for the species was in the Limpopo Province, however this species has also been detected in Gauteng. Owing to its cosmopolitan distribution, I predict that *C. capitata* would occur in both regions. In contrast, the distribution of *C. cosyra* would be linked to the distribution of its plant hosts. Lastly, I predict that *C. rosa*, would be found predominantly in the subtropical, milder areas at a lower altitude than its sister species, *C. quilicii*.

2.2 Materials and methods

2.2.2 Fruit fly collection

An exploratory array of traps to collect fruit flies was established on the University of Pretoria Hatfield campus (25° 45’ 21” S 28° 13’ 51” E; altitude: 1,339 m). This array was active from the 13th to the 26th of February 2018. This initial pilot phase was also used to determine the optimal trap layout and fruit fly lures that would be used for later collection in this project.

Fruit fly collection was conducted using yellow, McPhail-type bucket traps baited with one of the four lure dispensers. These lure dispensers included Biolure® (Suterra LLC, Bend, USA and distributed in South Africa by Chempac (Pty) Ltd., Suider Paarl), which is a three-component lure [ammonium acetate, trimethylamine hydrochloride and 1,4-diaminobutane (putrescine)] that has been proven to attract *Ceratitis* flies along with other tephritid species (Heath et al. 2007, Leblanc et al. 2010). The second was methyl eugenol (ME; Chempac, Suider Paarl), which attracts sexually mature, male *B. dorsalis* (Vargas et al. 2000). The third lure was enriched ginger oil lure (EGO lure; Insect Science, Tzaneen) which attracts males from the genus *Ceratitis* (Manrakhan et al. 2017). The last lure used was trimedlure (TML; t-butyl-2-methyl-4-chlorocyclohexanecarboxylate), which attracts males of *C. capitata* (Shelly and Pahio 2002). The exploratory array included traps baited with TML but based on the limited range of species that were trapped, a decision was made to use EGO lure due to its attraction to a wider variety of *Ceratitis* species (Table 2.1).

Table 2.1: Trap catches during the exploratory sampling that was conducted on the University of Pretoria Hatfield Campus during the period of 13th to 26th February 2018.

Species	Biolure	Methyl eugenol	Trimedlure
B. dorsalis		11	
C. quilicii	12		157
C. simi			1
Total	12	11	158

Fruit fly samples were subsequently collected in South Africa from eight separate locations (shown in Figure 2.1). These locations included four within the highveld region of South Africa within 100 km of Pretoria. The highveld sampling was conducted from 26th March to the 2nd of April 2018. These locations included the University of Pretoria Experimental Farm (25°45'10.0"S 28°14'46.0"E; altitude: 1327 m), which is a small-scale farm with crops such as

apples, citrus, pears, and avocadoes (fruit available at each site summarised in Table 2.2). The second location was the Tswaing Meteorite Crater (25°24'57.0"S 28°06'00.0"E; altitude: 1120 m). The nature reserve in which the meteorite crater is located hosts a large number of marula trees, *Sclerocarya birrea*, and is surrounded by the settlement of Soshanguve, which is known to have backyard and small-scale farms where a wide range of fruit and vegetables are grown (maize, potatoes, spinach, peaches, mangoes, apricots and bananas) (Van der Linde 2005). Magaliesmoot AH (25°42'54.7"S 28°02'46.8"E; altitude: 1271 m) comprised a series of small-scale farms with similar fruit and vegetables as those described for Soshanguve. The Mooinooi farm (25°46'25.4"S 27°36'27.6"E; altitude: 1290 m) on the outskirts of Brits represented the fourth location. This location is a citrus farm, with orange cultivars and lemons, as well as nectarines on neighbouring plots and wild fig trees, Transvaal milkplum, *Englerophytum magaliesmontanum*, and a few marula trees in the surrounding indigenous vegetation.

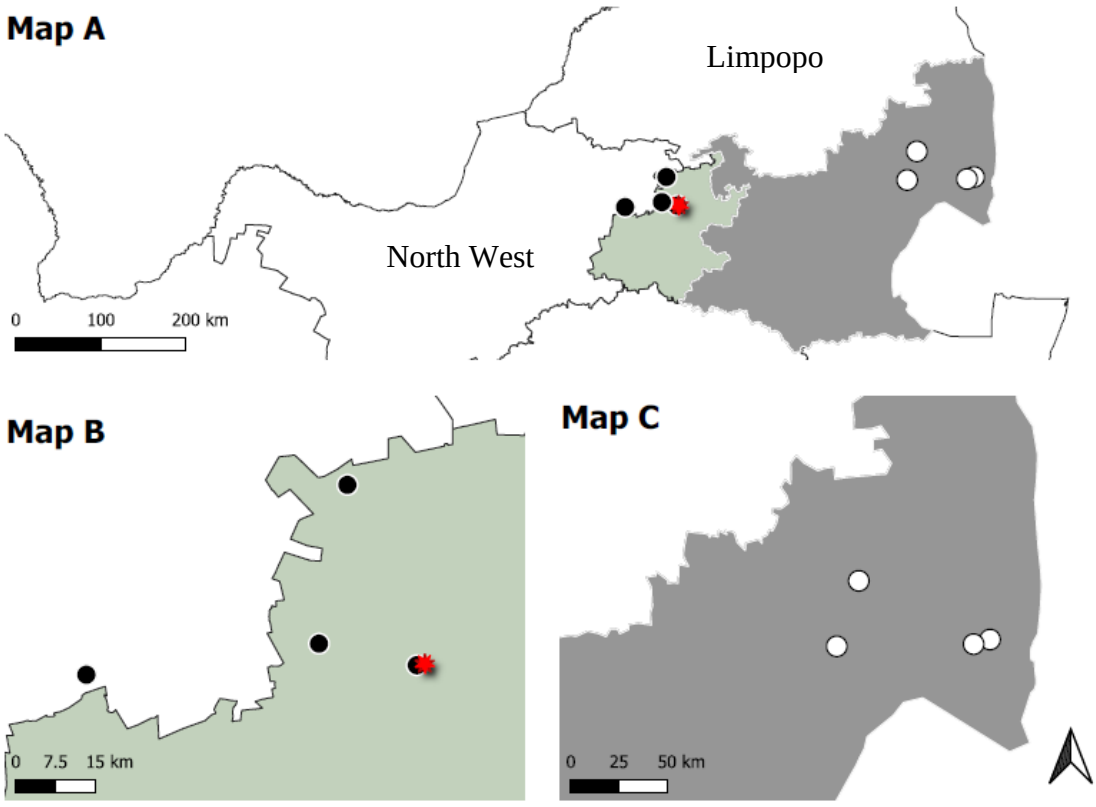


Figure 2.1: Map (A) showing the sampling locations in the highveld (black dots) and the lowveld (white dots) sampling regions with Gauteng (green) and Mpumalanga (grey) and surrounding provinces of North West and Limpopo. Map magnified to show the distribution of sampling sites in the highveld (B) and lowveld (C). The red star shows the location of Pretoria.

Four lowveld locations were sampled from the 3rd to the 11th of April 2018. These locations were situated within the area of the Mpumalanga province that lies within a 100 km radius of Mbombela (formerly Nelspruit). The Siyalima Boerdery (25°24'57.5"S 31°45'59.7"E; altitude: 530 m) and Hectorspruit Farm (25°26'23.3"S 31°40'51.8"E; altitude: 280 m) locations were both citrus farms. These farms produce a wide variety of orange cultivars, lemons and had large pine trees as windbreaks. The Hectorspruit citrus farm neighbours the Kruger National Park so there are probable refugia for pest fruit flies within a close distance to the farm. The ARC Institute for Tropical and Subtropical Crops research station sites, one in Mbombela (-25.45127 S 30.96919 E; altitude: 691 m) and the other in Burgershall (25°06'39.0"S 31°05'02.0"E; altitude: 759 m) host a wide variety of fruit trees namely, macadamia nuts, litchis, wild fig trees, bananas, coffee and papayas.

Table 2.2: Fruit trees located around the fruit fly sampling arrays at the various sampling locations in the highveld and lowveld regions

		Fruit trees near the various arrays		
Region	**Site name**	**Array 1**	**Array 2**	**Array 3**
Highveld				
	Tswaing Meteorite Crater	Marula tree	-	Marula tree
	Mooinooi Farm	Wild fig tree	Marula fruit tree	Oranges
	Magaliesmoot AH Farm	Tomatoes	Papaya and Mango	Cabbage and Okra
	UP Experimental Farm	Apples	Oranges and Lemons	Avocadoes
Lowveld	ARC-ITSC Nelspruit	Litchi Tree	Litchi Tree	Wild Fig Tree
	ARC-ITSC Burgershall	Coffee Tree	Banana	Avocado and Papaya
	Siyalima Boerdery	Wild Fig Tree and Orange Tree (Navels)	Orange Trees (Navels)	Orange Trees (Naval Turkeys)
	Hectorspruit Farm	Orange Trees (Navels Midnight)	Wild Fig Trees and Orange Trees (Navels Midnight)	-

The highveld and lowveld sampling locations were separated by altitude, which corresponds to differences in the known distribution of several pest tephritid fruit flies including *B. dorsalis*, *C. capitata*, *C. cosyra*, *C. quilicii*, and *C. rosa*. The altitude is also important in terms of lifespan especially for members of the *Ceratitis* species (Duyck et al. 2010). The March-May sampling

period also represents a peak in tephritid abundance in subtropical and temperate regions of South Africa because it coincides with the end of the summer fruit crop seasons (Manrakhan et al. 2012, Nyamukondiwa et al. 2013).

During fruit fly sampling, three clusters of three traps were placed at each location. Each trap within a cluster (Figure 2.2) was baited with one of either Biolure, methyl eugenol or EGOlure. Trap clusters were placed at a minimum distance of 100 m from each other. Traps within a cluster were placed 50 m from each other as per manufacturer recommendations. Traps in each cluster were deployed for seven days during which time they were emptied daily and collected specimens were separated and stored individually in absolute ethanol in 1.5 mL microcentrifuge tubes. Once stored, the collected specimens were transported to the Hatfield Campus of the University of Pretoria for morphological identification, which was performed using a dissecting microscope and with reference to White and Elson-Harris (1992), De Meyer et al. (2016) and the online identification tools available on https://fruitflykeys.africamuseum.be/.

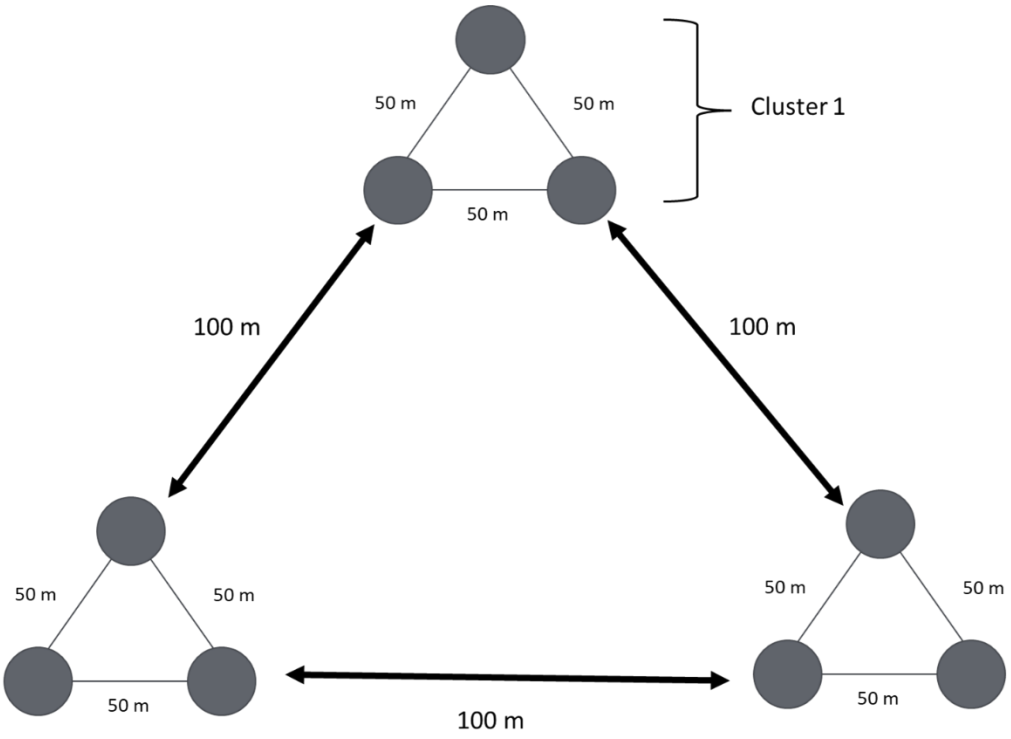


Figure 2.2: Diagram showing the trap layout used for sampling at the various sampling location. The three clusters together represent one replicate, this layout was repeated at each sampling location. The grey circles represent one of the three lures used (EGOlure, Biolure and methyl eugenol.

2.2.1 Data analyses

In order to verify the sex- bias for EGOlure and methyl eugenol and determine whether there was a significant sex-bias in traps baited with Biolure. A chi-squared tests with Yate's correction were used. The chi-squared tests were performed with a 95% confidence level (α = 0.05) using MS Excel® (version 1809, Microsoft). Fruit fly assemblage data from each trap cluster were summarised by calculating abundance (total number of individuals), species richness (total number of species) and two measures of species diversity. The first measure of diversity was the Shannon-Wiener diversity index, which evaluates the uncertainty associated with a prediction based on the proportion of species relative to the total number of species (Keylock 2005). The second diversity measure was Simpson's diversity index, which measures the probability that two individuals randomly selected from a sample belong to the same species (Hunter and Gaston 1988).

Normality for the aforementioned variables was inspected with Shapiro-Wilk tests in SPSS Statistics 26 (IBM Corporation). This normality test was used as the sample size was the 12 trap arrays (Mishra et al. 2019). Species richness and both diversity indices data were normally distributed according to the Shapiro-Wilk ($p>0.05$). Abundance was not normally distributed. To establish the effect of region (highveld or lowveld) on species richness, the Shannon-Wiener diversity index and Simpson's diversity index, a generalised linear model (GLZ) was used with Gaussian errors and identity link. Due to abundance representing count data and not being normally distributed, the effect of region on this response variable was determined using a GLZ with negative binomial distribution and log link. Wald chi-squared tests were used to summarise the effect of region on abundance, species richness and both diversity indices predicted by the GLZ models.

Species-by-trap cluster abundance data were used to perform a non-metric multidimensional scaling ordination (nMDS) to assess the effect of region on fruit fly assemblage structure using the 'vegan' library in R Studio (version 1.1.423, RStudio.Inc.) running R version 3.1.3.4 (Team 2013, Team 2015). Abundance of each fruit fly species caught by each cluster of traps was used to generate a dissimilarity matrix using the Chao dissimilarity index. This index was found to be the most appropriate dissimilarity index using 'rankindex' because it resulted in the highest rank-order with gradient similarity and permitted subsequent procedures. The dissimilarity index was reduced to two dimensions using 'metaMDS', then using the 'vegdist' procedure an analysis of similarity (ANOSIM) was conducted to determine whether there was

a significant effect of region on the observed fly assemblage structure. Ellipses plotted on the two-dimensional ordination were used to depict the 95% confidence interval of the centroid for each region.

2.3 Results

2.3.1 Fruit fly assemblage

Sampling in the highveld region resulted in the collection of 1508 tephritids belonging to the species *B. dorsalis*, *C. capitata*, *C. cosyra*, *C. pedestris* (Bezzi, 1924), *C. quilicii*, *C. quinaria* (Bezzi, 1918) and *D. ciliatus* (Loew, 1862) (summarised in Table 2.3). As female *C. rosa* and *C. quilicii* cannot be identified morphologically collection location was used to discern them. The fruit fly species caught in the lowveld were *B. dorsalis*, *C. capitata*, *C. cosyra*, *C, rosa* and *C. rubivora* (Coquillett, 1901), with a total catch of 1481 individuals. The lure that attracted the highest number of flies was EGOlure (n = 1989), followed by methyl eugenol (n = 806), and then Biolure® (n = 194). There was a significant sex bias towards males when using EGOlure (% male = 99.50; $\chi^2 = 1947.2$, df = 1, $P<0.001$) and methyl eugenol (% male = 99.88; $\chi^2 = 800.0$, df = 1, $P<0.001$). Biolure (% male = 52.58; $\chi^2 = 0.4$, df = 1, $P>0.05$) on the other hand showed no sex bias.

Table 2.3: Summary of nine fruit fly species caught during highveld (26 March – 2 April 2018) and lowveld (3 April – 11 April 2018) region sampling. The collections were conducted using Biolure, Methyl eugenol and Enriched Ginger Oil.

Region	Species	Biolure	Methyl Eugenol	Enriched Ginger Oil	**Total**
Highveld	*B. dorsalis*	-	24	-	**24**
	C. capitata	4	-	76	**80**
	C. cosyra	42	-	575	**617**
	C. pedestris	-	-	21	**21**
	C. quilicii	109	-	654	**763**
	C. simi	-	-	1	**1**
	D. ciliatus	2	-	-	**2**
Total		**157**	**24**	**1327**	**1508**
Lowveld	*B. dorsalis*	10	782	16	**808**
	C. capitata	4	-	101	**105**
	C. cosyra	1	-	427	**428**
	C. rosa	20	-	116	**136**
	C. rubivora	-	-	4	**4**
Total		**35**	**782**	**607**	**1481**

There was no significant effect of region on tephritid fly abundance (Fig. 2.2a; χ^2=0.002, df = 1, P = 0.965), species richness (Fig. 2.2b; χ^2 = 1.1, df=1, P =0.268) or the Shannon-Wiener index (Fig. 2.2c; χ^2 = 1.1, df = 1, P = 0.295). In contrast, region had a significant effect on Simpson's index (Fig. 2.2d; χ^2 = 3.9, df = 1, P =0.049), with Simpson's diversity being higher in the highveld when compared with the lowveld.

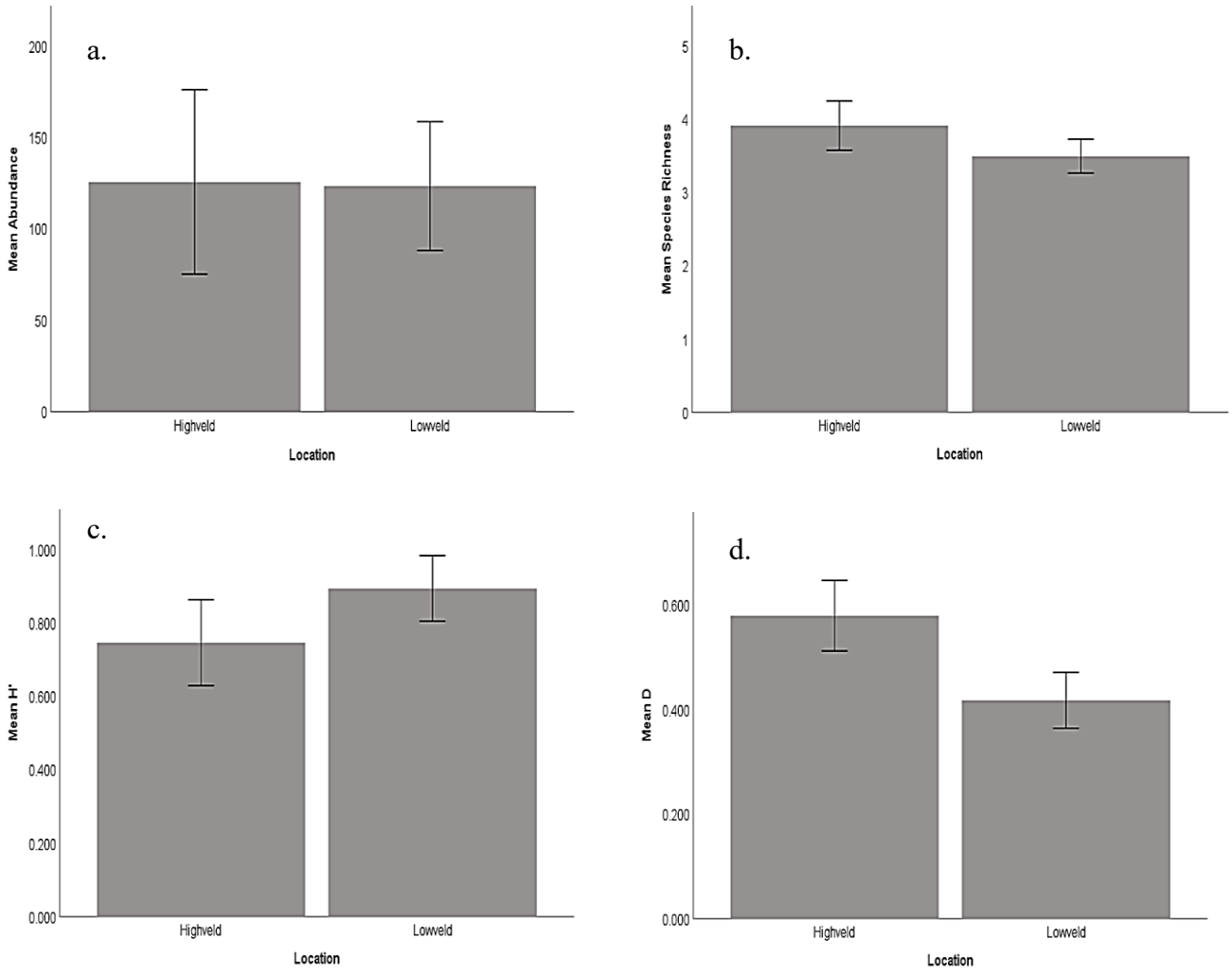


Figure 2.3: Mean tephritid fruit fly (a) abundance, (b) species richness, and species diversity calculated using the (c) Shannon-Weiner diversity index and (d) Simpson's diversity index for flies caught in clusters of traps placed in the four highveld and four lowveld of South Africa. Trap clusters (n =12) comprised three traps, each with a different lure (EGOlure, methyl eugenol and Biolure). Error bars represent ± 1 standard error.

The structure of fruit fly assemblages appeared to be influenced by the region of collection. Four species of fruit flies that were unique to their region are plotted outside of the 95% C. I of the centroid for each region, while the common species are located within close proximity to the ellipses drawn on the nMDS plot (Figure 2.3). This visual pattern was supported by ANOSIM, which indicated that assemblages differed between the highveld and the lowveld (Global R = 0.623, P = 0.001). The stress value, used to determine the goodness of fit for the nMDS, was 0.137, showing that there is a fair match between the data and the configuration of the nMDS (Kruskal 1964).

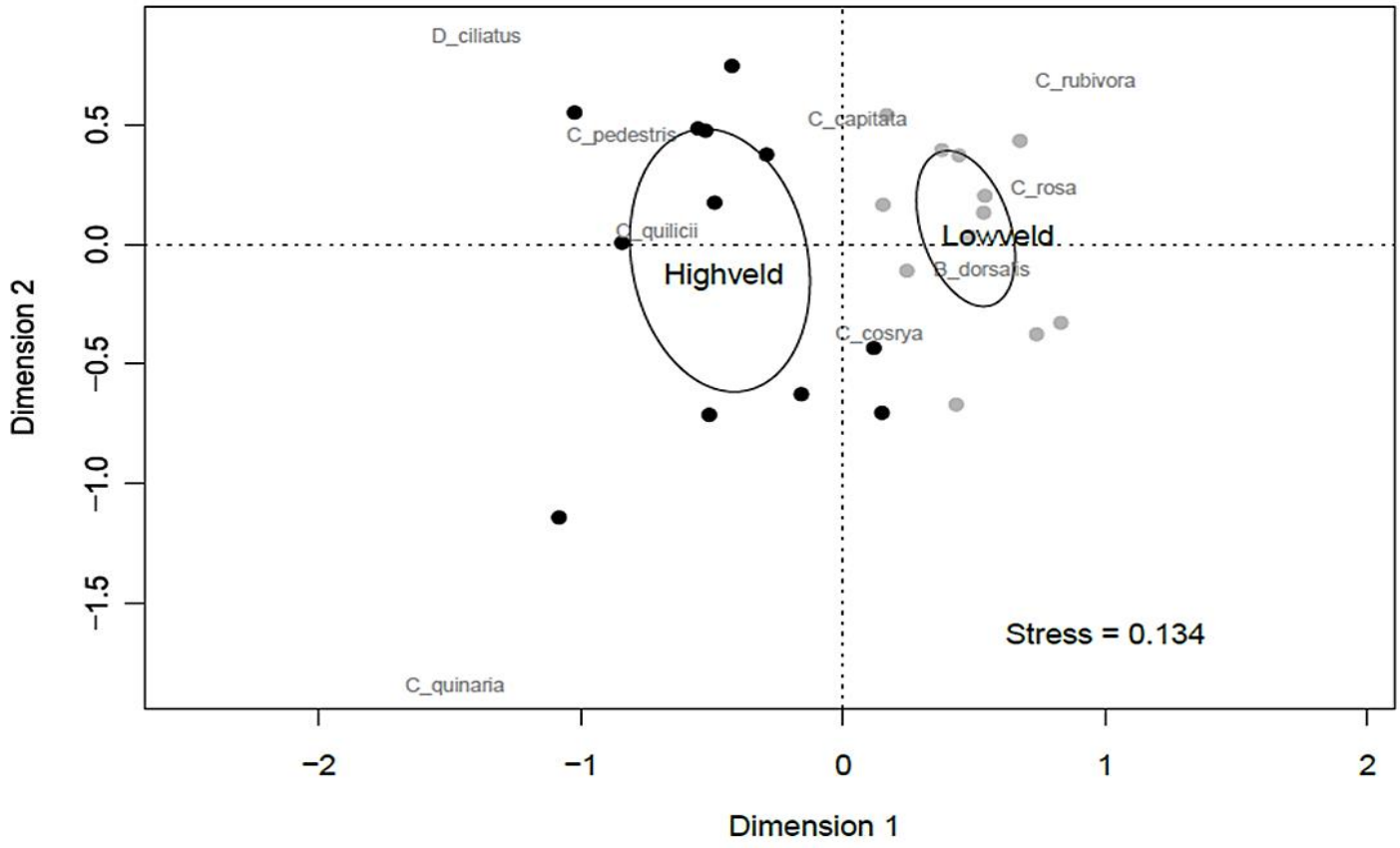


Figure 2.4: Non-metric dimensional scaling ordination of fruit fly species assemblages from the highveld and lowveld regions for 26 March - 11 April 2018 sampling period. Black markers represent species collected in the highveld, and the grey markers represent those collected in the lowveld. The ellipses represent the 95% confidence interval of the centroid for each region. The location of species names on the ordination plot indicates the sites with which species were most associated.

