Genomic insights into the evolution of the parasitoid *Microctonus aethiopoides*

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MEERAN HUSSAIN

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Abstract

Parasitoids are used as biocontrol agents in classical biological control programmes for controlling invasive pest populations. In Aotearoa New Zealand, there are two strains of *Microctonus aethiopoides* that have been used against two serious pest weevils of the *Sitona* group. However, there is limited understanding of *M. aethiopoides* adaptability, particularly in the area of reproductive mode and host associations. My thesis explores these gaps, using population and comparative genomic approaches to investigate the adaptability of *M. aethiopoides*.

Chapter 2 is a review that synthesises global research on parasitoid adaptation and identifies knowledge gaps across four key traits i.e., reproductive mode, olfaction, thermal tolerance, and microbiomes. Using the three *Microctonus* species in New Zealand as a focal example, this chapters explains why these traits matter for biocontrol and how genomic technologies reveal their underlying molecular mechanisms.

Chapter 3 explores the adaptation of Irish asexual *M. aethiopoides* in New Zealand by analysing whole-genome resequencing data from 43 individuals representing historic (Ireland) and contemporary populations. The study reveals two distinct genetic clusters, most likely reflecting the genetic ancestry of introduced populations rather than post-introduction geographic isolation. All populations exhibit very low diversity and limited population expansion since release, consistent with their asexual nature and suggesting limited adaptation. Asexual *M. aethiopoides* show some heterozygosity across populations, supporting automictic thelytoky, likely via central fusion, as the primary reproductive mechanism. However, linkage disequilibrium patterns resemble those of sexual organisms, raising the possibility of facultative sex. This chapter sheds light on the adaptation, spread, and reproductive strategies of asexual *M. aethiopoides* in New Zealand.

Chapter 4 further examines parasitoid adaptation, with a focus on host-associated divergence. Using eight newly assembled genomes from *M. aethiopoides* strains collected from three different hosts (*Hypera postica*, *Sitona discoideus*, *S. obsoletus*) and geographically distinct regions, this study investigates how host use and geography shape genomic structure. Comparative genomics reveals a clear host-associated phylogenetic split, with the *H. postica* lineage distinct from *Sitona*-associated lineages. Heterozygosity patterns support this separation, showing highest diversity in the *H. postica* group. However, gene families are mostly conserved across strains, with transposable element-related genes showing rapid evolution, suggesting their active role in genome restructuring. Chemosensory genes are also largely conserved, with only modest variation in odourant receptors that may reflect host-associated differences, although fragmentation and annotation bias cannot be ruled out. Overall, host association appears to be a key driver of divergence in *M. aethiopoides*, largely shaped by genome-level variation rather than major shifts in host-specific gene families.

Finally, **Chapter 5** assembles and characterises the first complete mitochondrial genome of *M. aethiopoides*, generated using a hybrid Oxford Nanopore-Illumina assembly. This study uncovers a long circular (33,173 bp) mitogenome with a unique gene arrangement, contributing a valuable genetic resource for future studies of mitochondrial evolution in parasitoids.

Overall, my thesis demonstrates how genomic technologies can be used to provide a more comprehensive understanding of the evolutionary factors shaping a species' genome and population structure. It provides a valuable genomic resource and sets a foundation of resources for continuing to build understanding of adaptation in the context of asexuality and host association in *M. aethiopoides*.

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Chapter 1

General introduction

When non-native species establish in new habitats and cause ecological or economic harm, they are termed invasive species. Invasive insects pose major challenges in Aotearoa New Zealand, with over 2,000 established species affecting biodiversity, ecosystems, and agriculture (Brockerhoff et al., 2010). Control methods include chemical (pesticides), cultural (crop rotation), and physical (traps) approaches each with varying success, costs, and environmental impacts. Genetic tools like RNA interference and gene drives show promise but face technical and ethical limitations. Thus, among these methods, biological control (biocontrol) stands out as a natural, sustainable, and often highly effective method for managing invasive species through their co-evolved natural enemies (i.e., predators, parasitoids, and entomopathogens).

Classical biological control is a key strategy for managing primarily invasive pests by introducing the natural enemies from their native range (Heimpel & Cock, 2018; Wyckhuys et al., 2024). It differs from augmentative control, as it relies on a one-time release for long-term impact, while the former requires periodic releases, making it more labour-intensive and costly (Bale et al., 2008). Parasitoids are used as biological control agents to manage arthropod pests in forest and agricultural ecosystems. Typically, the female parasitoid deposits its egg inside (endoparasitoid) or outside (ectoparasitoid) the host (mostly arthropods) and the parasitoid larva feeds on the host upon emergence. Such parasitoids are mainly from the orders Hymenoptera (75% of known parasitoids), Diptera, and Coleoptera and are the most successful agents in biocontrol programmes, playing important roles in regulating herbivorous insect populations (Blaimer et al., 2023; Eggleton & Belshaw, 1992).

1.1 Genetic diversity in asexual parasitoids and its implications for biological control

Parasitoids exhibit two principal reproductive modes, sexual (arrhenotoky) and asexual (thelytoky). In sexual species, fertilised eggs produce females and unfertilised eggs produce males, whereas in asexual species, females arise from unfertilised eggs and males are rare or absent (Adachi-Hagimori & Miura, 2020; Goldson et al., 1990). Asexual reproduction provides an advantage for biological control as every egg produces a female capable of parasitising hosts and enabling rapid population establishment even when host densities are low (Queffelec et al., 2021; Stouthamer, 1993). However, sexual reproduction is generally considered advantageous over evolutionary timescales, as meiosis and recombination promote genetic diversity, enhancing adaptive potential under changing environmental conditions (Muller, 1932, 1964). In contrast, the absence of recombination in asexual lineages often leads to reduced genetic diversity and accumulation of deleterious mutations, constraining long-term adaptability (Lynch, 1984; Otto & Michalakis, 1998).

Not all asexuals are strictly clonal. Some, such as automictic species, retain partial meiosis followed by fusion of meiotic products from the same individual. Depending on the mechanism (e.g., central or terminal fusion, gamete duplication), automixis can preserve varying levels of heterozygosity and therefore maintain limited genetic diversity despite the absence of fertilisation (Card et al., 2021; Lampert, 2008; Pearcy et al., 2011). Thus, sexual and asexual species can be expected to exhibit different patterns of genetic diversity across their genomes (Tvedte et al., 2019).

Although more than 500 Hymenopteran species are known to reproduce asexually (van der Kooi et al., 2017), the genetic consequences of asexuality in parasitoids remain poorly understood. Observations of declining efficacy in some asexual parasitoid biocontrol programmes, such as those using the parasitoid, *Microctonus hyperodae* Loan against the weevil *Listronotus bonariensis* (Kuschell) (Casanovas et al., 2018), raise important questions about whether reproductive mode influences the long-term success or failure of such control attempts. How these asexual lineages sustain effectiveness in dynamic and competitive ecological contexts is largely unexplored, despite their widespread use in biological control.

1.2 Host use as a driver of genetic differentiation and adaptation in parasitoids

Parasitoid divergence can be explained through two evolutionary frameworks - the adaptive radiation and oscillation hypotheses - which describe how host use contributes to parasitoid diversification. The adaptive radiation hypothesis suggests that diversification follows the emergence of key innovations. In parasitoids, such innovations might include the evolution of venom components that suppress host immunity, domesticated viral genes that facilitate parasitism, or structural changes in the ovipositor allowing egg deposition into concealed hosts (Hambäck et al., 2024; Maletti et al., 2021; Polaszek & Vilhemsen, 2023). These innovations could leave clear genomic signatures, such as the expansion or diversification of gene families related to host manipulation, sensory perception, or immune evasion (Hambäck et al., 2024). In contrast, the oscillation hypothesis proposes that diversification arises from ecological opportunity acting on standing genetic variation rather than new genetic inventions (Hambäck et al., 2024). For example, parasitoid lineages already possess a flexible "toolkit" of genes, enabling them to attack a variety of hosts during environmental shifts or changes in host availability that expose parasitoid populations to new hosts, leading to host-associated differentiation and eventual speciation (Hambäck et al., 2024). Genomically, this could be reflected in more subtle changes, such as gene copy number variation or selection acting on existing gene families, rather than the appearance of entirely new genes (Hambäck et al., 2024).

Hymenopteran parasitoids represent one of the most species-rich and ecologically diverse insect groups (Forbes et al., 2018). Yet, the mechanisms driving this extraordinary diversification remain only partly understood. Parasitoids in general possess a distinctive lifestyle that depends on insect hosts for survival and reproduction, where females locate and oviposit on or within living hosts. This intimate dependence on specific host species, coupled with exposure to varying environmental conditions, creates strong opportunities for divergent selection, leading to ecological specialisation and genetic differentiation among parasitoids exploiting different hosts (Feder & Forbes, 2010; Stireman et al., 2006; Tilmon, 2008). Endoparasitoids, in particular, tend to be more host-associated than ectoparasitoids because their internal development within the host demands highly specialised physiological and behavioural strategies to overcome host immune defences, often resulting in a narrower and more specific host range (Asgari, 2012; Moreau & Asgari, 2015).

While processes of host-associated differentiation and ecological speciation are well established in herbivorous insects (Funk, 2010; Knolhoff & Heckel, 2014; Michielini et al., 2024), similar mechanisms are increasingly recognised in parasitoids (Häner et al., 2024; Koppik et al., 2015; Phillips et al., 2008; Shaw, 1988; Zaviezo et al., 2021). Host-associated diversification in parasitoids differs from herbivores because host expansions in parasitoids may not only occur to encompass closely related host species but may also follow the host lifestyle or even the phylogeny of the host plant consumed by the herbivorous host (Nyman et al., 2015). In parasitoids, different hosts can act as potent selective agents shaping behavioural traits related to host recognition and oviposition, as well as physiological compatibility with host defences, thereby generating selection for adaptations finely tuned to specific hosts (Antolin et al., 2006; Mackauer et al., 1996). Such host-mediated divergent selection can also occur within a single species, where genetically distinct lines differ in host preferences. Increasing evidence supports this intraspecific variation as a key factor influencing traits critical for biological control, including host specificity (Goldson et al., 2003, 2005; Häner et al., 2024), life history strategies (Zaviezo et al., 2021), and learning behaviour (Koppik et al., 2015). Despite strong ecological evidence for host-associated differentiation in parasitoids, the link between these processes and genome evolution remains unclear. Comparing genomes of conspecific parasitoids reared on different hosts could provide a powerful way to bridge this gap.

1.3 *Microctonus aethiopoides*

Microctonus aethiopoides (Loan) (Hymenoptera: Braconidae) is an important endoparasitoid that parasitises adult weevils from the genera *Hypera* (Germar) and *Sitona* (Germar) (Coleoptera: Curculionidae). This parasitoid is native to Europe and North African regions and has been successfully used in classical biological control programmes against agricultural pests in North America (Loan, 1969; Radcliffe & Flanders, 1998; Rand et al., 2018), Australia, and New Zealand (Goldson et al., 2004; Vink et al., 2003).

Figure 1.1 *Microctonus aethiopoides* (Irish strain) ovipositing into its host, *Sitona obsoletus* (clover root weevil). (Image rights: Dr Trevor James, AgResearch)

In New Zealand, two strains of *M. aethiopoides* have been introduced to target two exotic weevil pests. A Moroccan sexual strain of *M. aethiopoides* was introduced in 1982 via Australia to control the lucerne weevil *Sitona discoideus* (Gyllenhal) (Stufkens et al., 1987). After the discovery of another pest clover root weevil (*Sitona obsoletus* (Gmelin)), and the subsequent finding that the Moroccan *M. aethiopoides* strain was ineffective (Barratt et al., 1997; McNeill et al., 2000; White et al., 2015), an intensive search for alternative biocontrol agents was initiated in the United States and Europe in 1998, with *M. aethiopoides* shown to be the prevalent parasitoid reared from wild-collected *S. obsoletus* in the European Union (EU) and United Kingdom (UK) (Goldson et al., 2001, 2004). In New Zealand quarantine, evaluation of the EU and UK *M. aethiopoides* populations showed that they could be successfully reared on New Zealand *S. obsoletus*. However, it was also found that sexually reproducing strains of *M. aethiopoides* could interbreed with the existing Moroccan population, resulting in a loss of efficacy and potentially compromising control of both *S. discoideus* in lucerne and *S. obsoletus* in pasture if the European strains of *M. aethiopoides* were also released (Goldson et al., 2003). To mitigate the risk, an asexual Irish strain of *M. aethiopoides* that had been collected as part of the European effort was selected for release in 2006 (Gerard et al., 2006; McNeill et al., 2006). Within a year, parasitism rates of up to 70% were observed in overwintering *S. obsoletus* populations, with subsequent field studies indicating significant declines in both adult and related larval densities (Gerard et al. 2007, 2011; McNeill et al. 2016) indicating the promise of the Irish *M. aethiopoides* as an effective biocontrol agent had been realised.

1.4 Thesis aims and objectives

Parasitoid wasps are key biological control agents that regulate pest populations in agricultural systems, and their long-term effectiveness depends on understanding their adaptability in various factors such as reproductive mode, host association, and ecological conditions influence their persistence. The endoparasitoid, *Microctonus aethiopoides*, provides an excellent system to explore the evolutionary dynamics of such factors. The apparent failure of asexual *M. hyperodae* to maintain effective biocontrol of *Listronotus bonariensis* in New Zealand has raised concerns about the long-term adaptability of asexual parasitoids (Casanovas et al., 2018). Therefore, the release of the asexual *M. aethiopoides* strain enables assessment of whether comparable genetic constraints could influence its future performance.

Investigating genomic diversity, population structure, and signatures of recombination of *M. aethiopoides* across time and space among New Zealand regional populations will reveal how asexual lineages maintain variation despite their reproductive mode (Mozhaitseva et al., 2023), while also offering insights into their adaptive potential and resilience under changing environmental conditions. Furthermore, because *M.*

aethiopoides strains are associated with multiple host species (Gerard et al., 2006; Steele et al., 2006; Sundaralingam et al., 2001), the species complex provides a unique system to explore genome-level divergence and to identify gene families linked to host-associated adaptation across strains. Finally, mitochondrial genomes provide valuable phylogenetic markers due to their maternal inheritance and frequent gene rearrangements, which are especially common in Hymenoptera (Li et al., 2016; Wei et al., 2010; Zheng et al., 2021). However, mitogenome data for the Euphorinae subfamily remain limited. By generating the complete mitogenome of *M. aethiopoides,* my thesis also contributes important genomic resources for the Euphorinae, a subfamily that includes important biological control agents, and supports future phylogenetic and evolutionary studies.

Ultimately, my thesis explores the genomic mechanisms shaping evolution and adaptability of *M. aethiopoides* across different contexts of biological control. Using a combination of population genomics, comparative genomics, and mitochondrial analyses, my research addresses four aims:

1) **To examine** how asexual reproduction influences genomic diversity and adaptive potential by exploring the spatial and temporal variation of the Irish *M. aethiopoides* populations across New Zealand;
2) **To evaluate** evidence of facultative sexuality in the Irish *M. aethiopoides* using genomic data;
3) **To produce** high-quality genome assemblies of *M. aethiopoides* strains using hybrid short- and long-read sequencing technologies;
4) **To assess** whether *M. aethiopoides* strains reared on different host species exhibit genomic and gene family differences indicative of host-associated adaptation and diversification;
5) **To assemble and characterise** the circular mitochondrial genome of *M. aethiopoides* and describe gene rearrangements with potential ecological and evolutionary implications.

1.5 Thesis outline

My thesis comprises six chapters, with Chapters 2-5 developed as independent research papers. Each of these chapters has been submitted, or is being prepared for submission, to peer-reviewed journals. As they are written as stand-alone studies, there may be some overlap in methodological details and introductory context.

Chapter 2 presents a comprehensive literature review on the adaptive traits underpinning the effectiveness of parasitoids used in classical biological control, with a particular focus on the *Microctonus* species introduced in New Zealand. The review highlights ongoing challenges in predicting the long-term success of parasitoid releases, as illustrated by the post-release decline in *M. hyperodae* efficacy and the geographically variable non-target impacts among *M. aethiopoides* strains. It synthesises research, primarily on Hymenopteran parasitoids, examining four key adaptive traits - reproductive mode, olfaction, thermal tolerance, and symbiont interactions - that collectively shape host specificity, adaptability, and persistence. Drawing from behavioural, ecological, and molecular studies, this chapter identifies general patterns and important knowledge gaps, particularly regarding trait-specific adaptive responses influencing host use and environmental tolerance. Finally, it underscores the importance of integrating behavioural, genomic, and ecological approaches to elucidate the molecular basis of adaptation and to enhance the long-term efficacy and safety of parasitoid-based biocontrol programmes in New Zealand.

Chapter 3 investigates population-level genetic differentiation and reproductive mechanisms in the asexual Irish strain of *M. aethiopoides*, a parasitoid introduced to New Zealand for biological control of clover root weevil. In this chapter, I analyse whole-genome resequencing data from individuals collected across four regional sites in New Zealand and from a quarantined Irish release population maintained at the Lincoln AgResearch facility. To assess spatial and temporal genetic variation following introduction, I examine patterns of genetic structure using PCA, admixture, and phylogenetic analyses, and explore genetic diversity metrics, such as nucleotide diversity (π) and Tajima's D to understand population diversity and expansion following introduction. I also investigate the mode of asexual reproduction and assess the potential for residual or facultative sexuality by analysing heterozygosity patterns and linkage disequilibrium. This study provides a genomic framework for understanding the evolutionary dynamics, reproductive mechanisms, and implications for the long-term adaptability of asexual biocontrol agents.

Chapter 4 builds upon the framework of parasitoid adaptation established in **Chapter 3**, shifting to host-associated adaptation and genomic divergence across *M. aethiopoides* strains through a comparative genomics approach. In this chapter, I build and analyse genome assemblies of *M. aethiopoides* strains reared from different host species and geographic regions to investigate the genetic basis of host adaptation. I use tools, such as OrthoFinder and CAFE, to compare gene families and identify rapidly evolving genes across strains. I then focus specifically on chemosensory gene families to determine whether variation in these genes reflects adaptation to host-specific strains. This study aims to understand how host associations drive genomic differentiation and contribute to the evolutionary diversification of *M. aethiopoides*.

Chapter 5 focuses on the assembly and characterisation of the mitochondrial genome of *M. aethiopoides* to explore its evolutionary and phylogenetic relationships within the Braconidae. In this short chapter, I assemble the first complete mitochondrial genome of *M. aethiopoides* using a hybrid approach that combines short and long read sequencing data from **Chapter 4** and annotate it for comparison of mitochondrial gene content, base composition, and gene order with other Braconidae species to investigate patterns of mitogenome conservation and rearrangement.

Finally, **Chapter 6** provides a synthesis of the key findings from the research chapters, discussing their main research outcomes and identifying the main conclusions about the evolutionary processes (such as reproductive mode and host use) that have shaped adaptation in *M. aethiopoides*. In addition, I focus on future research opportunities, delivering concrete suggestions for achieving a better understanding of parasitoid biocontrol systems in the context of their long-term persistence and efficacy in New Zealand.

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Chapter 2

Review: What makes a good biocontrol agent? A perspective on parasitoids

Meeran Hussain[1], Elahe Parvizi[1], Mark R. McNeill[2], Ang McGaughran[1]

[1]Te Aka Mātuatua - School of Science, University of Waikato, Private Bag 3105, Hamilton, 3240, New Zealand

[2] Bioeconomy Science Institute, Tuhiraki, Christchurch, New Zealand

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2.1 Abstract

Parasitoids are central to classical biological control, yet predicting their long-term effectiveness post release remains challenging. In Aotearoa New Zealand, three *Microctonus* (Hymenoptera: Braconidae) species have been used against pest weevils, but key aspects of their biology remain poorly understood. This is evident in post-release decline of *M. hyperodae* efficacy and geographically variable non-target impacts among *M. aethiopoides* strains. Such cases highlight knowledge gaps in host-searching behaviour, reproductive mode, adaptability to environmental conditions, and microbiome composition within parasitoids and hosts.

We review studies, mainly on Hymenopteran parasitoids, focusing on key adaptive traits of biocontrol: reproductive mode, olfaction, thermal tolerance, and symbiont interactions. Synthesising findings from host specificity, behavioural and molecular analyses we identify broader patterns and key gaps. While molecular research on *Microctonus* is limited, evidence from other systems shows trait-specific adaptive responses, with variation in olfactory sensitivity, reproductive strategies, and microbiome interactions influencing host use and persistence.

While field-level efficacy is often assessed, species-specific evaluation of life history traits under relevant environmental conditions is essential, followed by genomic and transcriptomic studies to reveal the molecular basis of adaptation. Integrating behavioural, molecular, and ecological insights will be essential for optimising parasitoid releases and ensuring long-term biocontrol success in New Zealand.