2.4 Discussion

A total of 2989 fruit flies belonging to nine species were collected from the highveld and lowveld regions of South Africa. Three sampled species were found across both regions, while the remaining species were unique to one of the two regions. Fruit fly abundance is strongly correlated with temperature and abiotic factors, and the availability of habitat, food, water, and oviposition substrates (Lopes et al. 2015). Drew and Hooper (1983) found that fly populations increased with the onset of higher temperatures and the beginning of the summer rain period. Furthermore peak trap captures corresponded with the fruiting times of their major hosts. Musasa et al. (2019) similarly reported higher fruit flies trapped per day (FTD) during the harvest months with a mean temperature of 19 °C. They also reported that they saw the highest FTD during the month of December. Our sampling period aligned with South African autumnal April temperatures, which vary between 17 - 30°C (Manrakhan and Addison 2007, Thomas et al. 2007).

More specifically, *C. capitata* populations are known to peak towards the end of the fruiting season, which occurs during the March to May period in South Africa, while *C. rosa* populations peak between February and April (Manrakhan et al. 2012). Our sampling sites also comprise commercial, research and small-scale farming locations with diverse host plant availability, the majority of which were at the end of their fruiting seasons (Manrakhan and Addison 2014, Grové et al. 2017). In addition, wild host plants play an important role in maintaining fruit fly populations, especially during periods when cultivated fruit and vegetable crops are scarce and out of season (Gnanvossou et al. 2017). A combination of these factors likely permitted the fruit fly populations to grow to large numbers, allowing for observation of multiple species near their peaks in abundance.

A comparison of fruit flies caught in the lowveld and the highveld revealed that the species richness and the Shannon-Wiener diversity index did not differ, however, the fruit fly assemblages from the regions were distinct. The lowveld had lower values for Simpson's diversity index, indicating that there is less species diversity in the region with the species present being more even in abundance (i.e., there is not one dominant species). This observation could be due to the relatively more homogenous habitat of the sites used in the lowveld relative to the highveld. In the lowveld, sampled sites comprised of citrus farms or agricultural research stations. Grové and de Beer (2019) sampled at four orchards in the Mbombela area and reported a greater species diversity in the lowveld than observed in our

study but this is likely owing to our two-week sampling period *versus* their four-year sampling period. Thus, a longer sampling period would be essential for future comparisons of species diversity in the highveld and lowveld. Furthermore, sampling through the various phases of the fruiting seasons is essential for monitoring fruit fly population fluctuations, and the effect of changes in fruit availability on fruit fly composition and competition. For example, there is growing evidence that *B. dorsalis* outcompetes *C. cosyra* in East Africa, leading to almost complete replacement of the latter in mangoes (Mwatawala et al. 2006a, Ekesi et al. 2009a). Similarly, competition between fruit flies has led to the displacement of *C. capitata* populations by *B. dorsalis* in Hawaii (Duyck et al. 2004). Similar studies on fruit fly competition in South African agroecosystems are necessary.

The large number of *B. dorsalis* found in the lowveld may be due to these sites being located near the suspected point of entry and suitability of the local environment. Furthermore, *B. dorsalis* is known predominantly as a lowland resident (Ekesi et al. 2006). Other reasons for their high abundance in the lowveld could be the quasi-absence of natural enemies and the polyphagous status of this formidable invasive species (Vayssières et al. 2009). Higher numbers of *B. dorsalis* were previously linked to the availability of ripe mangoes (*Mangifera indica*; Linnaeus, 1753), the preferred host, and guava (*Psidium guajava*; Linnaeus), the alternative host (Mwatawala et al. 2006b, Hill and Terblanche 2014). Theron et al. (2017) found that the adult *B. dorsalis* population was more influenced by temperature than host abundance, reporting that an increase in temperature correlated with an increase in the adult population. Our sampling was conducted following the month of February which was the month with the highest population pressure in the study conducted by Theron et al. (2017). In contrast the low numbers in the highveld may be due to pest control methods used on the commercial farms, namely bait sprays, removal of fallen fruit and other sanitation methods. The first adult *B. dorsalis* was detected in the City of Tshwane in 2010, and suppression programmes have been implemented since this detection, thus the low numbers may be linked to pest control (Manrakhan et al. 2015).

The collections of *C. capitata* align with this species being spread through a combination of the fruit fly's natural dispersal ability and human-mediated dispersal (De Villiers et al. 2013, Karsten et al. 2013). These dispersal methods are thought to contribute to the high levels of gene flow between *C. capitata* populations in South Africa (Karsten et al. 2013). The ability of the species to occur in different climates is attributed to the phenotypic plasticity of this species. *Ceratitis capitata* is able to tolerate local bioclimatic conditions owing to their thermal

tolerance and desiccation resistance (Weldon et al. 2016, Weldon et al. 2018). In South Africa, *C. capitata* is an important pest of citrus, deciduous, and subtropical fruit (De Villiers et al. 2013). This, coupled with the aforementioned plasticity, could help to explain the high numbers of *C. capitata* caught around the Mooinooi Farm area and the lowveld sites, as these were in close proximity to citrus farms. In a similar way, the distribution of *C. cosyra* in Southern Africa is limited to the subtropical regions where the marula tree (*Sclerocarya birrea*) is available (De Villiers et al. 2013). This fruit fly was abundant within the lowveld and the highveld, with the greatest abundance observed at the Tswaing crater and Mooinooi Farm where marula trees were present and typically dropping their fruit around one month before sampling occurred. In contrast, the low abundance observed at the UP Experimental Farm and Magaliesmoot Farm sampling sites may be linked to the absence of marula trees. This stark difference in abundance within the same region could indicate that *C. cosyra* is not as mobile as *C. capitata*.

Unlike *C. capitata*, which is widely distributed throughout agroecosystems in South Africa, *C. rosa s.s.* has a more limited distribution with *C. quilicii* being the more widespread species when compared to *C. rosa s.s.* (Karsten et al. 2016). Adult *C. capitata* and *C. cosyra* are more desiccation resistant than *C. rosa s.s* (Weldon et al. 2016). Furthermore, based on lower developmental threshold considerations, *C. quilicii* (egg, larva and pupa) are better adapted to low temperatures than *C. rosa* (Tanga et al. 2015). *Ceratitis rosa s.s*, has been reported as dominant in lower altitude areas of South Africa while *C. quilicii* is predominant in the high-altitude area in Réunion (Virgilio et al. 2013, Tanga et al. 2018). *Ceratitis quilicii* was found only in the highveld while *C. rosa s.s.* was found only in the lowveld. This separation in distribution is due to the fruit fly species being mostly associated with highland and lowland regions, respectively (Tanga et al. 2018). The distribution of *C. rosa* and *C. quilicii* is likely linked to their thermal tolerance and desiccation resistance, since *C. quilicii* has a wider climate tolerance range while *C. rosa* is restricted to the wetter regions in South Africa (De Villiers et al. 2013, Tanga et al. 2018).

The remaining fruit flies caught during this study were caught in only one of the two regions sampled. Species unique to one region were *C. pedestris*, *C. quinaria*, *C. simi* and *D. ciliatus* which were caught only in the highveld, whereas *C. rubivora* was caught only in the lowveld. *Ceratitis pedestris* is an occasional pest on stone fruits; in South Africa, this pest has been found in litchi orchards (Grové and de Beer 2012). This species is also strongly associated with

the plant genus *Strychnos* (Loganiaceae; Linnaeus, 1753), several species of which occur naturally in Southern Africa (Adebowale 2014), and tomato (Solanaceae; Linnaeus) (De Meyer and Freidberg 2005, Grové and de Beer 2012). *Dacus ciliatus* (Loew) is reported throughout Africa and can infest at least 16 host plants belonging to the Cucurbitaceae, with pest control research focussing primarily on melon, pumpkin, and squash production (Vayssières et al. 2008, Kamali et al. 2013). *Ceratitis rubivora* is a stenophagous species attacking solely representatives of the genus *Rubus* (Mwatawala et al. 2006a). South Africa is known to have both native and introduced *Rubus* species, these along with various hybrid taxa are recorded to occur in Gauteng, Mpumalanga and various other Provinces (Sochor et al. 2018). The distribution of *C. rubivora* is linked to regions of South Africa used for blackberry (*Rubus fruticosus*; Linnaeus), loganberry (*Rubus loganobaccus*; James Harvey Logan,1881) and raspberry (*Rubus idaeus*; Linnaeus) production and those with indigenous berries (Karsten et al. 2018, Sochor et al. 2018), and *C. rubivora* flies have been reared out of fruit from invasive plants (Malod et al. 2020).

Only one *C. quinaria* was collected during sampling. This is likely because this fruit fly species is only abundant in the dry season (Vayssières et al. 2009). Alternatively, the collection of a single *C. quinaria* may be due to sampling on and around citrus farms because this fruit fly does not naturally infest citrus and has a rather narrow host range with mango being the preferred cultivated host along with *Prunus armeniaca* and *P. guajava*. *Ficus* sp., found in bushveld vegetation, are the only known native hosts in South Africa (Manrakhan et al. 2020). *Ceratitis simi* (Munro, 1933), which was caught during the exploratory study, is another Afrotropical member of the *Ceratitis* genus, with plant hosts for this species including *Acokanthera longiflora* (Apocynaceae; Staph, 1922) and *A. schimperi* (Apocynaceae; Schweinf, 1891) (De Meyer 1998).

To conclude, the nine species of fruit flies caught during this study only provide a snapshot of the fruit fly population in the highveld and lowveld regions of South Africa. However, the results do suggest an important role for host availability and climate on the presence and abundance of different fruit fly species. Long-term sampling and monitoring would be necessary to detect other species present in these regions. Paired with a detailed survey of hosts and their fruiting phenology, this would allow for clearer conclusions on competition and resource partitioning between the various fruit fly species as there is overlap in terms of hosts.

A prolonged monitoring programme would allow for the quantification of outbreak occurrence along with the effect of abiotic and biotic variables on fruit fly populations.

Chapter 3. Mitochondrial *COI* gene diversity of tephritid fruit flies from the South African Highveld and Lowveld

3.1 Introduction

One of the greatest challenges in the natural sciences is the classification of biologically relevant entities (Heinrichs et al. 2011). Despite technological advances, species recognition and delineation remain fraught with difficulties for some taxa. Historically, species were separated when deemed to be sufficiently different according to morphological characteristics (Mayr 2000). However, the identification of insects on the basis of morphological characters alone often represents a challenging task requiring experienced taxonomists. In addition, molecular studies have revealed the existence of numerous biological species that have accumulated genetic divergence without accompanying morphological disparities (Fišer Pečnikar and Buzan 2014). The strengths of molecular techniques are that they not only differentiate between members of certain cryptic species complexes but also allow for species identification in instances where specimens may be damaged or incomplete. One such molecular technique involves the use of molecular short standardized DNA fragments that can be recovered and characterized as a unique identification marker for all species, termed DNA barcodes (Fišer Pečnikar and Buzan 2014). Hebert et al. (2003b) argued that the integration of DNA barcoding into traditional taxonomic tools could efficiently disclose hidden biodiversity more rapidly and more reliably than traditional methods alone. The ability of DNA barcoding to distinguish species from a range of taxa and to reveal cryptic species has been well documented (Kress et al. 2015).

Mitochondrial DNA (mtDNA) due to its simple, maternally transmitted inheritance, low recombination, fast rate of evolution and short coalescence time has been used as a marker of choice for barcoding of animals (Wallace 2007, Oliveira et al. 2008). A short DNA sequence of ~600 base pairs (bp) within the mitochondrial gene encoding cytochrome c oxidase subunit (*COI*) has been accepted as a practical, standardized, species-level DNA barcode for many groups of animals (Hebert et al. 2003b, Hebert and Barrett 2005, Valentini et al. 2009) and has spurred the establishment of an international initiative to generate barcodes for all life forms (https://ibol.org/). Whilst widescale application of this initiative has been highly successful, this barcode locus has been found to work poorly in some invertebrates (Evans and Paulay 2012), amphibians and reptiles (Vences et al. 2012).

DNA barcoding using the *COI* gene region has been useful in distinguishing blackfly (Simuliidae; Newman, 1834) species, a group for which fly identification was hampered by cryptic species and phenotypic plasticity (Rivera and Currie 2009). DNA barcodes can also be

applied as tools for addressing fundamental issues in ecology, evolution, and conservation biology (Kress et al. 2015). For example, they can be used to define species assemblages in communities, and which multispecies interactions occur in previously poorly investigated environments. They can also be used to infer the geographic pattern of genetic variation.

As species-level identification is crucial in many applications of economic and social importance, DNA barcoding has been used across a wide variety of insects. Many true fruit flies (Diptera: Tephritidae) are frugivorous insects that are considered arthropod pests due to the direct damage caused to crops when females oviposit and larvae develop in the fruit stems and seeds of the host plants (Aluja and Norrbom 1999). Female oviposition allows for the introduction of spoilage microbes, which lead to fruit breakdown and rot. Larval development is dependent on the ingestion of the flesh of the fruit. In the Western Cape Province of South Africa, economic losses to the value of US$3 million per annum have been attributed to two species of tephritid fruit flies – the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann, 1824) and the Natal fruit fly, *C. rosa* Karsch, 1887 (Mumford and Tween 1997). Enkerlin and Mumford (1997) reported that *C. capitata* damage amounted to US$192 million per year in Israel, Jordan and the Palestinian Territories owing to direct damage, which is yield loss and control costs, and indirect damage caused by environmental impact and market loss.

A 2014 study on the costs of pesticide usage in Ghana reported that, depending on the mode of spray used, the cost of fruit fly chemical control for an acre of mango plantation over two harvesting seasons ranges from US$688 to US$915.20 (Banson and Egyir-Yawson 2014). A subsequent analysis of 211 eradication programmes against 17 fruit fly species deduced that the cost of eradication ranged from US$0.1 million to US$240 million and averaged about US$12 million (Suckling et al. 2016). These high control costs have led to multiple fruit fly species being considered quarantine pests and to the imposition of quarantine restrictions on exporting countries (Verghese et al. 2004). Fruit transported in airline passenger baggage is also strictly regulated as this has been identified as a major invasion pathway (Liebhold et al. 2006). The management of harmful non-native species is normally considered the most cost-effective approach to prevent Tephritidae invasion.

Tephritidae is a large dipteran family with approximately 4,200 described species in 500 genera (White and Elson-Harris 1992). In Africa, there are 150 recorded tephritid genera and more than 50 species are considered to be of economic importance (Grové and de Beer 2012). These fruit flies belong to the genera *Bactrocera*, *Zeugodacus* (Hendel,1927), *Anastrepha* (Schiner,

1868), *Dacus* (Fabricius, 1805), *Trirhithrum* (Bezzi, 1918) and *Ceratitis* (Macleay, 1829) (Virgilio et al. 2009, Grové and de Beer 2012). Fruit flies found in South Africa belong to nine genera namely *Bactrocera, Ceratitis, Coelotrypes* (Bezzi, 1923), *Corpophthoromyia* (Hardy, 1977), *Dacus, Elaphromyia* (Bigot, 1859), *Munromyia* (Bezzi, 1922), *Perilampsis* (Bezzi, 1920) and *Trirhithrum* (Manrakhan et al. 2017, Grové and de Beer 2019). There are other tephritids in South Africa; however, they are far less studied and very little information is available, as they breed in plants and plant parts like leaves and flowers that are not of commercial value (Karsten et al. 2018).

The genus *Ceratitis* is a predominantly Afrotropical group and comprises an estimated 95 described species. This genus is hypothesised to have evolved in East Africa (Baliraine et al. 2004). For the cosmopolitan *C. capitata*, South Africa is considered part of its native range as there is no genetic difference between individuals found in South Africa and elsewhere in Africa (Karsten et al. 2015) and it is widely distributed throughout agroecosystems in South Africa. In contrast, the Natal fruit fly *C. rosa* (Karsch,1887), only occurs in cooler, wetter and more coastal parts of the country (De Meyer et al. 2008). Early documentation on *C. rosa s.l.* indicated that there was an "R2", "cool" or "highland" morphotype of *C. rosa* (Virgilio et al. 2013, Tanga et al. 2018). Subsequent investigations that considered behavioural (Juarez et al. 2015), genetic (Virgilio et al. 2018), morphological and physiological evidence (De Meyer et al. 2016) led to the proposal that the "R2" morphotype should be given species status, viz. *Ceratitis quilicii* (De Meyer, Mwatawala and Virgilio, 2018). This species prefers cool higher altitudes with *C. rosa s.s* preferring warm, lower altitudes (Mwatawala et al. 2015). However, in the northern parts of South Africa, *C. quilicii* and *C. rosa* occur sympatrically (Virgilio et al. 2013). The two species are morphologically similar, and the species can only be discerned on the basis of mid-tibia hair differences, and this is only in males. The marula fruit fly, *Ceratitis cosyra* (Walker, 1849), infests mango and guava, but its distribution in southern Africa is limited to the subtropical regions of the North East and East Coast following the distribution of the marula tree, *Sclerocarya birrea*, which is an important wild host (De Villiers et al. 2013).

In addition to these indigenous fruit flies, *Bactrocera dorsalis* (Hendel, 1912) a species endemic to Southeast Asia (Clarke et al. 2019), was recorded on the African continent in 2003 when it was detected in Kenya (Lux et al. 2003). In 2007 and 2008, single male specimens were intercepted on South Africa's northern border and the invasion was confirmed in 2010

when several individuals were intercepted in the provinces of Limpopo, North-West and Gauteng (Manrakhan et al. 2015). Ecological niche and climatic models based on the global distribution of *B. dorsalis* have predicted the potential for a southward range expansion in South Africa (Stephens et al. 2007, Hill and Terblanche 2014).

The DNA barcoding of tephritid fruit flies is an objective of international barcode campaigns, such as the Quarantine Barcoding of Life (QBOL, http://www.qbol.org/en/qbol.html) and the Tephritidae Barcode Initiative (TBI, Van Houdt et al. 2010), which was initiated by the Consortium for the Barcode of Life (CBOL, http://www.barcoding.si.edu/major_projects.html) in 2006 (Jiang et al. 2014). The Barcode of Life Database (BOLD, http://www.boldsystems.org) is another barcode repository which was officially established in 2007. This database is one of the most prominent informatics platforms for the acquisition, storage, analysis and publication of DNA barcode records (Ratnasingham and Hebert 2007). Multiple tephritids have been studied from the perspective of DNA barcoding including the identification of *C. capitata* (Barr et al. 2012), *B. tryoni* (Blacket et al. 2012), 81 fruit fly identifications in New Zealand as part of a biosecurity identification test (Armstrong and Ball 2005), and recovery of full DNA barcodes from museum tephritid fruit flies (Van Houdt et al. 2010).

Whilst *COI* barcoding has proven useful for delineating species, some challenges have been experienced with members of the *Ceratitis* FARQ complex (formerly FAR complex) which comprises of *C. fasciventris* (Bezzi), *C. anonae* (Graham), *C. rosa* (Karsch) and *C. quilicii* (De Meyer, Mwatawala and Virgilio) (Jiang et al. 2014). Although *COI* barcoding is unable to delineate members of the *Ceratitis* FARQ complex, it has been identified as a complementary tool that is useful for distinguishing between *C. cosyra* and *C. striatella* (Virgilio et al. 2017). In this study, I utilised *COI* barcoding to confirm the morphological identity of the fruit flies caught during our sampling. In addition, the *COI* barcodes generated in this study were used for phylogenetic analyses, to investigate haplotype diversity and to construct haplotype networks for each of the fruit fly species sampled in the highveld and lowveld of South Africa. I predict that *COI* barcoding will prove informative for delineating all species except those of the FARQ complex and that fruit flies that are indigenous to South Africa will have higher levels genetic diversity than invasive species. The use of *COI* data to construct haplotype networks will permit visualisation of genealogical relationships at the intraspecific level and

allow for inferences regarding the biogeography and history of populations (Leigh and Bryant 2015).

3.2 Materials and methods

3.2.1 Fruit fly collection

Fruit flies were collected using yellow, McPhail-type bucket traps baited with one of the four lure dispensers at four highveld and four lowveld sampling sites, as described in Chapter 2.

3.2.2 Nucleic acid extraction

Nucleic DNA was extracted from 211 specimens, one of which was *C. simi* collected on campus during experimental layout trial, using the NucleoSpin® tissue kit (MACHEREY-NAGEL, Germany). Preceding the use of the NucleoSpin tissue kit the fruit fly specimens were washed to remove environmental contaminants and serially rehydrated using double distilled water (ddH_2O). The rehydration process involved a triple replication of a 30-minute immersion of the whole fruit fly in the ddH_2O. The rehydration was done as the fruit flies were stored in absolute (100%) ethanol. Following the rehydration, the specimen was pierced multiple times using a sterile dissecting needle following which the extraction protocol was utilized according to manufacturer's instructions, with an overnight Proteinase K digestion at 56°C.

3.2.3 PCR amplification of COI gene region

COI barcoding was conducted using universal *COI* primers published by Folmer et al. (1994) that target a 750 bp region corresponding to the cytochrome oxidase I gene within the mitochondrial genome. PCR reactions were performed in a final volume of 40 µl, containing 1.5 U of DreamTaq (Thermo Fisher Scientific, USA), 1X buffer, 0.4 mM dNTPs (Fermentas Thermo Fisher, USA), 0.4 µM of the forward primer LCO1490 5'GGTCAACAAATCATAAAGATATTGG3', 0.4 µM of the reverse primer HCO2189 5' TAAACTTCAGGGTGACCAAAAAATCA3', and 3 µl of template DNA. Thermal cycling was performed on the ABI 3500 genetic analyser (Applied Biosystems) under the following conditions: an initial denaturation step at 96℃ for 10s, annealing at 48℃ for 30s, and elongation at 70℃ for 1 minute, this was repeated for 40 cycles. This was followed with a final elongation step at 70℃.

3.2.4 DNA sequencing

PCR products were electrophoresed through a 1.5% agarose gel against a 1kb DNA molecular weight marker (Thermo Fischer, USA) in order to estimate the product size. Agarose gels,

stained with Goldview (Guangzhou Geneshun Biotech Ltd.), were visualized by UV irradiation and PCR products of the expected size were purified directly from the tube using the Roche PCR Product Purification Kit (Roche Diagnostics GmbH, Manneheim, Germany), according to manufacturer's instructions. Bi-directional cycle sequencing was performed using the BigDye v.3.1 terminator cycle-sequencing kit (Perkin Elmer, USA), with each of the external PCR primers in separate reactions at the annealing temperature of 48°C. Unincorporated primers, dNTPs and fluorescently labelled ddNTPs were removed by sodium-acetate precipitation and the purified products were then submitted to the Core Sanger sequencing facility at the University of Pretoria. Sequence chromatograms were visualized and edited in the Chromas programme embedded in MEGA 7 (Kumar et al. 2016) and used to generate contiguous sequences (contigs). Each contig is a consensus sequence created through the combination of the forward and reverse sequenced PCR products.

3.2.5 Phylogenetic analyses: Maximum likelihood and Bayesian inference

Each contig was used in a nucleotide BLAST (Basic Local Alignment Search Tool) search against the Genbank database (https://blast.ncbi.nlm.nih.gov/) to identify the most closely related sequences. The final aligned dataset comprising 211 specimens and representatives of ten tephritid fruit fly species was used to infer an initial p-distance neighbour-joining tree. This assisted with identifying the total number of haplotypes (N*h*) and allowed for the removal of multiple representatives of the same haplotype from the same locality thereby reducing the dataset size to 127 sequences. The same 211 taxon dataset was run in DnaSP 6 (Rozas et al. 2017) to crosscheck the number of unique haplotypes and representative sequences for each. Subsequent analyses with the reduced 127 taxon dataset included maximum likelihood (ML) and Bayesian inference (BI) performed in MEGA 7 (Kumar et al. 2016) and MrBayes (Huelsenbeck and Ronquist 2001), respectively. The best-fit model of sequence evolution was identified under the corrected Akaike information criterion (AICc) in MEGA 7 (Kumar et al. 2016). The best-fit model also guided the selection of priors for BI. Two independent runs each comprising of four chains (one cold and three heated) were performed over 10 million generations, with sampling every 1000 generations. The initial 25% of each run were discarded as burn-in based on multivariate visualization run on MCMC Tracer Analysis Tool version 1.7 (Rambaut et al. 2018).

3.2.6 Haplotype networks and population genetic parameters

This final dataset comprising 211 specimens was partitioned at the species level and were used to compile single-species datasets for the nine species identified through *COI* barcoding. The only exception was the *Ceratitis* FARQ complex, for which data of the two representative species were combined. Three datasets were generated for each of the individual species/species-complex, viz. (i) all representative sequences for a species/species-complex, (ii) all unique haplotypes by sampling locality and (iii) unique haplotypes only. Datasets belonging to *C. simi*, *C. quinaria*, *C. rubivora* and *D. ciliatus* were not generated for haplotype network analysis due to the small samples size (Table 3.1). Thus, a total of five species were subjected to population-level analyses. DnaSP 6 (Rozas et al. 2017) was used to confirm haplotype diversity (h), and nucleotide diversity (π). The Mantel test was used to assess whether there was a significant relationship between geographic and genetic distance/isolation by distance (IBD). The test with 100 permutations was conducted using ARLEQUIN 3.5.2.2 (Excoffier and Lischer 2010). The minimum straight-line distance between the GPS coordinates of sampling sites were taken as the geographical distance.