Glossary

Term	Full Form / Explanation
Apomixis	Clonal asexual reproduction without meiosis or recombination.
Arrhenotoky	Sexual reproduction in Hymenoptera via haplodiploidy (unfertilised eggs → males; fertilised → females).
Automixis	Asexual reproduction involving meiosis and partial recombination, with diploidy restored.
CAT	Catalase: an antioxidant enzyme that decomposes hydrogen peroxide into water and oxygen.
GLVs	Green Leaf Volatiles: a group of volatiles released immediately upon plant damage, often early cues for parasitoids.
GST	Glutathione S-Transferase – an enzyme that detoxifies harmful byproducts of oxidative stress.
HIPVs	Herbivore-Induced Plant Volatiles: chemical compounds released by plants in response to herbivore feeding that attract parasitoids.
HSPs	Heat Shock Proteins: molecular chaperones that protect cells from thermal stress.
IHOE / OHOP	Inside-/Outside-Hosts-Overwintering Endoparasitoids: strategies for surviving winter inside or outside host bodies.
IRs	Ionotropic Receptors: a class of olfactory receptors involved in chemosensory detection.
MhFV	*Microctonus hyperodae* Filamentous Virus: a virus associated with *M. hyperodae*, possibly affecting parasitoid performance.
OBPs	Odourant-Binding Proteins: proteins involved in carrying odour molecules to receptors in insect antennae.
ODEs	Odourant-Degrading Enzymes: enzymes that degrade excess odourants to reset the olfactory system.
ORs	Odourant Receptors: proteins that detect odourant molecules and trigger neural responses.
ROS	Reactive Oxygen Species: chemically reactive molecules involved in oxidative stress.
SNMPs	Sensory Neuron Membrane Proteins: membrane proteins involved in pheromone detection.
SOD	Superoxide Dismutase: an enzyme that neutralises superoxide radicals.
sHsps	Small Heat Shock Proteins: a family of proteins involved in stress response.
Thelytoky	Asexual reproduction where females are produced from unfertilised eggs.
VLPs	Virus like particles: Resemble viruses but are not infectious because they lack genetic material

2.2 Introduction

With increasing global movement and trade, the rate of biological invasion is escalating globally (Epanchin-Niell et al., 2021), and Aotearoa New Zealand is no exception. Despite its small size, the country harbours nearly 27,000 introduced species (compared to ~80,000 native species) (Ministry of the Environment, 2021), some of which cause significant harm to ecosystems and economies (Goodenough, 2010) and can be classified as 'invasive'.

Invasive insect pests are a particular problem in New Zealand, with an estimate of more than 2,000 established species that range in impacts from ecological (e.g., competition among species leading to extinction of natives), to environmental (e.g., changes in ecosystem function), and economic (e.g., affecting human health or agriculture). For example, several key invasive arthropod pests, including the Argentine stem weevil (*Listronotus bonariensis* (Kuschel)), clover root weevil (*Sitona obsoletus* (Gmelin)), and lucerne weevil (*Sitona discoideus* (Gyllenhal)), have caused significant damage to New Zealand pastures (Eerens et al., 2005; Prestidge et al., 1991; Tomasetto et al., 2018). Based on 2018 estimates, Argentine stem weevil and clover root weevil incur annual costs of up to \$200 million and \$235 million, respectively, in improved agricultural grasslands (Ferguson et al., 2019).

2.2.1 Biological control

Classical biological control is a key strategy for managing invasive species by introducing the natural enemies from their native range (Heimpel & Cock, 2018; Wyckhuys et al., 2024). It differs from augmentative control, as it relies on a one-time release for long-term impact, while the former requires periodic releases, making it more labour-intensive and costly (Bale et al., 2008). In New Zealand, where many crops and their associated pests have been introduced without their natural predators, classic biological control has played an important role in pest management since the early days of European agriculture (Cameron et al., 1993). When successful, biological control provides an effective and economically viable method for controlling insect pests, with parasitoids often proving to be particularly valuable agents for agricultural insect pests.

The success of biocontrol is influenced by biotic and abiotic factors, including the ecological compatibility between the control agent and the target pest, climatic suitability, and release management, with host specificity being important for both establishment and the minimisation of non-target risks (Van Driesche, 1994; Goldson et al., 2014; Jervis et al., 2023). However, as outlined in the review by Leung et al. (2020), biological control, especially classical biological control, is facing challenges especially related to the ability to source biological material from overseas jurisdictions. Thus, new strategies to improve the efficacy of biocontrol agents increasingly use genetic and genomic approaches (Leung et al., 2020). In particular, understanding the heritability of desired insect biocontrol traits has the potential to improve control of insect pests (Chattington et al., 2025), although there are challenges in deciding which traits are the best to optimise (Leung et al., 2020; Segoli et al., 2023).

2.2.2 *Microctonus* parasitoids

Microctonus (Wesmael, 1835) (Hymenoptera: Braconidae: Euphorinae) is an important genus that parasitises weevils from the genera *Hypera* (Germar) and *Sitona* (Germar) (Coleoptera: Curculionidae) (Shaw, 1988). As endoparasitoids of adult weevils, several species of the genus have been used in classical biological control of these two weevil genera (Aeschlimann, 1980, 1983; Loan, 1967). These parasitoids attack the adult weevil by ovipositing their eggs into the host hemocoel, using the ovipositor to pierce the soft membrane between the sclerites. Though the weevils continue to feed while the parasitoid is developing inside the host, they are rendered sterile before the final instar emerges to pupate, thereby killing the weevil (Loan & Lloyd, 1974; Loan & Holdaway, 1961).

In New Zealand, three *Microctonus* species have been introduced to target three major exotic weevil pests, including two strains of *Microctonus aethiopoides* (Loan) (Gerard et al., 2006; Stufkens et al., 1987), and a single *M. hyperodae* (Loan) strain (Goldson et al., 1990). *Microctonus hyperodae*, is an asexual (thelytokous) parasitoid native to South America, introduced to New Zealand as a biological control agent targeting *L. bonariensis*. In its native range, *M. hyperodae* along with a range of other natural enemies suppresses weevil populations (Lloyd, 1966; Lloyd & Ahmad, 1972) but was found to be the most prevalent parasitoid attacking *L. bonariensis* (Loan & Lloyd, 1974). A classical biological control program initiated in 1988, resulted in the collection of over 13,000 adult *L. bonariensis* with unknown levels of parasitism from eight different locations across South America, which were imported into New Zealand invertebrate quarantine (Goldson et al., 1990). Parasitoid colonies from each South American location were reared and maintained as distinct ecotypes and, after a series of laboratory and non-target impact testing (Goldson et al., 1992), equal numbers of parasitoid were released across New Zealand in 1991 (Goldson et al. 1993; McNeill et al., 2002).

Microctonus hyperodae was able to successfully parasitise *L. bonariensis*, with overwintering parasitism rates exceeding 90% within three years of its release (Barker & Addison, 2006; Goldson et al., 1998). However, seven years post-release (c. 14 weevil generations), it was noted that *M. hyperodae* was losing its effectiveness as a biocontrol agent, based on reports of a notable recurrence of pasture damage by *L. bonariensis* (Popay et al., 2011). This decline in efficacy has been hypothesised to result from co-evolutionary dynamics, where the sexually reproducing host may be adapting more rapidly than the asexual parasitoid (Casanovas et al., 2018). Additionally, the recent finding of a novel *Microctonus hyperodae* filamentous virus (MhFV) (Inwood et al., 2023) has prompted speculation about its possible link to reduced parasitoid performance.

A Moroccan strain of *M. aethiopoides* was introduced into New Zealand via Australia in 1982 to control the lucerne weevil *Sitona discoideus* (Gyllenhal) (Stufkens et al., 1987). However, laboratory-based quarantine studies showed that this strain was ineffective against *Sitona obsoletus* (Gmelin) (Barratt, Evans, & Ferguson, 1997; McNeill et al., 2000), likely due to host-associated endosymbionts that encapsulate and destroy developing parasitoid eggs and larvae (White et al., 2015). Consequently, an intensive search for alternative biocontrol agents was initiated in the United States and Europe in 1998 (Goldson et al., 2001, 2004), with *M. aethiopoides* shown to be the prevalent parasitoid reared from *S. obsoletus* collected from several countries in the EU and UK.

In New Zealand quarantine, evaluation of the different *M. aethiopoides* populations showed that they could be successfully reared on New Zealand populations of *S. obsoletus*. However, it was also found that sexually reproducing (arrhenotokous) strains of *M. aethiopoides* could interbreed with existing Moroccan population (also arrhenotokous), resulting in a loss of efficacy and potentially compromising control of both *S. discoideus* in lucerne, and *S. obsoletus* in pasture, if the European strains of *M. aethiopoides* were released (Goldson et al., 2003). To mitigate these risks, an asexual (thelytokous) Irish strain of *M. aethiopoides* that had been collected as part of the European effort was selected for release in 2006 after further evaluation (Gerard et al., 2006; McNeill et al., 2006). Within a year, parasitism rates of up to 70% were observed in overwintering *S. obsoletus* populations, indicating the promise of *M. aethiopoides* as an effective biocontrol agent (Gerard et al., 2006, 2007, 2011).

Subsequent post-release monitoring has shown non-target parasitism of several native and exotic weevil species by both Moroccan and Irish strains of *M. aethiopoides* (Barratt et al., 1997; Ferguson et al., 2024). Some of the non-target parasitism was predicted from laboratory-based host range testing, which revealed considerable intraspecific variation in host preference and reproductive mode (Barratt, Evans, Ferguson, et al., 1997; Goldson et al., 2005). Field observations have also shown that the Moroccan strain parasitises a broader range of non-target endemic weevil species in New Zealand than it does in Australia, where it had been introduced against *S. discoideus* (Barratt, 2004; Barratt et al., 2005). Recent surveys in New Zealand have expanded this host range further, identifying new non-target associations with weevils in the genera *Chalepistes*, *Nicaeana*, and *Eugnomus* (Ferguson et al., 2024).

The Irish strain, although capable of non-target parasitism, has shown comparatively lower levels of impact (Ferguson et al., 2024) compared to the Moroccan. Non-target parasitism of the endemic weevil *Chalepistes aequalis* (Broun) by both strains of *M. aethiopoides* has the potential to displace the endemic *Microctonus zealandicus* (Shaw), (M. McNeill, unpublished data). Moroccan *M. aethiopoides* is also able to successfully develop in field populations of *L. bonariensis* (McNeill et al., 2002) and recent studies in Otago have indicated that Irish *M. aethiopoides* may be competing with *M. hyperodae* in parasitising *L. bonariensis* (B. Barratt, unpublished data). In comparison, *M. hyperodae* exhibits a narrow host range in the field and supports the quarantine-based results (Goldson et al., 1992).

As well as general parasitism differences, the three parasitoids differ in two key aspects. *M. hyperodae* is a solitary endoparasitoid that can discriminate between parasitised and unparasitised hosts (McNeill et al., 1996). Moroccan *M. aethiopoides* is also a solitary endoparasitoid but exhibits a low level of discrimination for parasitised and non-parasitised hosts (McNeill & Baird, 2010; M McNeill, unpublished data). By comparison, Irish *M. aethiopoides* is a gregarious parasitoid and appears to favour oviposition of previously parasitised hosts (McNeill & Baird, 2010). Meanwhile, virus like particles (MaVLP) have been found in ovaries of Moroccan *M. aethiopiodes* but are absent in European strains of *M. aethiopoides* (Barratt et al., 2006). They are also absent from *M. hyperodae* and the endemic *M. zealandicus* (Barratt et al., 1999). However, the role of MaVLP in successful parasitism compared to *Microctonus* species lacking MaVLP has yet to be explored.

Other parasitoid species introduced into New Zealand have also failed to establish, highlighting the complex ecological hurdles faced by biocontrol agents in novel environments. For example, *Anaphes atomarius (Brèthes),* a polyphagous egg parasitoid initially considered for controlling *L. bonariensis* in 1967, showed a high parasitism rate but failed to overwinter successfully (Cameron et al., 1989). A potential reason for this failure was hypothesised to be its inability to establish due to the lack of alternative hosts besides

*L. bonariensi*s during winter (June-August). Similarly, *Heteronychus arator (Fabricius)*, a scarab pest affecting pastures and some crop plants in New Zealand, has been the target of multiple biocontrol programmes, most of which were unsuccessful (Cameron et al., 1989). One such programme involved the introduction of *Microphthalma europaea (Egger),* a tachinid parasitoid*,* between 1967 and 1969, and although this species could be successfully reared on *H. arator* under laboratory conditions, it failed to establish in the field (Cameron et al., 1989). Similarly, other introduced parasitoids, such as *Tiphia* (*J.C.Fabricius) sp.* and the tachinid fly *Dexia uniseta (Curran)*, failed to parasitise *H. arator* effectively (Cameron et al., 1989). Meanwhile, as noted above for *M. hyperodae* and *L. bonariensis*, some parasitoids can experience a decline in efficacy over time (Shields et al., 2022), although worldwide examples are rare (e.g., Kraaijeveld & Godfray, 2009) and often related to the presence of endosymbionts (Hsiao & Hsiao, 1985; Käch et al., 2018; Oliver et al., 2003).

Collectively, studies on the *Microctonus* genus in New Zealand highlight several important but underexplored factors that may influence the success and specificity of parasitoid-based biocontrol. These include the observed decline in parasitoid effectiveness over time (Popay et al., 2011), potentially linked to reproductive mode mismatches with evolving sexual hosts (Casanovas et al., 2018), the emerging role of the novel virus in *M. hyperodae* (Inwood et al., 2023, 2024), and the occurrence of higher non-target impacts exhibited by geographically distinct strains of the same parasitoid species, as seen with *M. aethiopoides* (Barratt, 2004; Barratt et al., 2005; Ferguson et al., 2024). These findings point to the significant role of underlying biological variation in shaping host-parasitoid dynamics. Yet, several important traits, such as olfactory behaviour and its molecular basis, reproductive mode - specific biological characteristics, endosymbiont composition, and environmentally driven adaptability - remain poorly understood within the *Microctonus* group. The following sections examine key biotic and abiotic biocontrol-relevant components that characterise parasitoids, and the traits that can potentially be targeted to improve biological control outcomes (Fig. 2.1).

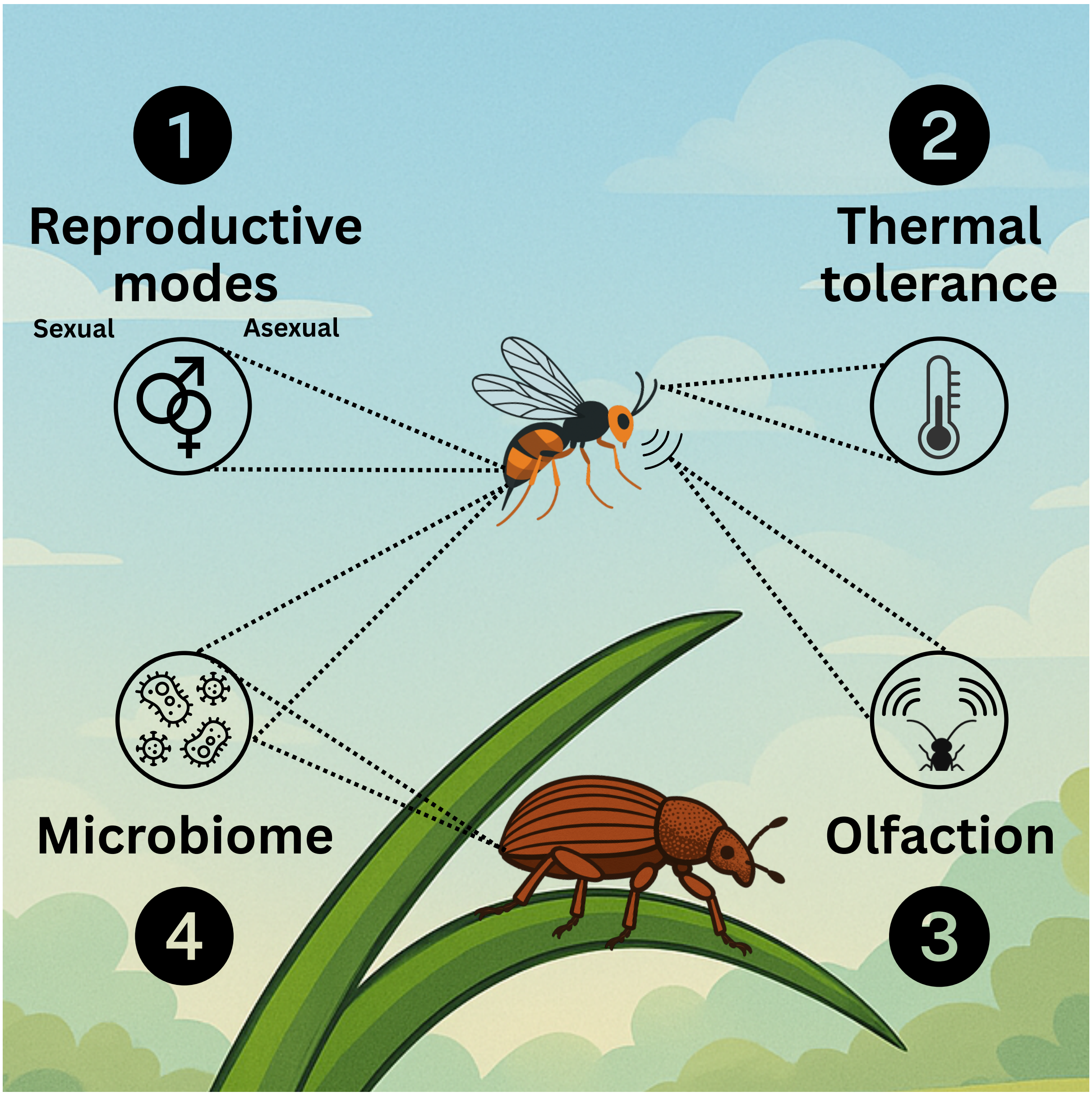

Figure 2.1 Conceptual overview of key traits examined in this review that influence parasitoid-host interactions in biocontrol systems: (1) reproductive modes (sexual or asexual); (2) thermal tolerance; (3) olfaction for host detection; and (4) microbiome. These traits collectively help improve the parasitoids' efficiency in locating, parasitising, and successfully developing in its host (e.g., weevil). Some icons were created with use of ChatGPT, and the complete figure was assembled using Canva.

2.2.3 Biocontrol success

For parasitoids, successful biological control involves overcoming several challenges (Vinson, 1984). The first challenge covered in this review is reproductive mode and its influence on genetic variation within populations, which can thereby affect the rate and direction of evolutionary adaptation (Casanovas et al., 2018; Moritz et al., 1990).

Our second focus is olfaction. Selectively locating the host in complex environments with mixed signals primarily relies on the recognition of cues associated with the host and its environment through sensory modes. This includes olfaction and vision, with olfaction playing a dominant role (Freund & Olmstead, 2000; Gonthier et al., 2023; Lim & Ben-Yakir, 2020). Once the host is located, the parasitoid lays its egg, initiating development either within the host's body (endoparasitoid) or externally on its surface (ectoparasitoid). This stage relies on the parasitoids' ability to overcome the host immune system and, particularly for endoparasitoids, the presence of virulence factors (e.g., polydnavirus and non-polydnavirus, (Quicke & Butcher, 2021; Strand & Pech, 1995) and venom proteins (Poirié et al., 2014; Yang et al., 2021)).

Classical biological control aims for long-term pest suppression, so biocontrol agents must be evaluated not only for short-term efficacy but also for their capacity to persist under varying biotic and abiotic conditions. Thus, our third focus is thermal tolerance, given that temperature alters insect physiology and can also facilitate the spread of invasive species (Furlong & Zalucki, 2017; Hance et al., 2007).

Finally, we focus on the microbiomes within both parasitoids and their hosts, which can also significantly influence biocontrol processes (Bourne et al., 2023; Martinez et al., 2012). For example, bacterial communities can directly impact a parasitoids' development by hindering its growth within the host, and some can even modify the reproductive strategies of both the host and the parasitoid (Werren et al., 2008).

In this review, we examine the unique contributions of *Microctonus* parasitoids in biocontrol, exploring four key traits (Fig. 2.1), to highlight research gaps and propose potential avenues for future study. By addressing these areas, we seek to identify strategies that could enhance the effectiveness of biocontrol programmes and improve the long-term persistence of these parasitoids in pest management strategies to ensure more sustainable and resilient outcomes for insect pest management.

2.3 Reproductive modes

While most eukaryotes reproduce sexually through 'amphimixis' (diplodiploidy), sexual reproduction in Hymenoptera most commonly occurs via 'arrhenotoky' (haplodiploidy), which is believed to be the ancestral state (Cook, 1993) and occurs when fertilised eggs develop into diploid females and unfertilised eggs develop into haploid males (Adams & Tariq, 2024). In contrast, asexual reproduction occurs by 'thelytoky', where diploid females develop from unfertilised eggs and males are comparatively rare (Moritz et al., 1990; Schön et al., 2009). Based on the above definitions, we will hereafter refer to arrhenotoky as 'sexual reproduction' and thelytoky as 'asexual reproduction'.