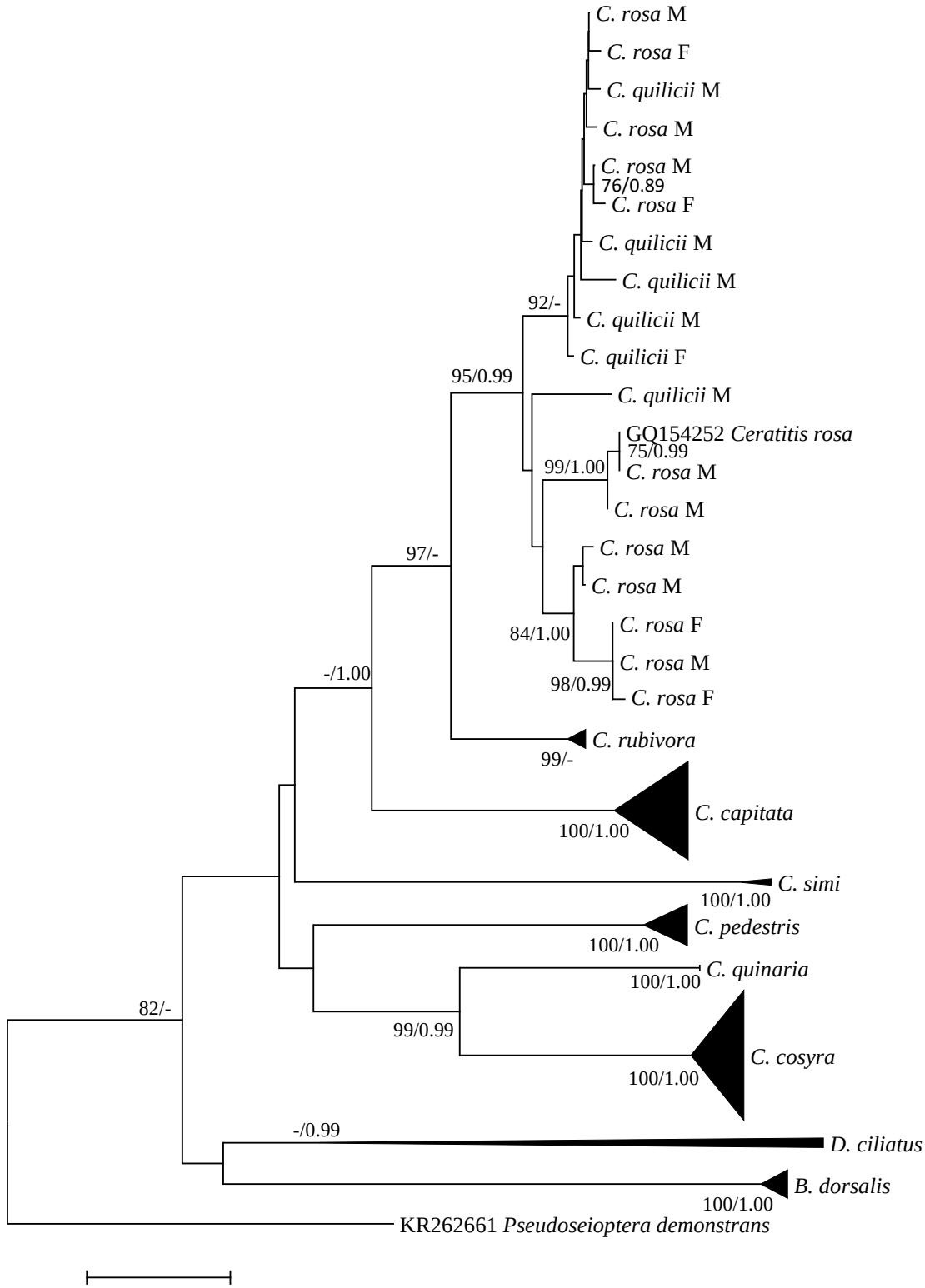
Haplotype accumulation curves were generated in R Studio (version 1.4.1103, Rstudio, Inc.) version 3.4.0.3 (Team 2013, Team 2015). The 'spider' package was used to compute and plot haplotype accumulation curves through the 'HaploAccum' function, 1000 permutations were used. The 'chaoHaplo' function was used to calculate Chao1 which is an estimate of the number of haplotypes in the population based on the total number of haplotypes present, while accounting for the number of singletons and doubletons in the dataset. The output result of the chaoHaplo function is an estimate of the total number of haplotypes in the population, within the lower and upper 95% confidence limits. An 'abline' was generated to show an estimated haplotype diversity where each sample collected is a new haplotype. The five created datasets were used for the visualization of the haplotype networks using Population Analysis with Reticulate Trees (POPART). A TCS haplotype network was constructed using an agglomerative approach where clusters are progressively combined with one or more connecting edges (Leigh and Bryant 2015).

3.3 Results

3.3.1 *COI* phylogenetic analyses

Sequences generated in this study were used in nucleotide BLAST (blastN) searches to identify the closest sequence matches available in the Genbank database. Duplicate sequences for each

haplotype were removed, resulting in a dataset comprising of 127 sequences (summarized in Table 3.3) that was trimmed to a length of 604 bp. The best-of-fit model of sequence evolution selected under the AICc was the GTR+G+I; the general time reversible model with a gamma distribution shape parameter (G) of 1.00 and proportion of invariant sites (I) of 0.56. The *COI* gene phylogeny (Figure 3.1) confirmed that the barcode region accurately assigned *B. dorsalis*, *C. capitata*, *C. cosyra*, *C. pedestris*, *C. quinaria*, *C. simi* and *D. ciliatus* fruit fly species (100% accuracy for each). However, *COI* barcodes could not discern species that formed part of the *Ceratitis* FARQ complex to which *C. quilicii* and *C. rosa* belong.



3.3.2 Haplotype analyses and networks

COI barcodes generated for 47 *B. dorsalis* specimens, recovered five haplotypes and a haplotype diversity of 0.549, , a nucleotide diversity (π) of 0.0036 (Table 3.1). For the invasive *B. dorsalis*, the haplotype network reveals two dominant haplotypes, BdH1 and BdH5, with the former being more abundant and more broadly distributed (occurring at all eight sites) than BdH5 (detected only at 5 sites). These two haplotypes are separated by four mutational steps and likely correspond to two separate introductions that occurred Haplotypes BdH3 and BdH4, link respectively to BdH1 and BdH5 with one mutational step and are likely to have arose from each of these introduced haplotypes. All other haplotype linkages are separated by three or more mutational steps.

Members of the *Ceratitis* genus are characterised by high haplotype diversity (h) >0.85. For *C. capitata* 30 unique haplotypes were recovered from 40 specimens that were sequenced with a haplotype diversity of 0.945, a nucleotide diversity (π) of 0.00661 and a Chao1 estimate of 353 haplotypes. From 53 *C. cosyra* specimens sequenced, 35 unique haplotypes and a haplotype diversity of 0.978, a nucleotide diversity (π) of 0.009 and a Chao1 estimate of 98 haplotypes were recovered. Eleven haplotypes were identified from the 19 *C. pedestris* sequences generated with haplotype diversity of 0.860, a nucleotide diversity (π) of 0.0049 and a Chao1 estimate of 47. For the sister complex of *C. quilicii* and *C. rosa*, 17 haplotypes were identified from the 52 sequences generated, a nucleotide diversity (π) of 0.01443 and the haplotype diversity was found to be 0.916 and a Chao1 estimate of 29. For *C. rubivora* and *D. ciliatus* four and two haplotypes were recovered, respectively, whereas *C. quinaria* and *C. simi* had only one haplotype, corresponding to the single sample collected for each. The sequences for the latter four species were excluded from further analysed as there were too few representatives.

Table 3.1: Summary of the number of specimens characterised for each species/species complex and the corresponding dataset (A-D) used for haplotype analyses. For the *C. quilicii* and *C. rosa* species complex, data were pooled (dataset E) for the analyses.

DNA extracted fruit flies				**Dataset statistics**						
Species	**Highveld**	**Lowveld**	**Total (N)**	**Dataset**	**Length (bp)**	**N*h***	***π***	***h***	**Chao1 estimated N*h***	**Mantel – regression coefficient**
B. dorsalis	21	26	47	A	599	5	0.0036	0.549	12	0.000194
C. capitata	17	23	40	B	551	30	0.0066	0.945	353	0.000325
C. cosyra	45	8	53	C	521	35	0.0090	0.978	98	-0.000002
C. pedestris	19	0	19	D	551	11	0.0440	0.860	47	0.002971
C. quilicii	0	29	29	E	608	17	0.0144	0.916	29	-0.000328
C. rosa	23	0	23							
C. rubivora	0	4	4							
C. quinaria	0	1	1							
C. simi	0	1	1							
D. ciliatus	2	0	2							

Nh: number of haplotypes, h: haplotype diversity, π: nucleotide diversity

No discernible correlation between genetic and geographic distances was recovered from the regression coefficient of the Mantel Test, for any of the five datasets indicating an absence of isolation by distance. Haplotype networks were inferred using POPART following the completion of the haplotype accumulation curves. The angle for the curve for *B. dorsalis* (dataset A; Fig 3.2) is far from the 1:1 ratio where every new sample represents a new haplotype, indicating a low haplotype diversity. This is supported by a Chao1 estimate of 12 haplotypes. For *C. capitata, C. cosyra* and *C. pedestris*, the angle of the haplotype accumulation curve is close to a 1:1 ratio. This is indicative of a high haplotype diversity and is also evident in the Chao1 estimates of 353, 98 and 47 haplotypes for each of the three species (Figure 3.3, 3.4 and 3.5). In the instance of the *C. quilicii* and *C. rosa* (dataset E; Fig 3.6), the angle of the haplotype curve is closer to the 1:1 ratio dotted line than for *B. dorsalis* but not as close as for the other *Ceratitis* species. This is indicative of moderate diversity but not as high as observed for the other *Ceratitis* species. The Chao1 estimates that there are 29 haplotypes, thus 11 more than the 17 identified in this study when using dataset E (*Ceratitis rosa* s.s. and *C. quilicii* combined).

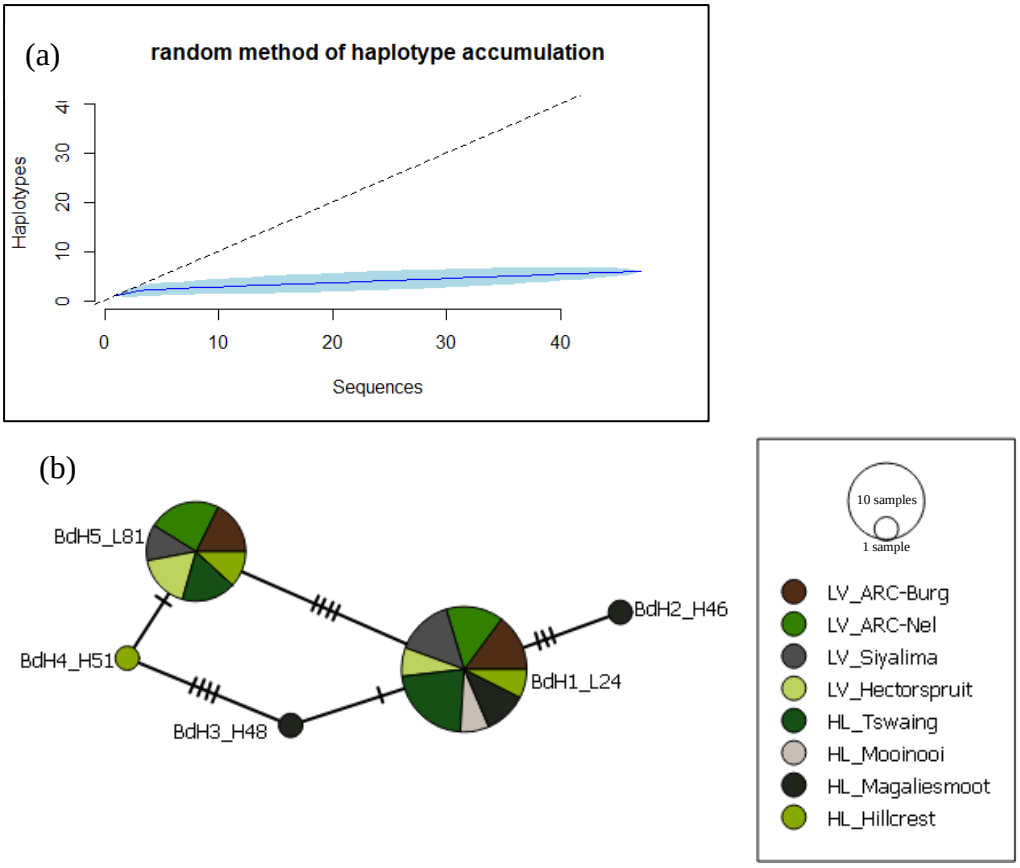


Figure 3.2: (a) Haplotype accumulation curve for *B. dorsalis* with a dotted line showing a 1:1 occurrence of haplotypes per sampled sequence. (b) Haplotype network showing the relatedness and distribution of the five haplotypes found in the 47 *B. dorsalis* fruit flies. Highveld sampling sites have been demarcated with HL and lowveld with (LV) in the colour-code key to the right. The circle size corresponds to the number of specimens and the horizontal bars indicate the number of mutations separating haplotypes.

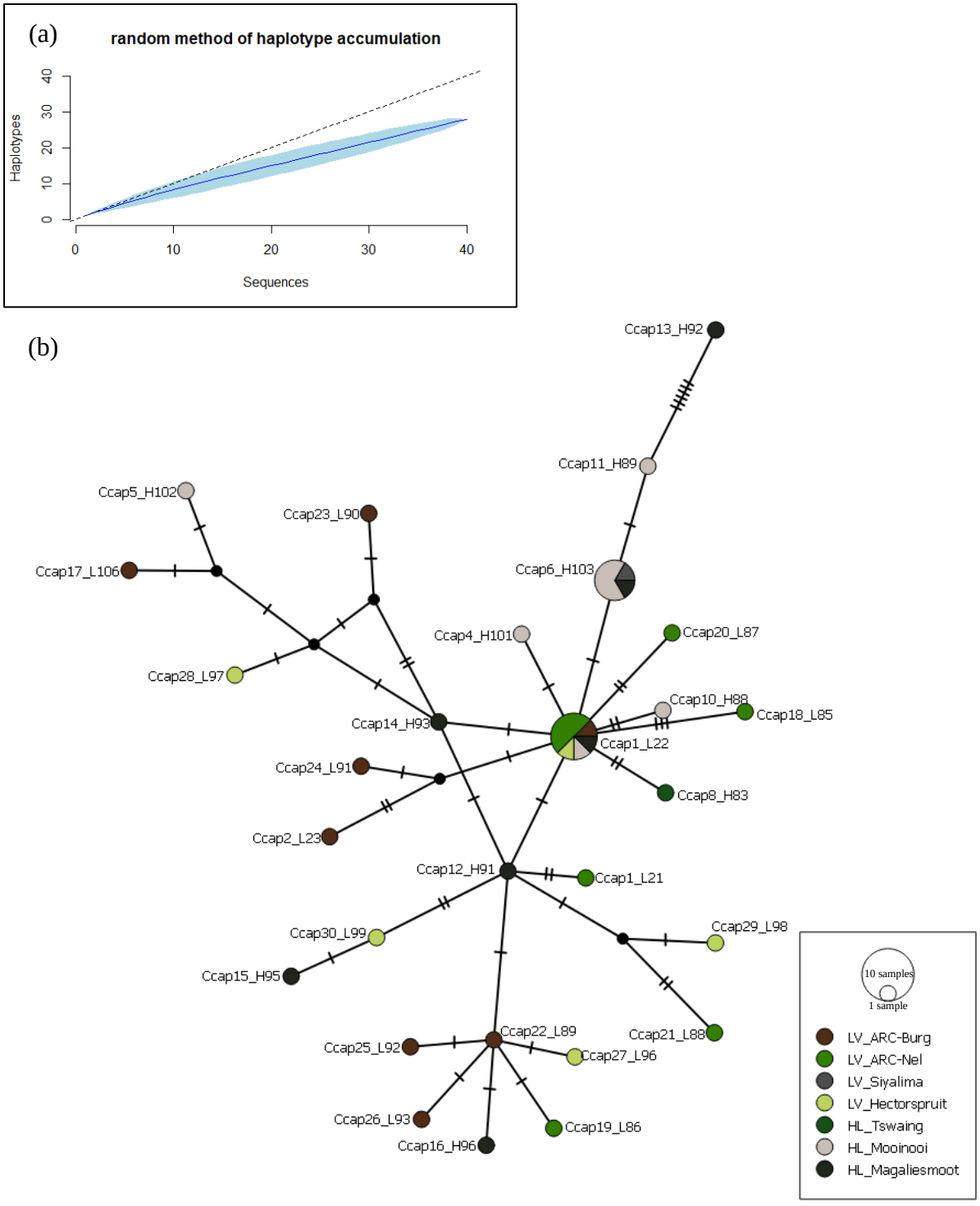


Figure 3.3: (a) Haplotype accumulation curve for *C. capitata* with the dotted line showing a 1:1 occurrence of haplotypes per sampled sequenced. (b) Haplotype network showing the relatedness and distribution for the 30 haplotypes found in the 40 *C. capitata* fruit flies in the Highveld (HL) and Lowveld (LV). Circle size corresponds to the number of specimens and the horizontal bars indicate the number of mutations separating each haplotype.

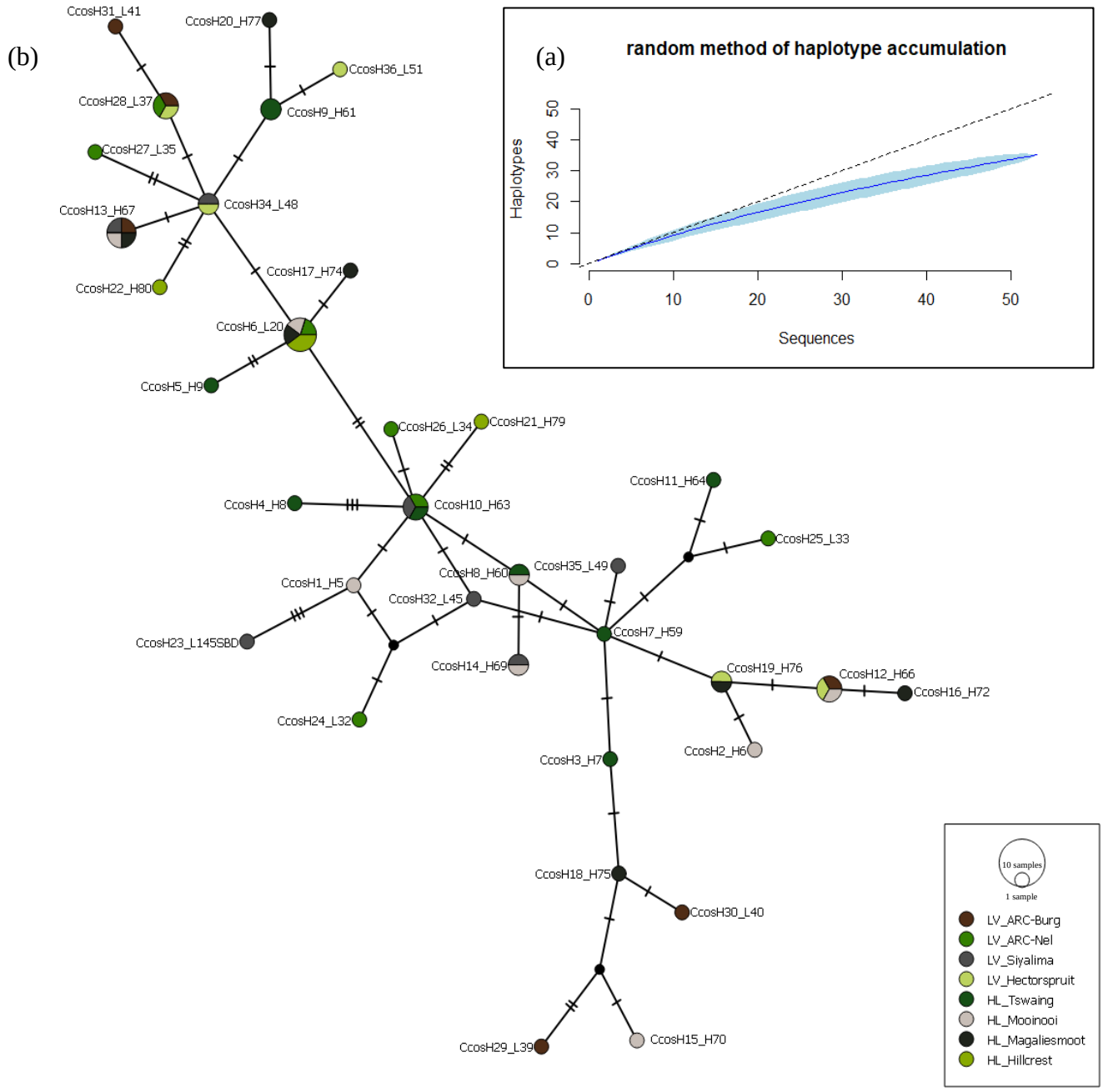
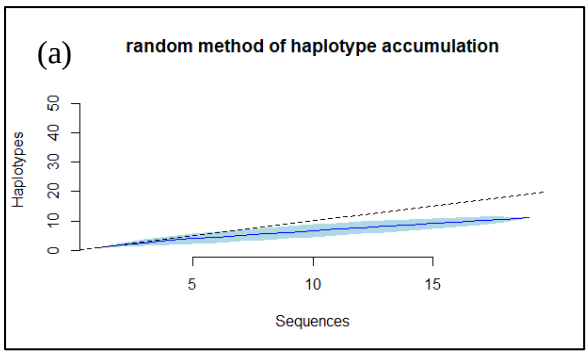


Figure 3.4: (a) Haplotype accumulation curve for *C. cosyra* with the dotted line showing a 1:1 occurrence of haplotypes per sampled sequenced. (b) Haplotype network showing the relatedness between the 38 haplotypes found in the 40 *C. cosyra* fruit flies in the highveld (HL) and lowveld (LV). Circle size corresponds to the number of specimens and the horizontal bars indicate the number of mutations separating each haplotype.

(a)



(b)

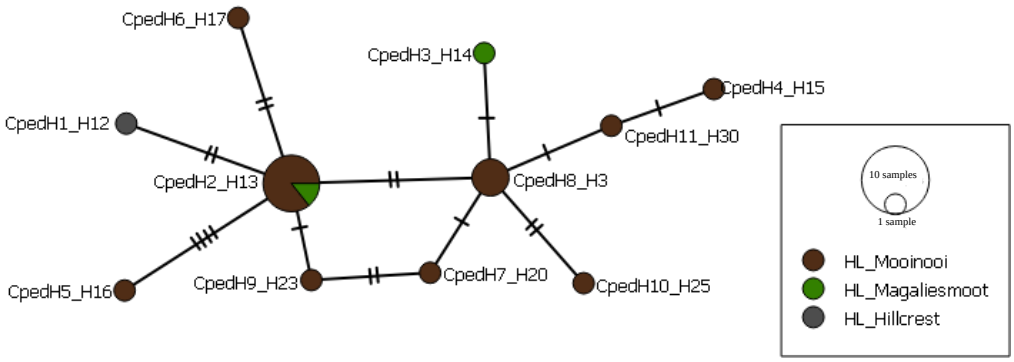


Figure 3.5: (a) Haplotype accumulation curve for *C. pedestris* with the dotted line showing a 1:1 occurrence of haplotypes per sampled sequenced. (b) Haplotype network showing the relatedness between the 12 haplotypes found in the 19 *C. pedestris* fruit flies in the Highveld (HL). There are no representatives from the Lowveld. Circle size corresponds to the number of specimens and the horizontal bars indicate the number of mutations separating each haplotype.

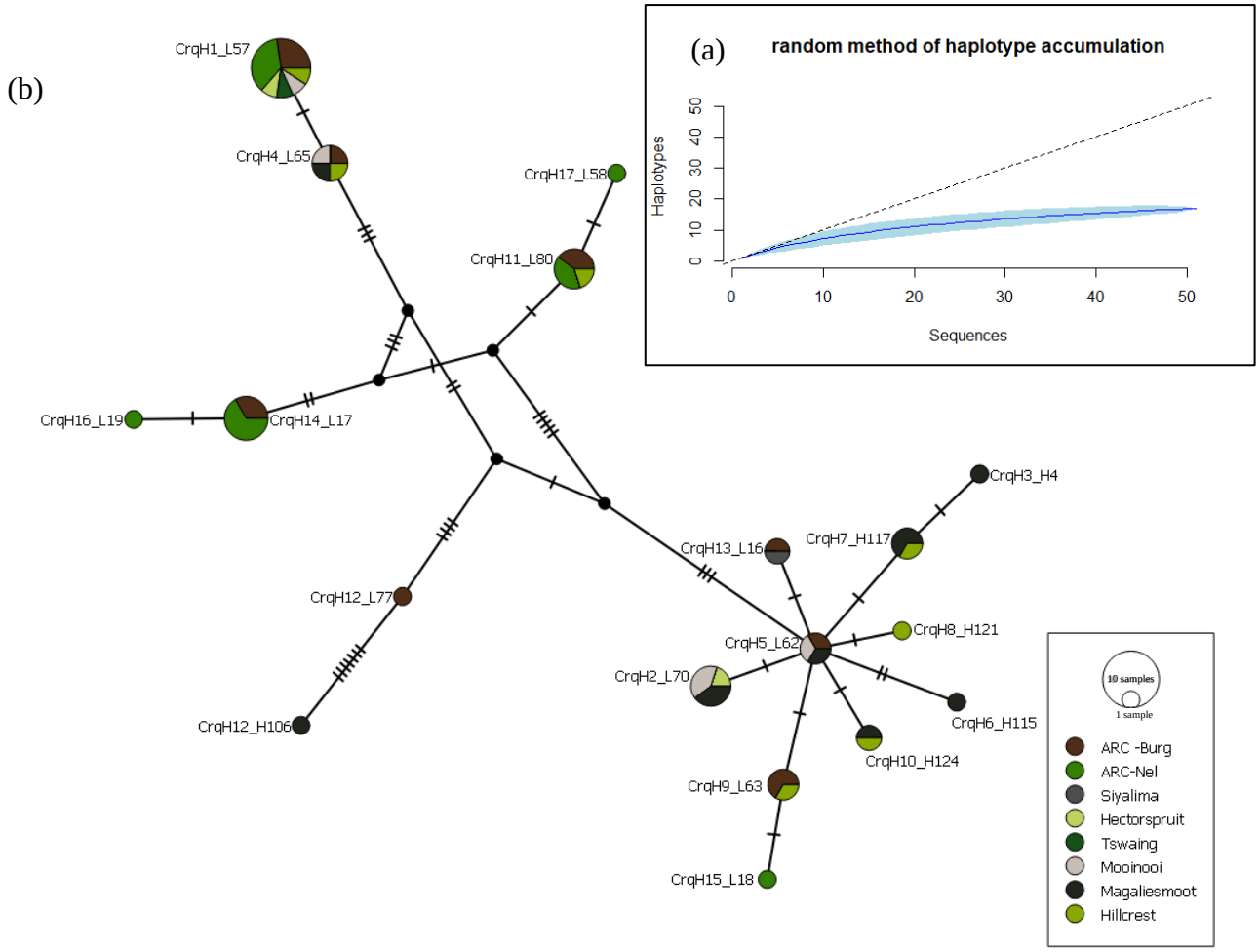


Figure 3.6: (a) Haplotype accumulation curve for *C. rosa* and *C. quilicii* with the dotted line showing a 1:1 occurrence of haplotypes per sampled sequenced. (b) Haplotype network showing the relatedness between the 17 haplotypes found in the 52 *C. rosa* and *C. quilicii* representative fruit flies in the Highveld (HL) and Lowveld (LV). Circle size corresponds to the number of specimens and the horizontal bars indicate the number of mutations separating each haplotype.

3.4 Discussion

Cytochrome *c oxidase I* (COI) barcodes generated for the fruit flies accurately identified all fruit flies with the exception of those species belonging to a complex of closely related species. Species assignment accuracy with and without the representatives of the FARQ complex was 63% (*C. quilicii* and *C. rosa* incorrectly sorted) and 100%, respectively. A feasibility test was conducted on the molecular identification of European fruit flies based on *COI* barcode to identify species from 555 sequences of 135 ingroup species from three subfamilies and 42 genera with the mandatory outgroup (Smit et al. 2013). This study reported an accurate identification of 73.3%. This low identification percentage was improved to 87.1 % through the removal of singletons and problematic groups (Smit et al. 2013). It was evident in the generated *COI* phylogenetic tree, that *COI* barcodes for *C. quilicii* and *C. rosa* could not be separated. Jiang et al. (2014) reported that *COI* barcoding is incapable of identifying and separating members of the *Ceratitis* 'FARQ' complex, where the aforementioned species are members. This aligns with the proposition of Hebert et al. (2003a) that *COI* barcoding should act together with traditional taxonomic practices. A better-suited molecular method would be microsatellite profiling which has been shown to be capable of delineating the members of the *Ceratitis* 'FARQ' complex. Owing to the economic importance of multiple fruit flies, tephritids are a focal group for DNA barcoding studies. Although, *COI* barcodes could not separate *C. rosa* and *C. quilicii*, the remaining *Ceratitis* species were readily identified with high levels of support (bootstrap values and posterior possibility values >70% and >0.95, respectively; Figure 3.1.

The *COI* gene tree (Figure 3.1) shows the accurate separation of *B. dorsalis* and *D. ciliatus*. This aligns with studies that report a 91.2% rate of successful identification using *COI* barcoding (You et al. 2014). Krosch et al. (2020) reiterated the *COI* barcoding could not successfully separate fruit flies in complexes, in this case the *B. tryoni* complex. The researchers surmised that this may be due to nuclear mitochondrial DNA (numt) similarity amongst *B. xanthodes, B. passiflorae* and *B. musae* (Krosch et al. 2020). To mitigate the inefficiency of *COI* barcoding, internal primers were used and they successfully amplified *COI* barcodes for which full length fragments could not be amplified (Krosch et al. 2020). Although, *COI* barcoding was not entirely successful for species delineation, the gene region did allow for the identification of multiple haplotypes from the separate sites.

Haplotype networks drawn using the TCS network are constructed using an agglomerative approach where clusters are progressively combined with one or more connecting edges (Clement et al. 2002, Leigh and Bryant 2015). The haplotype network for *B. dorsalis* (Fig 3.2) identified five unique haplotypes (with a haplotype diversity = 0.549, Table 3.1) which was the lowest level of haplotype diversity recovered for the five species evaluated. The low levels of haplotype diversity in the lowveld may be due to the relatively recent (2010) entry of *B. dorsalis* into the Limpopo, North-West and Gauteng provinces (Manrakhan et al. 2015). Wu et al. (2012) reported that *B. dorsalis* populations in their early stages of invasion are less genetically diverse than those that have long been established. This is further supported by the low number of mutational steps separating the five haplotypes as well as the low level of haplotype diversity observed for the *B. dorsalis* dataset (dataset A).

In contrast, high genetic and haplotype diversity was observed in both the highveld and lowveld for *C. capitata* and *C. cosyra*. A similarly high genetic diversity was observed in a study by Karsten et al. (2013) conducted using samples collected from locations in the Western Cape, Northern Cape, Eastern Cape, Limpopo and Gauteng Provinces in South Africa. In that study it was suggested that South Africa is part of the native range of *C. capitata* given that there is no genetic difference between individuals found in South Africa and elsewhere in Africa (Karsten et al. 2015). A later study by Karsten et al. (2018) suggested that this pattern of no differentiation may be due to inland dispersal of *C. capitata*. Our analyses identified 30 haplotypes from 40 *C. capitata* specimens (Fig 3.3; Haplotype diversity = 0.945). *Ceratitis cosyra* (Fig 3.4; Haplotype diversity = 0.978), *C. quilicii and C. rosa* (Fig 3.6; Haplotype diversity = 0.916) were reported to have rather high haplotype diversity throughout sub-Saharan Africa, however this high haplotype diversity was reported from small sample sizes (Elfekih and Haymer 2010). *Ceratitis quilicii and C. rosa* have been considered as a complex due to incomplete lineage sorting using *COI* barcoding. The haplotype network generated from this complex also shows that there is an overlap of haplotypes between the highveld and lowveld, this is of particular interest as *C. quilicii* was collected in the highveld and *C. rosa* in the lowveld. Furthermore, there was no correlation between the genetic and geographic distances. The separation of the two species is supported by the distribution of these fruit flies as being mostly associated with highland and lowland regions, respectively (Tanga et al. 2018). *Ceratitis pedestris* also has a high genetic diversity with 12 haplotypes being identified from 19 specimens (Haplotype diversity = 0.860). This species was only collected in the highveld

region. This species like other members of the genus *Ceratitis* in this study are of Afrotropical origin with occurrence records in South Africa.

Phylogenetic trees when drawn using *COI* barcodes sourced from BOLD systems (https://v3.boldsystems.org/), revealed the existence of haplotypes that were not detected in our sampled population. For the species *B. dorsalis* 98 haplotypes were recovered from 308 samples with most of the unique samples originating from Asia (Appendix Fig 1). Similarly, high diversity is observed in *C. capitata* (43 haplotype recovered from 81 samples; Appendix Fig 2), *C. cosyra* (84 haplotypes recovered from 169 samples, Appendix Fig 3), *C. pedestris* (15 haplotypes recovered from 24 samples; Appendix Fig 4), and the joint *C. quilicii* -*C. rosa* (26 haplotypes from 78 samples; Appendix Fig 5) when globally sourced samples are considered. Another observation from these phylogenetic trees was that most of the haplotypes recovered in my study were shared with those uploaded onto the repository, thus our samples are reflective of results found globally. However, this was a cursory assessment and a more in-depth analysis of haplotype diversity across the species distributional range may reveal more unique and shared haplotypes.

The results suggest that indigenous or longer-term invasive fruit flies have been able to radiate separate populations from the original one. This is further suggested when *B. dorsalis* haplotype diversity and entry into South Africa are taken into consideration. A clearer understanding of this phenomenon may be observed through a long-term haplotype diversity investigation study. For this aforementioned project and future studies, I would suggest larger samples sizes thus the haplotype accumulation curves may have a more evident plateau which indicates that sampling has been done to a point of "near-saturation", thus allowing for a more accurate identification of the haplotype diversity for each species. Additionally, I would suggest a survey or monitoring programme throughout South Africa specifically along the distribution of certain fruit flies to determine whether they are limited to these regions, this may allow for a more accurate inference of the relationship between the genetic diversity and geographic distance.

Chapter 4. Molecular detection and characterisation of *Wolbachia* in fruit flies from South Africa

4.1 Introduction

Symbiotic bacteria are omnipresent in nature, having a significant impact on eukaryotic evolution and diversity (Kikuchi 2009). Some symbiotic bacteria are harmful or even lethal and referred to as parasites or pathogens, while others are beneficial to hosts and are known as mutualists (Kikuchi 2009). The historical emphasis on pathogenic bacteria and their diseases led to an assumption that genes encoding virulence factors are specific to those relationships. However, several of the cellular and molecular mechanisms that underlie interactions between an animal and its beneficial microbiota are remarkably similar to those found in pathogenic bacteria (Ruby et al. 2004). Molecular methods have played a key role in shedding light on these relationships.

The class Insecta accounts for >90% of known animal species and dominates a variety of terrestrial habitats. Insects are colonised by benign and beneficial microorganisms (Douglas 2014), with approximately half of all insect species presumed to harbour endosymbiotic bacteria (Kikuchi 2009, Douglas 2014). As a consequence, microbial interactions with insects can affect behaviour, tolerance to environmental perturbations, resistance to pathogenic microbiota, and, the maintenance and/or development of the immune system (Bahrndorff et al. 2016).

Microbial protection in insects is a well-documented phenomenon. Piel (2002) reported that *Pseudomonas* sp. (Migula, 1894) in *Paederus* (Fabricius, 1775) rove beetles synthesize "pederin", which the beetles use as protection from predation. Another bacterium of the genus *Spiroplasma* (Saglio, 1973), protects *Drosophila hydei* (Sturtevant, 1921) against parasitic wasps (Xie et al. 2010) and *Drosophila neotestacea* (Grimaldi, James & Jaenike, 1992) against *Howardula* (Mason & Heinz, 2012) nematode parasites (Jaenike et al. 2010). Another fascinating interaction is between aphids and *Buchnera* (Munson *et al*., 1991), where the maternally inherited bacterial symbionts aid in metabolite synthesis (Douglas 1998, 2014). As symbiotic microbes can influence the ability of their host to transmit pathogens, they hold potential for developing disease control strategies that harness the role of symbiotic microbes in reducing vector-borne pathogen transmission by an insect host (Weiss and Aksoy 2011).

4.1.1 *Wolbachia*

Wolbachia (Hertig, 1936) is a genus of endosymbiotic alpha-proteobacteria that together with the pathogenic tick-borne genera *Ehrlichia* (Moshkovis, 1945) and *Anaplasma* (Theiler, 1910), comprise the family Anaplasmataceae. Anderson and Karr (2001) reported that *Wolbachia* has

been found to share a recent common ancestor with *Ehrlichia*, with the separation between the two owing to the derived state of *Wolbachia* where it is able to manipulate host reproduction. The principal hosts of *Wolbachia* are terrestrial arthropods, and initial estimates for the number of infected species ranged from 20% to 76% (Werren et al. 1995, Jeyaprakash and Hoy 2000). Recently this range has been refined to around 50% of arthropod species being infected with *Wolbachia*, primarily due to more efficient molecular techniques and the broader assessment of insect hosts (Zug and Hammerstein 2012, Weinert et al. 2015). *Wolbachia* are present in most tissues of infected individuals, including ovaries and testes (Dobson et al. 1999, Cheng et al. 2000). It been suggested that *Wolbachia* to rely on their ability to alter the host phenotype to perpetuate their own spread (Moran et al. 2008). Such phenotypic alterations generally result in a reduction in reproductive opportunities for non-infected individuals, giving a relative advantage to infected individuals to pass the symbiont to their offspring (Correa and Ballard 2016).

The genus *Wolbachia* is delineated into 16 'supergroups': A-F and H-Q (Gerth et al. 2014, Bleidorn and Gerth 2017). The term supergroup is employed to avoid confusion with the designation of more closely related groups (Zhou et al. 1998b). Supergroups are associated with host-specificity and the type of symbiotic relationship that occurs between the host and bacteria (Bleidorn and Gerth 2017). Supergroup A and B belong to the same clade and share their ability to adapt to a wide arthropod host range (Table 3.1; Gerth et al. 2014). Supergroups C and D are only found in filarial nematodes, while Supergroup E occurs in Collembola and Supergroup F is present in both arthropods and two nematodes of the genus *Mansonella* (Manson, 1891) (Casiraghi et al. 2005, Mercot and Poinsot 2009).

Table 4.1: Summary of the hosts and the lifestyle of 13 of the 16 *Wolbachia* supergroups (adapted from Lo *et* al. (2002), Werren *et* al. (1995) and Wang *et* al. (2016)). The lifestyles for supergroups H-Q have not been reported.

Supergroup	Host/s	Lifestyles
A and B	Arthropods	Parasitism; facultative, proximate, and obligate mutualism
C and D	Filarial nematodes	Obligate mutualism
E	Springtails and mites	Parasitism with evolved dependencies
F	Termites and nematodes	Parasitism; obligate mutualism
H	Termites	
K	Mite (*Bryobia*)	
M	Aphid (*Cinara* and *Toxoptera*)	
N	Aphid (*Toxoptera*)	
O	Silverleaf whitefly (*Bermisi tabaci*)	
P	Mites (*Syringophilopsis* and *Torotrogla*)	
Q	Mites	

Wolbachia are vertically transmitted bacteria, thus they are entirely dependent on the host for survival and transmission. The presence of a *Wolbachia* infection has been suggested to have links to the provision of nutrients, vitamins, and proteins to the host (Zug and Hammerstein 2015, Kamtchum-Tatuene et al. 2017). *Wolbachia* has been implicated in the mediation of host protection against arboviruses, which stems from competition for resources, the pre-activation of the host immune system, and/or the induction of microRNA (miRNA) (Zug and Hammerstein 2015, Kamtchum-Tatuene et al. 2017).

While *Wolbachia* infection can have positive outcomes for the host, these bacteria can have detrimental effects as well, including changes to the reproductive system of the host to ensure own propagation (Kamtchum-Tatuene et al. 2017). These alterations to the host reproductive system include male killing, parthenogenesis induction, and feminisation, all of which skew the sex ratio in favour of more females (Anderson and Karr 2001). Additionally, some strains of *Wolbachia* are known to bring about the phenomenon of cytoplasmic incompatibility (CI). Cytoplasmic incompatibility is separated into two variations, unidirectional CI occurs when infected males mate with uninfected females; this cross results in aborted fertilization while fertilization and infection persistence occurs when the female is also infected. Bi-directional CI occurs when males and females are infected with different and mutually incompatible *Wolbachia* strains, leading to reduced hatch rates in both directions of the cross (Mercot and Poinsot 2009).

Frentiu et al. (2014) reported that *Aedes aegypti* (Linnaeus, 1762) infected with the *wMel* strain of *Wolbachia* showed reduced replication and transmission of the dengue virus (*Flavivirus*, Flaviviridae) in laboratory trials. Similar replication reduction had been reported in other RNA viruses such as yellow fever (van den Hurk et al. 2012), chikungunya (Moreira et al. 2009) and West Nile (Hussain et al. 2013). The current iteration of the Eliminate Dengue Programme has been expanded to develop *Wolbachia* as an intervention to control mosquito-transmitted viral diseases which include dengue, Zika and chikungunya, and this expanded programme was renamed the World Mosquito Programme (WMP; https://www.worldmosquitoprogram.org) (O'Neill 2018). The WMP reported that in areas where there are high levels of cytoplasmic incompatibility inducing *Wolbachia* (surpassing an unstable equilibrium point estimated to be less than 0.3 for the *wMel* strain of *Wolbachia*) are present, reduced dengue virus transmission were observed (O'Neill 2018, https://www.worldmosquitoprogram.org). However, O'Neill (2018) reported that while *Wolbachia* is maintained at a very high frequency in wild mosquito populations it is rarely at complete fixation.

The occurrence of a *Wolbachia* infection can be confirmed through the amplification and sequencing of the gene region encoding the *Wolbachia* surface protein (*wsp*). The *wsp* gene has been routinely used for infection confirmation and supergroup designation (Baldo et al. 2005). However, *wsp* sequences can be highly recombinant making *wsp*-based supergroup designation unreliable (Baldo et al. 2005). This prompted the development of a multilocus sequence typing (MLST) approach for *Wolbachia* infection confirmation and supergroup assignment. Baldo et al. (2006) developed a *Wolbachia* MLST comprising of five conserved genes namely *gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*. These genes are housekeeping genes that are broadly distributed across the genome and subjected to purifying selection (Baldo and Werren 2007). The aforementioned housekeeping genes have been shown to be consistent in supergroup classification of a strain (Baldo et al. 2006), thus allowing for confirmation that initial *Wolbachia* surface protein (*wsp*) supergroup assignment was correct.

Multilocus sequence typing was meant to account for the *wsp* shortfall, however false positives and negatives have been reported for MLST assays performed with the, *ftsZ* and *gltA* housekeeping genes (Casiraghi et al. 2005). The 16S rRNA gene region, although highly conserved, has been reported as informative about infection status, gene transfer and supergroup designation (Morrow et al. 2015). Thus, a combination of 16S rRNA-*wsp* screening

and MLST approaches would be the beneficial for *Wolbachia* infection confirmation and the initial delineation of strains into the various supergroups.

To evaluate the suitability and effectiveness of pest control in programmes that rely on *Wolbachia* infection, the occurrence of infection in the pest populations before and during the programme needs to be accurately measured. Recording of both the proportion of infected individuals within the population (prevalence) and the change in levels of infection over time are important parameters describing the infection frequency of *Wolbachia*.

4.1.2 *Wolbachia* in the Tephritidae

In Africa there are 150 recorded tephritid genera and more than 50 species that are considered to be of economic importance (Grové and de Beer 2012). Fruit flies found in South Africa are classified within nine genera namely *Bactrocera* (Macquart, 1835), *Ceratitis* (Macleay, 1829), *Coelotrypes* (Bezzi, 1923), *Corpophthoromyia* (Hardy, 1977), *Dacus* (Fabricius, 1805), *Elaphromyia* (Bigot, 1859), *Munromyia* (Bezzi, 1922), *Perilampsis* (Bezzi, 1920) and *Trirhithrum* (Bezzi, 1918) (Manrakhan et al. 2017, Grové and de Beer 2019).

In the Tephritidae, several studies have detected *Wolbachia* strains in important pest species. In tephritid fruit flies, PCR and sequencing approaches have found that ~66% of 87 screened tephritid species have at least one record of positive *Wolbachia* presence; all *Wolbachia* detections were found to belong to the A and B Supergroups (Mateos et al. 2020). Twelve *Anastrepha* (Schiner, 1868) species in South America were reported as being infected with the *Wolbachia Wmel* strain belonging to supergroup A, thus showing that there is a high occurrence of *Wolbachia* in Tephritid fruit flies (Coscrato et al. 2009). Similarly, using a 16S rRNA screening assay, Rocha et al. (2005) detected *Wolbachia*, belonging to supergroup A, in three *Ceratitis capitata* (Wiedemann, 1824) collected in oranges from São Bento do Sapucaí, State of São Paulo, Brazil.

In the oriental fruit fly, *Bactrocera dorsalis* (Hendel, 1912), *Wolbachia* has been reported at low prevalence in populations from China (1.27%) and Thailand (1.15%) (Kittayapong et al. 2000, Sun et al. 2007). Gichuhi et al. (2019) reported a prevalence of 3.6% in the period between 2005 and 2009, and 1.1% in 2017 when 357 *B. dorsalis* individuals, collected in Tanzania, Uganda, and Sudan, were tested for infection and ten were positive for *Wolbachia*.

Overall low prevalence has also been reported in eight *Bactrocera* species and *Dacus axanus* (Hering, 1938) from specimens that were collected in tropical far north Queensland during

summer (Morrow et al. 2014). Prevalence rates were reported as *B. bryoniae* (Tryon, 1927) (7.85%), *B. decurtans* (May, 1965) (16.7%), *B. frauenfeldi* (Schiner, 1868) (14.7%), *B. neohumeralis* (Hardy, 1951) (9.8%), *B. peninsularis* (Drew & Hancock, 1981) (0%), *B. perkinsi* (Drew & Hancock, 1981) (100%, only five flies tested), *B. strigifinis* (Walker, 1861) (13.5%), *B. tryoni* (Froggatt, 1897) (4.1%), and *D. axanus* (10%). *Wolbachia*-infected fruit flies may be co-infected with multiple strains. In this regard, a single *Bactrocera ascita* (Hardy, 1983) individual was found to be infected with five *Wolbachia* strains (Jamnongluk et al. 2002) belonging to supergroups A and B.

The association between *Wolbachia* and tephritid flies is important due to the potential for *Wolbachia*-mediated pest control. However, the success of the approach hinges on two main requirements, the first being that *Wolbachia* strains are transferable between hosts and express the expected phenotype (Schneider et al. 2013). Multiple researchers have reported the successful transfer of *Wolbachia* by micro-injection from donor to recipient hosts within the same insect order, this was followed by the confirmation of their phenotype in the novel host (Poinsot et al. 1998, Zabalou et al. 2004, McMeniman et al. 2009). The second requirement is that the transferred infection and expected phenotypes persist in a stable manner in the novel host. Schneider et al. (2013) demonstrated that infections by multiple *Wolbachia* strains can shift in prevalence after artificial host transfer, they suggest that this may be due to either stochastic or selective processes.

The current study aims to determine the prevalence and diversity of *Wolbachia* in tephritid fruit flies from the lowveld and highveld regions of South Africa. Based on previous reports of *Wolbachia* prevalence and diversity in tephritid flies, I predict that (i) *Wolbachia*, if present, will occur at a low prevalence and (ii) any detected *Wolbachia* will belong to either the A or B supergroups.

4.2 Materials and methods

4.2.1 Fruit fly collection

Fruit fly samples were collected in South Africa from eight separate locations (Figure 2.1). These locations included four within the highveld region of South Africa, within 100 km of Pretoria. The highveld sampling was conducted from 26th March to the 2nd of April 2018. These locations included the University of Pretoria Experimental Farm (25°45'10.0"S 28°14'46.0"E;

altitude: 1327 m). The second location was the Tswaing Meteorite Crater (25°24'57.0"S 28°06'00.0"E; altitude: 1120 m). The third location was Magaliesmoot AH (25°42'54.7"S 28°02'46.8"E; altitude: 1271 m). A farm in Mooinooi (25°46'25.4"S 27°36'27.6"E; altitude: 1290 m) on the outskirts of Brits was the fourth location.

Four lowveld locations were sampled from the 3rd to the 11th of April 2018. These locations occur within the area of the Mpumalanga province and are situated within a 100 km radius of Mbombela (formerly Nelspruit). The Siyalima Boerdery (25°24'57.5"S 31°45'59.7"E; altitude: 530 m) and Hectorspruit Farm (25°26'23.3"S 31°40'51.8"E; altitude: 280 m) were sampled first, followed by two ARC Institute for Tropical and Subtropical Crops research stations sites, one in Mbombela (-25.45127 S 30.96919 E; altitude: 691 m) and the other in Burgershall (25°06'39.0"S 31°05'02.0"E; altitude: 759 m).

Fruit fly sampling was conducted using yellow, McPhail-type bucket traps (Chempac bucket trap, Chempac (Pty) Ltd., Suider Paarl) baited with one of three lure dispensers. Biolure® (Suterra LLC, Bend, USA and distributed in South Africa by Chempac (Pty) Ltd., Suider Paarl) is a three-component lure [ammonium acetate, trimethylamine hydrochloride and 1,4-diaminobutane (putrescine)] that attracts *Ceratitis* flies along with other tephritid species (Heath et al. 2007). Methyl eugenol (ME; Chempac, Suider Paarl) attracts sexually mature, male *B. dorsalis* (Vargas et al. 2000). Enriched ginger oil (EGO) lure (Insect Science, Tzaneen) attracts males of *Ceratitis* species (Manrakhan et al. 2017). During the sampling, three clusters of three traps with each lure type represented were placed at a minimum distance of 100 m from each other. This layout was selected based on the results of the pilot study described in Chapter 2. Traps within a cluster were placed 50 m from each other as per manufacturer recommendations. Traps in each cluster were deployed for seven days, during which time they were emptied daily and collected specimens were separated and stored individually in absolute ethanol in 1.5 mL microcentrifuge tubes. Once stored, the collected specimens were transported to the Hatfield Campus of the University of Pretoria for morphological identification, performed using a dissecting microscope and with reference to published taxonomic keys (White and Elson-Harris 1992, De Meyer et al. 2016).

4.2.2 *Wolbachia* screening

Initial screening was conducted on a subset of 246 fruit flies (Summarised in Table 4.2), sampled from four lowveld and four highveld sampling sites and representative of five morphologically identified species (Chapter 2). Nucleic DNA was extracted using the

NucleoSpin® tissue kit (MACHEREY-NAGEL, Germany). Preceding the use of the NucleoSpin tissue kit the fruit fly specimens were washed and serially rehydrated using double-distilled water (ddH_2O). The rehydration process involved a triple replication of a 30-minute immersion of the whole fruit fly in the ddH_2O. Following the rehydration, the specimen was pierced multiple times using a sterile dissecting needle and incubated overnight at 56°C proteinase K digestion.

Table 4.2: Fruit flies used for an initial *Wolbachia* screening phase (n = 246). Fruit fly species (*C. quinaria*. *C. rubivora*, *C. simi* and *D. ciliatus*) for which sample size was <5 are not included.

Locality		Morphological identification									
Highveld		*B. dorsalis*		*C. capitata*		*C. cosyra*		*C. quilicii / rosa*		*C. pedestris*	
	Site	M	F	M	F	M	F	M	F	M	F
	Tswaing Meteorite Crater	10	-	2	-	6	3	2	1	-	-
	Magaliesmoot Farm	5	-	9	-	6	-	8	1	3	-
	Mooinooi Farm	3	-	10	-	6	2	6	3	17	1
	UP Experimental Farm	5	-	3	-	6	-	8	-	1	-
Lowveld											
	Siyalima Boerdery	6	-	1	-	7	-	1	-	-	-
	Hectorspruit Farm	6	-	6	-	6	-	2	-	-	-
	ARC-ITSC Nelspruit	6	1	9	2	6	1	11	3	-	-
	ARC-ITSC Burgershall	6	2	2	1	6	-	10	3	-	-

Subsequent to the initial screening of 246 specimens, a second extraction method, a modified version of the Boom et al. (1990) protocol (henceforth Boom extraction) was used for an expanded locality-specific (Mooinooi) assessment. The NucleoSpin® tissue kit extraction and the Boom extraction are both chaotropic, guanidine-based methods in which nucleic acids are bound to silica beads and inhibitors are removed through successive washes. For the NucleoSpin® tissue kit extraction, nucleic acid release from cells relied on overnight proteinase K digestion, whereas for the Boom extraction method relied on tissue pulverisation and lysis. Prior to extraction using the latter, fruit flies (n =235) were subjected to serial rehydration as described previously. The whole fruit fly was then ground to a powder using liquid nitrogen and a pestle, and a 10% W/V homogenate was prepared by addition of phosphate buffered saline (PBS) solution.