Reproductive mode can significantly impact the genomic and physiological characteristics of insects (Adams & Tariq, 2024; Sandrock et al., 2011). Asexual reproduction typically reduces genetic diversity compared to sexual reproduction (Moritz et al., 1990), potentially limiting potential to adapt to changing environments or host defences over time. This raises an important question: How do different modes of reproduction influence the long-term effectiveness of classic biological control? The following sections address this question by comparing sexual and asexual strains of various parasitoid species and exploring key mechanisms underlying sexuality and asexuality.

2.3.1 Sexual vs asexual

Female parasitoids are the most valuable in pest biocontrol because they lay their eggs in or on the host to ultimately result in host death (Eggleton & Gaston, 1990). Asexually reproducing parasitoids can offer advantages over their sexually reproducing counterparts. For example, they have the potential to reduce production costs by utilising all eggs and host resources to generate exclusively female offspring. They can also rapidly establish sustainable populations even in environments with low host densities, as a single female is sufficient to initiate a new population (Queffelec et al., 2021; Stouthamer, 1993). Once established, these populations can also experience weaker Allee effects compared to sexual species during early population growth (Queffelec et al., 2021). However, over the long-term, sexual reproduction is considered advantageous because it involves meiosis and recombination - processes which help to maintain genetic diversity, which is necessary for fostering adaptive potential in changing environments (Muller, 1932, 1964). By comparison, asexual reproduction typically results in clonal offspring that lack genetic diversity, potentially hindering adaptive processes (Lynch, 1984; Otto & Michalakis, 1998). Thus, sexual and asexual species can be expected to exhibit different patterns of genetic diversity across their genomes (Tvedte et al., 2019).

In addition to intraspecific diversity, a model study on host-parasitoid coevolution in New Zealand ecosystems suggests that differences in reproductive modes between the host and its parasitoid can create an asymmetry in additive genetic variance (the combined effects on a trait, stemming from one or more gene loci), which is important for adapting to selective pressure (Taft & Roff, 2012). Over time, this asymmetry may negatively impact the biological control system, as a sexually reproducing host can evolve more rapidly than its asexual parasitoid (Casanovas et al., 2018; Crow & Kimura, 1965), potentially leading to the development of host resistance. This mechanism is considered a contributing factor in the decline in *M. hyperodae* parasitism rates in *L. bonariensis* (Goldson & Tomasetto, 2016; Shields et al., 2022). Sexual parasitoids with higher genetic variance can also encounter less host resistance and stabilise host/parasitoid interactions faster, while asexual parasitoids with lower genetic variance can be more likely to face significant host resistance with associated declines in parasitism rates (Casanovas et al., 2018). Thus, parasitoid populations that lack genetic diversity may further struggle to adapt and efficiently target new pests compared to preferred or familiar hosts (Baker et al., 2003).

2.3.2 How is genetic diversity maintained in asexual strains?

On the surface, sexual parasitoids seem to be better equipped to manage resistance dynamics compared to asexual parasitoids. But this poses questions as to the effectiveness of asexual parasitoids in biocontrol systems, and the concern of failure in the long term. However, it turns out that such species can employ adaptive mechanisms to maintain genetic diversity despite their asexual reproductive mode.

Despite the name, asexual reproduction is not always completely clonal; it can persist in different forms with varying cytological mechanisms (Engelstädter, 2017; Rabeling & Kronauer, 2013) (Fig. 2.2). In Hymenoptera, asexual reproduction can have a genetic basis, or it can be induced by endosymbionts. The genetic basis of asexual reproduction involves apomixis or automixis. Apomixis is mitotic (clonal) i.e., it lacks meiosis and the fusion of meiotic products, so offspring genotypes are identical to those of the mother (Fig. 2.2), resulting in low genetic diversity and potentially low adaptive potential (Barker, 2013; Nakamura et al., 2021). Automixis involves partial meiosis that retains recombination, with diploidy restored via the fusion of sister or non-sister nuclei containing recombinant chromosomes. Thus, automixis produces offspring that may be genetically distinct from the mother, though total homozygosity is increased (Moritz et al., 1990; Schön et al., 2009), meaning the overall population viability and genetic stability can suffer if heterozygosity continues to be lost over time (Goudie et al., 2012; Pearcy et al., 2011) (Fig. 2.2).

The most common form of asexual reproduction induced by endosymbionts is a type of automixis termed 'gamete duplication', which mostly results from infection by the bacteria, *Wolbachia* (Pannebakker et al., 2004). *Wolbachia* induces asexuality by duplicating the entire genome of haploid eggs, resulting in homozygous females within a single generation (Stouthamer & Kazmer, 1994) (Fig. 2.2). Another endosymbiont, *Rickettsia,* has been shown to use a distinct apomictic cloning mechanism in the parasitoid *Neochrysocharis formosa* (Westwood) that nevertheless maintains diploidy to preserve heterozygosity over generations (Adachi-Hagimori et al., 2008).

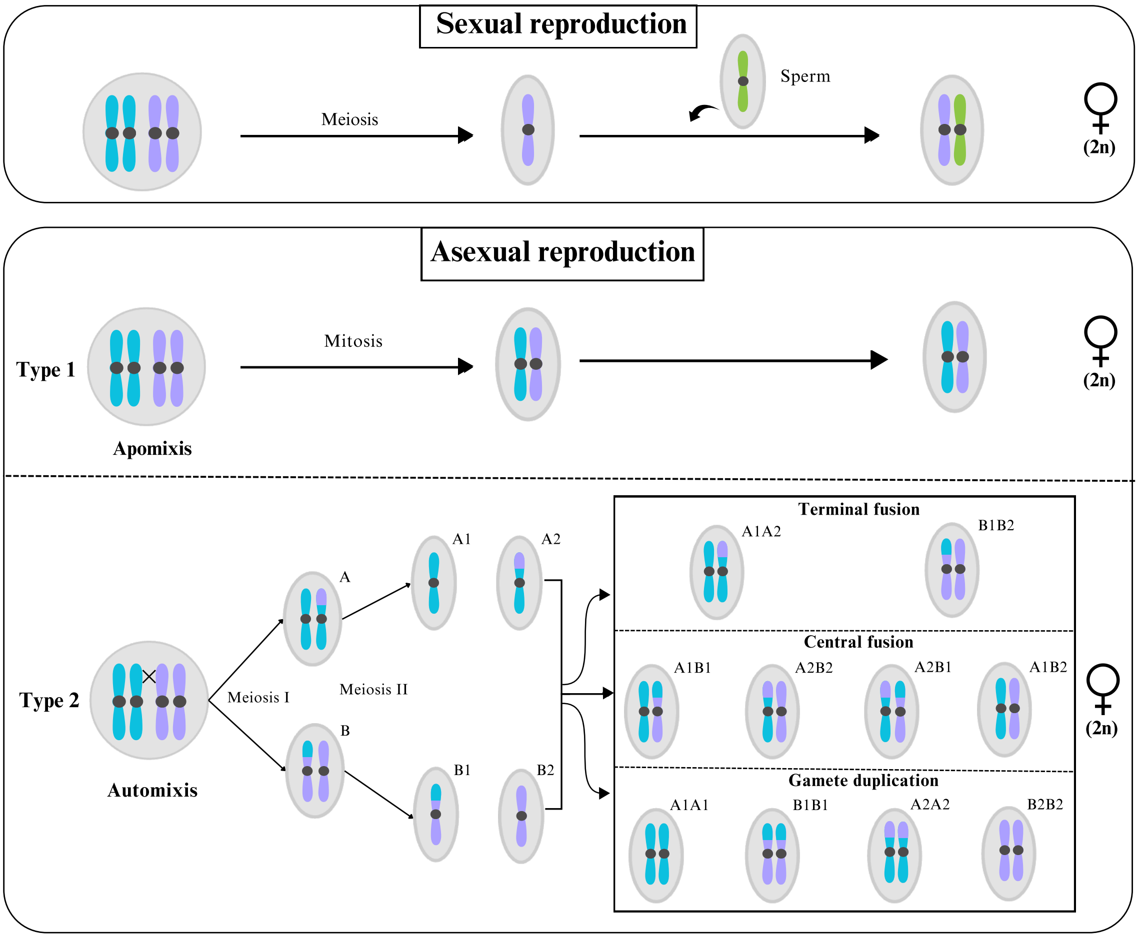

Figure 2.2 Modes of Hymenoptera reproduction. Sexual reproduction is primarily driven by meiosis, involving the fusion of male and female gametes, resulting in offspring that are typically female. In contrast, asexual reproduction occurs via several modes, broadly

grouped into apomixis and automixis. In central fusion automixis, meiosis happens normally to create four haploid nuclei. Diploidy is restored by the fusion of the egg pronucleus with the polar body from meiosis I. Without recombination between a locus and its centromere, the maternal alleles at this locus are preserved, maintaining heterozygosity near the centromere *(*Heimpel & de Boer, 2008; Moritz et al., 1990). However, if crossover events occur between the locus and centromere, maternal heterozygosity can be reduced, resulting in offspring that are homozygous for one allele. In terminal fusion automixis, the egg pronucleus merges with its sister nucleus in the second-division polar body to form the zygote. In this process, offspring from heterozygous mothers will become homozygous for either allele if there is no recombination, or maternal heterozygosity may be retained near the telomere *(*Heimpel & de Boer, 2008; Moritz et al., 1990*)*. See the main text for further information. Image inspired from: Capdevielle Dulac et al., (2022) & Rabeling & Kronauer, (2013) and created using Canva.

Recombination plays a crucial role in defining the mutational load and genetic health of asexual populations, which tend to accumulate more deleterious mutations over time in a phenomenon known as Muller's ratchet (Muller, 1964). This effect is particularly significant in asexual species that do not undergo recombination. However, even minimal recombination and segregation (as seen in automixis) can lead to fewer deleterious mutations compared to those in apomixis reproduction, as these processes help maintain genetic diversity and reduce mutational load (Browne, 1992; Charlesworth et al., 1993; Green & Noakes, 1995a; Haccou & Schneider, 2004; Neiman & Taylor, 2009; Pamilo et al., 1987). For example, central fusion with increased recombination in asexual strains of the solitary parasitoid *Venturia canescens* (Gravenhorst) and the Cape honeybee (*Apis mellifera capensis* Eschscholtz) have been shown to reduce mutational load (Beukeboom & Pijnacker, 2000; Schneider et al., 2002; Tucker, 1958; Verma & Ruttner, 1983).

Asexual reproduction can be obligate or facultative, with facultative asexuality allowing organisms to switch between sexual and asexual reproduction and gene flow between them introducing genetic variation that is important for their persistence over time (Sandrock et al., 2011; Simon et al., 2003; West et al., 1999). This process has contributed to genetic exchange between individuals in species such as bdelloid rotifers, *Adineta vaga* (Davis) (Vakhrusheva et al., 2020) and *Macrotrachella quadricornifera* (Milne) (Laine et al., 2022; Signorovitch et al., 2015).

In some species, rare males in asexual lines mate with related female sexual lines, resulting in new asexual lineages via a process known as contagious parthenogenesis (Maccari et al., 2014; Sandoval & Vickery, 1996). In natural conditions, such gene flow is possible when the asexual and its sexual conspecifics co-exist in the same environment (Belshaw et al., 1999; Delmotte et al., 2001; Sandrock et al., 2011). For example, *V. canescens* can undergo gene transfer from sexual males to stable asexual females, resulting in the transmission of the male genome to the offspring in about 30% of mating events (Schneider et al., 2003); such gene flow is also seen in several *Trichogramma* parasitoids (Stouthamer, Luck, et al., 1990; Stouthamer, Pinto, et al., 1990; Stouthamer & Kazmer, 1994; Stouthamer & Werren, 1993). In these cases, rare males in otherwise asexual populations (Adachi-Hagimori & Miura, 2020; Goldson et al., 1990; Ma et al., 2015; Sandrock et al., 2011; van der Kooi & Schwander, 2014; Wachi et al., 2021) are mostly haploid and arise due to the occasional failure of central fusion (Sandrock et al., 2011), or are diploid and arise via homozygosity at complementary sex determination loci (Engelstädter et al., 2011; Heimpel & de Boer, 2008).

Rare sex in asexual species has been shown to significantly mitigate Muller's ratchet by allowing gene flow, even with low recombination rates (Charlesworth et al., 1993; Som et al., 2007). Beneficial mutations can become fixed in populations with low levels of sexual reproduction, with genetic diversity thereby increased (Green & Noakes, 1995; Hedrick & Whittam, 1989). However, asexual species that lack recombination and chromosomal re-assortment can still acquire independent irreversible mutations in homologous chromosomes (i.e., Muller’s ratchet) that accumulate over time to enhance diversity - a phenomenon known as the Meselson effect (Mark Welch & Meselson, 2000). This effect is commonly associated with apomixis due to the complete lack of recombination but can also be observed in certain forms of automixis-based reproduction, particularly where recombination is limited (Du et al., 2023; Wachi et al., 2021).

2.3.3 Life history traits

Along with genetic diversity, reproductive mode has been shown to affect the life-history traits and behaviours of parasitoids (Ameri et al., 2015; DU et al., 2023; Miura & Tagami, 2004; Silva et al., 2000; Tenguri et al., 2016; Wang et al., 2024; Wang & Smith, 1996; Ye et al., 2023). In particular, dual sexual and asexual reproductive capability can impact key parameters, such as developmental time, fecundity, longevity, sex ratio, generation time, reproduction rate, parasitism rate, host feeding, stinging, and host killing (Ameri et al., 2015;

Du et al., 2023; Miura & Tagami, 2004; Wang et al., 2024; Ye et al., 2023) though the extent and nature of these impacts can be variable among species. For example, developmental time of asexual parasitoids is shown to be higher than that of sexual individuals in *Lysiphlebus fabarum* (Marshall) (Hymenoptera: Braconidae) (Ameri et al., 2015), *Trichogramma minutum* (Riley) (Hymenoptera: Trichogrammatidae) (Wang & Smith, 1996), and *Trichogramma kaykai* (Pinto and Stouthamer) (Hymenoptera: Trichogrammatidae) (Miura & Tagami, 2004), but not in *Neochrysocharis formosa* (Westwood) (Hymenoptera: Eulophidae) (Ye et al., 2023), or *Diglyphus wani* (Liu, Zhu & Yefremova) (Hymenoptera: Eulophidae) (Du et al., 2023). Adult longevity is similar for most of these species, except the asexual strains of *N. formosa* and *L. fabarum*, which have a longer life span (Ameri et al., 2015; Ye et al., 2023). Asexual strains of *L. fabarum, N. formosa* and *D. wani* also show higher fecundity as well as higher parasitism rates - resulting in more offspring and providing significantly better biocontrol potential of their relevant hosts (Du et al., 2023; Tenguri et al., 2016; Wang et al., 2024). In contrast, asexual strains of *T. minutum, T. cordubensis* (Vargas and Cabello), and *T. deion* (Pinto and Oatman) (Silva et al., 2000) have lower fecundity and a lower biocontrol potential than their sexual counterparts (Silva et al., 2000; Wang & Smith, 1996).

Endosymbionts (microorganisms that live inside the cells or tissues of insects), can significantly influence the biocontrol potential of parasitoids (see section 2.6: Microbiome) and their life history traits, particularly through mechanisms such as inducing asexuality (Adachi-Hagimori & Miura, 2020; Fuxelius et al., 2007; Werren et al., 2008), as mentioned above. For example, in *N. formosa*, the endosymbiont bacteria *Rickettsia* (da Rocha-Lima), benefits by using host amino acids and ATP to fuel its incomplete metabolic pathways, which may cause the asexual *N. formosa* to feed on more hosts to supply extra nutrients (Adachi-Hagimori et al., 2008; Fuxelius et al., 2007; Ye et al., 2023). Meanwhile, another endosymbiont bacteria *Wolbachia* (Hertig) can negatively affect life history traits in *T. cordubensis* by reducing fecundity, longevity, and pre-adult mortality, compared to uninfected individuals (Silva et al., 2000).

Despite the above noted trends, it is challenging to generalise the effects of reproductive mode on biocontrol potential based on biological characteristics alone, as these traits vary idiosyncratically between species. Complex biotic and abiotic factors, such as geographic origin, presence of endosymbiont bacteria, microclimate conditions, and host environment further add to this complexity.

2.3.4 Future aspects

Asexual and sexual reproduction each have their own advantages and disadvantages, as discussed in the previous sections (see section 2.3.1: Sexual vs asexual). However, understanding the long-term effectiveness of these reproductive modes requires examining their ability to adapt to environmental conditions and stresses. It is well established that asexual species typically exhibit lower genetic diversity than their sexual counterparts (see section 2.3.3: How is genetic diversity maintained in asexual strains?). But how does this difference impact their capacity to adapt to adverse conditions? This question has received relatively little attention, especially in studies directly comparing sexual and asexual individuals. With advancements in RNA sequencing technologies (Leung et al., 2020), this aspect can now be explored in greater depth, which will undoubtedly shed light on how parasitoids respond to biotic and abiotic factors and uncover further variability in these responses between sexual and asexual individuals.

It is now understood that the co-existence of sexual and asexual strains or ecotypes can potentially facilitate gene flow (see section 2.3.3: How is genetic diversity maintained in asexual strains?). However, research on this phenomenon remains limited, and it is not yet fully confirmed whether such gene flow occurs between sexual and asexual parasitoids. Furthermore, an important question arises: could this gene flow influence the effectiveness of biocontrol? Addressing this gap in knowledge is important for understanding the broader implications of reproductive strategies in biocontrol programmes.

In New Zealand, genetic exchange in *M. aethiopoides* could enable asexual populations of the Irish strain to adapt to new environmental conditions by acquiring advantageous genetic variations from the sexual Moroccan strain. However, laboratory experiments have shown that inter-ecotype mating between sexual strains of *M. aethiopoides* (Moroccan vs. European strains) can produce hybrids which, though viable, are less effective at parasitising their respective hosts (Goldson et al., 2003). This raises a primary concern about the potential for interbreeding between sexual and asexual populations in the wild, resulting in hybrid offspring with reduced biocontrol efficacy. While genetic markers exist to differentiate between strains (Phillips et al., 2006), more research is needed to assess the impact of hybridisation on biocontrol effectiveness. However, during initial collections of *M. hyperodae* from South America, four males were reared from two South America *L. bonariensis* populations being held in quarantine (Goldson et al., 1990). Although wing fanning was observed by one male towards a virgin female, and all males were placed with virgin females for 24 hours, no males emerged from the offspring (M. McNeill, unpublished data). Nor were males subsequently found during the mass-rearing programme as part of research and commercial releases of *M. hyperodae* (McNeill et al., 2002). Thus, gene flow may be limited across reproductive modes, although further research is needed to confirm this.

Key future research areas will include identifying genomic regions subject to introgression, detecting the presence of hybrid individuals, and understanding the extent of gene flow between asexual and sexual strains. Monitoring allele frequency changes and selection pressures across the genome will also aid in predicting evolutionary responses to the New Zealand environment, as well as the stability and effectiveness of *M. aethiopoides* and other parasitoids in biocontrol. Functional genomic studies (e.g., RNA-Seq) targeting stress response genes, olfactory genes, and other relevant pathways could provide further insights into how hybridisation might affect parasitoid efficiency and host-finding abilities, helping to inform targeted strategies that improve biological control outcomes.

2.4 Olfaction

Parasitoids use different sensory modes including, olfaction, vision, and phonotaxis (hearing), to locate their hosts in the field (Frederickx et al., 2014; Walker, 1993; Xiaoyi & Zhongqi, 2008). Among these, olfaction is the primary mechanism, with the effectiveness of biological control often depending on a parasitoid's ability to detect and respond to chemical cues from both the host and the environment, such as plant volatiles (Frederickx et al., 2014; Kruidhof et al., 2015; Mattiacci et al., 1999).

Visual cues can also significantly influence parasitoid behaviours for host selection. For example, *Microctonus* parasitoids(Cournoyer & Boivin, 2005; Loan & Holdaway, 1961; Smith, 1952) can use environmental landmarks, such as plant shapes or colours, to memorise and relocate potential host sites (Cournoyer & Boivin, 2005; Loan & Holdaway, 1961; Smith, 1952; (Desouhant et al., 2010; Fischer et al., 2001; Nussenbaum et al., 2024; Vinson, 1976). *Diachasmimorpha longicaudata* (Ashmead), a parasitoid of fruit flies, can associate specific colours (i.e., fruit colours representing the host habitat) with host presence through associative learning (Nussenbaum et al., 2024). This results in increased oviposition attempts and higher offspring production when visual cues match previously rewarding experiences (Nussenbaum et al., 2024). Also, many studies have shown that Hymenopteran parasitoids possess trichromatic vision, enabling them to detect ultraviolet, blue, and green wavelengths and, in some cases, discriminate shapes, with distinct visual adaptations reflecting their ecological roles and behavioural needs (Chen et al., 2016; Desouhant et al., 2010; Lim & and Ben-Yakir, 2020; Pérez et al., 2012). In keeping with the scope of this review, further discussion on visual cues will be limited. Instead, we will focus on the role of olfaction in parasitoid biocontrol, highlighting its central importance throughout the parasitoid life cycle.

2.4.1 Olfaction cues

After mating, female parasitoids use chemical cues, such as herbivore-induced plant volatiles (HIPVs), green leaf volatiles (GLVs), and kairomones to locate their hosts. Plant-associated cues, such as HIPVs and GLVs, are abundant and can attract parasitoids over long distances (Fig. 2.3), but they are often unreliable indicators of host presence in complex environments, whereas host-derived cues (i.e. kairomones) are more reliable but effective only at short range (Fig. 2.3), where their concentration is higher (Gómez-Cabezas et al., 2023). Thus, successful parasitism largely depends on the combined ability of the parasitoid to detect and respond to chemical cues emitted by both host and the plant, which varies among species based on differences in how their olfactory systems process these chemical cues (Das et al., 2017; Dillman et al., 2012).

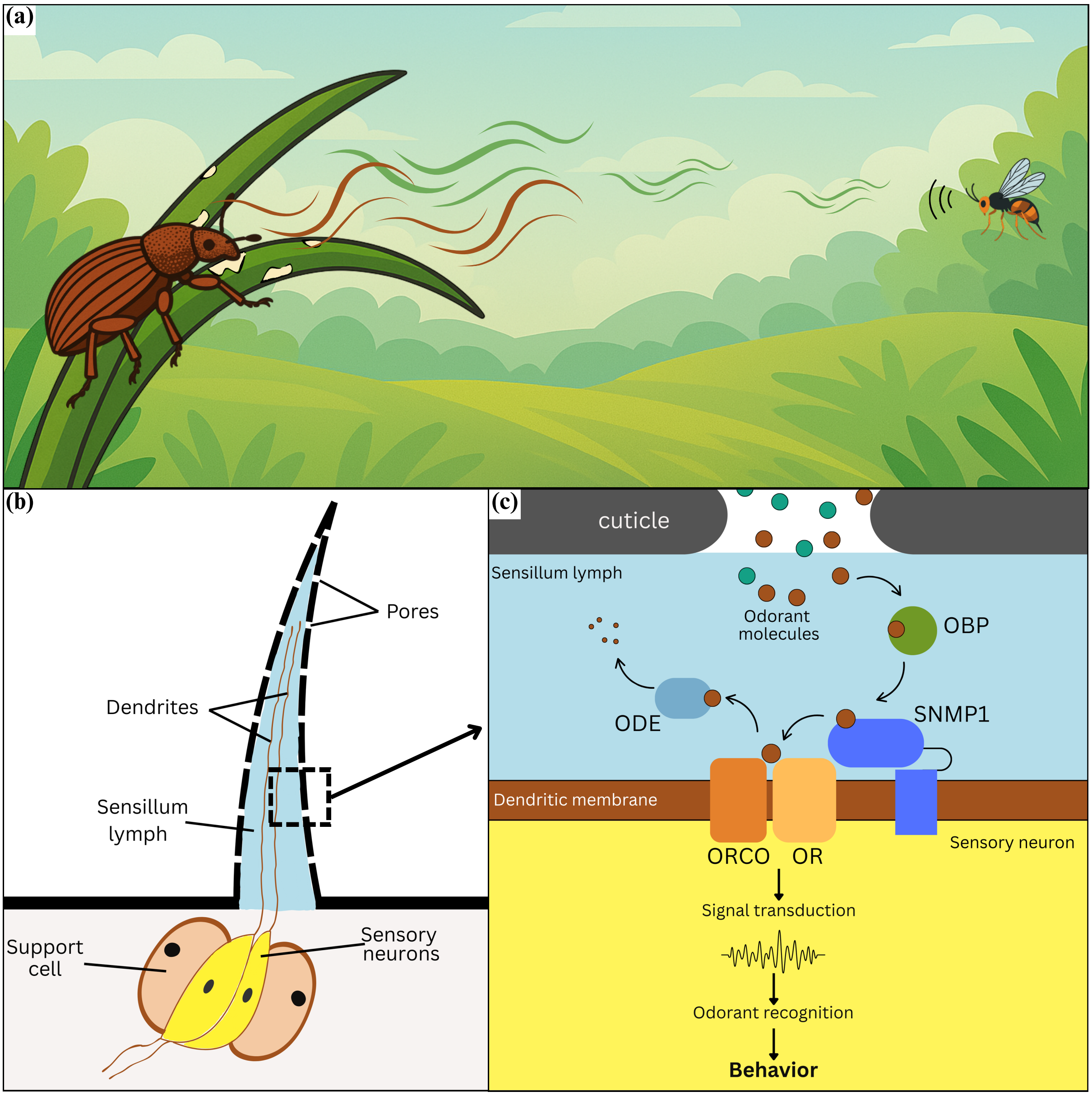

Figure 2.3 Schematic representation of the olfactory detection process in parasitoids. (a) Chemical cues released by both the host insect (weevil) and the host-infected plant. Plant-derived odours act as long-distance attractant cues, while host-derived odours function as short-distance attractants. These chemical cues travel through the air and are detected by the parasitoid; (b) Cross-sectional view of an antenna sensillum, showing how odourants enter through surface pores and diffuse into the sensillum lymph, reaching the dendrites of sensory neurons; (c) Molecular pathway of odour recognition, odourant-binding proteins (OBPs) transport odour molecules to odourant receptors (ORs) (in some cases, this can involve other

olfactory receptors, such as ionotropic receptors (IRs)), odourant receptor co-receptor (ORCO) and sensory neuron membrane protein 1 (SNMP1). Odourant-degrading enzymes (ODEs) break down excess molecules. The interaction at the OR-ORCO complex initiates signal transduction, leading to odour recognition and ultimately behavioural responses. Some icons were created with use of ChatGPT, and the complete figure was assembled using Canva.

When fed upon, plants use direct and indirect defence mechanisms to protect themselves against herbivores. Direct defences include physical barriers, such as trichomes, and chemical defences, such as toxic compounds, that harm herbivores (Karban et al., 2000; Sharma et al., 2009). On the other hand, indirect defences involve the release of volatile chemical compounds, such HIPVs and GLVs, to attract parasitoids or predators of herbivores to specific habitats (Kessler & Baldwin, 2001; War et al., 2011).

GLVs are a specific group of volatile organic compounds that are quickly released by plants when their tissues are damaged, typically due to mechanical injury or herbivore feeding (Scala et al., 2013). These compounds, primarily composed of six-carbon (C6) molecules like (Z)-3-hexenol, (Z)-3-hexenal, and (Z)-3-hexenyl acetate, serve as early attraction signals (Scala et al., 2013). In contrast, HIPVs are a broader category of volatiles emitted specifically in response to herbivore attacks. While HIPVs can include GLVs, they also encompass a wider range of compounds, such as terpenoids (e.g., β-ocimene), fatty acid derivatives, and phenolics (Turlings et al., 1990; War et al., 2011). Many Hymenopteran parasitoids are attracted to these chemical cues, but their sensitivity and the strength of their chemotactic response can vary depending on the composition of the cues, the type of parasitoid, and the developmental stage of the host (larvae or adult), among other factors (González et al., 2018; Greenberg et al., 2023; Wäschke et al., 2013).

Plants can change the blend of volatile components they release in response to damage, either by producing new compounds not emitted by undamaged plants (qualitative changes) or by varying the amounts of the primary volatiles (quantitative changes) (Dicke, 1999; Lin et al., 2008; Rasmann & Turlings, 2008). These changes depend on plant traits, the site of damage, and the specific herbivore species involved, which can also influence the differential attraction of the parasitoids (Hoballah & Turlings, 2001). For example, *Cotesia vestalis* (Haliday) requires a mixed blend of four HIPVs (*n*-heptanal, *α*-pinene, sabinene, and (*Z*)-3-hexenyl acetate) to elicit a strong response towards infested cabbage plants (*Brassica oleracea*), whereas the individual compounds do not trigger a response (Shiojiri et al., 2010). Other parasitoids, such as *Anagrus nilaparvatae* (Pang & Wang), *Cotesia sesamiae* (Cameron), and *Campoletis chlorideae* (Uchida) show strong attraction to a single HIPV (e.g., linalool, (Z)-jasmone; Sun et al., 2019; Tamiru et al., 2015; Xiao et al., 2012). Alternatively, egg deposition over plants can trigger the production of volatiles or alter leaf chemistry, leading plants to attract specific parasitoids (Meiners & Hilker, 1997; Mumm et al., 2003).

Oviposition by insect herbivores can also affect the release of plant volatiles. For example, the chemical profile of volatiles has been shown to change when comparing herbivory alone to herbivory combined with oviposition, although the effect on parasitoid attraction may vary (Michereff et al., 2011). The composition of HIPVs also changes based on the parasitism status of the herbivore that is feeding on the plant (Bonaventure et al., 2011). Some parasitoids can detect these changes, enabling them to distinguish between parasitised and unparasitised hosts, which helps them avoid superparasitism (ovipositing into an already parasitised host) thereby enhancing their reproductive success (Kafle et al., 2020; Rana et al., 2002).

Parasitoid use of HIPVs for host finding shows notable differences between generalist and specialist parasitoids (Ngumbi & Fadamiro, 2012; Peñaflor et al., 2011). Generalist parasitoids, which have broad host ranges, tend to respond to more general host-related volatiles (i.e., GLVs or common HIPVs) that are not specific to a particular host (Ngumbi & Fadamiro, 2012). As such, they often rely on an innate ability to detect and respond to these chemical cues across a variety of host plants, allowing the efficient location of potential hosts without the need for prior learning (Peñaflor et al., 2011). In contrast, specialist parasitoids target specific hosts and are attracted to highly specific HIPVs that are directly associated with their individual host species (Ngumbi & Fadamiro, 2012; van Oudenhove et al., 2017). Specialists therefore often need to learn to recognise and respond to specific HIPVs, especially when their hosts are polyphagous and feed on multiple plant species (Peñaflor et al., 2011). This learning enables specialist parasitoids to adapt to different plant environments and effectively exploit odours from various plants or genotypes that harbour their desired host, ensuring precise host location (Peñaflor et al., 2011).

While HIPVs play a crucial role in plant defence, they can sometimes have the opposite effect - benefiting the herbivore instead. Certain components of some HIPVs, such as (E)-2-hexenal and (Z)-3-hexenol, are ineffective at attracting parasitoids like *C. chlorideae*, even though they are major constituents of HIPVs from maize infested by *Mythimna separata* (Walker) (Yan & Wang, 2006), i.e., the parasitoid's specific host.

Similarly, methyl salicylate is a common emission from pest-infested plants that can negatively affect the attraction of parasitoids like *Diadegma semiclausum* (Hellén) potentially helping herbivores escape parasitism (Snoeren et al., 2010). Exposure to specific HIPVs can also enhance herbivore resistance or immunity to parasitoids (Ghosh & Venkatesan, 2019; Hu et al., 2021). For example, exposure to indole (a common HIPV) can increase the resistance of *Spodoptera littoralis* (Boisduval) caterpillars, significantly reducing the parasitism success of *Microplitis rufiventris* (Kokujev) (Ye et al., 2018).

Kairomones are chemical compounds that mediate interspecific interactions, benefiting the organism of another species upon detection, with the 'eavesdropper' exploiting the cue to then locate their host or prey (Ayelo et al., 2021). Kairomones can be volatiles that are directly released by (e.g., faecal volatiles), or indirectly associated with (e.g., substrates the host feeds on or compounds from their developmental stages) the host (Steidle & Van Loon, 2003; Vet & Dicke, 1992). They are generally distinct from plant background odours and thus constitute the most reliable sources of chemical cues for parasitoid host detection (Ayelo et al., 2021; Lewis et al., 1975).

Volatile kairomones serve as long-range cues, while non-volatile kairomones act as contact cues by relying on specific parasitoid behaviours, such as probing (exploring surfaces with the ovipositor), antennating (tapping surfaces with antennae), drilling (penetrating host or substrate with the ovipositor), or drumming (tapping surfaces with legs or antennae) (Bénédet et al., 2002; Bleeker et al., 2004; Hendry et al., 1973; Rostás et al., 1998). For example, the parasitoid *Encarsia formosa* (Gahan) is attracted to honeydew volatiles, which serve as long-range cues from its whitefly host, *Trialeurodes vaporariorum* (Westwood) (Ayelo et al., 2022). Upon landing, *E. formosa* then responds to nonacosane (a contact chemical), which is released from the whitefly and acts as an arrestant, causing the parasitoid to remain in the area and increasing its chances of successful parasitism (Ayelo et al., 2022). Similarly, hosts use alarm pheromones as chemical signals to warn their conspecifics of potential danger, and some parasitoids have developed the ability to exploit these pheromones to identify and locate their hosts (Jiang et al., 2023; Micha & Wyss, 1996). For example, the aphid parasitoid, *Aphidius gifuensis* (Ashmead), can sense the alarm pheromone (E)-β-farnesene, which is released by aphids when under threat from parasitoids (Jiang et al., 2023).

2.4.2 Olfactory systems

The olfactory recognition system greatly affects foraging, parasitism, oviposition, and other behaviours of insects (Dillman et al., 2012; Fleischer et al., 2018; Freund & Olmstead, 2000) and undoubtedly plays an important role in biocontrol. The olfactory system in insects is based on odourant detection, primarily through sensory structures on the antennae, where various proteins, such as odourant-binding proteins (OBPs), odourant-degrading enzymes (ODEs), Niemann-Pick proteins type C2 (NPC2s), odourant receptors (ORs), ionotropic receptors (IRs), chemosensory proteins (CSPs), and sensory neuron membrane proteins (SNMPs) mediate the sensation and transmission of odours that lead to physiological and behavioural responses (Leal, 2013; Pelosi, Iovinella, et al., 2018; Steinbrecht, 1998; Vinson, 1984).

Volatile odourants from the environment are generally very hydrophobic, so must be solubilised and ferried from the external environment to the membrane of chemosensing neurons. This process is carried out by OBPs or CSPs or NPC2s, which are small proteins abundantly expressed in insect antennae (Blomquist, 2003; Pelosi, Iovinella, et al., 2018). ORs or IRs located on the dendritic membrane within olfactory sensilla detect volatile compounds and convert them into electrical signals, which are transmitted to the insect brain to regulate behaviour (Fleischer et al., 2018; Sato et al., 2008). Subsequently, ODEs locate odourants and degrade them, thereby cleaning the olfactory system for a new cycle of detection (Ishida & Leal, 2005; Robertson et al., 2003; Vogt et al., 1985).

ODEs represent a functional category of enzymes encompassing several distinct gene families, including cytochrome P450s, carboxylesterases, alpha-esterases, aldehyde oxidases, glutathione S-transferases, and alcohol dehydrogenases (Chertemps & Maïbèche, 2021; Kang et al., 2021). ORs and IRs specialise in discriminating between different odourant molecules, with sensitivity towards detecting short-chain organic acids, amines, and aldehydes (Pelosi et al., 2018; Schmidt & Benton, 2020). Meanwhile, SNMPs particularly SNMP1 play a crucial role in pheromone perception (Vogt et al., 2009).

In addition to those discussed above, other sensory-related gene families, such as transient receptor potential channels, ammonium transporters, and pickpocket receptors, are also increasingly recognised for their potential roles in chemosensation and host detection (H. Lin et al., 2005; Mao et al., 2020; Wulff et al., 2024).

Variation in the number of OBPs across different parasitoid species reflects significant differences in their olfactory capabilities, which may be associated with their ecological niches and behavioural needs, and thus likely plays a role in biocontrol. For example, parasitoids that exist in complex, open environments tend to have a larger number of these proteins (e.g., *Nasonia vitripennis* (Walker), n=90; Vieira et al., 2012), than those in more concealed and homogenous habitats (e.g., *Copidosoma floridanum* (Ashmead), n=8; Donnell & Strand, 2006). This variation can derive from adaptive evolutionary processes, with gene gain and loss events, ecological pressures, and life history strategies shaping the OBP families of each species (Campanini & de Brito, 2016; Sánchez-Gracia & Rozas, 2008).

The expression profiles of OBPs across different parasitoid developmental stages and tissues further highlight their specialised roles. Typically, OBPs are highly expressed in the antennae. However, they can also be expressed in other tissues, such as the female abdomen in *Sclerodermus* species, where they may play roles in oviposition or other reproductive behaviours (Zhou et al., 2015). Stage-specific expression is another important aspect, with some OBPs uniquely expressed in larvae or pupae, suggesting roles in developmentally relevant olfactory processes (He et al., 2020; Zhou et al., 2015). Sexual dimorphism in OBP expression arises due to the distinct behavioural roles of male and female parasitoids, with female-specific proteins mainly involved in host location and male-specific proteins involved in sex-pheromone detection and mating behaviour (Michereff et al., 2011; Zhou, 2010).

The ORs, another important mediator of olfaction responses, found in olfactory receptor neurons, can detect a wide range of volatile chemicals, including pheromones and general odours. Variation in OR functionality and specificity between species underlines their potential importance in biocontrol. PxylOR11, a type of odourant receptor in *Plutella xylostella* (Linnaeus) is specifically expressed in female antennae and is tuned to aromatic compounds like benzyl alcohol, phenylacetaldehyde, and salicylaldehyde, which are HIPVs released by host plants (Liu et al., 2022). These compounds can either repel or attract natural enemies (Liu et al., 2022; Shiojiri et al., 2010), an important consideration in pest management. Moreover, some olfactory receptors specifically detect the sex pheromones of their hosts. This includes the specific olfactory receptor (MmedOR49) of the parasitoid *Microplitis mediator* (Haliday), which responds strongly to a key sex pheromone component (Z5-10) of its target pests (e.g., *Agrotis segetum* and *Agrotis ipsilon*); the receptor shows increased expression in adult female parasitoids, suggesting its importance in host-seeking behaviour immediately after emergence (Shan et al., 2023).

Understanding the role of OBPs and ORs in species-specific interactions enhances knowledge about the selective responses of parasitoids to odourant molecules, which can help refine biocontrol strategies (Huang et al., 2023; Leung et al., 2020). For example, this knowledge can be applied to develop more targeted biocontrol for specific hosts that integrates gene editing techniques to enable modulation of the expression of genes that code for odourant functions (Huang et al., 2023). Similarly, knowledge of the chemical cues associated with host searching/parasitoid attraction can enhance biocontrol efficiency. For example, Lewis et al. (1975) found that application of tricosane (a kairomone) to plants in the field increased the parasitism of *Trichogramma* species. Beyond enhancing biocontrol efficacy, this approach can also minimise impacts on non-target species by encouraging natural enemies to remain in specific areas where such chemical cues are applied (Ayelo et al., 2021).

2.4.3 Future aspects

Despite the significance of olfaction in biocontrol, its research remains highly limited within the context of New Zealand biocontrol systems (Saunders et al., 2024). Although some biological studies have been conducted on *M. aethiopoides* (Fusco & Hower, 1974; Loan & Holdaway, 1961), research on its interactions with host species, particularly mechanisms of host location, remains largely unexplored (but see Steele et al., 2006).

Climate change is driving increasingly unpredictable and extreme environmental conditions in New Zealand's pastures, with rising CO_2 concentrations in the atmosphere and associated warming (Chapman et al., 2024) that could modify the olfaction-based behaviour of parasitoids (Pinto-Zevallos & Blande, 2024). Thus, understanding the olfactory mechanisms used by biocontrol organisms is essential. By investigating the role of olfactory-related genes in biocontrol success, future work will likely identify the molecular components that enable parasitoids to detect and locate their hosts. Understanding these mechanisms would furthermore open up the possibility of behavioural conditioning, where parasitoids are exposed to specific pest-related odourants to strengthen their host-finding ability through learned responses. This could make parasitoids more selective by increasing their attraction to the volatiles associated with the target pest and decreasing responses to non-target species (Kruidhof et al., 2019). Additionally, uncovering the specific plant volatiles or pheromones that guide parasitoid behaviour could help in the design of strategies to enhance pest suppression, such as using synthetic odour blends to attract parasitoids into crop areas where pests are prevalent (Uefune et al., 2021). Ultimately, this knowledge could be applied to refine biocontrol programmes, ensuring parasitoids are not only effective at locating and parasitising their intended hosts, but also minimising unintended impacts on non-target organisms.

2.5 Thermal tolerance

Unfavourable climatic conditions can lead to the extinction and migration of key species, altering community structures (Frölicher et al., 2018; Shi et al., 2021). Insects, particularly parasitoids, are vulnerable to thermal stress due to their complex physiology (Paaijmans et al., 2013). To be an effective biocontrol agent, parasitoids must withstand environmental stress when released. At the molecular level, an organism's stress response is multifactorial, involving various mechanisms that work together to counteract the stress it experiences (Blagojević, 2007; Feder & Hofmann, 1999; Peric-Mataruga et al., 2006; Schlinger & Hall, 1960; Wen et al., 2016; Yuan et al., 2021). Understanding these mechanisms is crucial for developing effective solutions to mitigate stress-related challenges. To provide clarity and a focused perspective, this review focuses on thermal tolerance and the mechanisms associated with it. Although these mechanisms often overlap with responses to other stressors (e.g., humidity, pesticides, UV light etc; Csorba et al., 2024; Li et al., 2024; Rakes et al., 2024), our emphasis on thermal stress allows for a more comprehensive exploration of this specific adaptation.