The homogenate was stored in 1.5 mL microcentrifuge tubes and at -20°C until use. In a 1.5 mL microcentrifuge tube, a 200 µL aliquot of each homogenate/specimen was combined with a 940 µL L6 lysis buffer and silica solution and regularly inverted/mixed over a period of five minutes to ensure adequate mixing and binding of nucleic acids to the silica. The DNA-silica matrix was pelleted by centrifugation, after which the supernatant that was discarded and 900 µL of L2 wash buffer was then added to the pellet. After adequate mixing, centrifugation and supernatant removal, two wash steps with 800 µL of 70% ethanol and 700 µL of acetone were performed. The nucleic acids were then eluted by addition of 30 µL 1xTE and incubation at 56ºC to ensure release of the DNA from the silica beads. The silica was pelleted by centrifugation and the supernatant containing the eluted nucleic acids was transferred to a sterile microcentrifuge tube. The extracted nucleic acid products were stored at -20ºC for further analysis. A subset of samples (n=4) were prepared using both the Boom et al. (1990) extraction technique and the NucleoSpin® tissue kit to allow for comparison of the methods.

Polymerase chain reaction (PCR) screening was used to confirm the presence of the *Wolbachia* genome. This was done using primers targeting three gene regions, *viz*. *16S rRNA, ftsZ* and *wsp* (Zhou et al. 1998b, Riegler and Stauffer 2002; Table 3.3). The mitochondrial *COI* gene region of the fly host was amplified using universal *COI* gene primers (Folmer et al. 1994) that target a 710 bp barcode region. This assay was run in conjunction with the *Wolbachia* PCR-screening to confirm DNA integrity and to preclude the possibility of false negatives arising from poor template quality. Three 16S rRNA assays (T-V) were initially evaluated. The T reaction comprised of two universal bacterial primers (27F and 1513R/1492R) that target a ~1500 bp

region (primer sets T1 and T2), primer set U (27F and EHR16S R) targets a 790 bp region of the 5' end of the gene, and primer set V (EHR 16S D and 1492 R) targets a 1030 bp region of the 3' end of the gene. The *ftsZ* assay, primer set F (FtsZ F and FtsZ R), targets a 1055 bp region of the *ftsZ* gene. Finally, the *wsp* assay using primer set W (81F and 691R) targets genes encoding the *Wolbachia* surface protein (Table 3.3). A touchdown PCR thermal cycling approach was used for each primer set, which involved an initial denaturation performed at 96℃ for 10s, three 30s annealing phases at temperatures specific for each primer pair, as summarised in Table 3.3, with elongation at 70℃ fixed at 1 minute for all 40 cycles. A final elongation at 70℃ for 1 minute constituted the final step. DNA extracted from a *Wolbachia*-positive fig wasp (*Alfonsiella pipithiensis*) served as a positive control.

Table 4.3: Summary of primers and thermal cycling conditions used for molecular detection of *Wolbachia* genome and for amplification of the mitochondrial *COI* gene barcoding region of fruit fly host genome (*COI* amplification did not follow a touchdown PCR cycling profile).

	Primer set Primer name: sequence (5' – 3')	Reference	Gene targeted	Amplicon size (bp)	Touchdown PCR Ta (°C) ×2, ×3, ×35
COI	LCO1490: GGTCAACAAATCATAAAGATATTGG (F) HCO2189: TAAACTTCAGGGTGACCAAAAAATCA (R)	(Folmer et al. 1994)	*COI*	~710	48 (initially 44)
F	*ftsZ* f1: GTTGTCGCAAATACCGATGC (F) *ftsZ* r1: CTTAAGTAAGCTGGTATATC (R	(Werren et al. 1995)	*ftsZ*	~524	60, 59, 58
W	Wsp81F: TGGTCCAATAAGTGATGAAGA AAC (F) Wsp691R: AAAAATTAAACGCTACTCCA (R)	(Zhou et al. 1998b)	*Wsp*	~600	56,55,54
T1	pA (27F): AGAGTTTGATCMTGGCTCAG (F) 1513 R: ACGGYTACCTTGTTACGACTT (R)	(Edwards et al. 1989) (Weisburg et al. 1991)	*16S rRNA*	~1500	60,59,58
T2	pA (27F): AGAGTTTGATCMTGGCTCAG (F) pH (1492 R): TACGGYTACCTTGTTACGACTT (R)	(Edwards et al. 1989) (Reysenbach et al. 1992)	*16S rRNA*	~1500	60,59,58
U	pA (27F): AGAGTTTGATCMTGGCTCAG (F) EHR 16SR: GTAATCGTGGATCATCATGC (R)	(Edwards et al. 1989) (Parola et al. 2000)	*16S rRNA*	~790	56,55,54
V	EHR 16SD: GGTACCYACAGAAGAAGTCC (F) pH (1492 R): TACGGYTACCTTGTTACGACTT (R)	(Parola et al. 2000) (Reysenbach et al. 1992)	*16S rRNA*	~1030	58, 57,56

Prevalence was calculated according to the Zug and Hammerstein (2012). Where prevalence is represented by:

$$Prevalence = \frac{Infected\ individuals\ in\ the\ species}{Total\ number\ of\ individuals\ in\ the\ species}$$

4.2.3 Nucleotide sequencing and phylogenetic analyses

PCR products were electrophoresed through a 1.5% agarose gel against a 1kb DNA molecular weight marker (Thermo Scientific Fisher, United States). Agarose gels were stained with Goldview (Guangzhou Geneshun Biotech Ltd.) to allow for visualization by UV irradiation. This allowed for PCR products of the expected size to be selected for purification, with the Roche PCR Product Purification Kit (Roche Diagnostics GmbH, Manneheim, Germany), according to the manufacturer's instructions. Bi-directional cycle sequencing was performed using the BigDye v3.1 terminator cycle-sequencing kit (Perkin Elmer, USA), using each of the external PCR primers in separate reactions. Cycle-sequencing was performed the primer pair-specific annealing temperature indicated in Table 4.3. Sodium-acetate precipitation was used to remove unincorporated primers, dNTPs and fluorescently labelled ddNTPs. Once purified, the products were submitted to the Core Sanger sequencing facility of the University of Pretoria for sequencing. Sequence chromatographs were visualised and edited in the Chromas programme embedded in MEGA 7 (Kumar et al. 2016) and used to generate contiguous sequences (contigs).

4.2.4 Sequence analyses based on PCR-Sanger sequencing data

The generated 16S rRNA and *wsp* gene contigs were used in nucleotide blast searches against the Genbank database (https://www.ncbi.nlm.nih.gov/blast) to identify the most closely related sequences in the database. The final, aligned dataset for each gene region was used to infer an initial p-distance neighbour joining (NJ) tree to evaluate the uncorrected genetic divergence between the *Wolbachia* sequences generated in this study and homologous, publicly available sequences in the Genbank database. Sequences for the remaining five MLST targets were extracted from the PacBio long read data and each gene region was supplemented with reference sequences for each of the supergroups included in Baldo et al. (2006). Reference sequences were downloaded from Genbank and crosschecked with publications to confirm the Supergroup designations; these have been summarised in Appendix Table 4.1 (*wsp* reference table) and Table 4.2 (*16S rRNA* reference table).

4.2.5 Next generation sequencing

A DNA aliquot of the confirmed *Wolbachia* positive fruit fly was subjected to whole genome amplification using the Genomiphi v2 Amplification kit (henceforth referred to a gDNA). For quality control purposes, the gDNA concentration was calculated using the Nanodrop and confirmed with a Qubit. Next generation sequencing through the SMRTcell 8M on the Sequel IIe system requires at least 300 ng in 200 µL.

Once the above-mentioned conditions were met the gDNA was submitted to Inqaba Biotech® for low input workflow PacBio sequencing. Multilocus sequence typing (MLST) targets that were not amplified through PCR-sequencing, viz. *coxA* (cytochrome oxidase I subunit, 1551 nt), *fbpA* (ferric binding protein, 900 nt), *ftsZ* (*ftsZ* gene region, 1197 nt), *gatB* (Glutamyl-TRNA Amidotransferase Subunit B, 1425 nt) and *hcpA* (Helicobacter Cysteine-rich Protein A, 741 nt) were extracted from the draft genome compiled from the long-read PacBio data using the relevant gene regions of the annotated genome in the most closely related Wolbachia genome, detected in Diaphorinia citri (CP048819). As a control the *wsp* and 16S rRNA data generated by PCR-Sanger sequencing were also used in nucleotide blast searches against the draft genome. Ultimately, a concatenated dataset comprising of six gene regions was generated for supergroup delineation as per the guidelines of Baldo et al. (2006).

4.2.6 Concatenated dataset for MLSA

Following confirmation that the *16S rRNA* and *wsp* nucleotide sequences generated by Sanger sequencing were identical to the corresponding sequence generated by PacBio, a concatenated dataset was created using a combination of Sanger and PacBio MLST target reads. The dataset was complemented with homologous reference sequences summarised in Table 4.5 (concatenated MLSA reference table). Subsequently the maximum likelihood (ML) and Bayesian inference analyses were performed in MEGA 7 (Kumar et al. 2016) and MrBayes (Huelsenbeck and Ronquist 2001), respectively. The best-fit model of sequence evolution was determined for each individual and the concatenated dataset under the Akaike information criterion (AICc) in MEGA 7 (Kumar et al. 2016) and used for the ML analysis. For Bayesian Inference (BI), two independent runs, each with one cold chain and three heated chains at default heat settings, were executed in MrBayes (Huelsenbeck and Ronquist 2001). Sampling occurring every 100^{th} iteration over the 10 million generation run, resulting in 100,000 datapoints. The initial 25% of each run was discarded as burn-in based on multivariate visualization run on MCMC Tracer Analysis Tool version 1.7 (Rambaut et al. 2018). Nodal

support was assessed through 5000 bootstrap replicates for ML and from posterior probability for BI.

4.3 Results

4.3.1 *Wolbachia* PCR screening and assay optimisation

The initial screening of 246 samples from the eight sampling sites revealed difficulties with the specificity of certain primer sets. Universal *16S rRNA* primer set T1 (27F+1513R), and T2 (27F+1492R) were used to confirm DNA integrity and to confirm primer binding efficiency. Owing to the suboptimal performance of the T1 primer set (27F+1513R), the reverse primer 1492R and the forward primer 27F, were respectively used for the forward-reverse combinations with the Anaplasmataceae-specific primers, EHR-16SD and EHR-16S-R (Parola et al. 2000). This resulted in the amplification of two overlapping fragments (Table 4.3, U and V) which once sequenced and combined, produced a near-complete 16S rRNA gene fragment sequence of ~1500 bp. Primer set F (*ftsZ*) whilst amplifying fragments of the expected size, were shown, through Sanger sequencing of the purified products, to be false positives. Due to the poor specificity, and the inability to detect the positive *Wolbachia* genome of the positive control, which was readily confirmed by *16S rRNA* primer set (U and V) and *wsp* primer set (W) amplification and sequencing, primer set (F) was excluded from the *Wolbachia* PCR-screening assay panel. Although primer set U detected the *Wolbachia*-positive fruit fly, the primer set also resulted in non-target amplification of other bacteria, despite optimisation attempts to improve specificity (Appendix Table 6). The non-target bacteria amplified with primer set U, included *Klebsiella oxytoca* (Flügge, 1886), *Cronobacter* sp. (Iversen *et al*., 2008), *Bacilius safensis* (Satomi *et al*., 2006), *Dysgonomonas oryzarvi* (Kodama *et al*., 2012) and *Lactococcus laudensis* (Summarised in Appendix Table 6) based on nucleotide sequence identity values > 98%. Similarly, non-target amplification detected with F primer included *Thermus thermophilius* (Yoshida and Oshima, 1974), whilst the universal 16S rRNA primer set T detected *Citrobacter* sp. (Werkman and Gillen, 1932), and *Enterobacter cloacae* (Jordan, 1890). Based on these results, all further screening was conducted using two primer sets V and W, which target a 1030 bp and 600 bp fragment of the 16S rRNA and *wsp* genes respectively. Only one *C. cosyra* (LR2EGO H66) fruit fly of the initial 246 samples from eight localities and representative of five species was found to be positive for the *Wolbachia* genome.

4.3.2 *Wolbachia* screening

A total of 246 fruit fly samples from the highveld and lowveld regions were initially screened for the presence of *Wolbachia*, of which 246 DNA extracts were prepared with the NucleoSpin® tissue extraction kit and a further 235 with the Boom method. A single positive was detected in a male *Ceratitis cosyra* from the Mooinooi Farm in the initial phase during which DNA extracts were performed with NucleoSpin® tissue extraction kit. Based on this result, further molecular efforts focused on fruit flies collected from the Mooinooi Farm site and screening was expanded so that of the 738 fruit flies collected from this site, 616 (84.5 %) were assessed for *Wolbachia* genome presence. Based on this, the prevalence (Table 4.4) for the 191 *C. cosyra* fruit flies evaluated was 0.005%. No other fruit fly species from the Mooinooi, or other sampling sites were found to be positive for *Wolbachia*.

Table 4.4: Fruit flies collected at the highveld Mooinooi Farm (n = 738), proportion screened for *Wolbachia* infection (n = 616) using primers targeting the *wsp* and 16S rRNA gene regions. Species identification was based on morphology.

Fruit fly species	Number of caught during sampling	Number (percentage) screened for *Wolbachia* presence	Prevalence
B. dorsalis	3	3 (100%)	0
C. quilicii	418	355 (84.9%)	0
C. capitata	62	52 (83.9%)	0
C. cosyra	237	191 (90.6%)	0.005
C. pedestris	18	15 (83.3%)	0
Total	**738**	**616 (83.5%)**	

Table 4.5: Summary of the three datasets (*wsp* and two 16S rRNA datasets) used for initial assignment of *Wolbachia* supergroup based on sequences generated by PCR-Sanger sequencing and complemented with reference sequences

Dataset	Gene region	Length in nucleotides (bp)	Number of taxa	AICc best-fit model[1]	G	GC content	R Value
[A]	*wsp*	495	18	T92+G	0.548	0.37	2.0305
[B]	*16S rRNA*	358	25	K2G	0.169	0.50	3.8033
[C]	*16S rRNA*	899	20	K2G	0.192	0.50	3.8550

An initial 608 bp dataset created from the bi-directional sequencing of the product amplified using the W primer set was complemented with reference sequences to create dataset A, which was trimmed to remove end-unaligned sequences, resulting in a final dataset that was 495 bp in length (Table 4.5). A 16S rRNA dataset, 1001 nucleotides in length was compiled based on overlapping sequences generated from the U, and V primer sets. This sequence was used to compile two datasets B and C for phylogenetic analysis. Dataset B was reduced to 358 nucleotides after end-unaligned sequences were trimmed, as the available reference sequences for supergroups F, H, A and G were of this shorter length. Dataset C represents the longer 16S rRNA dataset (899 bp) that excludes these short sequences and representatives of the afore-mentioned supergroups. The tree generated using the *wsp* gene primer set (Dataset A; Figure 4.1) indicates that the positive fly from Mooinooi (LR2Ego *C. cosyra wsp*) falls into the Supergroup B. In contrast, the *16S rRNA* gene trees inferred with datasets B and C, indicate that the sequence we generated is most closely related to a Supergroup N sequence and clusters within a well-supported clade (92%) containing B, K and N supergroup representatives. The lack of supergroup clustering with the 16S rRNA datasets is likely due to the short length (358 bp and 899 bp respectively), and the lack of sufficient informative sites within this highly conserved gene.

[1] [1] T92 +G - Tamura 3-parameter with a discrete gamma distribution, K2G – Kimura 2 parameter with a discrete gamma distribution and GTR + G+ I – General Time Reversable with a discrete gamma distribution and invariant sites

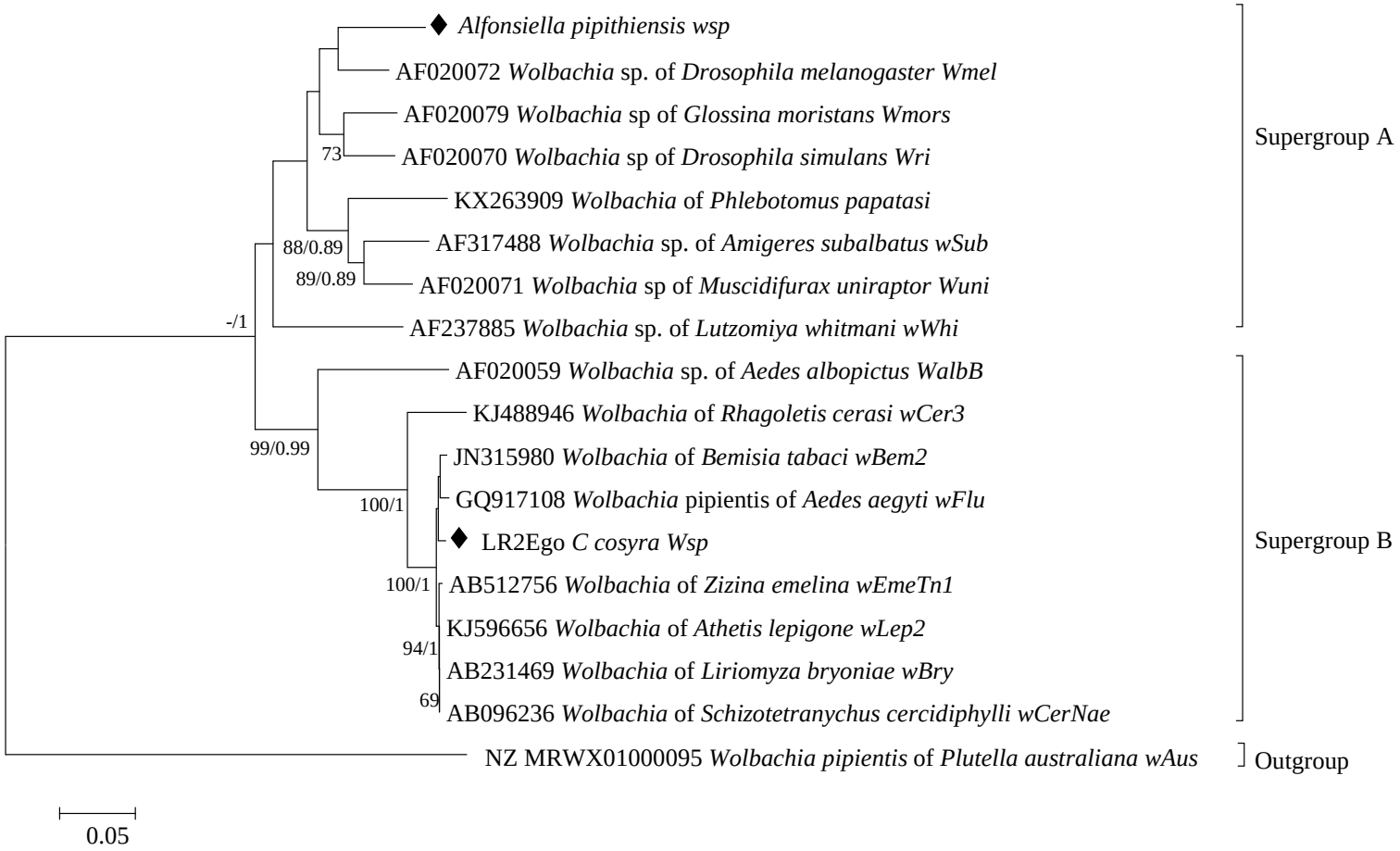


Figure 4.1: *Wolbachia* surface protein (*wsp*) gene region maximum likelihood tree compiled using dataset A (495nt in length). Tree includes Bayesian inference posterior probability. Tree showing Supergroup A and B of *Wolbachia* along with the sequence (highlighted with ♦) generated for the positive control (*Alfonsiella pipithiensis*) and for the *Ceratitis cosyra*. Figure shows bootstrap values > 65% and posterior probability > 0.90.

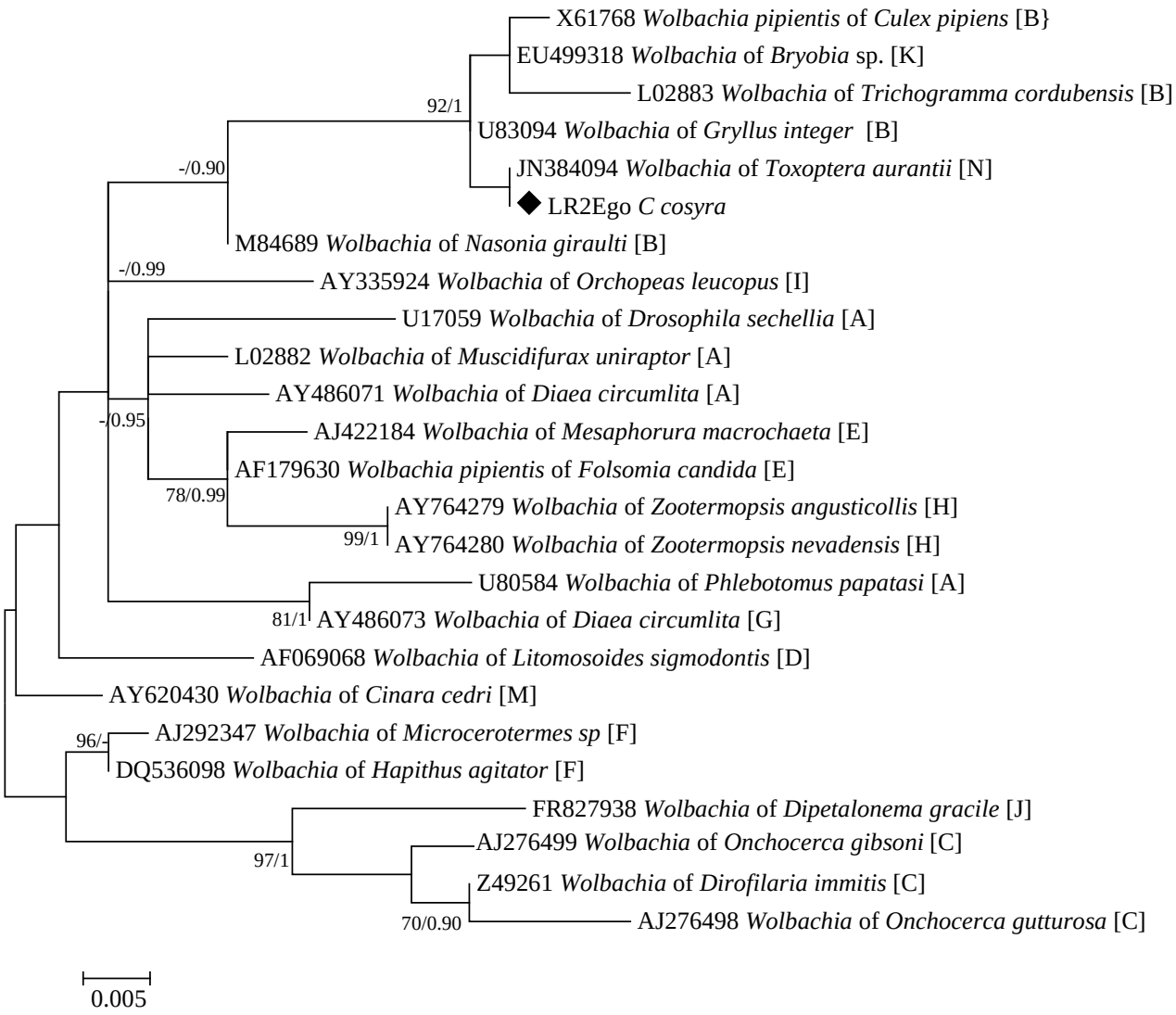


Figure 4.2: 16S rRNA gene region maximum likelihood (ML) tree compiled using dataset B (358 nt in length). Nodal support values are from ML that are ≥65% (from 10000 bootstrap replicates) and posterior probabilities ≥0.90 from Bayesian inference (BI) are indicated ML/BI on the relevant nodes. The supergroup designation for each reference strain is provided in square brackets.

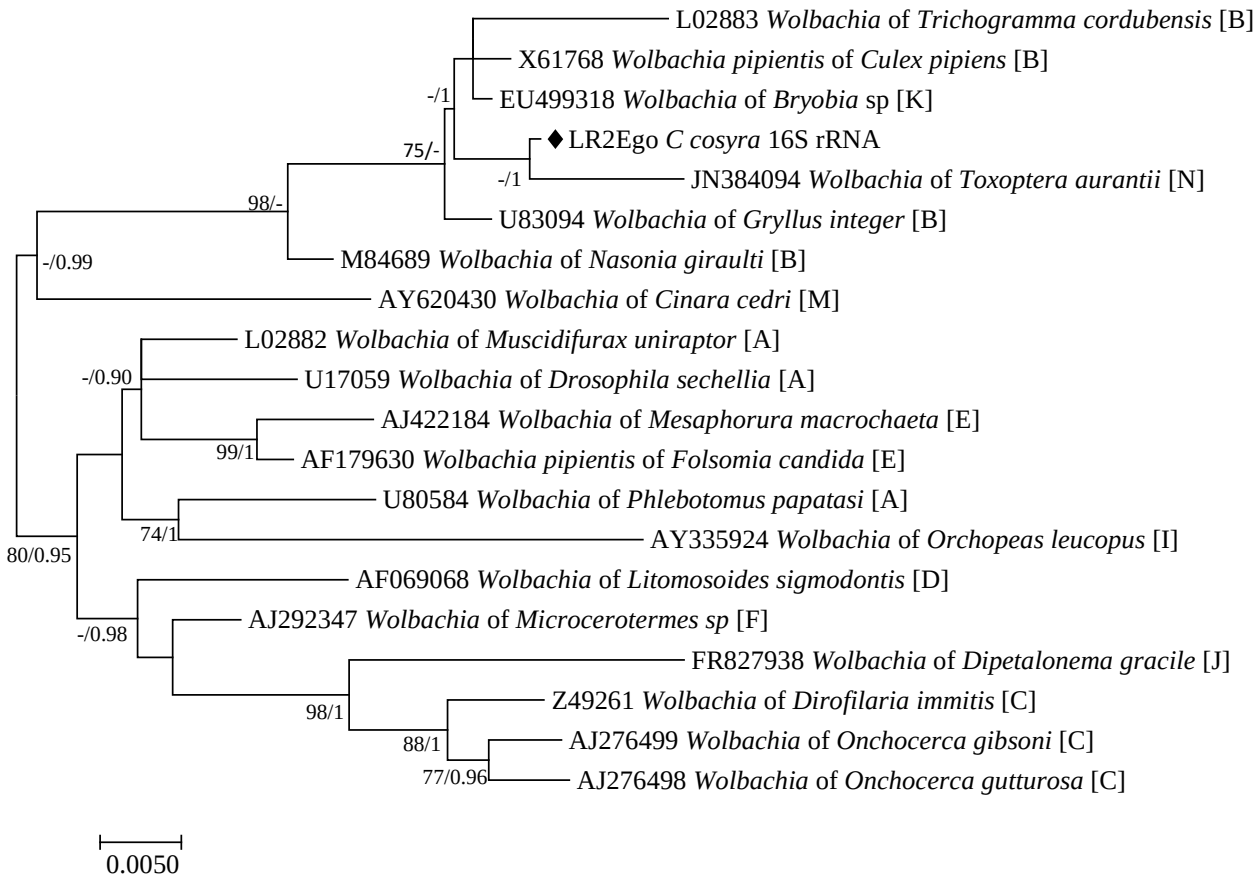


Figure 4.3: 16S rRNA gene region maximum likelihood (ML) tree compiled using dataset C (899 nt in length). Tree includes Bayesian inference (BI) results as posterior probability transferred onto the relevant nodes, with nodal support values being ML/BI. Tree showing Supergroups A – F, I-J, M and N reference sequences with generated sequence (highlighted with ♦). Figure shows bootstrap values > 65% and posterior probability > 0.90.

4.3.3. Concatenated dataset for MLSA

DNA prepared for the single positive, male *C. cosyra*, was submitted for PacBio sequencing. A total of 1,970,302 reads were produced and the reference-based assembler, rebaler (Baeza 2020), was able to extract three quarters of the *Wolbachia* genome. On generation of a partial draft genome, each the MLST gene targets of the most closely related annotated genome in Genbank, were used in blast searches to recover the corresponding homologous data in the *Wolbachia*-positive Mooinooi sample. The MLST gene regions for five genes (*gatB, coxA, hcpA, ftsZ* and *fbpA*; summarised in Table 4.6) of the closest *Wolbachia* reference genome (CP048819) were used in nucleotide blast search against the partial draft genome to recover the corresponding *Wolbachia* gene sequences of *C. cosyra* strain (summarised in Table 4.6) to create the concatenated dataset. The MLST primer sets included the *fts*Z gene region corresponding to the fragment targeted by primer set F (Table 4.3) and the *wsp* gene generated through Sanger sequencing. The best-fit model of nucleotide sequence evolution for the concatenated dataset was selected according to the AICc and the model was determined to be the General Time Reversable (GTR) model with a discrete gamma distribution (G =0.44) and invariant sites (I = 0.42).

Table 4.6: Multi-locus sequence typing gene regions used for the creation of the concatenated dataset. Information about fragments extracted include fragment length, the number of taxa and the fragment range as per CP048819 *Diaphorina citri* reference sequence

Gene region	Fragment length (bp)	Number of taxa	Fragment range as per CP048819
gatB	369	37	413868 – 414236
coxA	405	37	407814 – 408213
hcpA	430	37	1356753 – 1357182
ftsZ	435	37	658521 – 658955
wsp	467	37	983377 – 983893
fbpA	429	37	1233381 – 1234271
Concatenated dataset [D]	2535	37	N/A

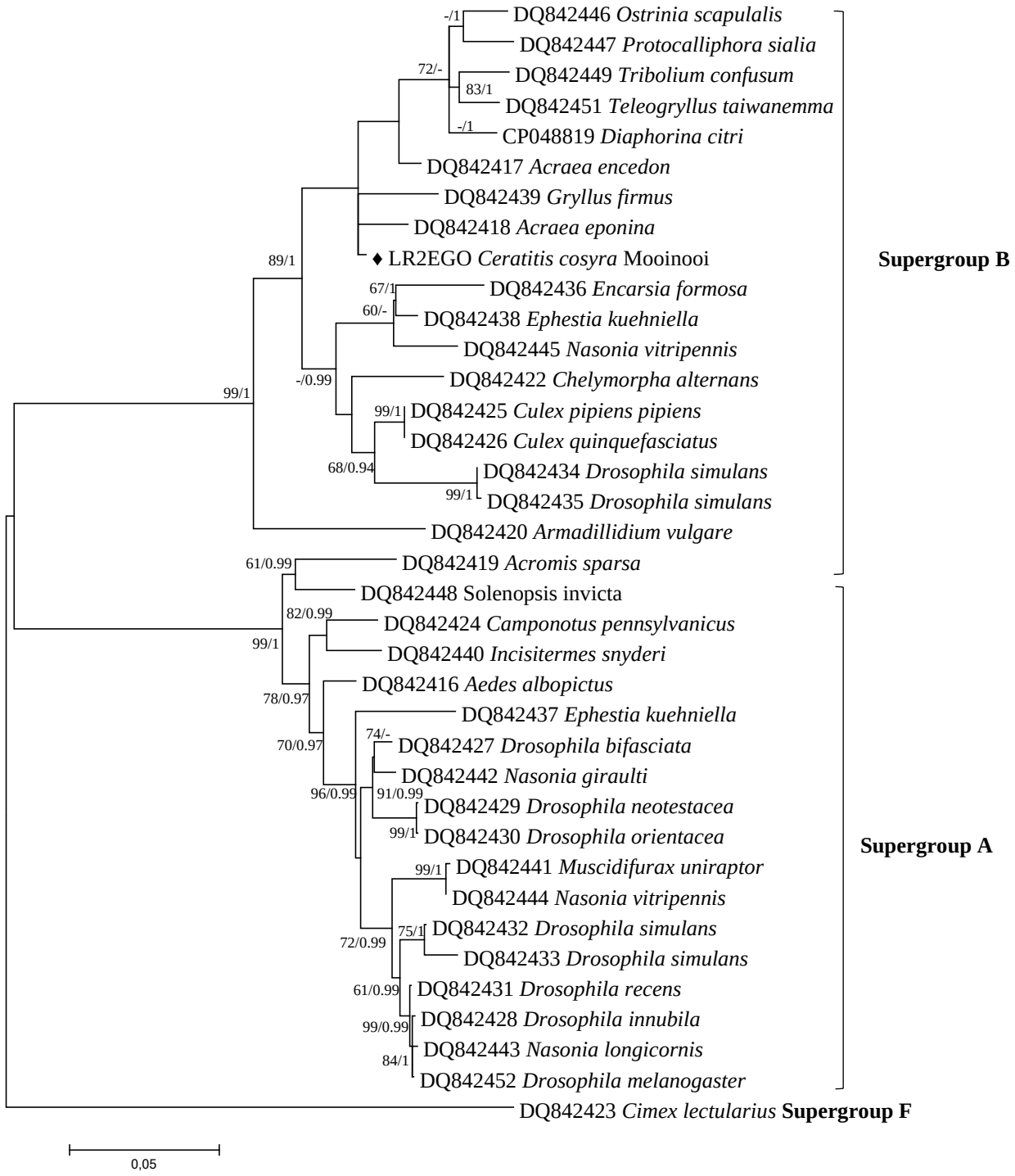


Figure 4. 4: Maximum likelihood (ML) and Bayesian inference (BI) for MLST gene phylogeny inferred with concatenated *coxA*, *fbpA*, *ftsZ*, *gatB*, *wsp* and *hcpA* gene sequences (dataset D, 2535 nt in length) depicting the relatedness with *Wolbachia* sequence in *Ceratitis cosyra* (highlighted with ♦), characterised in this study and supergroup A, B and F reference

sequences. Bootstrap values > 70% from ML and posterior probability values > 0.90 from BI are indicated ML/BI next to the relevant node.

The tree generated using the six concatenated MLST gene targets (Dataset D, Figure 4.4) confirm the supergroup B assignment obtained with *wsp* alone (Dataset A). Moreover, the concatenated dataset successfully separated the four supergroups into their respective clades. Sequences used for compilation of dataset D are provided in the appendix (Supp Table 4.3 - Concatenated dataset D Reference sequences).

4.4 Discussion

Wolbachia was detected in a single specimen of *C. cosyra*, of a total of 191 conspecifics from the Mooinooi area. This corresponds to a species- and site-specific prevalence of 0.52%. *Wolbachia* was not detected in *Bactrocera dorsalis* (3), *C. capitata* (52), *C. pedestris* (15) or *C. quilicii* (355) from Mooinooi. Based on a review of PCR and sequencing approaches (Mateos et al. 2020), *Wolbachia* presence was confirmed in ~66% of the 87 tephritid species that were screened. This estimate is based on confirmation of at least one positive *Wolbachia* specimen per species (Mateos et al. 2020). Positive infections were recorded in *Anastrepha* (Supergroup A) (Coscrato et al. 2009), *Bactrocera* (Supergroup A/B) (Kittayapong et al. 2000, Gichuhi et al. 2019), *Ceratitis* (Supergroup A) (Rocha et al. 2005, Coscrato et al. 2009), and *Rhagoletis* (Supergroup A/B) (Mateos et al. 2020). However, Mateos et al. (2020) report that surveys conducted on lab colonies and wild populations indicate that *Wolbachia* is absent from numerous localities in different continents. *Wolbachia* was also detected in *Dacus* species, and *Zeugodacus cucurbitae* (Coquilett, 1849) (formerly *Bactrocera cucurbitae* (Coquilett, 1899)) and *Z. diversa* but the supergroups to which these strains belong were not identified (Mateos et al. 2020).

It may be that failure to detect *Wolbachia* in other species sampled in the course of my study was due to the low number of flies sampled or the limited duration of sampling. In *Anastrepha*, a genus of tephritid fruit flies from the American tropics and subtropics, approximately 10 species were shown to harbour at least one population with a prevalence approaching 100% (Coscrato et al. 2009, Mateos et al. 2020). In *Bactrocera*, one population of *B. caudata* (Fabricius, 1805) had a 100% prevalence, whereas all other species exhibited low *Wolbachia* prevalence (Mateos et al. 2020). In *B. dorsalis* populations in China, Sun et al. (2007) reported low prevalence (0.7-3%) in four out of five Chinese populations evaluated, while a later study by Liu et al. (2016) reported the absence of *Wolbachia* in 15 wild populations. Liu et al. (2016) suggested that a potential reason for the difference between their results and the earlier study was that the *Wolbachia* infection may have been resolved rather than heading to fixation in the population. From these reports, it is clear that *Wolbachia* prevalence in tephritid populations is highly variable and that seasonal fluctuations may occur.

Our *Wolbachia* screening was performed using a two-gene PCR screening and sequencing approach. The *16S rRNA* gene was unable to assign supergroup with confidence and the sequence generated for the infected C. cosyra male clustered within a clade comprising of

supergroups B, N and K representatives (Fig 4.2 and 4.3). Given the high level of sequence conservation within the 16S rRNA gene, it is possible that unrelated strains may group together. To overcome this, Rodriguez-R et al. (2018) suggested the use of a percentage of 98.5% or 99% *16S rRNA* gene identity and query cover for the recovery of more accurate average nucleotide identity (ANI), which is a more accurate proxy for species. Our initial *16S rRNA* of 1001 bp, approximating 67% full-gene length/query cover, whereas the reduced datasets (358 bp and 899 bp, denoted B and C, respectively in Table 4.5), fall well below the ideal query coverage, rendering them of limited use for species and supergroup assignment. Konecka and Olszanowski (2021) used two *16S rRNA* targeting primers as well as those targeting the house keeping gene regions namely *fbpA*, *ftsZ*, *gatB*, *gltA* and *hcpA*, to create a longer concatenated dataset that would be more informative as to which Supergroup the *Wolbachia* they detected in *Hypochthonius rufulus* (Acari: Oribatida) belongs to. A similar approach was used for supergroup assignment of the *Wolbachia* detected in the *C. cosyra* fruit fly from Mooinooi.

The *wsp* assay shows that the sequence detected belongs to a clade comprised of supergroup B sequences. Supergroup-typing of *Wolbachia* strains has been mostly based on phylogenetic inference of the *Wolbachia* surface protein (*wsp*) (Baldo et al. 2005). Gichuhi et al. (2019) used a PCR-based *wsp* assay to identify four strains of *Wolbachia* infecting two *B. dorsalis* individuals, all strains that were detected belonged to Supergroup B. WSP-typing detected strains belonging to Supergroup A in *Anastrepha* fruit flies and their associated braconid wasps (Mascarenhas et al. 2016). The aforementioned study also found two samples of *Anastrepha obliqua* and one sample of *Doryctobracon brasiliensis* that had multiple infections (Mascarenhas et al. 2016). *Wolbachia* infected fruit flies being identified as members of both Supergroup A and B is not out of the ordinary as Supergroups A and B are associated with most arthropods (Gerth et al. 2014). In our study, a single *Wolbachia* genome was detected using NGS and targeted PCR-sequencing. This result aligns with reports that a single infection is common. Hence, even though Mascarenhas et al. (2016) detected multiple infections in one sample, they also demonstrated that the majority of positive samples correspond to single infections.

Although *wsp* strain inference has been used for the WSP strain typing methodology, Baldo and Werren (2007) suggested that it would be more informative and beneficial when used in conjunction with full MLST characterization. This suggestion comes as the extensive

recombination of *wsp* can lead to inaccuracy of supergroup placement. Thus, to get a clearer indication of which supergroup the detected *Wolbachia* strain is the MLST and *wsp* strain inference techniques should be used simultaneously. Karimi and Darsouei (2014) used this joint strain typing method and successfully identified two new strain types belonging to Supergroup A, while the remaining strains were identified as *wCer6* and *wVes1* and were found in *Rhagoletis cerasi* and *Carypomya vesuviana*, respectively. In the current study, we created a concatenated dataset for six MLST gene targets, *coxA*, *fbpA*, *ftsZ*, *gatB*, *wsp* and *hcpA* from a draft, partial genome generated from PacBio long reads. The supergroup B delineation inferred with this multi-gene dataset supports the supergroup B delineation recovered with the *wsp* region.

Wolbachia detection through a PCR-based method also has the shortcoming of false negatives due to the presence of inhibitors, and low concentration/poor quality of the target DNA molecule. Simões et al. (2011) tested primer sets targeting the *16S rRNA* gene regions, and primers sets FbpA, FtsZ-1/2, CoxA, GatB, HcpA and WSP. They reported that certain primers sets are significantly more efficient than others but that no single protocol can be utilised for the detection of all known *Wolbachia* infections (Simões et al. 2011). Mateos et al. (2020) suggested that negative *Wolbachia* detections should be validated through the evaluation of the quality of the DNA extract, through positive amplification of a host-encoded gene. In keeping with this the mitochondrial Cytochrome Oxidase subunit I (*COI*) was used for the validation of DNA integrity in our study. Similarly, the initial use of universal bacterial primers confirmed DNA integrity and primer binding efficiency, allowing us to control for the other shortcomings outlined by these authors.

The possibility for the positive *Wolbachia* genome detection being due to the presence of an endo-parasitic host is dispelled as *COI* barcoding only detected *C. cosyra* (*COI* primers) and *Wolbachia COI* (*coxA* primers). The current study differed from Towett-Kirui et al. (2021) as they detected the mitogenome of endoparasitic strepsipteran, *Dipterophagus daci*, from 13 tephritid fruit flies. This detection was through whole genome sequencing through Illumina HiSeq 2500 sequencing (Towett-Kirui et al. 2021).Therefore, future studies that detect the presence of *Wolbachia* infection should consider the possibility of a *Wolbachia* positive endoparasitoid. Another factor that we would have to account for is horizontal gene transfer (HGT). Horizontal gene transfer from *Wolbachia* to the eukaryotic host has been confirmed in *Aedes aegypti*. Klasson et al. (2009) found that adjacent *A. aegypti* genes AAEL004181 and

AAEL004188 show similarity with genes in the genome of *Wolbachia* strains *wPip* and *wMel*. Our findings do not dispel the possibility for HGT.

Thus, for future studies, we would suggest the continued use of MLST gene targets to infer concatenated phylogenies that can assign supergroups with confidence. The two-gene screening approach that includes the *wsp* gene region is valuable for reducing the possibility of false negatives, whilst sequencing of *wsp* provides an initial indication of supergroup assignment that should be validated through amplification and sequencing of additional MLST markers. We concur with the suggestion that DNA integrity should be confirmed in parallel with *Wolbachia* screening to exclude the possibility of false negatives. In this study, a *COI* validation assay was run in parallel to *Wolbachia* screening. The primer used to target *COI* provided valuable insights into fruit fly diversity in South Africa, when sequenced (Chapter 3). The two-step process used here, viz. (i) two-gene screening for *Wolbachia* genome presence in combination with host fly mitochondrial *COI* gene amplification to preclude false negatives and (ii) focussed MLST gene characterisation of *Wolbachia*-positive samples, through PCR-sequencing and/or NGS approaches, represents a cost-effective approach for establishing *Wolbachia* prevalence and diversity in under-studied taxa/regions. It is clear that longer-term studies are needed to determine the occurrence of *Wolbachia* in the different regions of South Africa and to assess seasonal fluctuations. These data are essential to guide the use of *Wolbachia* for pest management.

Chapter 5. General Discussion

5.1 Implication of research in the context of incompatible insect technique

The incompatible insect technique (IIT) strategy involves the control of insect populations through bacterium-mediated reproductive suppression. Tephritid fruit flies are among the most serious agricultural pests worldwide, and their control and management demand large and costly global efforts. There is a clear need for cost-effective and environmentally friendly integrated pest management (IPM) strategies. *Wolbachia*-mediated incompatible insect technique shows promise as a biological control method for various insects. This is based on the phenotypic effects of *Wolbachia* on the infected insect, specifically the occurrence of uni-directional cytoplasmic incompatibility (uni-CI) and bi-directional CI (bi-CI). Prior to the implementation of the IIT programme certain questions have to be answered. Firstly, the morphological and genetic diversity of tephritid fruit flies in the fruit production areas from the South African highveld and lowveld regions should be established. Secondly, the prevalence and diversity of *Wolbachia* in naturally-infected populations should be determined. Through this project I aimed to answer these questions and others that became apparent during the project.

Accurate identification of a host is crucial for an understanding of the viability of the IIT programme. During the project, a total of 2989 fruit flies belonging to nine species were collected throughout the highveld and lowveld sampling. Three of these species were found across the two regions while the remaining species were unique to one region or the other. The sampling sites comprised of commercial, research and small-scale farming locations with diverse host plant availability, the majority of which were at the end of their various fruiting seasons (Manrakhan and Addison 2014, Grové et al. 2017). Wild host plants play an important role in maintaining fruit fly populations, especially during periods when cultivated fruit and vegetable crops are scarce and out of season (Gnanvossou et al. 2017).

In regard to future studies, I suggest that these include an investigation of fruit trees found in and around the sampling area, and the collection of weather data, specifically temperature and rainfall. A study by Tiring and Satar (2021) highlighted the importance of alternative hosts and the effects of temperature on population fluctuations. They found that *C. capitata*, collected in Turkey, fluctuated outside of the harvest period based on the availability of unharvested grapefruits and temperatures of between 24-26°C (Tiring and Satar 2021). Further, the collection of the aforementioned information allows us to create a summary of the

characteristics of fruit flies that are in the area thus I can identify any anomalous behaviour that might be accredited to a potential *Wolbachia* infection.

A comparison of fruit flies collected in the lowveld and highveld revealed that species richness and the Shannon-Wiener diversity index did not differ, however, the fruit fly assemblages from the regions were distinct with certain species being unique to the region they were collected in. Meanwhile, the Simpson's diversity index indicated that in the lowveld there is less species diversity in the region with the species present being more even in abundance. The continuous monitoring and surveillance of fruit flies permits an understanding of any intra- and interspecies competition. Duyck et al. (2004) reported that competition among fruit flies led to *C. capitata* being displaced by *B. dorsalis*.

Cytochrome c oxidase I (*COI*) barcoding of 211 fruit flies confirmed the morphological identifications, except in the case of *C. rosa* and *C. quilicii* which form part of a sister complex. Other authors report similar challenges when using the COI barcoding region for fruit flies belonging to the same sister complex (Smit et al. 2013, Jiang et al. 2014). Despite this limitation, barcoding allowed assessment of haplotype diversity and structure, and provided preliminary insights into the invasion history of *B. dorsalis*. Of the nine species of fruit flies collected, three of the most abundant were afrotropical species that displayed high levels of haplotype diversity and no evidence of isolation by distance. The absence of region-specific haplotypes also applied to the two sister species of the FARQ complex, for which documented habitat preferences exist. These observations suggest that the natural dispersal abilities of lfies together with human-facilitated fruit fly dispersal through the transportation of infested fruits and vegetables (Dominiak and Daniels 2012, Louzeiro et al. 2021) are likely explanations for the lack of geographical structuring.

The results also showed greater haplotype diversity in fruit flies indigenous to the area than those associated with recent invasions, this was also suggested as the reason behind the high haplotype diversity of *C. capitata* (Karsten et al. 2015). Thus, a larger and more diverse sampling area would allow for more accurate estimates of the haplotype diversity across an altitudinal gradient from the highveld leading into the lowveld thus allowing for a more accurate summation of region-specificity in fruit fly haplotypes and environmental factors. *COI* barcodes sourced from BOLD (Supp Table 3.1 and Supp. Figures 3.1 to 3.5) revealed higher diversity in *B. doralis* in Asia, and more haplotypes were detected from sampling across the indigenous habitats. The *B. dorsalis* sequences generated in our study together with those

sourced from BOLD revealed that our sequences form part of two distinct clades and that the BdH2 haplotype is basal to a clade containing the BdH3 and BdH5 haplotypes. This suggests three possible scenarios (i) rapid diversification from the initial haplotype introduced, (ii) longer field presence of the invasive fruit fly than what is on record, and (iii) a separate later introduction. Furthermore, the *B. dorsalis COI* tree makes it clear that the source of the invading haplotype/s will be impossible to discern using *COI* as the two dominant haplotypes are detected in several East and West African countries. The non-dominant haplotype is unique to South Africa and likely evolved in situ from the two dominant haplotypes that were introduced.

The BOLD sourced sequences also revealed that in *C. capitata* three haplotypes detected in our study were previously detected in Uganda, Mozambique, and Zambia, but that the overwhelming majority are unique to South Africa. In the species, *C. cosyra*, haplotypes recovered in our study are similar to those detected in Kenya, South Africa, Mozambique, Madagascar, Democratic Republic of Congo, Ethiopia, and the Ivory Coast. *Ceratitis pedestris* haplotypes detected in our study were a perfect match to a haplotype in Kenya but the remaining haplotypes are unique.

A total of 616 fruit flies were screened for the presence of *Wolbachia* using a two-gene approach in which the 16S rRNA gene and wsp gene targeted for amplification. Although alternative PCR assays for the same and different genes were assessed, they were excluded based on the high levels of false positives due to non-target amplification. *Wolbachia* was detected in a single male *C. cosyra* collected from the Mooinooi Farm, and this positive detection was identified as belonging to supergroup B. For future studies, I would suggest an initial PCR screening for the presence of *Wolbachia*. To overcome the shortcoming of false negatives and positives, I would suggest the inclusion of barcoding *viz*. *COI* barcoding that was used in this study to confirm DNA integrity of nucleic acid extracts, the use of primer assays that have high levels of specificity and the inclusion of a positive control. Collaborations with other researchers investigating the pest control potential of the detected *Wolbachia* strain would advance the research beyond supergroup typing and genome characterisation. Furthermore, I would suggest sample sizes of about 50 specimens per species as this would permit the calculation of a more accurate representation of the prevalence of *Wolbachia*. Lastly, the sequencing of all amplicons that are the right size as they may be transient infections or short fragments from a previous *Wolbachia* infection. Mateos et al. (2020) underscored the

importance of determining the strain identity and the phenological effects of the strain on the fruit fly host. A combination of prolonged fruit fly sampling, *Wolbachia* screening and *Wolbachia* typing to strain level would allow for: (i) monitoring of the spread of the *Wolbachia* infections, and (ii) identification of the strains detected and the fitness consequences of the detected strains. Ultimately, research on the feasibility of implementing a uni-CI or bi-CI, and trans-infection success are needed to harness the biological control potential of *Wolbachia*.

In conclusion this dissertation reports on a snapshot of *Wolbachia* presence in the South African highveld and lowveld. *Wolbachia* was only detected in one specimen thus showing an absence in most species assessed and a very low prevalence in *C. cosyra*, which suggests that uni-CI would be the suitable programme to be utilized. The results obtained from the current study shows that *Wolbachia*-mediated IIT may be viable if the aforementioned expanded studies are implemented. Furthermore, future research would then have to focus on rearing infected males and sterile females for each of the target species, ascertain whether *Wolbachia* strain *wCer2*, which has been identified as capable of CI and other fitness consequences such as embryonic mortality, could be introduced into the environment for the control of fruit fly populations, and investigate the interactions between the native *Wolbachia* and the released *Wolbachia*.