Thermal tolerance can be classified into two categories: heat tolerance and cold tolerance. Heat tolerance is crucial for parasitoids to survive in regions where high temperatures could otherwise limit their effectiveness. Cold tolerance is essential for adapting to cooler climates as well as for improving cold storage techniques that are used during parasitoid movement and storage for biocontrol purposes (Colinet & Boivin, 2011). Despite their importance, there is a notable lack of comprehensive review papers addressing both heat and cold tolerance in a unified framework. This section of the review aims to fill this gap by providing an overview of thermal tolerance mechanisms in insect parasitoids.

2.5.1 Heat and cold stress

An insect’s ability to withstand cold temperatures can be classified into three groups: freeze-tolerant, freeze-avoidant, and chill-susceptible. Freeze-tolerant insects survive by controlling internal ice formation, while freeze-avoidant insects prevent freezing altogether by supercooling their body fluids and using cryoprotectants. Chill-susceptible insects lack these mechanisms, making them vulnerable to damage at suboptimal temperatures (Clark & Worland, 2008; Hance et al., 2007). Parasitoids are exposed to low temperatures naturally throughout their lifecycle, especially during overwintering, and artificially as part of the cold storage processes involved in biological control rearing programmes. Overwintering is a natural adaptation where insects enter diapause, a behavioural and physiological strategy to survive unfavourable climatic conditions (Andreadis & Athanassiou, 2017; Denlinger, 2002). This diapause capability is also leveraged in the cold storage method, a valuable tool in biological control (Colinet & Boivin, 2011). Cold storage extends the shelf life of parasitoids, allowing enough to be preserved and released in the field at optimal times. This approach also helps balance the production of natural enemies with the availability of their hosts, ensuring cost-effective and efficient biological control solutions, particularly for large-scale releases (Colinet & Boivin, 2011).

Overwintering strategies can either correspond to inside-hosts-overwintering endoparasitoids (IHOE) or outside-hosts-overwintering parasitoids (OHOP). IHOEs, typically koinobionts, remain at immature stages within their hosts during winter, leveraging the host's morphology, physiology, and behaviour to occupy sheltered habitats (Babendreier et al., 2003; Humble, 2006). In contrast, OHOPs overwinter outside their hosts, either as immature stages or adults, often facing exposure to unpredictable climatic conditions (D'Ottavio et al., 2024; Scherr et al., 2023).

Low temperature impacts parasitoids in various ways, often influencing their survival, reproductive traits, and developmental stages. For example, short-term exposure to low temperatures hampers growth and population dynamics, as evidenced by the decreased reproductive success of *Trichopria drosophilae* (Perkins) and associated lower female offspring proportions under extreme cold (Chen et al., 2024; Yan et al., 2023). However, some species and/or life stages are more tolerant to cold than others. For example, *T. drosophilae* exhibit greater cold tolerance during its immature (non-adult) developmental stages (Amiresmaeili et al., 2020). Moderately low temperatures (8–12°C) have minimal adverse effects on *Psix saccharicola* (Mani) L. pupae, preserving adult emergence rates and balanced sex ratios (Forouzan et al., 2018). In contrast, exposure to more extreme cold temperatures (4–5°C) significantly reduces adult emergence, shifts sex ratios toward a male bias, and negatively impacts fecundity and parasitism rates in both *P. saccharicola* and *Habrobracon hebetor* (Say) (Askari Seyahooei et al., 2018; Forouzan et al., 2018). Thus, cold tolerance of parasitoids can vary across different species, developmental stages, temperatures, and durations of exposure. Understanding the optimal conditions and exposure times is crucial for minimising negative effects while preserving key life-history traits, whether during cold storage or when releasing parasitoids into cold environments.

Insects are vulnerable to heat stress due to their complex physiology (Paaijmans et al., 2013), with parasitoids more sensitive to temperature fluctuations than their hosts

(Furlong & Zalucki, 2017). Heat stress has been shown to have profound effects on the biology and efficacy of parasitoids, influencing various physiological, reproductive, and developmental traits (Alloui-Griza et al., 2022; Oliveira et al., 2017; Spahn & Lill, 2022; Wang & Keller, 2020; Zhang et al., 2021). Reproductive traits are particularly sensitive, as heat shock in adult male parasitoids, such as *Anisopteromalus calandrae* (Howard) and *N. vitripennis*, induces sterility (Chevrier et al., 2019; Hidalgo et al., 2019). This is likely because, under such stressful conditions, organisms are hypothesised to reallocate energy reserves designated for reproductive functions into cell maintenance and protection (Harshman & Zera, 2007).

Parasitoid efficacy is generally affected by heat stress, though the impact varies across species. For instance, higher temperatures reduce the efficacy of the parasitoid *Diadegma insulare* (Cresson) in controlling the pest *Plutella xylostella* (Linnaeus) (Spahn & Lill, 2022). In contrast, the parasitoid *Neochrysocharis formosa* (Westwood) maintains its biocontrol potential against agromyzid leafminers even under elevated temperatures (Jing-Li et al., 2022), showcasing species-specific resilience. Mortality rates and life history traits are negatively impacted by brief exposure to high temperatures (Alloui-Griza et al., 2022). In the aphid parasitoid *Aphidius avenae* (Haliday), heat stress leads to increased mortality, reduced reproductive output, shortened lifespan, and impaired progeny development (Roux et al., 2010). Similarly, developmental traits are adversely affected, with high temperatures hindering the growth and development of the parasitoid *Aenasius arizonensis* (Girault) (M. N. et al., 2024; Zhang et al., 2021). In *Eretmocerus warrae* (Nauman and Schmidt), elevated temperatures result in reduced body size, fecundity, and longevity, further diminishing fitness (Wang & Keller, 2020). Heat stress can also influence the venom composition of parasitoids, which plays a crucial role in host-parasitoid immune interactions. In *Leptopilina boulardi*, venom composition was found to vary significantly with temperature (Cavigliasso et al., 2021), potentially altering the parasitoids' ability to suppress host defences. Overall, heat stress challenges the adaptability and ecological roles of parasitoids, with diverse impacts on their biology and interactions with hosts.

2.5.2 Heat shock proteins (HSPs)

Protein denaturation can occur at both high and low temperatures, though it is more pronounced at high temperatures (Tsai et al., 2002). At high temperatures, HSPs play an important role in mitigating stress-induced denaturation and maintaining protein stability

(Evgenev et al., 2014; Feder & Hofmann, 1999). As well as in response to thermal stress, HSPs are produced during developmental processes or diapause, with their levels varying across life stages (Evgenev et al., 2014).

In insects, there are four major families of HSPs that include the small heat shock proteins (sHsps), Hsp60, Hsp70, and Hsp90. HSP40 proteins function independently or in coordination with HSP70 to facilitate ATP hydrolysis and prevent the aggregation of denatured proteins (Huang et al., 2009; Suh et al., 1998), while other chaperones, such as HSP60, HSP70, and HSP90, assist in refolding damaged proteins. Together, they work to repair or degrade misfolded proteins under stress, preventing their accumulation and maintaining intracellular homeostasis (Feder & Hofmann, 1999; Sørensen et al., 2003).

HSPs are often used as molecular markers or to study gene expression patterns related to thermal tolerance. Research on parasitoid species, such as *Pteromalus puparum* (Linnaeus) and *Cotesia chilonis* (Munakata), highlights the dynamic regulation of HSP expression in response to heat or cold stress, with variations depending on developmental stage and environmental conditions. For example, *P. puparum* induces six HSPs under temperature extremes (H. Wang et al., 2012), while *C. chilonis* exhibits stage-specific and stress-type-dependent HSP expression (Song et al., 2020). Similarly, studies on *Aphelinus asychis* (Walker) have demonstrated sex-specific gene expression under high temperatures (Liu et al., 2020). Though primarily studied in response to heat stress, HSPs are also induced during cold stress and play a crucial role in mitigating protein damage caused by freezing (Štětina et al., 2015; Teets et al., 2011; Štětina et al., 2015). The upregulation of HSP genes has also been associated with diapause during winter, highlighting their importance in cold adaptation (Flannagan et al., 1998; Rinehart et al., 2007).

2.5.3 Antioxidants

Reactive oxygen species (ROS) are byproducts of oxidative metabolism and include molecules such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), and singlet oxygen (O) (Livingstone, 2001). They are primarily generated in the mitochondria and microsomes via the electron transport chain and are involved in various cellular processes like embryonic development, cell differentiation, and cell signalling (Apel & Hirt, 2004; Hernández-García et al., 2010). When excess ROS are produced during thermal stress, they can cause significant cellular damage, such as DNA mutations, strand breaks, and apoptosis/cell death (Storey & Storey, 2010). Additionally, ROS contribute to lipid peroxidation (LPO), especially targeting polyunsaturated fatty acids in cell membranes, which disrupts membrane fluidity (Apel & Hirt, 2004; Paital et al., 2016). Elevated LPO levels are indicative of oxidative stress and lead to the production of malondialdehyde, a marker of this damage (Lopez-Martinez et al., 2008; Meng et al., 2009).

To reduce the level of ROS, insects rely on antioxidants. A small portion of ROS is neutralised by dietary antioxidants, such as ascorbic acid and carotenoids, but the majority are eliminated by antioxidant enzymes (Aucoin et al., 1991; Felton & Summers, 1995; Moreira et al., 2021). Insects have developed a sophisticated antioxidant system to counteract oxidative stress. This system includes enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and glutathione-S-transferase (GST) (Aucoin et al., 1991; Yang et al., 2010). SOD plays a key role by converting superoxide anions into hydrogen peroxide and molecular oxygen, which are then broken down by CAT and POD into water and oxygen (Ahmad, 1992; Ahmad et al., 1991; Felton & Duffey, 1991; Yang et al., 2010). GST helps detoxify products of lipid peroxidation, further protecting cells from damage (Koirala et al., 2022). Additionally, there are non-enzymatic antioxidants, such as lipids and water-soluble (ascorbate, glutathione, etc.) compounds, that support ROS protection and contribute to the adaptive responses of insects to cold stress (Felton & Summers, 1995). Thus, the antioxidant system is vital for combating the harmful effects of ROS and maintaining cellular integrity under thermal stress.

2.5.4 Cryoprotectants and metabolic response

Cryoprotectants, including sugars (such as trehalose, maltose, and glucose) and polyols (such as glycerol and sorbitol), are essential for combating low-temperature stress (Storey & Storey, 1991). Generally, the ability to withstand cold temperatures, known as cold hardiness, begins with preparation before winter (cold acclimation), via the storage of sufficient nutrients, such as lipids and carbohydrates, to sustain winter metabolism and support developmental growth in the spring (Andreadis & Athanassiou, 2017; Storey & Storey, 2012). This preparation is accompanied by the production of cryoprotectants, which regulate ice-nucleating agents, adjust water content, and modify cellular membrane composition to various extents for freeze tolerant and freeze avoidance insects (Clark & Worland, 2008; Hance et al., 2007). The degree of supercooling (the temperature at which a liquid transition to ice) influences ice crystal formation, with low-temperature nucleation producing small crystals capable of penetrating tissues through gap junctions to increase the risk of intracellular damage during rapid freezing (Denlinger & Lee Jr, 2010; Lee, 1991). However, in freeze-tolerant insects, ice-nucleating agents regulate supercooling, slowing crystal formation and thus reducing freezing damage (Toxopeus & Sinclair, 2018). Freeze-avoidant insects can actually prevent ice formation in their body fluids and tissues by eliminating ice-nucleating agents and producing antifreeze proteins, which help maintain their body fluids in a liquid state and prevent crystallisation (Lee, 1991; Zachariassen, 1985).

Homeoviscous adaptation is a shared strategy to maintain cell membrane integrity by regulating lipid composition in response to temperature extremes (Sinensky, 1974). During cold stress, membranes become rigid, reducing enzyme access to lipid stores and impairing basal metabolism (Enriquez & Teets, 2023). To counteract this, insects increase the degree of unsaturation in membrane lipids, incorporating polyunsaturated fatty acids to preserve fluidity and prevent phase transitions, which is essential for cold tolerance (Kostal & Simek, 1998; Overgaard et al., 2005; van Dooremalen et al., 2011). This process includes changes in fatty acid composition, such as increases in palmitoleic acid, which enhances membrane stability (Atapour et al., 2007; Zhang et al., 2024). Other fatty acids, like linolenic acid and stearic acid, may decrease to support this restructuring (Atapour et al., 2007; Canavoso et al., 2001; Zhang et al., 2024). In contrast to low temperature, high temperatures increase membrane fluidity, which can disrupt the function of lipid-associated proteins and enzymes. To counter this, insects elevate the proportion of saturated fatty acids to stabilise their membranes (Hazel & Williams, 1990; van Dooremalen et al., 2011; van Dooremalen & Ellers, 2010). This ability to fine-tune lipid composition allows insects to maintain adequate membrane fluidity and functionality across a wide range of temperatures (Kostal & Simek, 1998; van Dooremalen et al., 2011).

Insects can also synthesise cryoprotectants and free amino acids (such as proline and β-alanine), to protect cells from damage during cold stress (Storey & Storey, 1991). For example, trehalose acts by reducing the supercooling point (Olsson et al., 2016; Storey & Storey, 2012), thereby improving cold tolerance, while glycerol contributes to cryoprotection depending on the type of cold stress (Gokulanathan et al., 2024; Wen et al., 2016; Xue et al., 2024). Despite being termed 'cryoprotectants', these compounds are not exclusive to cold stress. Under heat stress, trehalose plays a vital role in regulating cytoplasmic viscosity during thermal stimulation, helping to maintain cytoplasmic homeostasis and stabilise cellular reaction rates (Persson et al., 2020; Wang et al., 2021). These adaptations, involving both membrane restructuring and cryoprotectant accumulation, are important for improving thermal tolerance in insects.

2.5.5 Neural responses

Responses to temperature are further fine-tuned through neural mechanisms that involve thermoreceptor neurons, thermal projector neurons, neurotransmitters, neuromodulators, neurohormones, and the central nervous system (Osborne, 1996; Peric-Mataruga et al., 2006). Thermoreceptor neurons serve as the primary sensors of temperature change, detecting shifts through ion movements across neuronal membranes. These neurons generate action potentials, triggering the release of neurotransmitters and neuromodulators, which facilitate communication with thermal projector neurons (Kumar et al., 2024; Xiao & Xu, 2021). Thermal projector neurons communicate these signals to integrative centres in the central nervous system, such as the protocerebrum, lateral horn, and mushroom bodies (Chiang et al., 2023). Here, temperature information is processed to coordinate appropriate responses, including behavioural actions like thermotaxis, heat avoidance, and temperature preference, as well as physiological adjustments like the production of HSPs and the modulation of metabolism (Kumar et al., 2024; Xiao & Xu, 2021).

Neurohormones are activated during prolonged or severe thermal stress, orchestrating systemic and long-term adaptations. They regulate processes like metabolism, diapause, and developmental timing, helping insects survive beyond immediate behavioural and physiological adjustments (González-Tokman et al., 2020; Kumar et al., 2024). For instance, elevated temperatures can increase levels of juvenile and moulting hormones, modulating responses that align with the insect’s overall stress management strategy (González-Tokman et al., 2020). Together, these chemical mediators ensure an integrated and effective response to thermal extremes.

Biogenic amines are essential for regulating basic life processes in insects (Farooqui, 2012; Sinakevitch et al., 2018). They function as neurotransmitters and neuromodulators in the nervous system, and in some cases, they act as neurohormones by being released into body fluids (Sinakevitch et al., 2018). The main biogenic amines in insects include octopamine, serotonin, dopamine, histamine, and tyramine (Blenau & Baumann, 2001). When insects experience thermal stress, biogenic amines are quickly released to help them respond. For example, changes in biogenic amine levels help insects survive harsh environments or prepare for prolonged stress (Armstrong & Robertson, 2006; Isabel et al., 2001). Interestingly, this release appears to occur regardless of the specific type of stress, such as heat or starvation (Gruntenko et al., 2004; Orchard et al., 1981).

Release of biogenic amines is often accompanied by increased activity or production of enzymes involved in biogenic amine synthesis, along with their precursors and metabolites (González-Tokman et al., 2020). For instance, octopamine helps locusts cope with temperature extremes by regulating key physiological processes like ion balance and energy maintenance (Armstrong & Robertson, 2006). In addition to biogenic amines, neuropeptides play a role in stress responses, often working alongside biogenic amines (Altstein & Nässel, 2010; Schoofs et al., 2017). For example, neuropeptide F influences behaviours such as feeding and stress management. When hungry, some insects (e.g., flies), may forage in cold conditions, but reduced neuropeptide F levels in the larvae can prevent feeding in such environments (Lingo et al., 2007).

In insects, exposure to low temperatures can lead to a state of neuromuscular paralysis known as chill coma, which is triggered by a rapid loss of nervous system function and a shutdown of the central nervous system (Andersen et al., 2023; Robertson et al., 2017, 2020, 2023). Remarkably, chill coma is reversible if the insect is returned to a warmer, permissive temperature quickly enough (Overgaard & MacMillan, 2017). However, prolonged cold exposure can exacerbate the effects of chill coma by disrupting ion gradients, causing extracellular hyperkalemia and depolarising excitable tissues (MacMillan et al., 2014). Even when recovery is possible, severe cold exposure often results in behavioural impairments, locomotor defects, and, in extreme cases, mortality (Andersen & Overgaard, 2019; MacMillan & Sinclair, 2011). Therefore, neurological responses to thermal stress highlight the importance of understanding how insects adapt to extreme temperatures and the role of neural mechanisms in this process.

2.5.6 Morphological and behavioural responses

Some parasitoids can use morphological measures to escape extreme temperatures (Schlinger & Hall, 1960) and/or modify the behaviour of their host (Brodeur & McNeil, 1989; Krespi et al., 1994; Lagos et al., 2001; Langer & Hance, 2000). Morphological traits like colour variation play a key role, with species like *Trioxys utilis* (Hymenoptera: Braconidae) producing light-coloured cocoons to reflect heat in warm conditions and darker cocoons to absorb heat in the cold, thus optimising thermoregulation (Schlinger & Hall, 1960). Similarly, parasitoids can influence host behaviour, causing them to seek favourable environments. For example, *Aphidius ervi* (Haliday) can guide its host aphids to warmer canopy surfaces that increase its own development rate (Lagos et al., 2001), while *Aphidius* *nigripes* (Ashmead) induces mummification of its host in sheltered microhabitats to avoid freezing (Brodeur & McNeil, 1989).

2.5.7 Future aspects

In contrast to the other sections, much of the focus of this section has necessarily been on insects versus parasitoids due to the current research focus. Indeed, most studies on parasitoids have primarily focused on HSPs as key indicators of thermal tolerance (Ding et al., 2021; Liu et al., 2020; Song et al., 2020), leaving the broader genetic and molecular landscape underlying stress responses less studied for this group.

To gain a more holistic understanding, future work should examine multiple genes and pathways involved in stress responses – especially thermal stresses. For example, advanced transcriptomic approaches offer the potential to uncover complex regulatory networks and provide deeper insights into the integrated mechanisms driving stress resilience in parasitoids (Ashraf et al., 2022; Xiong et al., 2024). Indeed, one such study on *Tamarixia radiata* (Waterston) under heat stress revealed the upregulation of molecular chaperones, immune response pathways, stress signalling, and oxidative resistance genes, including HSPs, ATPases, and detoxifying enzymes (Ashraf et al., 2022). Another study on *P. puparum* revealed a complex regulatory network involving both HSPs and lipogenesis genes in response to heat stress (Xiong et al., 2024). These findings suggest that integrating broader transcriptomic analyses will provide deeper insights into the interactions among pathways and identify novel targets for advancing our understanding of stress resistance mechanisms in parasitoids.

2.6 Microbiome

An insect's microbiome plays an important role in modulating the biology and physiology of its hosts, influencing various biological processes. These microbial interactions range from mutualistic to parasitic, offering potential for species-specific pest management strategies. Microbial symbionts are broadly classified as endosymbionts or ectosymbionts based on whether they reside inside the insect's cells or externally (Zhang et al., 2022). Further, endosymbionts are categorised as obligate (primary) or facultative (secondary), each with distinct roles and impacts on insect ecology and evolution.

Obligate symbionts are maternally inherited and provide essential nutrients lacking in the host's diet (Baumann, 2005; McNeill et al., 2014). They reside in specialised host organs called bacteriomes and form a mutualistic relationship important for host survival (Baumann, 2005). These symbionts are typically found among beta- and gamma-proteobacteria. In contrast, facultative symbionts are non-essential for host survival, resulting in a more flexible relationship and host impacts that range from neutral to beneficial or even detrimental. Facultative symbionts often reside in the haemolymph and consist of alpha- and gamma-proteobacteria (McNeill et al., 2014). Unlike obligate symbionts, facultative symbionts can be horizontally transferred between hosts (Huigens et al., 2004; Kraaijeveld et al., 2011; Werren et al., 1995). Microbial symbionts profoundly impact parasitoid fitness, host interactions, reproduction, and immunity, making them integral to the parasitoid's' life history and role in pest management. This section primarily focuses on elucidating the diverse roles and impacts of microbial symbionts on parasitoids and their effects on biocontrol.

2.6.1 Reproductive manipulation

Symbionts, especially endosymbionts, induce various reproductive alterations, including cytoplasmic incompatibility, parthenogenesis, feminisation, and male-killing. Common bacterial endosymbionts mediating these processes include *Wolbachia* (Hertig), *Rickettsia* (da Rocha-Lima), and *Cardinium* (Zchori-Fein) (Haghshenas-Gorgabi et al., 2023; Owashi et al., 2024; Richardson et al., 2023). Cytoplasmic incompatibility occurs when modifications induced by endosymbionts in the sperm of infected males are not complemented by the egg of uninfected females or females infected with an incompatible strain. It results in reduced offspring viability when infected males mate with uninfected females, as seen in the parasitoid *Nasonia vitripennis* (Bordenstein et al., 2001).

Parthenogenesis involves the induction of asexual reproduction, leading to all-female populations, and has been extensively studied in some parasitoids including *Trichogramma* spp., following infection with *Wolbachia* (Guo et al., 2023), and *Encarsia* spp*.,* harbouring *Cardinium* (Giorgini et al., 2009). Feminisation is the conversion of genetic males into functional females and has been observed in the parasitoid *Encarsia pergandiella* (Howard), when infected by *Cardinium* (Giorgini et al., 2009), resulting in skewed sex ratios. Finally, male-killing bacteria are endosymbionts that cause female hosts to produce female-biased broods by eliminating male offspring during embryogenesis (Hurst & Jiggins, 2000). For example, in the parasitoid *Pteromalus venustus* (Walker), male embryo mortality is induced by *Arsenophonus nasoniae* (Gherna) (Goerzen & Erlandson, 2018).

While reproductive manipulations by endosymbionts can enhance biocontrol by increasing female populations, they may also have evolutionary consequences by altering host mating systems and their spread (Brenninger et al., 2025), reducing genetic diversity due to prolonged asexual reproduction (see section 2.3: Reproductive modes), and compromising the long-term stability of parasitoid populations. Apart from reproductive manipulation, symbionts can contribute directly or indirectly to host-parasitoid interactions; they can be beneficial or detrimental to biocontrol, as discussed in the following sections.

2.6.2 Host detection

Symbionts, whether associated with the herbivorous host insect or the plant it inhabits, can aid parasitoids in locating their hosts by enhancing host visibility and susceptibility through the modulation of chemical and visual cues. For example, the presence of the endosymbiont *Rickettsiella* spp. changes the colouration of pea aphids, making them more vulnerable to parasitism (Tsuchida et al., 2010). Symbionts can also influence parasitoid behaviour to enhance host-seeking efficiency. For example, gut microbiota may produce metabolic byproducts (i.e., microbial volatile organic compounds) that amplify olfactory responses, helping parasitoids locate suitable hosts (Castelo & Crespo, 2022). Certain bacterial strains are known to produce compounds that have been shown to either attract or repel the aphid parasitoids *Aphidius colemani* (Viereck) and *Diaeretiella aphidum (McIntosh)*, thereby influencing parasitoid host-location behaviour through microbial cues (Goelen et al., 2020). Additionally, microbiomes associated within host plants can contribute to improved parasitoid host detection. For example, the fungal root symbiont *Trichoderma harzianum* (Rifai) enhances plant defence by priming the production of volatile organic compounds, particularly terpenoids, through activation of the salicylic acid pathway, a metabolic shift that increases the attraction of the parasitoid *A. ervi* to aphid-infested plants and thereby boosts biocontrol effectiveness (Coppola et al., 2017). However, in some cases, a repellent effect may occur, as demonstrated by *Pseudomonas fluorescens* (Flügge), which alters the volatile profiles of aphid-infested plants by suppressing the jasmonic acid pathway, thereby reducing the plant's attractiveness to the aphid parasitoid *Diaeretiella rapae* (McIntosh) (Pineda et al., 2013).

2.6.3 Stress adaptation

We explored above how insects respond to stress, using thermal stress as an example (see section 2.5: Thermal tolerance), with a focus on physiological responses. Additionally, symbionts contribute to the acquisition of essential nutrients, thereby enhancing survival, stress adaptation, and overall fitness in insects. However, studies specifically examining the nutritional role of symbionts in parasitoids remain limited. Understanding how endosymbionts in particular support parasitoid nutrition and stress adaptation is important, as these factors directly influence biocontrol efficacy. For example, in the parasitoid *Asobara tabida* (Nees), *Wolbachia* plays an essential role in iron homeostasis, reducing labile iron concentrations to protect parasitoid cells from oxidative stress and apoptosis, thereby ensuring successful egg development (Kremer et al., 2009). Similarly, the endosymbionts of parasitoid *N. vitripennis* have been shown to enhance cold tolerance during diapause by modulating glucose and glycerol levels in larvae (Dittmer & Brucker, 2021).

2.6.4 Defensive mechanisms

Endosymbionts can modulate host-parasitoid interactions by providing defensive benefits to their parasitoid hosts that can significantly reduce the effectiveness of parasitoid-based biocontrol by interfering with parasitoid development and survival. For instance, *Hamiltonella defensa* (Moran) produces toxins that impair *A. ervi* parasitoid larval development within the parasitoid’s host, effectively reducing parasitism success (Oliver et al., 2005, 2012). Similarly, the endosymbiont *Spiroplasma poulsonii* competes with the parasitoid larvae of *Leptopilina boulardi* (Barbotin, Carton & Keiner-Pillault) and *A. tabida* for essential lipids in *Drosophila melanogaster* (Meigen), ultimately leading to larval starvation and death (Paredes et al., 2016). These defence strategies can alter host-parasitoid dynamics and present challenges for the long-term success of biological control programmes. However, some parasitoids use superparasitism as a strategy to overcome symbiont-mediated protection. For example, *A. ervi* increases its parasitism success by laying multiple eggs in aphids infected with *H. defensa*, while typically depositing a single egg in uninfected aphids, suggesting that parasitoid oviposition behaviour is influenced by the host's infection status (Oliver et al., 2012).

As well as physiological traits, endosymbiotic microbes can significantly influence host behaviour by altering defensive responses. These behavioural modifications can either enhance or suppress the host's ability to evade parasitism, directly impacting parasitoid-host dynamics. For example, *H. defensa* reduces defensive behaviours in aphids, making them less likely to escape parasitoid attacks (Dion et al., 2011), whereas *Regiella insecticola* (Moran) enhances aphid defensive behaviours that improve their chances of avoiding parasitism (Bauwens et al., 2015). Additionally, some endosymbionts like *H. defensa*, can influence aphid responses to alarm pheromones by altering locomotor activity, further shaping group behaviours (Badji et al., 2021).

2.6.5 Future aspects

Research on the microbiome of *Microctonus* parasitoids is still in its early stages, with recent foundational work including the development of high-quality genome assemblies for *M. hyperodae* and *M. aethiopoides* (Inwood et al., 2023). These genomic resources have enabled investigations into pathogen associations, including the identification of *Microctonus hyperodae filamentous virus* (MhFV) (Inwood et al., 2023), which is actively transmitted to *L. bonariensis* during both successful and interrupted parasitism, and may contribute to premature host mortality, suggesting a potential role in enhancing biocontrol efficacy (Inwood et al., 2024). Future research should focus on elucidating the functional role of MhFV within *M. hyperodae* and determining its presence across hosts, non-host weevil species, and other *Microctonus* parasitoids in New Zealand to better understand its transmission and ecological spread.

Microbiome research of *S. obsoletus*, has revealed the presence of defensive endosymbionts, including *Wolbachia* and multiple *Rickettsia* strains (White et al., 2015). These endosymbionts may be associated with the failure of the Moroccan strain of *M. aethiopoides* to successfully parasitise *S. obsoletus* (due to premature mortality of larvae in the weevil; (McNeill et al., 2000, 2014) and may also contribute to regional variation in parasitism success by the Irish strain of *M. aethiopoides*, suggesting a possible defensive role against parasitoids (White et al., 2015). The presence of *Rickettsia* in *S. obsoletus* (White et al., 2015), also raises the possibility of shared endosymbionts between host and parasitoid, potentially through horizontal transmission. However, further validation is needed to confirm the presence and specific strain associations in both the Irish strain of *M. aethiopoides* and *S. obsoletus*.

To advance this area of research, further characterisation of microbiomes associated with these parasitoids and their hosts using comprehensive approaches such as 16S rRNA amplicon sequencing is essential. Coupling this with functional symbiont assays, such as antibiotic treatment to eliminate specific symbionts in the species and assess their functional contribution, and volatile organic compound identification to determine symbiont-mediated effects on parasitoid attraction or host-parasitoid interactions, will further promote understanding of their functional roles and may offer new ways to enhance biocontrol by improving parasitoid performance.

2.7 Conclusion

The effectiveness of classical biological control lies not only on initial establishment and parasitism rates, but also on the long-term adaptability and ecological compatibility of the parasitoid species. While the introduction of three *Microctonus* species has played a significant role in managing weevil pests in New Zealand, the biological and ecological complexity underpinning their effectiveness in the long term remains insufficiently explored.

This review identifies reproductive mode, olfactory behaviour, stress responses, and symbiotic microbial associations as some of the key adaptive traits influencing host specificity, parasitoid persistence, and overall insect pest control outcomes. Drawing on studies from diverse Hymenopteran systems, we show that integrative approaches, combining behavioural, ecological, and molecular data, can (or in future, will) yield valuable insights into parasitoid biology and performance.

Classical biological control programmes involve a series of steps to ensure that outcomes are positive and effective. This includes correct identification of the exotic insect when detected in its new geographic range, identification of its geographic origin and the biological control agents acting on the insect in that country of origin, and the identification and selection of the most effective biocontrol agent(s) in the new country. Thereafter, quarantine-based studies provide the opportunity to undertake further research on host range, particularly non-target effects on endemic or beneficial species. Within the framework of future biological control programs research that includes molecular and transcriptomic analyses to uncover the mechanistic basis of traits like host recognition, reproductive strategy, and thermal resilience will be valuable components to achieving sustainable and effective pest control.

Ultimately, bridging the gap between trait-level understanding and ecological outcomes (i.e., host-parasitoid dynamics in the field) will help improve the predictive power and sustainability of parasitoid-based biocontrol moving forward, for example by helping to refine release strategies, such as matching strain traits to specific environments or host populations. These integrated approaches hold the promise of improving our understanding of adaptive potential in *Microctonus* and other parasitoid species, and informing the design of more resilient, targeted, and sustainable classical biological control programmes in New Zealand.

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Chapter 3

Using genomic data to infer evolutionary processes in the asexual parasitoid *Microctonus aethiopoides*

Meeran Hussain[1], Elahe Parvizi[1], Mark R. McNeill[2], Ang McGaughran[1]

[1]Te Aka Mātuatua - School of Science, University of Waikato, Private Bag 3105, Hamilton, 3240, New Zealand

[2] Bioeconomy Science Institute, Tuhiraki, Christchurch, New Zealand

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3.1 Abstract

Biological control offers a sustainable alternative to pesticides, with asexual parasitoids favoured for their ability to reproduce without males and produce all-female offspring. However, asexuality may limit the parasitoids' long-term adaptability to parameters, such as climate changes or physiological or behavioural changes in the host, reducing the potential effectiveness of the biocontrol.

In this study, we performed whole-genome resequencing on 43 individuals of the Irish strain of the asexual (thelytokous) endoparasitoid *Microctonus aethiopoides*, which was introduced to New Zealand in 2006 to control clover root weevil (*Sitona obsoletus*). We sampled from five parasitoid populations, one from the original collection location in Ireland and four release locations in New Zealand, to assess spatial and temporal genetic variation and investigate the genetic basis for its reproductive mechanism.

Population structure analyses revealed two distinct genetic clusters, likely reflecting the differential establishment of haplotypes introduced from Ireland rather than geographic isolation. Though one haplotype appeared more widespread, particularly in the South Island, further sampling is needed to confirm this. All populations exhibited uniformly low genetic diversity, with Tajima's D values decreased in contemporary populations, indicating population expansion since introduction. Patterns of high heterozygosity and short homozygous segments support automictic thelytoky, likely via central fusion, as the primary

mode of asexual reproduction. However, linkage disequilibrium rates resembled those of sexual populations, suggesting possible facultative sexual reproduction in this species.

Overall, our genomic data provide new insights into how asexual biocontrol agents persist and evolve post-release and shed light on this species' long-term viability and reproductive strategy.

3.2 Introduction

Classical biological control is a key strategy for managing invasive species by introducing natural enemies from their native range. In New Zealand, exotic and accidentally introduced weevils, such as *Listronotus bonariensis* (Kuschel) (Argentine stem weevil) and *Sitona obsoletus* (Gmelin) (clover root weevil), are among the most serious pests of ryegrass (*Lolium* L. spp.) and white clover (*Trifolium repens* L.), respectively. These pests have been estimated to cause up to NZ$235 million per year in damage to dairy, sheep, and beef pastures (Ferguson et al., 2019). Parasitoids are particularly valuable biocontrol agents in agriculture (Seehausen et al., 2021), and classical biological control programmes targeting both *L. bonariensis* (Barker & Addison, 2006; Goldson et al., 1998) and *S. obsoletus* (Gerard et al., 2011; Goldson et al., 2005) have played an important role in their management. However, not all classical biocontrol efforts are effective.

Various factors can impact biocontrol success, including climate incompatibilities, mismatches between parasitoid strains and target hosts, physiological defences of the host, Allee effects, and the influence of endosymbionts (Cameron et al., 1993; Hoelmer & Kirk, 2005; Seehausen et al., 2021). An interesting example of apparent failure after initial success was the biocontrol of *L. bonariensis* by *Microctonus hyperodae* Loan (Hymenoptera: Braconidae). Although initially successful (Barker & Addison, 2006; Goldson et al., 1998), levels of parasitism showed a progressive decline seven years after initiation (Goldson et al., 2014), with a key hypothesis being that the asexual parasitoid was being outcompeted by its sexual host because of avoidance behavioural changes (Casanovas et al., 2018).

Being asexual clearly has certain advantages in terms of negating the need to find a mate under stable environments (Amat et al., 2017) and allowing for rapid population growth (Heimpel & de Boer, 2008). Indeed, some 500 Hymenopteran species have been identified as having thelytokous asexual reproduction (van der Kooi et al., 2017). But how asexual parasitoids maintain their effectiveness in dynamic and competitive ecological environments is relatively unexplored. Considering the apparent reduced effectiveness of *M. hyperodae* (Casanovas et al., 2018), this sets up an intriguing hypothesis about the role of reproductive mode in driving success/failure outcomes of classical biological control programs.

A key limitation of asexuality is the potential associated reduction in genetic diversity. In the absence of a male partner, the lack of allelic reshuffling through meiosis between two parents results in offspring that are genetically similar to their mother, potentially limiting the population's overall ability to adapt to changing environmental conditions or evolving host defences (Normark et al., 2003; Tvedte et al., 2019). In contrast, sexual reproduction introduces genetic recombination through meiosis, which enhances genetic diversity and provides greater potential for adaptation to environmental changes and host resistance (Heimpel & de Boer, 2008; Otto & Lenormand, 2002).

Yet, asexual reproduction is not universally clonal. Some asexual systems possess mechanisms that can preserve or generate genetic variation. For instance, in apomictic (strictly clonal) asexuals, new genetic variation arises only through the accumulation of spontaneous mutations, which is typically a slow process. However, in automictic asexuals, reproduction involves partial meiosis followed by the fusion of meiotic products from the same individual. Depending on the mechanism (central fusion, terminal fusion, or gamete duplication), automixis can retain varying degrees of heterozygosity, thereby maintaining some genetic diversity despite the absence of fertilisation (Card et al., 2021; Lampert, 2008; Pearcy et al., 2011).

Patterns of linkage disequilibrium (LD) can be used to provide insights into reproductive mode, as LD tends to break down in sexually reproducing populations due to recombination, whereas in asexual or inbred populations, LD remains high - reflecting reduced genetic reshuffling and the accumulation of linked alleles, a consequence similar to that observed under strong inbreeding (Balloux et al., 2003; Hartfield et al., 2018). Another strategy is facultative sexuality, in which organisms predominantly reproduce asexually but switch to sexual reproduction under certain conditions. This dual mode allows occasional gene flow when the asexual population co-exists with its sexual conspecifics, enabling the retention or reintroduction of genetic diversity when needed (Adachi-Hagimori et al., 2011; Sandrock et al., 2011; Schneider et al., 2003). This diversity of asexual mechanisms highlights that even in the absence of regular outcrossing, some asexual organisms can mitigate the genetic limitations typically associated with clonal reproduction.

Microctonus aethiopoides (Hymenoptera: Braconidae) is an important endoparasitoid attacking the adult stage of *Hypera* and *Sitona* species (Aeschlimann, 1983; Loan, 1975; Loan & Holdaway, 1961; Shaw, 1988). Some strains have been successfully used in classical biological control programs against agricultural pests in North America (Loan, 1969; Radcliffe & Flanders, 1998; Rand et al., 2018). In New Zealand, two strains of *M. aethiopoides* have been introduced to control two *Sitona* species. The Moroccan strain was introduced to New Zealand in 1982 to control *Sitona discoideus* Gyllenhal (lucerne weevil), a major pest of lucerne (*Medicago sativa* L.) (Stufkens et al., 1987). Meanwhile, *Sitona obsoletus* (clover root weevil) was first detected in New Zealand in 1996 (Barratt et al., 1996). By 2006, it had rapidly spread to all parts of the North Island (Gerard et al., 2010) and it had reached most of the South Island by 2012 (Ferguson et al., 2012). Quarantine laboratory-based studies showed that the Moroccan strain of *M. aethiopoides* was ineffective against *S. obsoletus* (Barratt et al., 1997; McNeill et al., 2000), and this was attributed to a host physiological response mediated by endosymbionts that encapsulated and killed the developing eggs and larvae (White et al., 2015). Following extensive evaluation in quarantine (Goldson et al., 2005), an Irish strain of *M. aethiopoides* was thus released in 2006 (Gerard et al., 2006, 2007) and, within a year, parasitism rates of up to 70% were recorded in overwintering *S. obsoletus* populations (Gerard et al., 2011). There are distinct differences in reproductive mode between the two parasitoid strains. While Moroccan *M. aethiopoides* is a sexually reproducing (arrhenotokous) solitary endoparasitoid, the Irish strain is an asexually reproducing (thelytokous) and gregarious endoparasitoid (McNeill & Baird, 2010). Although biocontrol of *S. obsoletus* is currently effective, the parasitoid's asexual reproductive mode and the sexual mode of its host, suggests that degradation in the effectiveness of biocontrol may be a potential concern.

Here, we used genomic data to evaluate population diversity and differentiation patterns and explore reproductive mechanisms in the *M. aethiopoides* strain released against *S. obsoletus*. First, we assessed within and between population variation across space and time, using key population genomic metrics to assess the extent of genetic changes since the initial New Zealand releases in 2006. Second, we investigated patterns of LD and heterozygosity as informative metrics to infer whether recombination rates are suggestive of different forms of asexual reproduction in *M. aethiopoides*. Collectively, our approach provides a comprehensive understanding of how genomic factors may influence the long-term effectiveness of this parasitoid as a biocontrol agent.

3.3 Materials and methods

3.3.1 Sample collection

Historic samples of *M. aethiopoides* (the Irish strain) that were preserved in 95% ethanol and stored at -80°C at the AgResearch Lincoln facility were obtained from collections made during quarantine-based research conducted between 2000 and 2006 in England and New Zealand (Goldson et al., 2004; McNeill et al., 2006). Contemporary samples were reared from *S. obsoletus* collected from pastures containing white clover (*T. repens*) across four regions of New Zealand. In both Ireland and New Zealand, a modified blower-vac (G-vac) was used to collect litter from ryegrass-white clover pasture (McNeill & van Koten, 2020). The full sample collection and parasitoid extraction procedures are provided in Appendix 2.

In the laboratory, *S. obsoletus* adults were separated from the litter, and parasitoids subsequently reared from weevils following established protocols described by McNeill et al., (2006). A total of 44 parasitoids were included in this study, comprising nine historic samples from Athenry, Ireland (IRE) and contemporary samples from Mangōnui (MAN, n = 5), Hamilton (HAM, n = 10), Lincoln (LIN, n = 10), and Dunedin (DUN, n = 10) (Fig. 3.1a; Table A3.1). Contemporary parasitoid samples were preserved in 95% ethanol and stored at -20°C until DNA extraction.

3.3.2 DNA extraction and sequencing

Prior to DNA extraction, parasitoid samples were washed in deionised distilled water. Total genomic DNA was extracted from homogenised parasitoid samples using a column-based proteinase K digestion method with the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. Extracted DNA was quantified using the Qubit dsDNA High Sensitivity (HS) Assay Kit (Invitrogen) and a Qubit Fluorometer, yielding DNA concentrations ranging from 113-1320 ng (Table A3.1). Illumina sequencing was performed to achieve approximately 30-fold genome coverage. Paired-end sequencing (2 × 150 bp) was conducted using PCR-Free library preparation on a NovaSeq 6000 by Livestock Improvement Corporation (LIC) Genomics Facility (Hamilton, New Zealand).