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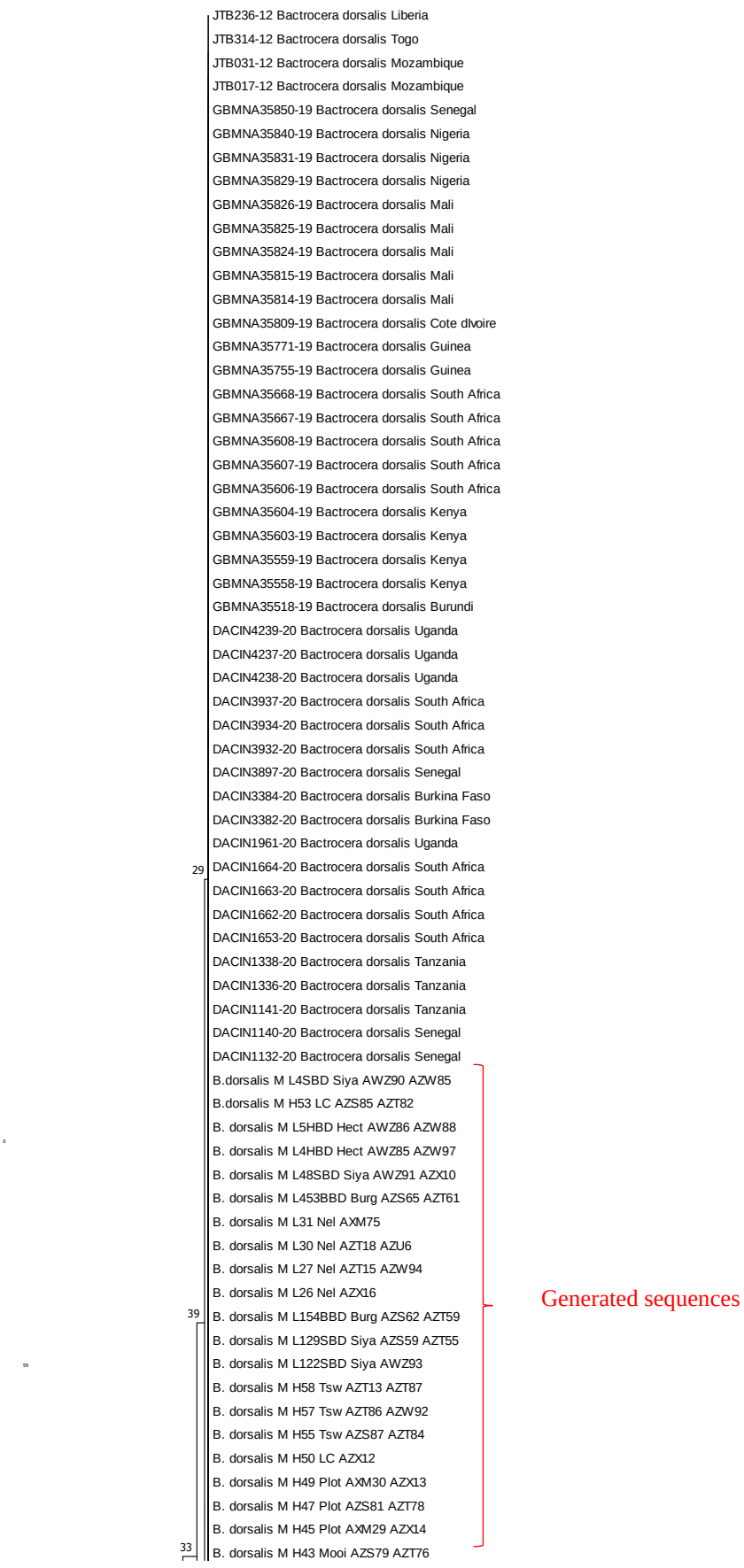
Appendix

Supplementary Tables and Figures for Chapter 3.

Supp Table 3.1 Summary information for the Neighbour joining trees that revealed haplotype diversity of samples sourced from BOLD.

Species	Amplicon size (bp)	Number of Taxa	Shared haplotypes
B. dorsalis	599	29	2
C. capitata	551	81	3
C. cosyra	521	169	10
C. pedestris	602	24	2
C. quilicii – C. rosa	572	78	7
C. rubivora	531	6	0

Shared haplotypes refers to haplotypes that were detected in our study that were matched to those sourced from Genbank.


JTB236-12 Bactrocera dorsalis Liberia
JTB314-12 Bactrocera dorsalis Togo
JTB031-12 Bactrocera dorsalis Mozambique
JTB017-12 Bactrocera dorsalis Mozambique
GBMNA35850-19 Bactrocera dorsalis Senegal
GBMNA35840-19 Bactrocera dorsalis Nigeria
GBMNA35831-19 Bactrocera dorsalis Nigeria
GBMNA35829-19 Bactrocera dorsalis Nigeria
GBMNA35826-19 Bactrocera dorsalis Mali
GBMNA35825-19 Bactrocera dorsalis Mali
GBMNA35824-19 Bactrocera dorsalis Mali
GBMNA35815-19 Bactrocera dorsalis Mali
GBMNA35814-19 Bactrocera dorsalis Mali
GBMNA35809-19 Bactrocera dorsalis Cote dIvoire
GBMNA35771-19 Bactrocera dorsalis Guinea
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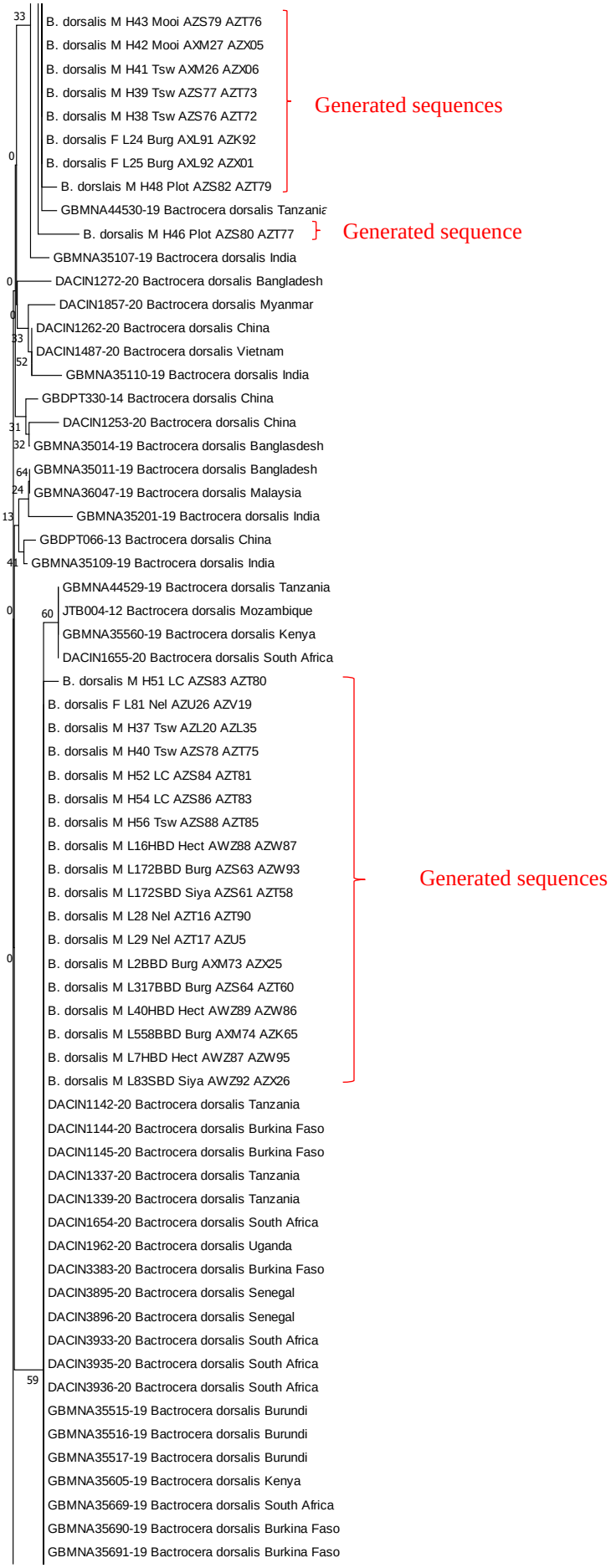
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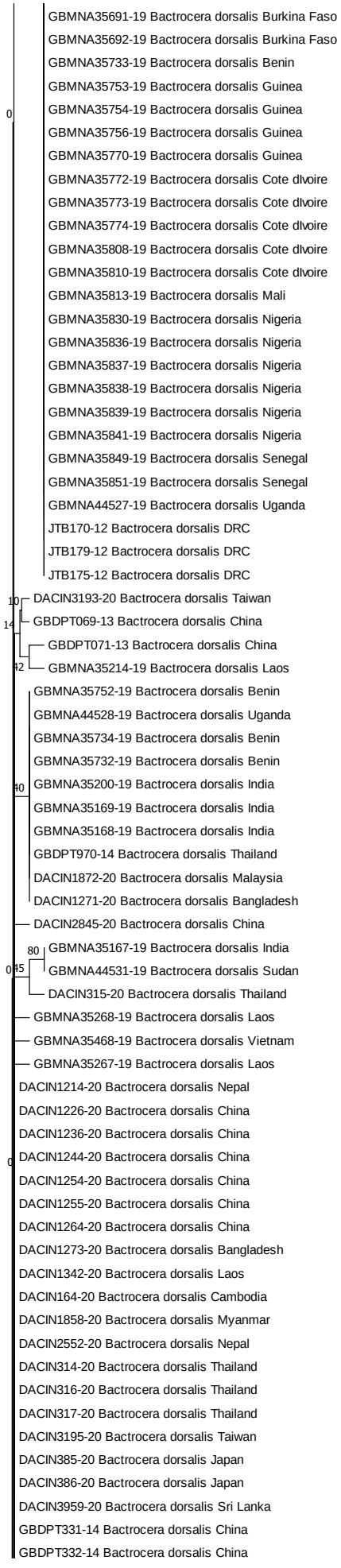
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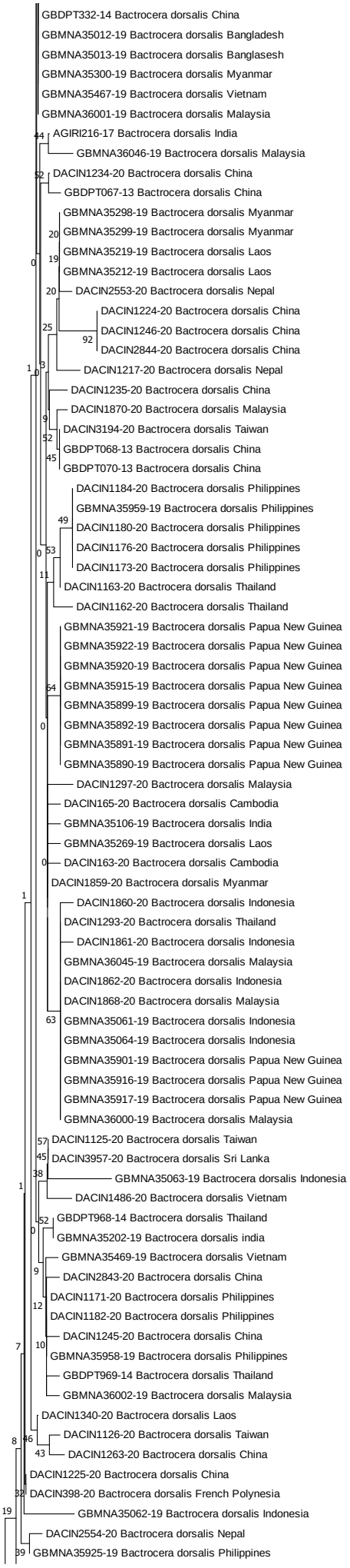
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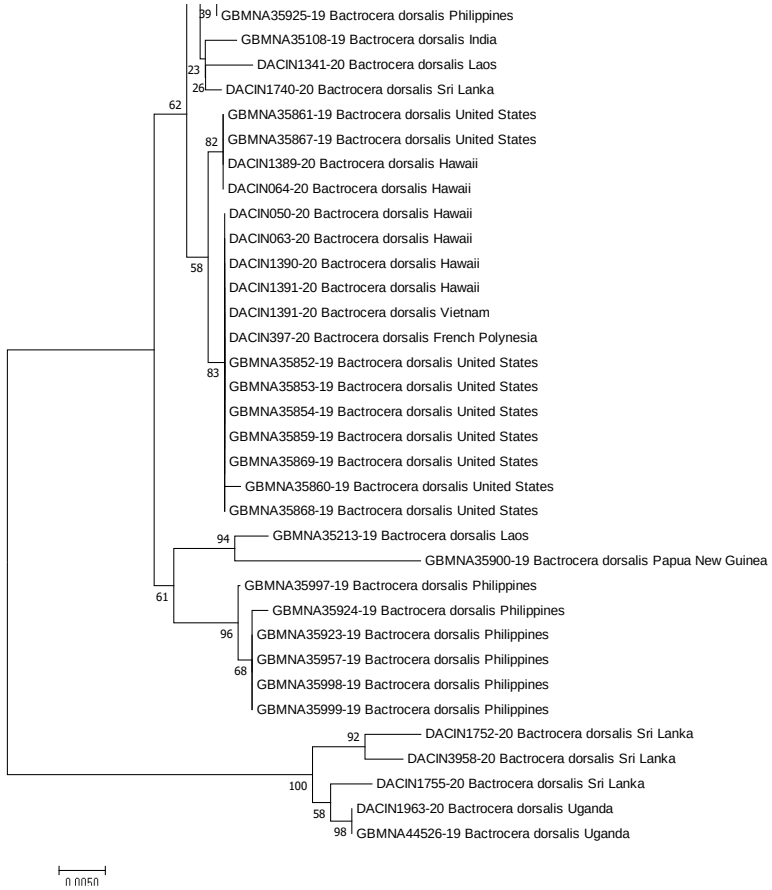
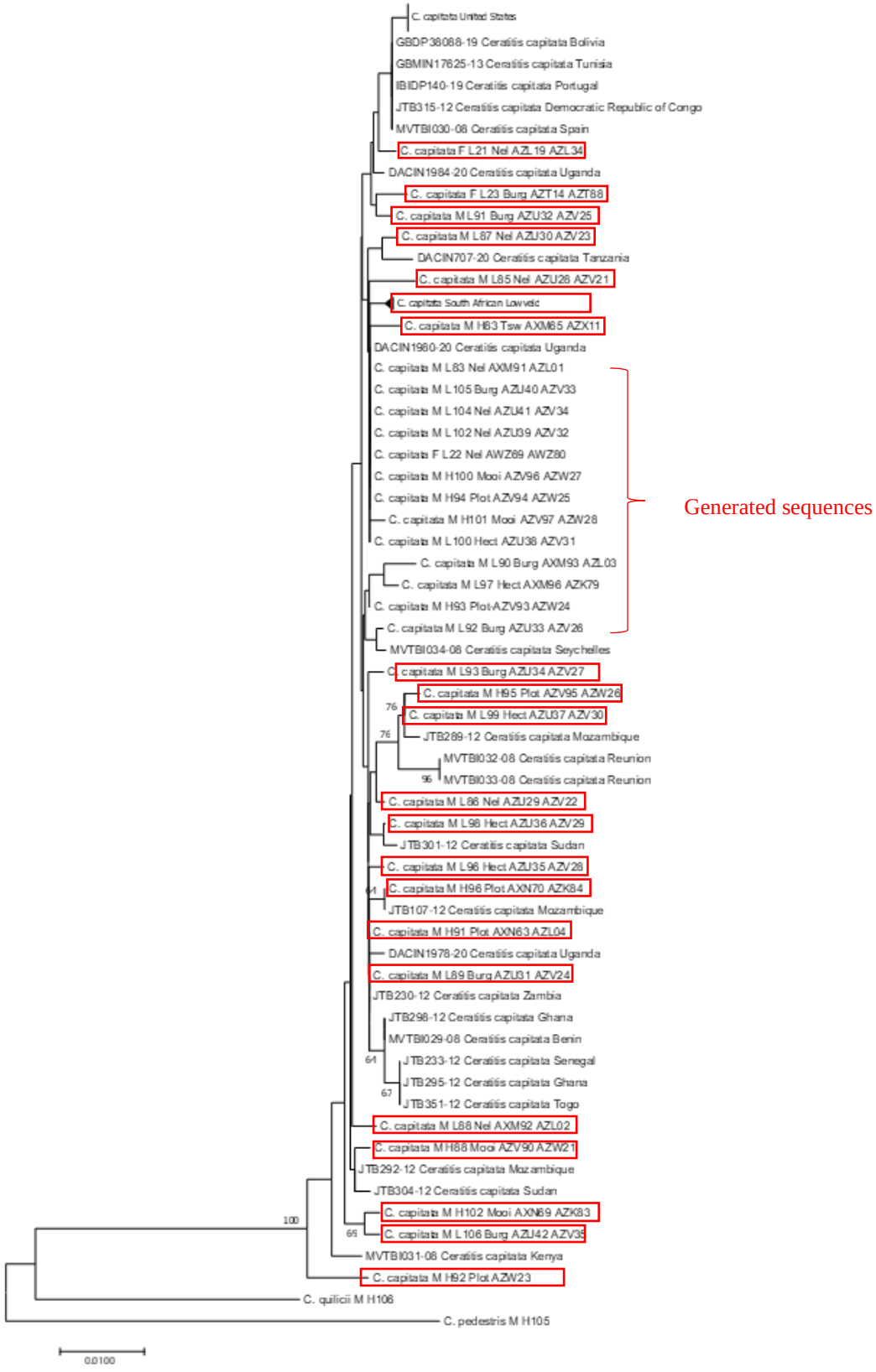
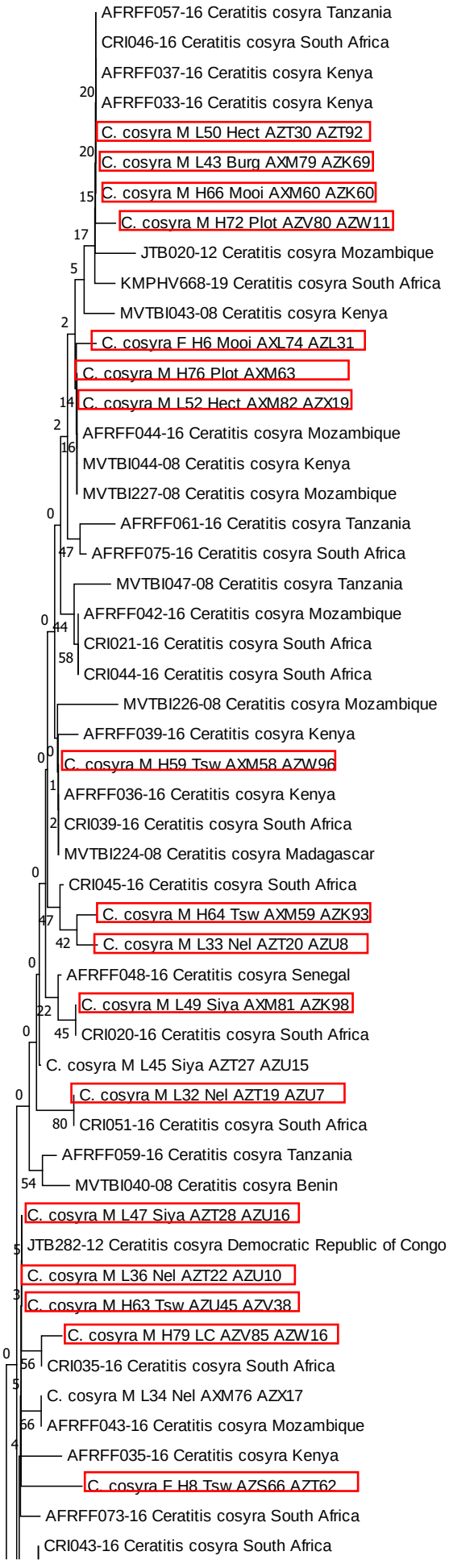


Supp. Figure 3.1: *Bactrocera dorsalis COI* barcodes neighbour-joining tree, ran for 10,000 bootstraps, showing the various haplotypes available in sequences sourced from BOLD and generated during our study (n = 308). As the tree is rather large, it has been split into five sections. Sequences generated in this study marked by red brackets.



Supp. Figure 3.2: *COI* barcode Neighbour- joining tree (10,000 bootstraps) for *C. capitata* drawn showing the various haplotypes extracted from samples collected during this studies