3.3.3 Data analysis

A high-quality improved genome assembly of the Irish ecotype of *M. aethiopoides*, developed using Oxford Nanopore Technologies and Illumina sequencing for ongoing comparative genomic analysis (unpublished), was used in this study. The genome (131.1 Mb, comprising 231 scaffolds with 8 pseudo-chromosomes) was assembled via ragtag scaffolding based on a previously Hi-C scaffolded genome assembly (Inwood et al., 2023) and consists of 13,881 predicted genes with a BUSCO completeness score of 95.0%.

Of the 44 samples processed, 43 were successfully sequenced, while one sample from the Irish population (IRE) failed to yield usable data (Table A3.1). Raw Illumina reads from the remaining samples were quality-filtered using Trim Galore (v0.6.10) (https://github.com/FelixKrueger/TrimGalore) with a minimum quality score (-q) of 20 and minimum read length (-length) of 100 bp. Clean reads were aligned against the assembled reference genome using BWA-MEM (v0.7.17) with M and R flags to mark secondary reads and add read groups, respectively (Li, 2013). The resulting SAM files were processed with SAMtools (v1.16.1) to remove duplicate reads (markdup) and sort alignments (Danecek et al., 2021). Only uniquely mapped reads (-F 4) were retained for variant calling. SNPs were called using BCFtools (v1.19) with a minimum mapping quality (--min-MQ) and base quality (--min-BQ) of 20 (Danecek et al., 2021). Biallelic SNPs were filtered to retain variants with a minimum minor allele frequency (MAF) > 0.05. Further filtering was performed on the resulting VCF file using PLINK v1.9 to retain SNPs with at least a 90% genotyping rate (--geno 0.1) (Purcell et al., 2007). Additionally, individuals with more than 90% missing data (--missing-indv) were excluded using VCFtools (v0.1.15) (Danecek et al., 2011). These processing steps were integrated into a Snakemake pipeline, publicly available at (https://github.com/meeranhussain/Population_genomic_analysis). A total of 133,329 high-quality SNPs were identified following these filtering steps (Table A3.2).

The population structure of *M. aethiopoides* was inferred using principal component analysis (PCA), implemented with the R package adegenet (v2.1.1) (Jombart & Ahmed, 2011), and sparse non-negative matrix factorisation (sNMF) analysis, performed with the R package LEA (v2.8.0) (Frichot & François, 2015). Prior to these analyses, variants underwent a stringent filtering step to reduce LD using PLINK v1.9. Specifically, SNPs were filtered based on pairwise LD (--indep-pairwise) with a window size of 50 SNPs, a step size of 5 SNPs, and an r^2 threshold of 0.2, resulting in a total of 37,285 SNPs retained for population structure analyses. The sNMF analysis was conducted by evaluating up to eight putative clusters (K-values ranging from 1 to 8), with 50 iterations of the algorithm performed per K-value. Cross-entropy results were plotted for each K-value to determine the optimal number of genetic clusters.

Tajima's D, nucleotide diversity (π), and individual-level heterozygosity were calculated across the genome in 100 kb sliding windows using VCFtools based on the full dataset of 133,329 high-quality SNPs. Heterozygosity for each individual was estimated using the "--het" option and observed heterozygosity (Obs_Het) was calculated using the formula: Obs_Het = 1 - (O(HOM) / N_SITES), where O(HOM) is the number of observed homozygous sites and N_SITES is the total number of sites analysed. Genetic differentiation (F_{ST}) was assessed using pixy (v1.2.11. beta1) (Korunes & Samuk, 2021). First, an all-site VCF file was prepared to include both variant and invariant sites, following the official pixy documentation (pixy.readthedocs.io/en/latest/generating_invar/generating_invar.html). Weir and Cockerham's F_{ST} was then calculated using a sliding window size of 100 kb and results were plotted to visualise the outlier windows for each population.

To infer reproductive mode information from genetic data, LD decay was calculated using the full filtered dataset (133,329 SNPs). To account for uneven sampling among populations, five individuals were randomly selected from each population for this analysis. LD decay was calculated for all SNPs within 500 kb windows using the OutStat command implemented in PopLDdecay (v.3.31) (Zhang et al., 2019) and decay curves were plotted up to 10 kb to allow better resolution of the decay trend. Runs of homozygosity (ROH) were called across the full genome using PLINK v1.9 on a less filtered dataset without MAF filtering (to retain information on rare alleles) while applying a genotype missingness filter (--geno 0.2), resulting in 160,071 SNPs. ROH segments were called using the --homozyg function with the following parameters: --homozyg-kb 20, --homozyg-snp 50, --homozyg-window-het 1, --homozyg-density 40, --homozyg-gap 100, and --allow-extra-chr. Using the results, F_{ROH} (a measure of inbreeding that quantifies the proportion of the genome made up of homozygous segments) was calculated as the total length of ROHs per individual divided by the total genome size (~130 Mb). Additionally, a phylogenetic tree was constructed to examine the genetic relationships among *M. aethiopoides* individuals/populations**.** The VCF file was converted to PHYLIP format using vcf2phylip (v.2.8) (Ortiz, 2019), and a maximum-likelihood tree was inferred with IQ-TREE (v.2.2.2) (Minh et al., 2020) using 1,000 ultrafast bootstraps (-bb 1000). The resulting tree was visualised and annotated using iTOL (v.6) (Letunic & Bork, 2024). To evaluate the statistical significance of differences (e.g., Tajima's D, genetic diversity and ROH values) between populations, we used Wilcoxon rank-sum tests with Bonferroni correction for multiple comparisons.

3.4 Results

3.4.1 Population diversity and differentiation

Nucleotide diversity (π) across the sampled populations of *M. aethiopoides* was consistently low, with median values ranging from $2.70e^{-05}$ to 3.24^{e-05} and similar levels of genetic variation across both contemporary and historic samples ($p > 0.05$ in all Wilcoxon pairwise comparisons; Fig. 3.1b). While Tajima's D median values were close to zero across all populations, we observed a significant decrease in values for the New Zealand contemporary versus the historic Irish (IRE) populations, indicative of population expansion (Fig. 3.1c).

The population structure analysis indicated that *M. aethiopoides* in New Zealand most likely comprises two distinct lineages (Fig. 3.1d). PCA showed clear clustering of the Hamilton (HAM) and Irish (IRE) populations along the first principal component (PC1), which explained 6.06% of the genetic variance. Individuals from Mangonui (MAN), Lincoln (LIN), and Dunedin (DUN) grouped closely in a second cluster, indicating minimal genetic differentiation among these contemporary populations. PC2, accounting for an additional 3.27% of the total variance, further differentiated the HAM and IRE populations.

The sNMF analysis supported the PCA results, with population admixture plots generated for K = 2-5. Although the cross-entropy analysis did not show a clear optimal value (Fig. A3.1), K = 2 was used for interpretation following the recommendation of Janes et al. (2017). At K = 2, the HAM and IRE populations were clearly distinguished from the remaining populations, with individuals in MAN, LIN, and DUN showing up to 90% genomic apportioning to the light blue cluster (Fig. 3.1e). Increasing the number of clusters (K = 3 to 5) showed finer-scale differentiation within contemporary populations; however, overall patterns consistently highlighted the genetic uniqueness of the HAM and IRE populations compared to the other contemporary populations.

Pairwise F_{ST} values indicated moderate genetic differentiation between populations, with values ranging from 0.009 to 0.139 (Table 3.1). Consistent with PCA and ADMIXTURE results, the lowest differentiation was observed between MAN and LIN ($F_{ST} = 0.009$) and the highest between HAM and DUN ($F_{ST} = 0.139$). The Irish population (IRE) showed moderate differentiation from the contemporary New Zealand populations (F_{ST} = 0.106–0.127), suggesting the occurrence of some genetic divergence post-introduction.

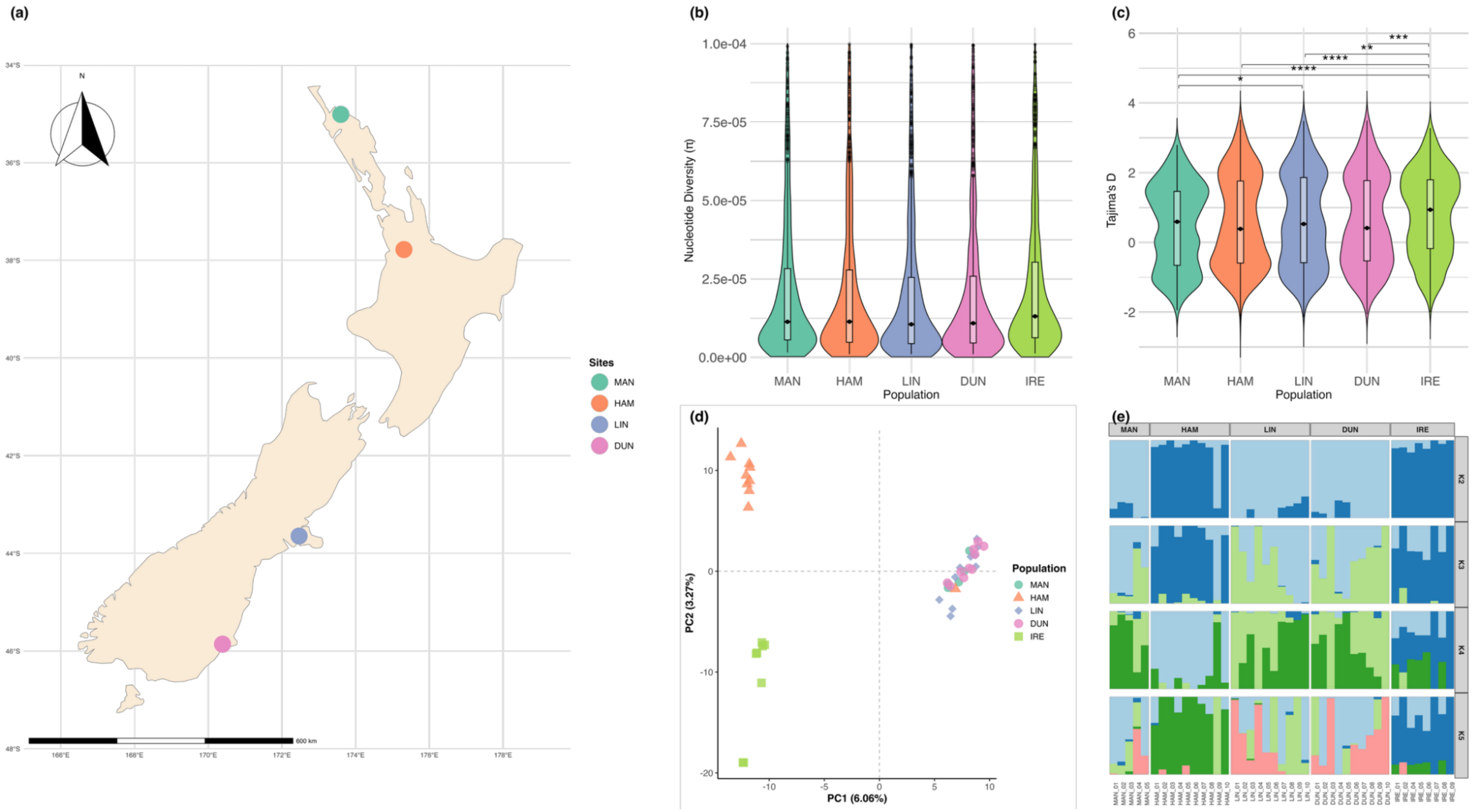
(a)
34°S
36°S
38°S
40°S
42°S
44°S
46°S
48°S
166°E
168°E
170°E
172°E
174°E
176°E
178°E
N
600 km
Sites
MAN
HAM
LIN
DUN
(b)
Nucleotide Diversity (π)
1.0e-04
7.5e-05
5.0e-05
2.5e-05
0.0e+00
MAN
HAM
LIN
DUN
IRE
Population
(c)
Tajima's D
6
4
2
0
-2
*
****
****
**
***
MAN
HAM
LIN
DUN
IRE
Population
(d)
PC2 (3.27%)
PC1 (6.06%)
10
0
-10
-20
-10
-5
0
5
10
Population
MAN
HAM
LIN
DUN
IRE
(e)
MAN
HAM
LIN
DUN
IRE
K2
K3
K4
K5

Figure 3.1 Sampling sites, genetic diversity and population structure of *M. aethiopoides* (Irish) populations in New Zealand: (**a**) Map of New Zealand showing contemporary sampling sites used in this study: Mangōnui (MAN), Hamilton (HAM), Lincoln (LIN), and Dunedin (DUN). The base map was generated using Natural Earth data in R; (**b**) Violin plots of nucleotide diversity (π) per 100 kb window across five populations and (**c**) Violin plots of Tajima's D values per 100 kb window across five populations. Pairwise comparisons across populations were tested using the Wilcoxon rank-sum test with Bonferroni adjusted p-values, significance levels are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; comparisons without asterisks are not statistically significant ($p \geq 0.05$); (**d**) Principal Component Analysis (PCA) showing genetic clustering of individuals from five populations; (**e**) ADMIXTURE analysis results for $K = 2$ to $K = 5$ ancestral clusters. Each vertical bar represents an individual, with colours indicating the proportion of ancestry assigned to each cluster. Individuals are grouped by population.

Genome-wide F_{ST} analysis revealed that outlier loci were widely dispersed across the genome and were primarily observed in population comparisons that involved HAM and IRE (Fig. A3.2, A3.3). However, the observed pattern showed no clear evidence of increased differentiation in any specific genomic region.

Table 3.1 Pairwise genetic differentiation (mean Weir and Cockerham's F_{ST}) among *M. aethiopoides* populations, calculated using variant and invariant sites across five populations.

	DUN	**HAM**	**IRE**	**LIN**
HAM	0.139			
IRE	0.127	0.106		
LIN	0.009	0.137	0.124	
MAN	0.014	0.135	0.117	0.012

3.4.2 Genomic patterns of linkage and recombination

All populations exhibited similar levels of heterozygosity, with median heterozygosity ranging between ~0.63 and 0.67 (Fig. 3.2a). Based on F_{ROH} estimates, approximately 5–10% of the genome consisted of continuous runs of homozygosity across all populations (Fig. 3.2b), with the highest proportion of genome-wide homozygosity and the greatest number of ROH segments observed in the DUN and MAN populations (Fig. 3.2c), while IRE exhibited the lowest proportion of homozygosity. This aligned with the heterozygosity results, as increased heterozygosity likely breaks up long runs of homozygosity. Finally, 50–100% of the observed ROH segments across all populations were relatively short (0.01–0.5 Mb) (Fig. 3.2d), indicating that recombination is frequently breaking long homozygous tracts. Notably, the IRE population had fewer ROH and higher heterozygosity compared with the contemporary populations.

LD decay for all populations displayed a rapid initial decline within the first 1 kb, followed by a plateau around $r^2 \approx 0.22$ (Fig. 3.2e), suggesting the presence of recombination, which breaks down LD over time. This pattern was consistent across historic and contemporary populations.

The phylogenetic tree showed three major clusters (Fig. A3.4), consistent with the patterns observed in the PCA. The topology placed individuals within contemporary populations together, with shorter branch lengths - particularly for individuals within the MAN, LIN, and DUN populations. The HAM and IRE populations were clearly separated from the rest and showed longer branch lengths.

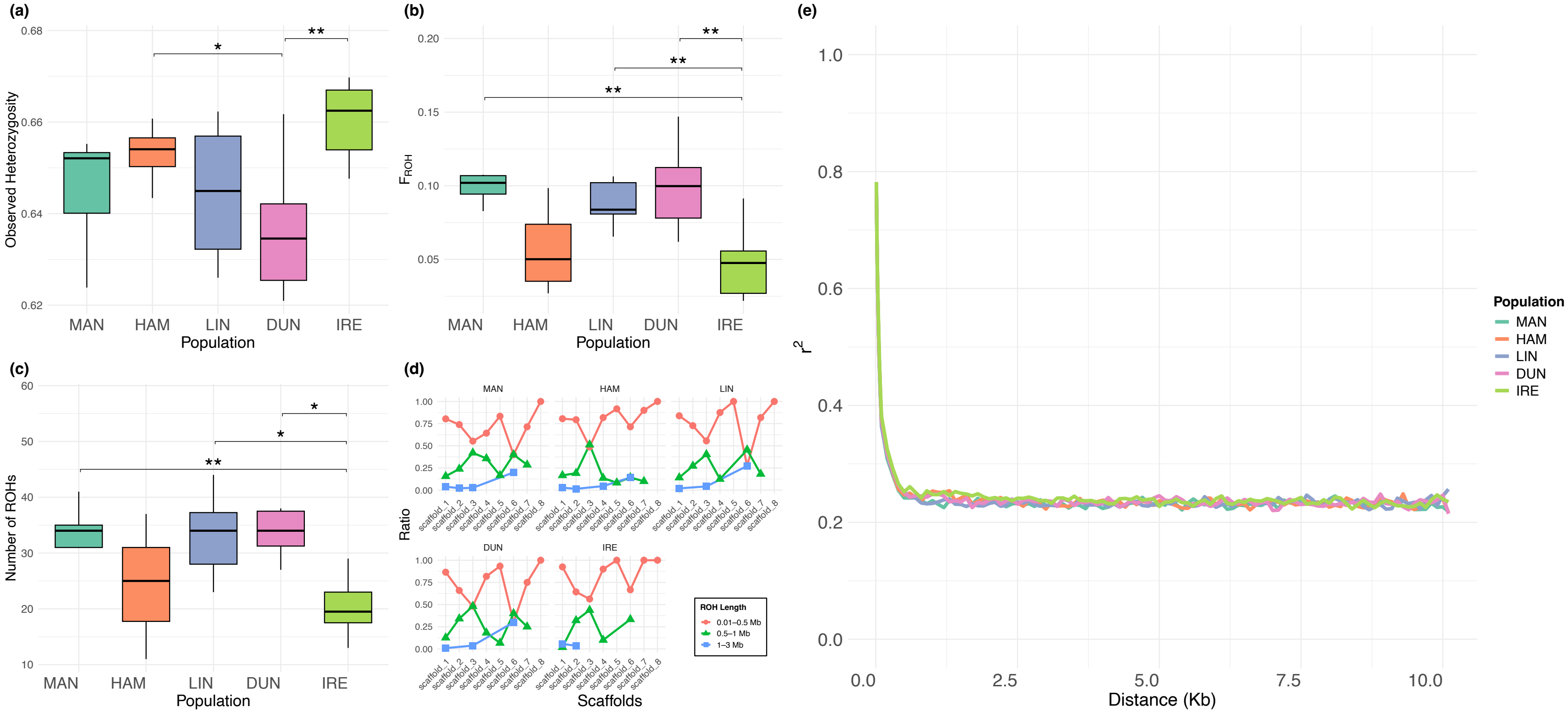

Figure 3.2 Heterozygosity, homozygosity, and linkage estimates across *M. aethiopoides* populations: **(a)** Observed heterozygosity per individual; **(b)** Genomic inbreeding coefficients (F_{ROH}); **(c)** Total number of runs of homozygosity (ROH) segments per individual; **(d)** Proportional distribution of ROH lengths across three size classes (0.001–0.5 Mb, 0.5–1 Mb, and 1–3 Mb) for each individual. Pairwise comparisons across populations were tested using the Wilcoxon rank-sum test with Bonferroni adjusted p-values. Significance levels are indicated as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, comparisons without asterisks are not statistically significant ($p \geq 0.05$); **(e)** Decay of linkage disequilibrium in the five *M. aethiopoides* populations. The plot shows the average r^2 values between SNP pairs as a function of physical distance (kb).

3.5 Discussion

Our analyses indicated the presence of two distinct New Zealand *M. aethiopoides* genetic clusters, with contemporary populations showing little suggestion of adaptation and diversification following initial release of the Irish strain. Additionally, genomic patterns of heterozygosity, homozygosity, linkage disequilibrium (LD), and branch length differences in the phylogenetic tree may indicate potential facultative sexuality in *M. aethiopoides*, which may have important implications on biocontrol potential in the longer term.

Population structure can arise due to a combination of environmental pressures and intrinsic genetic mechanisms, including variation in reproductive mode (Savolainen et al., 2013; Tvedte et al., 2019). In our study, *M. aethiopoides* exhibited two distinct genetic populations in New Zealand. The Hamilton population clustered closely with the historic Irish population, while the other contemporary populations formed a distinct genetic cluster. This pattern, along with the lack of strong evidence for spatial or temporal divergence in the selection analysis, suggests that environmental factors are not the primary driver of genetic structure. Instead, the observed genetic structure may reflect the distribution and establishment of different released *M. aethiopoides* haplotypes. According to McNeill et al., (2006), eight populations were originally sourced from Ireland, four of which (Crossnacreevy, Athenry, Oakpark, and Solohead) were introduced into New Zealand. These comprised two mitochondrial cytochrome oxidase I (COI) haplotypes (Phillips et al., 2006; Vink, 2012), which were released across the North and South Islands. Our data may suggest that one of these haplotypes dispersed more effectively than the other, particularly in the South Island. However, more extensive sampling across both islands is needed to confirm this pattern.