sampling and those sourced from BOLD (n = 81). Generated sequences highlighted with red rectangles and red brackets.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C. cosyra M H66 Mooi AXM60 AZK60
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JTB020-12 Ceratitis cosyra Mozambique
KMPHV668-19 Ceratitis cosyra South Africa
MVTBI043-08 Ceratitis cosyra Kenya
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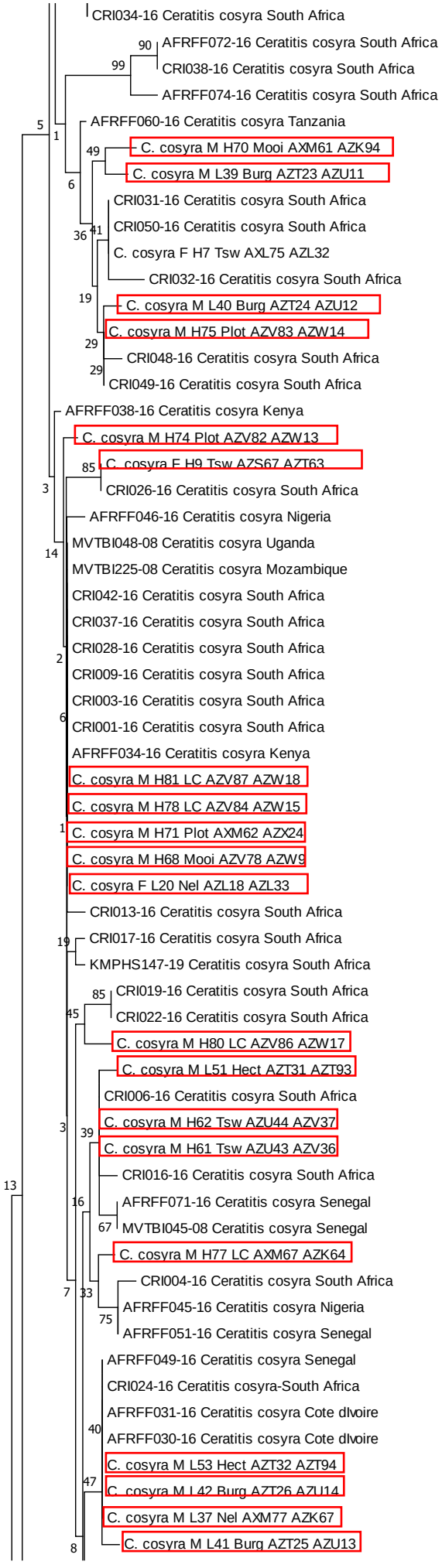
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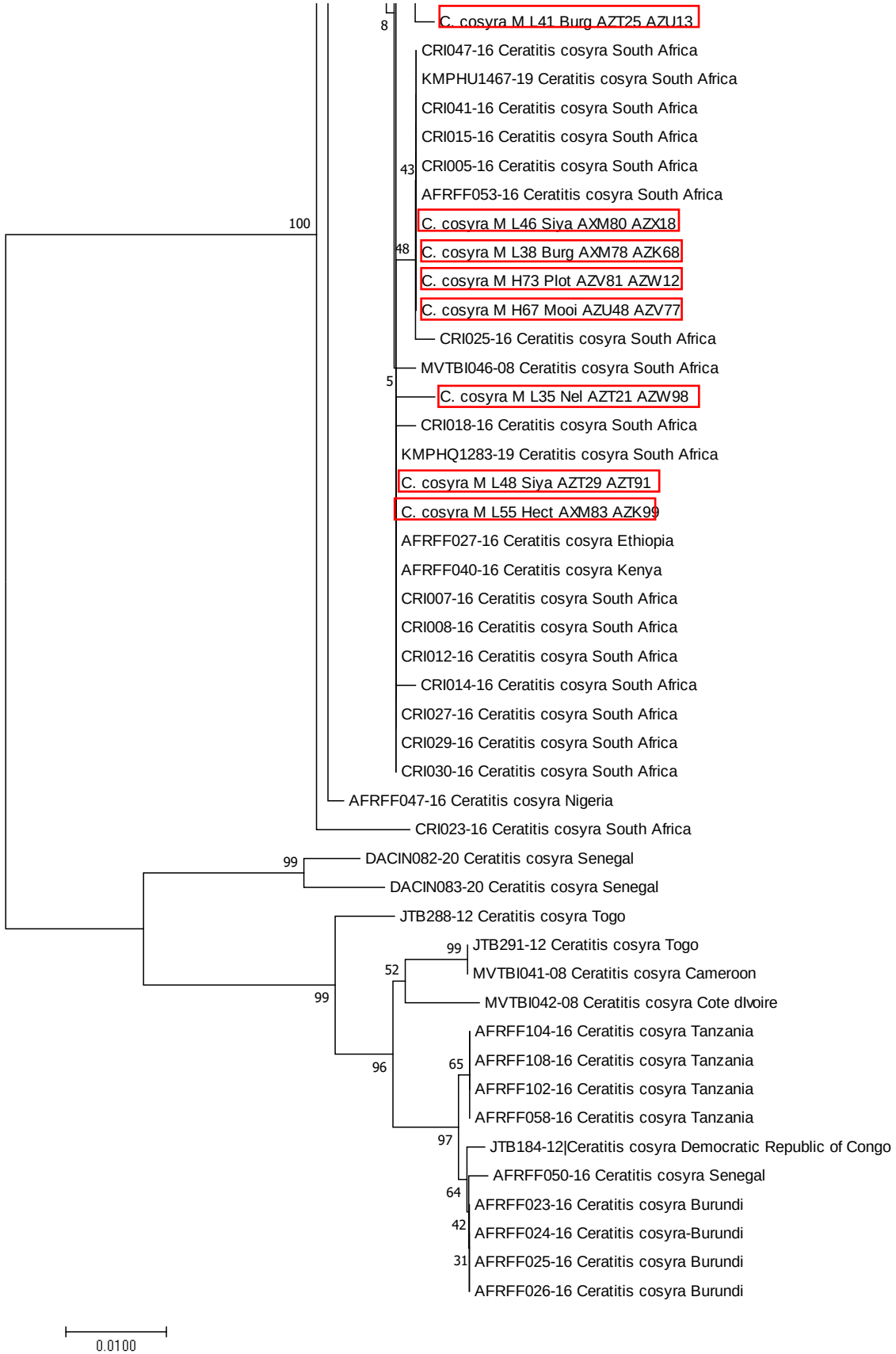
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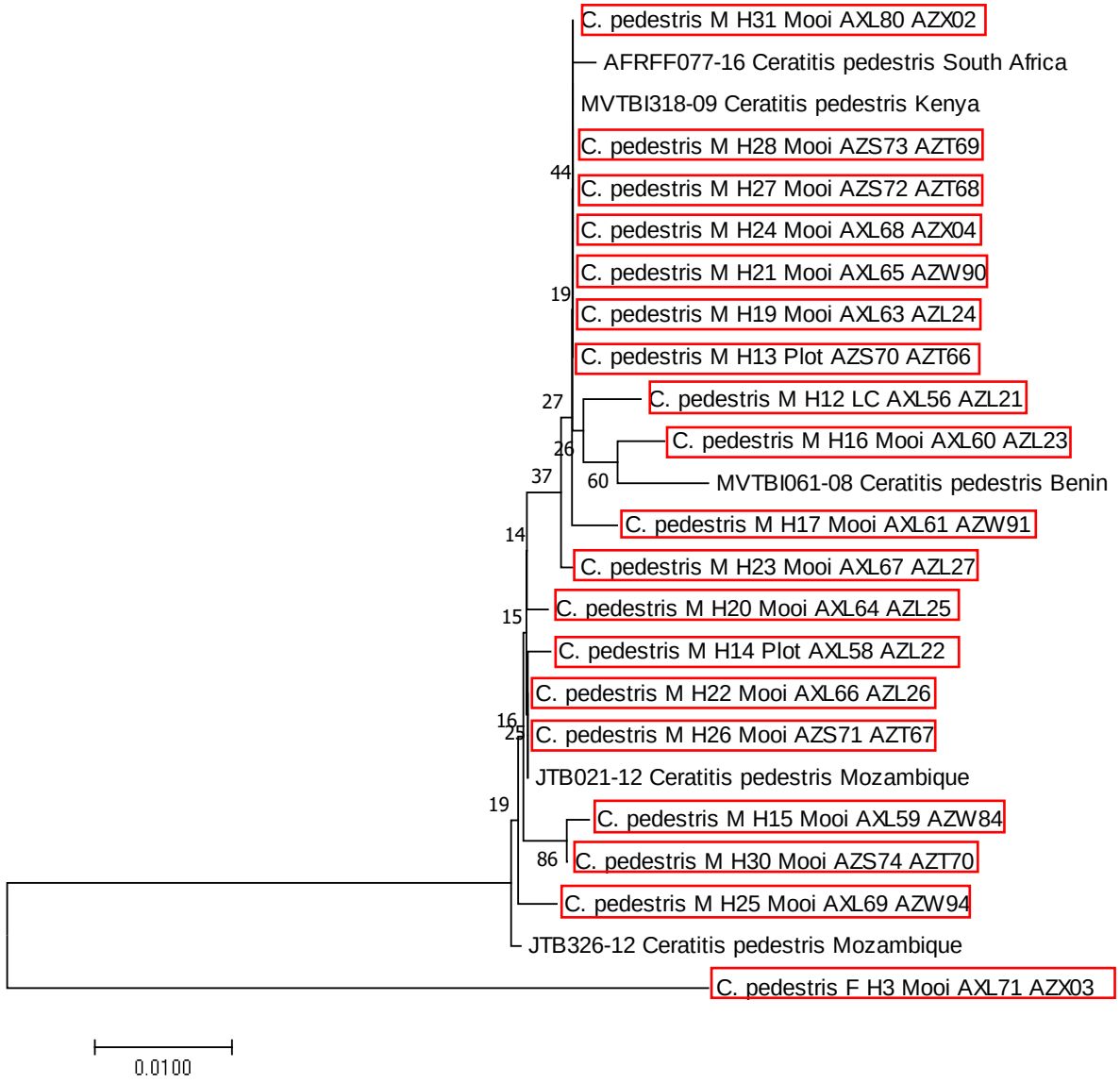
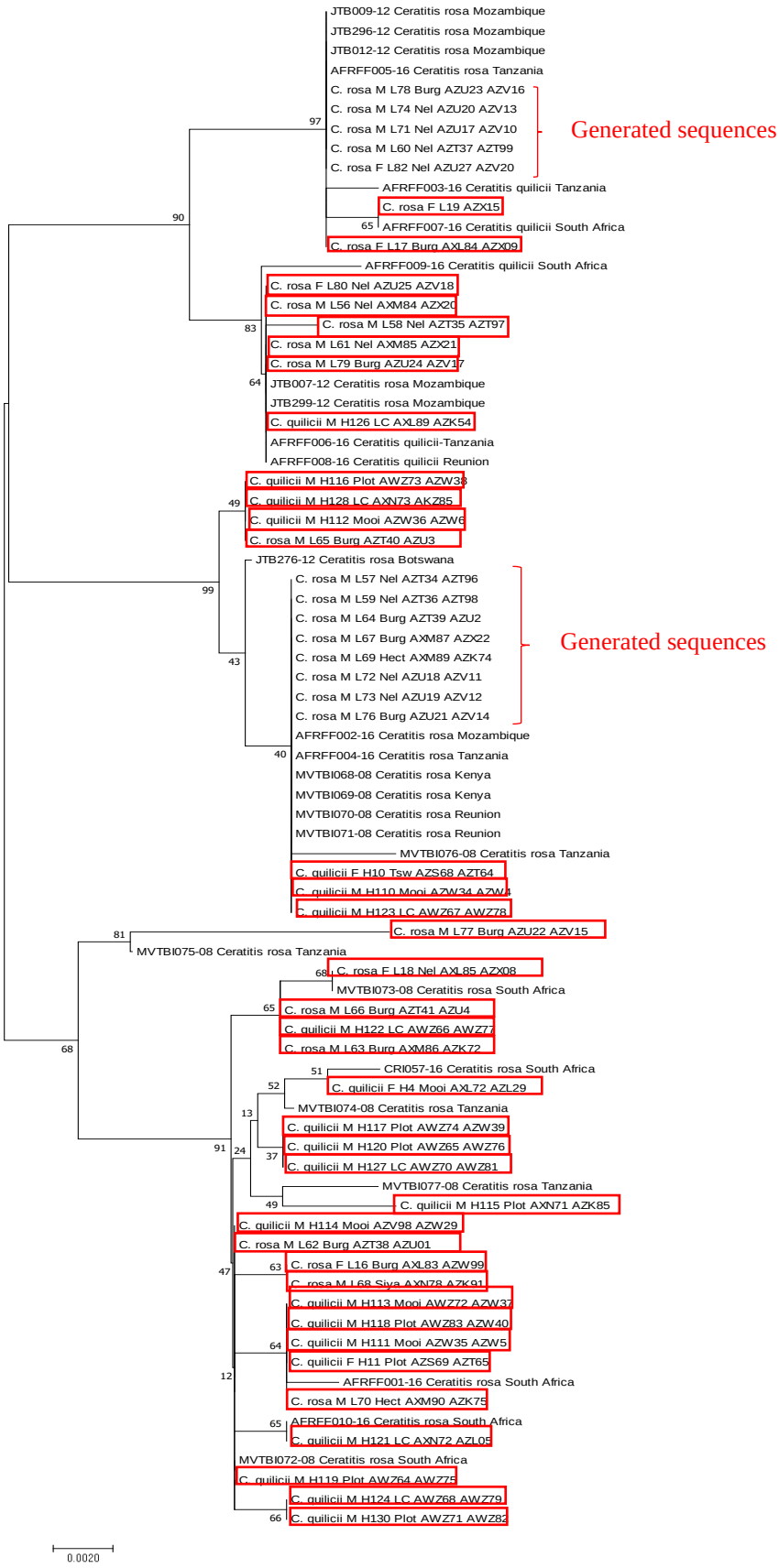
AFRFF050-16 Ceratitis cosyra Senegal
AFRFF023-16 Ceratitis cosyra Burundi
AFRFF024-16 Ceratitis cosyra-Burundi
AFRFF025-16 Ceratitis cosyra Burundi
AFRFF026-16 Ceratitis cosyra Burundi
8
43
100
48
5
99
99
52
99
96
65
97
64
42
31
0.0100

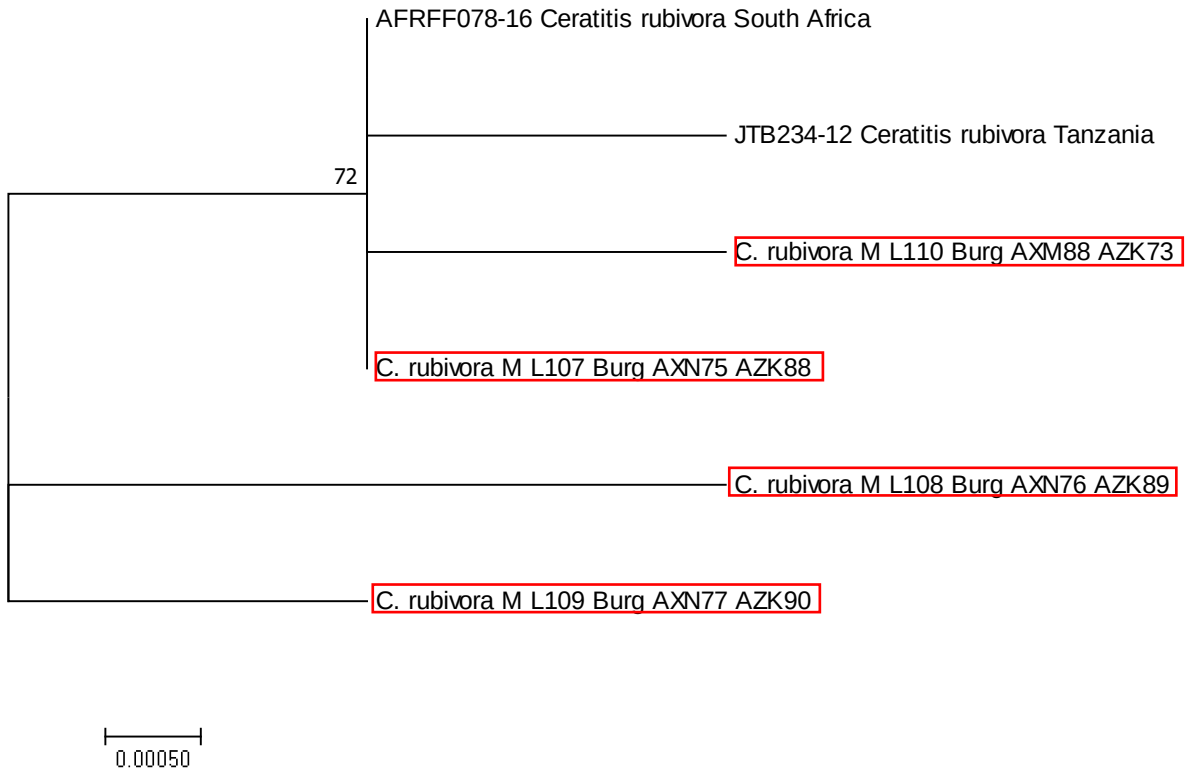
Supp. Figure 3.3: Neighbour-joining tree for *C. cosyra* showing the haplotypes recovered from *COI* barcodes sourced from BOLD and sampling during this study (n = 169). Generated sequences highlighted in red rectangular boxes.



Supp. Figure 3.4: *Ceratitis pedestris* neighbour-joining tree showing the haplotypes recovered from *COI* barcodes generated during this study and sourced from BOLD (n = 24). Generated sequences highlighted in red rectangular boxes.


JTB009-12 Ceratitis rosa Mozambique
JTB296-12 Ceratitis rosa Mozambique
JTB012-12 Ceratitis rosa Mozambique
AFRFF005-16 Ceratitis rosa Tanzania
C. rosa M L78 Burg AZU23 AZV16
C. rosa M L74 Nel AZU20 AZV13
C. rosa M L71 Nel AZU17 AZV10
C. rosa M L60 Nel AZT37 AZT99
C. rosa F L82 Nel AZU27 AZV20
Generated sequences
AFRFF003-16 Ceratitis quilicii Tanzania
C. rosa F L19 AZX15
AFRFF007-16 Ceratitis quilicii South Africa
C. rosa F L17 Burg AXL84 AZX09
AFRFF009-16 Ceratitis quilicii South Africa
C. rosa F L80 Nel AZU25 AZV18
C. rosa M L56 Nel AXM84 AZX20
C. rosa M L58 Nel AZT35 AZT97
C. rosa M L61 Nel AXM85 AZX21
C. rosa M L79 Burg AZU24 AZV17
JTB007-12 Ceratitis rosa Mozambique
JTB299-12 Ceratitis rosa Mozambique
C. quilicii M H126 LC AXL89 AZK54
AFRFF006-16 Ceratitis quilicii-Tanzania
AFRFF008-16 Ceratitis quilicii Reunion
C. quilicii M H116 Plot AWZ73 AZW38
C. quilicii M H128 LC AXN73 AKZ85
C. quilicii M H112 Mooi AZW36 AZW6
C. rosa M L65 Burg AZT40 AZU3
JTB276-12 Ceratitis rosa Botswana
C. rosa M L57 Nel AZT34 AZT96
C. rosa M L59 Nel AZT36 AZT98
C. rosa M L64 Burg AZT39 AZU2
C. rosa M L67 Burg AXM87 AZX22
C. rosa M L69 Hect AXM89 AZK74
C. rosa M L72 Nel AZU18 AZV11
C. rosa M L73 Nel AZU19 AZV12
C. rosa M L76 Burg AZU21 AZV14
Generated sequences
AFRFF002-16 Ceratitis rosa Mozambique
AFRFF004-16 Ceratitis rosa Tanzania
MVTBI068-08 Ceratitis rosa Kenya
MVTBI069-08 Ceratitis rosa Kenya
MVTBI070-08 Ceratitis rosa Reunion
MVTBI071-08 Ceratitis rosa Reunion
MVTBI076-08 Ceratitis rosa Tanzania
C. quilicii F H10 Tsw AZS68 AZT64
C. quilicii M H110 Mooi AZW34 AZW4
C. quilicii M H123 LC AWZ67 AWZ78
C. rosa M L77 Burg AZU22 AZV15
MVTBI075-08 Ceratitis rosa Tanzania
C. rosa F L18 Nel AXL85 AZX08
MVTBI073-08 Ceratitis rosa South Africa
C. rosa M L66 Burg AZT41 AZU4
C. quilicii M H122 LC AWZ66 AWZ77
C. rosa M L63 Burg AXM86 AZK72
CRI057-16 Ceratitis rosa South Africa
C. quilicii F H4 Mooi AXL72 AZL29
MVTBI074-08 Ceratitis rosa Tanzania
C. quilicii M H117 Plot AWZ74 AZW39
C. quilicii M H120 Plot AWZ65 AWZ76
C. quilicii M H127 LC AWZ70 AWZ81
MVTBI077-08 Ceratitis rosa Tanzania
C. quilicii M H115 Plot AXN71 AZK85
C. quilicii M H114 Mooi AZV98 AZW29
C. rosa M L62 Burg AZT38 AZU01
C. rosa F L16 Burg AXL83 AZW99
C. rosa M L68 Siya AXN78 AZK91
C. quilicii M H113 Mooi AWZ72 AZW37
C. quilicii M H118 Plot AWZ83 AZW40
C. quilicii M H111 Mooi AZW35 AZW5
C. quilicii F H11 Plot AZS69 AZT65
AFRFF001-16 Ceratitis rosa South Africa
C. rosa M L70 Hect AXM90 AZK75
AFRFF010-16 Ceratitis rosa South Africa
C. quilicii M H121 LC AXN72 AZL05
MVTBI072-08 Ceratitis rosa South Africa
C. quilicii M H119 Plot AWZ64 AWZ75
C. quilicii M H124 LC AWZ68 AWZ79
C. quilicii M H130 Plot AWZ71 AWZ82
0.0020

Supp Figure 3.5: Neighbour joining tree drawn from the *COI* barcodes generated during this study and sources from BOLD. Tree shows the various haplotypes for a combination of *C. quilicii* and *C. rosa* that can be retrieved from the dataset.



Supp. Figure 3.6: Neighbour joining tree (10,000 bootstraps) compiled for *C. rubivora* using *COI* barcodes sourced from BOLD and generated during this study (n = 6).

Supp. Figure 3.7: Neighbour joining trees (10,000 bootstraps) constructed using *COI* barcodes for *D. ciliatus* from this study and BOLD (n = 29)

Supplementary Figures and Tables for Chapter 4

Supp. Table 4.1: 16S rRNA Reference datasets with the supergroup the sequence was identified to belong and the publication from which it was sourced.

Name	Gene region	Host	Supergroup	Accession number	Publication
	16S	*Phlebotomus papatasi*	A	U80584	(Lo et al. 2002)
	16S	*Muscidifurax uniraptor*	A	L02882	(Stouthamer et al. 1993)
	16S	*Drosophila sechellia*	A	U17059	(Giordano et al. 1995)
wDiacir1	*16S*	*Diaea circumlita*	A	AY486071	(Rowley et al. 2004)
	16S	*Culex pipiens*	B	X61768	(O'Neill et al. 1992)
	16S	*Gryllus integer*	B	U83094	(Giordano et al. 1997)
	16S	*Trichogramma cordubensis*	B	L02883	(Stouthamer et al. 1993)
	16S	*Nasonia giraulti*	B	M84689	(Breeuwer et al. 1992)
	16S	*Onchocera gibsoni*	C	AJ276499	(Casiraghi et al. 2001)
	16S	*Onchocera gutturosa*	C	AJ276498	(Casiraghi et al. 2001)
	16S	*Dirofilaria immitis*	C	Z49261	(Sironi et al. 1995)
	16S	*Litomosoides sigmodontis*	D	AF069068	(Lo et al. 2002)

Name	Gene region	Host	Supergroup	Accession number	Publication
	16S	*Folsomia candida*	E	AF179630	(Vandekerckhove et al. 1999)
	16S	*Mesaphorura macrochaeta*	E	AJ422184	(Czarnetzki and Tebbe 2004)
	16S	*Microcerotermes*	F	AJ292347	(Lo et al. 2002)
	16S	*Hapithus agitator*	F	DQ536098	(Panaram and Marshall 2007)
	16S	*Diae acircumlita*	G	AY486073	(Rowley et al. 2004)
	16S	*Zootermopsis angusticollis*	H	AY764279	(Bordenstein and Rosengaus 2005)
	16S	*Zootermopsis nevadensis*	H	AY764280	(Bordenstein and Rosengaus 2005)
	16S	*Orchopeas leucopus*	I	AY335924	(Dittmar and Whiting 2004)
	16S	*Dipetatonema gracile*	J	FR827938	(Ferri et al. 2011)
	16S	*Bryobia sp.*	K	EU499318	(Ros et al. 2009)
	16S	*Cinara cedri*	M	AY620430	(Gomez-Valero et al. 2004)
	16S	*Toxoptera aurantii*	N	JN384094	(Augustinos et al. 2011)

Supp. Table 4.2: Reference sequences used in the construction of the *Wolbachia* surface protein (*wsp*), information included is the supergroup, accession number and the publication from which it was sourced.

Name	Gene region	Host	Supergroup	Accession number	Publication
wSub	*wsp*	*Amigeres subalbatus*	A	AF317488	(Ruang-Areerate et al. 2003)
wMel	*wsp*	*Drosophila melanogaster*	A	AF0200072	(Bozorg-Omid et al. 2020)
wRi	*wsp*	*Drosophila simulans*	A	AF020070	(Braig et al. 1998)
wMors	*wsp*	*Glossina moristans*	A	AF020079	(Zhou et al. 1998b)
wWhi	*wsp*	*Lutzomiya whitmani*	A	AF237885	(Ono et al. 2001)
wUni	*wsp*	*Muscidifurax uniraptor*	A	AF020071	(Zhou et al. 1998b)
	wsp	*Phlebotomus papatasi*	A	KX263909	(Karimian et al. 2018)
wFlu	*wsp*	*Aedes aegypti*	B	GQ917108	(Moreira et al. 2009)
wAlbB	*wsp*	*Aedes albopictus*	B	AF020059	(Zhou et al. 1998a)
wBem2	*wsp*	*Bermisi tabaci*	B	JN315980	(Henri and Mouton 2012)
wBry	*wsp*	*Liromyza bryoniae*	B	AB231469	(Tagami et al. 2006)
wCer3	*wsp*	*Rhagoletis cerasi*	B	KJ488946	(Arthofer et al. 2009)

Name	Gene region	Host	Supergroup	Accession number	Publication
wcerNae	wsp	*Schizotetranychus cercidiphyll*	B	AB096236	(Gotoh et al. 2003)
wEmeTn1	*wsp*	*Zizina emelina*	B	AB512756	(Sakamoto et al. 2011)
wLep2	*wsp*	*Athetis lepigone*	B	KJ596656	(Chen et al. 2017)
wAus	wsp	*Plutella australiana*	Outgroup	NZ_MRWX01000095	(Ward and Baxter 2017)
	wsp	*Alfonsiella pipithiensis*			This study
	wsp	*Ceratitis cosyra*			This study

Supp. Table 4.3: Reference sequences used in the construction of the multi-locus sequence typing analysis, information included is the sequences from *coxA*, *fbpA*, *ftsZ*, *gatB* and *hcpA*.

Wolbachia infection Host (Isolate number)	*coxA* – cytochrome oxidase, subunit I	*fbpA* – fructose-bisphosphate aldolase	*ftsZ* – Cell division protein	*gatB* – Glutamyl-tRNA (Gln) amidotransferase, subunit B	*hcpA* - Conserved hypothetical protein
Diaphorina citri	CP048819	CP048819	CP048819	CP048819	CP048819
Aedes albopictus (12)	DQ842268	DQ842342	DQ842305	DQ842416	DQ842379
Acraea encedon (22)	DQ842269	DQ842343	DQ842306	DQ842417	DQ842380
Acraea eponina (23)	DQ842270	DQ842344	DQ842307	DQ842418	DQ842381
Acromis sparsa (3)	DQ842271	DQ842345	DQ842308	DQ842419	DQ842382
Armadillidium vulgare (28)	DQ842272	DQ842346	DQ842309	DQ842420	DQ842383
Brugia malayi (37)	DQ842273	DQ842347	DQ842341	DQ842421	DQ842384
Chelymorpha alternans (19)	DQ842274	DQ842348	DQ842310	DQ842422	DQ842385
Cimex lectularius (36)	DQ842275	DQ842349	DQ842311	DQ842423	DQ842386
Camponotus pennsylvanicus (4)	DQ842276	DQ842350	DQ842312	DQ842424	DQ842387
Culex pipiens pipiens (29)	DQ842277	DQ842351	DQ842313	DQ842425	DQ842388
Culex pipiens quiquefasciatus (3)	DQ842278	DQ842352	DQ842314	DQ842426	DQ842389
Drosophila bifasciata (5)	DQ842279	DQ842353	DQ842315	DQ842427	DQ842390
Drosophila innubila (6)	DQ842280	DQ842354	DQ842316	DQ842428	DQ842391
Drosophila neotestacea (7)	DQ842281	DQ842355	DQ842317	DQ842429	DQ842392
Drosophila orientacea (8)	DQ842282	DQ842356	DQ842318	DQ842430	DQ842393
Drosophila recens (9)	DQ842283	DQ842357	DQ842319	DQ842431	DQ842394

Wolbachia infection Host (Isolate number)	*coxA* – cytochrome oxidase, subunit I	*fbpA* – fructose-bisphosphate aldolase	*ftsZ* – Cell division protein	*gatB* – Glutamyl-tRNA (Gln) amidotransferase, subunit B	*hcpA* - Conserved hypothetical protein
Drosophila simulans (10)	DQ842284	DQ842358	DQ842320	DQ842432	DQ842395
Drosophila simulans (11)	DQ842285	DQ842359	DQ842321	DQ842433	DQ842396
Drosophila simulans (26)	DQ842286	DQ842360	DQ842322	DQ842434	DQ842397
Drosophila simulans (27)	DQ842287	DQ842361	DQ842323	DQ842435	DQ842398
Encarsia Formosa (33)	DQ842288	DQ842362	DQ842324	DQ842436	DQ842399
Ephastia kuehniella (13)	DQ842289	DQ842363	DQ842325	DQ842437	DQ842400
Ephastia kuehniella (31)	DQ842290	DQ842364	DQ842326	DQ842438	DQ842401
Gryllus firmus (24)	DQ842291	DQ842365	DQ842327	DQ842439	DQ842402
Incisitermes synderii (14)	DQ842292	DQ842366	DQ842328	DQ842440	DQ842403
Muscidifurax uniraptor (15)	DQ842293	DQ842367	DQ842329	DQ842441	DQ842404
Nasonia giraulti (17)	DQ842294	DQ842368	DQ842330	DQ842442	DQ842405
Nasonia longicornis (16)	DQ842295	DQ842369	DQ842331	DQ842443	DQ842406
Nasonia vitripennis (18)	DQ842296	DQ842370	DQ842332	DQ842444	DQ842407
Nasonia vitripennis (34)	DQ842297	DQ842371	DQ842333	DQ842445	DQ842408
Ostrinia scapulalis (32)	DQ842298	DQ842372	DQ842334	DQ842446	DQ842409
Protocalliphora sialia (21)	DQ842299	DQ842373	DQ842335	DQ842447	DQ842410
Solenopsis Invicta (2)	DQ842300	DQ842374	DQ842336	DQ842448	DQ842411

Wolbachia infection Host (Isolate number)	*coxA* – cytochrome oxidase, subunit I	*fbpA* – fructose-bisphosphate aldolase	*ftsZ* – Cell division protein	*gatB* – Glutamyl-tRNA (Gln) amidotransferase, subunit B	*hcpA* - Conserved hypothetical protein
Tribolium confusum (20)	DQ842301	DQ842375	DQ842337	DQ842449	DQ842412
Teleogryllus taiwanemma (25)	DQ842303	DQ842377	DQ842339	DQ842451	DQ842414
Drosophila melanogaster (1)	DQ842304	DQ842378	DQ842340	DQ842452	DQ842415
Fragment Length (bp)	409	429	435	369	430