Additive genetic variance (the heritable component of genetic variation that fuels adaptation), is important for the success of biocontrol programs, as noted by several authors (Heimpel & Lundgren, 2000; Hopper et al., 1993; Stouthamer et al., 1992). Casanovas et al. (2018) further emphasised that additive genetic variance in a biocontrol agent should ideally exceed that of the target host to ensure effective long-term control. But, in asexual biocontrol agents, such variance is often limited due to low overall genetic diversity, thereby reducing adaptive potential and contributing to inconsistent outcomes compared to their sexual counterparts. Consistent with this, studies on other parasitoids have highlighted that populations with reduced genetic diversity are less capable of adapting to novel environments or evolving host populations (Hopper et al., 2019; Li et al., 2024; Phillips et al., 2008). This is concordant with our findings in *M. aethiopoides*, where low genetic diversity, weak population differentiation, and the absence of spatial or temporal divergence in selection analyses across all regional populations in New Zealand suggests minimal local adaptation. While there is currently no evidence of declining parasitism rates in *S. obsoletus*, the low genetic diversity of the *M. aethiopoides* populations in New Zealand underscores the importance of regular monitoring to detect any sustained reduction in biocontrol effectiveness. Strategically, any early indication of a breakdown in control could prompt targeted efforts to augment genetic diversity through renewed collections of the parasitoid from Ireland.

Genomic data provides a powerful framework for exploring reproductive modes by assessing patterns of homozygosity and heterozygosity across the genome - an approach that has been applied in several other species across multiple studies (Card et al., 2021; Freitas et al., 2023; Levine & Booth, 2025; Mozhaitseva et al., 2023; Sun et al., 2023). Previous studies on the Irish strain of *M. aethiopoides* showed the presence of conserved and actively expressed meiotic genes, along with detectable heterozygosity in the genome, suggesting that this species retains the potential for sexual reproduction (Inwood et al., 2023). In the current study, we observed similarly high heterozygosity in *M. aethiopoides* populations, suggesting that this species mostly reproduces via automixis with central fusion rather than terminal fusion (which would lead to a more rapid loss of heterozygosity) (Alavi et al., 2018; Card et al., 2021; Pearcy et al., 2006). Similar findings have been also observed in the ant *Cataglyphis cursor* (Fonscolombe), where central fusion results in a gradual decline in heterozygosity at rates consistent with theoretical expectations (Pearcy et al., 2006, 2011).

Phylogenetic relationships can reflect the underlying genetic topology of sexual and asexual populations: sexual species with active recombination typically form discrete clades with longer branch separations, while asexual populations more commonly exhibit shorter branches and less discrete structure due to reduced recombination (Tang et al., 2014). In this study, the phylogeny placed MAN, LIN, and DUN individuals in a close cluster with shorter branch lengths, whereas HAM and IRE formed separate clusters with longer branch lengths. This pattern likely represents distinct genetic lineages, however, due to the absence of known sexual populations in our dataset, the potential effects of recombination on the branch lengths of the phylogenetic tree cannot be confidently inferred.

Patterns of LD can provide further valuable insights into reproductive modes, as they reflect the extent of recombination and genetic reshuffling (usually driven by sexual reproduction) within a population (Hartfield et al., 2018). In particular, analysing LD decay patterns can help differentiate between obligate and facultative types of asexual reproduction (Freitas et al., 2023; Jaron et al., 2022). For example, in obligate clonal asexual populations, LD typically remains high ($r^2 \approx 1$) due to the complete absence of recombination, whereas in sexual populations, LD decays rapidly ($r^2 < 0.1$) (Freitas et al., 2023). The relatively low LD values we observed here, together with the high heterozygosity and short homozygous stretches, suggests that *M. aethiopoides* may exhibit facultative sexuality (whereby individuals can alternate between sexual and asexual reproduction depending on host population or environment cues), similar to that seen in some stick insects (Freitas et al., 2023; Jaron et al., 2022). Such patterns could be expected if LD breaks down rapidly (i.e., high LD decay, as observed here) via recombination (Freitas et al., 2023). Our findings align with the theoretical model by Kuhn et al. (2021), which shows that automictic reproduction, particularly when associated with high heterozygosity and low recombination, can mimic the genetic patterns typically attributed to sexual reproduction. This similarity makes it difficult to detect cryptic sex or transitions between reproductive modes with our current dataset.

To more definitively determine whether sexual reproduction occurs in the Irish strain, it will be necessary to compare both sexual and asexual populations using hybridisation experiments or machine learning-based genomic models (Kuhn et al., 2021; Levine & Booth, 2025; Sun et al., 2023). Such analyses will provide a powerful framework to detect cryptic sex (Freitas et al., 2023; Kuhn et al., 2021; Wachi et al., 2021), characterise the reproductive system, and assess its implications for the long-term effectiveness of *M. aethiopoides* as a biological control agent. Simulation-based genomic approaches (e.g., using tools like SLiM4; Haller & Messer, 2023) could also enable future studies to explore how different parasitoid asexual reproductive modes (such as automixis with central fusion, automixis with terminal fusion, gamete duplication, and apomixis), compare with sexual reproduction in shaping long-term adaptation alongside a sexually reproducing host under coevolutionary pressures. Similar approaches have been used recently to predict species invasiveness (e.g., Camus et al., 2024).

In the context of biological control, the reproductive dynamics of introduced parasitoid populations can be important for determining their long-term adaptability and effectiveness at both intra- and inter-specific levels. In New Zealand, the sexually reproducing Moroccan strain may be found co-occurring with the Irish strain of *M. aethiopoides* in pastures (McNeill et al., 2002). If the Irish strain retains the potential for sexual reproduction, as may be suggested by our data, then occasional gene flow with co-existing sexual strains could enhance its genetic diversity and potentially improve adaptive capacity (Halkett et al., 2008; Li et al., 2015; Schneider et al., 2003). However, such gene flow also poses risks for biocontrol, as laboratory studies have shown that inter-ecotype hybridisation can reduce host specificity and undermine the effectiveness of *M. aethiopoides* as a biological control agent (Goldson et al., 2003).

Alongside genetic diversity, gene flow, and reproduction are other factors that may contribute to biocontrol success. For example, environmental stress, including changes in temperature, can affect key life history traits, such as fecundity, lifespan, and development (Amat et al., 2006, 2017; Du et al., 2023; Tenguri et al., 2016; Wang & Smith, 1996). In the parasitoid wasp, *Venturi canescens* (Gravenhorst), sexually-reproducing (arrhenotokous) females exhibited greater behavioural flexibility under thermal stress, increasing their foraging activity in response to temperature drops, whereas asexual (thelytokous) females showed little behavioural change, suggesting enhanced thermal responsiveness in the sexual strain (Amat et al., 2006). In contrast, a study on the parasitoid wasp, *Diglyphus wani* Liu, Zhu & Yefremova, found that asexual strains outperformed sexual strains, exhibiting higher fecundity and reproductive rates, and thus potentially greater biocontrol effectiveness under stable environmental conditions (Du et al., 2023). Such studies provide valuable insights into how reproductive mode can influence ecological adaptability, highlighting the role of species-specific reproductive biology in shaping responses to environmental stresses and informing the effectiveness of biocontrol. Integrating molecular approaches, such as gene expression profiling via PCR and RNA sequencing, into such work could offer deeper insights into the underlying stress responses and adaptive potential of asexual and sexual lineages at the molecular level (Ding et al., 2021; Liu et al., 2020; Song et al., 2020; Xiong et al., 2024).

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Chapter 4

Comparative genomics reveals host-mediated genomic changes in *Microctonus aethiopoides* strains

Meeran Hussain[1], Elahe Parvizi[1], Mark R. McNeill[2], Ang McGaughran[1]

[1]Te Aka Mātuatua - School of Science, University of Waikato, Private Bag 3105, Hamilton, 3240, New Zealand

[2] Bioeconomy Science Institute, Tuhiraki, Christchurch, New Zealand

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4.1 Abstract

Parasitoids possess a life cycle that closely relies on its host. In classical biological control, on during the introduction to new environments, such associations create opportunities for divergent selection with respect to host and/or environment. The parasitoid *Microctonus aethiopoides* serves as a valuable model for investigating divergent selection, as its use as a biocontrol agent against weevils of the genera *Hypera* and *Sitona* has revealed clear intraspecific variation largely driven by adaptation to the different host taxa.

This study examined *M. aethiopoides* individuals reared from different host species (*Hypera postica*, *Sitona discoideus*, *Sitona obsoletus*) and originating from distinct geographic regions. We generated and compared eight high-quality genome assemblies of *M. aethiopoides* strains with each other, and with two publicly available genomes.

Comparative analyses revealed a strong host-associated phylogenetic structure separating the *H. postica* associated lineage from those associated with *Sitona* species. This separation was accompanied by distinct heterozygosity patterns, with the *H. postica* lineage showing the highest heterozygosity, followed by *S. discoideus* and *S. obsoletus*, and the lowest heterozygosity observed in the Irish lineage, most likely due to its asexual nature (unique among the tested strains). Further comparisons indicated that most gene families were conserved across strains, with rapid evolution of transposable element-related genes suggesting ongoing genome restructuring. Chemosensory gene family classification showed broad conservation of these genes across strains, with modest variation in odourant receptor copy numbers and an expansion of this gene family in the post-release New Zealand strain

(MaNZI) compared to its initial release strain (MaIRE) - a pattern that may reflect fine-tuning to local host cues following release.

Overall, our study highlights that host associations are key drivers of divergence within *M. aethiopoides*, with adaptation primarily driven by genome-level variation and limited evidence for major changes at the gene family level.

4.2 Introduction

Classical biological control is a long-standing strategy in integrated pest management, involving the deliberate introduction of natural enemies called biocontrol agents to suppress invasive pest populations (Heimpel & Cock, 2018; Wyckhuys et al., 2024). The nature of the process is grounded in the assumption that an introduced agent's host specificity and performance remain consistent with the targeted host, however, introduced populations are exposed to varying environmental conditions, alternative hosts, and fluctuating host densities that impose distinct selective pressures - driving population-level divergence over time (Wright & Bennett, 2018; Zepeda-Paulo et al., 2013). Divergent natural selection can promote local adaptation, conferring greater fitness on populations within their natal environment compared to alternative environments (Kawecki & Ebert, 2004; Nosil et al., 2009). Such selection reshapes the genome, favouring locally advantageous genes and contributing to genome-wide divergence among populations (Ebert & Fields, 2020; Hartmann et al., 2018; Nosil et al., 2009; Via, 2012).

Parasitoids are examplar biocontrol agents that exhibit a distinctive lifestyle characterised by their dependence on host insects for survival and reproduction, in which females locate and lay their eggs on or within a living host. Such dependence on insect hosts, combined with exposure to different environmental conditions, creates strong opportunities for divergent selection, driving ecological specialisation and genetic differentiation among populations that exploit different hosts (Feder & Forbes, 2010; Stireman et al., 2006; Tilmon, 2008). While host race formation and ecological speciation have been widely documented in herbivorous insects (Funk, 2010; Knolhoff & Heckel, 2014; Michielini et al., 2024), host associated differentiation is also recognised in other insects, including parasitoids (Häner et al., 2024; Koppik et al., 2015; Phillips et al., 2008; Shaw, 1988; Zaviezo et al., 2021).

In parasitoid associations, different hosts can act as potent selective agents - influencing both behavioural traits linked to host recognition and oviposition, and physiological compatibility with host defences - thereby generating pressure for adaptations finely tuned to specific hosts (Antolin et al., 2006; Mackauer et al., 1996). Adaptive pressure is also observed within parasitoid species, where genetically distinct lines can show differences in traits like host preference/specificity, fecundity, life history strategies, and learning behaviour; and there is growing evidence of associated impacts on biological control (Goldson et al., 2003, 2005; Häner et al., 2024; Koppik et al., 2015; Zaviezo et al., 2021). Thus, local adaptation of parasitoids driven by both host and geography can influence the overall host-parasitoid interaction, as well as their successful establishment and persistence in new environments (Phillips et al., 2008). Overlooking this variation risks underestimating non-target impacts or selecting biocontrol release strains with poor establishment potential.

Microctonus aethiopoides (Hymenoptera: Braconidae) is an important endoparasitoid that attacks the adult stage of *Hypera* and *Sitona* weevil species (Aeschlimann, 1983; Loan, 1975; Loan & Holdaway, 1961; Shaw, 1988). Native to Europe and North African regions, *M. aethiopoides* has been successfully used in classical biological control programs against agricultural pests in North America (Loan, 1969; Radcliffe & Flanders, 1998; Rand et al., 2018), Australia, and New Zealand (Goldson et al., 2004; Vink et al., 2003). In New Zealand, the Moroccan strain was introduced in 1982 to control *Sitona discoideus* Gyllenhal (lucerne weevil), a major pest of lucerne (*Medicago sativa* L.) (Stufkens et al., 1987), and an Irish strain was introduced in 2006 to control *Sitona obsoletus* Gmelin (clover root weevil) (formerly *S. lepidus)*, a major pest of white clover (*Trifolium repens* L.) (Gerard et al., 2006; McNeill et al., 2006).

Among the different *M. aethiopoides* strains, intraspecific variation has been shown to arise through host specificity (Barratt et al., 1997; Ferguson et al., 2024; Sundaralingam et al., 2001), while individual strains also show differences in morphology (Adler & Kim, 1985; Aeschlimann, 1983; Loan, 1975), development rate (Aeschlimann, 1983), and mating preferences (Goldson et al., 2003; Sundaralingam et al., 2001). These trait differences are supported by phylogenetic studies, which confirm clear genetic differences between *M. aethiopoides* populations associated with host taxa (Phillips et al., 2008; Vink et al., 2003).

Gene family analysis of parasitoids has to date highlighted the role of chemosensory genes in influencing behaviour during foraging, mating, and oviposition (Fleischer et al., 2018; Sheng et al., 2017; Wang et al., 2015; Zhao et al., 2016). These genes enable the parasitoid to recognise specific chemical cues that distinguish the correct host from others, allowing a high degree of host specificity (Wang et al., 2015; X. Zhu et al., 2024). Some of the major classes of chemosensory genes include odourant receptors (ORs), ionotropic receptors (IRs), odourant-binding proteins (OBPs), chemosensory proteins (CSPs), gustatory receptors (GRs), and sensory neuron membrane proteins (SNMPs). Among these, ORs play a major role in the recognition of host-associated cues, enabling the detection of a wide range of volatile chemicals, including pheromones and general odours (Pelosi et al., 2018; Schmidt & Benton, 2020; Yin et al., 2025). Variation in these chemosensory gene families are often associated with host range expansion, ecological specialisation, and divergent selection, reflecting their central role in mediating host recognition and adaptation in parasitoids.

In this study, we examined ten strains of *M. aethiopoides*. These include strains reared from three different host species *H. postica* (L. Gyllenhal), *S. discoideus*, and *S. obsoletus*. Within the strain parasitising *S. obsoletus*, six distinct geographic lines are considered (commonly referred to as biotypes in the literature). Although the terms “strain” and “biotype” are sometimes used interchangeably in published studies, this thesis uses the term *strain* consistently to describe each distinct lineage, whether it originates from a different host species or represents one of the six geographic lines associated with the clover root weevil (*S. obsoletus*). As well as differences in host specificity, these individual strains differed in reproductive modes, with eight and two sexual (arrhenotokous) and asexual (thelytokous) strains, respectively. We hypothesised that the different strains would show genomic variation and that there would be differences in gene families among strains that would associate with their host use and/or reproductive mode.

4.3 Materials and methods

4.3.1 Samples

This study included ten strains of *M. aethiopoides*, representing a broad geographic range and environmental conditions. Strains from the USA (MaUSA), Wales (MaWAL), Scotland (MaSCO), England (MaENG), Norway (MaNOR), Romania (MaROM), and Ireland (MaIRE) (Fig. 4.1) were sourced from the Lincoln Research Facility in New Zealand. Individuals from these strains were diploid females, except for the Romanian sample, which was a haploid male (due to the unavailability of female individuals from that population). In addition, a New Zealand-released Irish strain (MaNZI) was collected in 2024 from Dunedin, following the collection protocol outlined in Appendix 2. For each of these eight strains, a single individual was used for DNA extraction and whole genome sequencing. Two further *M. aethiopoides* strains from France (MaFRN) (GCA_030272935.1) and Morocco (MaMOR) (GCA_030272655.1), and an outgroup species (*Microctonus hyperodae;* Mhyp) (GCA_030347285.1) were downloaded from NCBI for inclusion in our analyses.

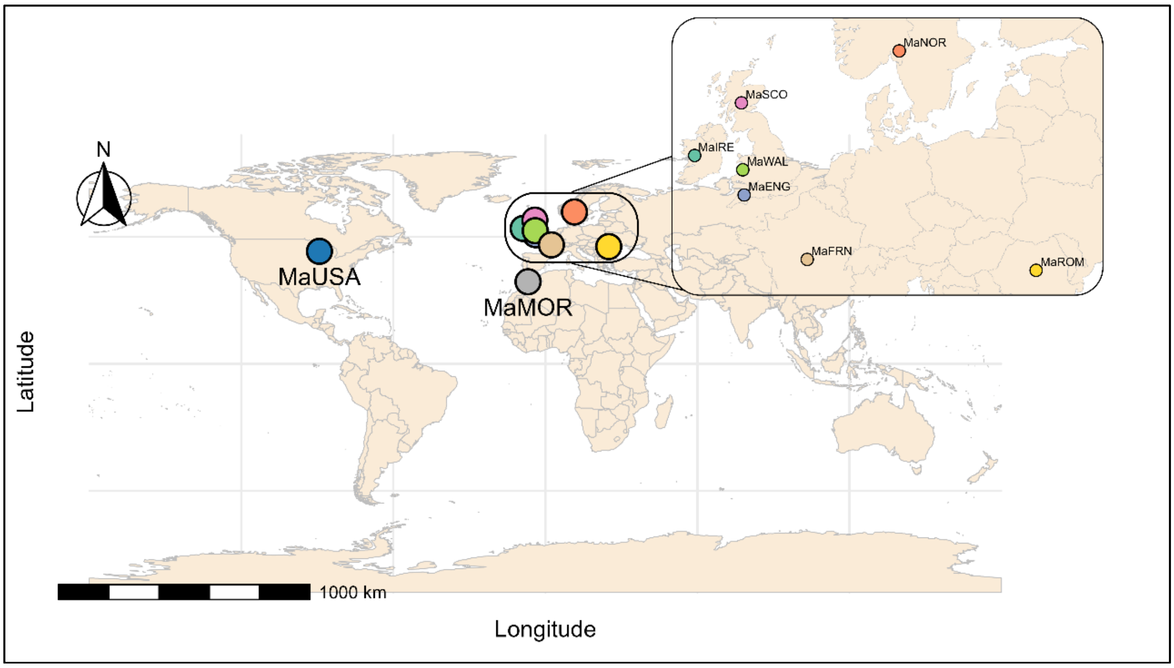

Figure 4.1 Geographic origin of all *Microctonus aethiopoides* strains used in this study. The map shows the collection or reference locations of eight strains: MaUSA (United States), MaENG (England), MaSCO (Scotland), MaWAL (Wales), MaNOR (Norway), MaFRN (France), MaROM (Romania), and MaMOR (Morocco). In addition, the New Zealand released Irish strain (MaNZI) was used for post release comparison with MaIRE.

4.3.2 Laboratory procedures and sequencing

Prior to DNA extraction, parasitoid samples were rinsed in deionised distilled water. Each sample was then flash-frozen in liquid nitrogen and homogenised using a sterile plastic pestle to maximise DNA yield. Genomic DNA was extracted from whole-body homogenates using a column-based proteinase K digestion protocol (DNeasy Blood & Tissue Kit, Qiagen) following the manufacturer's instructions. The nucleic acid purity (OD260/280 and OD260/230), concentration, and absorption peak were evaluated by Nanodrop. Quantification of extracted DNA was performed using a Qubit dsDNA High Sensitivity (HS) Assay Kit (Invitrogen) and Qubit Fluorometer.

For short-read sequencing, a minimum of 50 ng of DNA was allocated per sample and libraries were prepared using the Illumina DNA Prep kit and sequenced as paired-end reads (2 × 150 bp) on an Illumina NovaSeq platform at the Livestock Improvement Corporation Genomics Facility, Hamilton, New Zealand. The remaining DNA was then concentrated for long-read sequencing using a custom in-house SPRI bead mix prepared with Sera-Mag™ Magnetic SpeedBeads™, following the standard SPRI bead mix protocol (Jolivet & Foley, 2020), yielding a total final volume of 30 µL. The concentrated DNA was sequenced on an Oxford Nanopore Technologies PromethION platform, with libraries generated using DNA Ligation Sequencing Kits (SQK-LSK114) and R10.4.1 flow cells at Bragato Research Institute, Lincoln, New Zealand.

4.3.3 Bioinformatics processing

For the Illumina dataset, read quality was assessed using FastQC (v0.12.1) (Andrews, 2017). Quality trimming was performed using Trim Galore (v0.6.10) (Andrews et al., 2015), removing bases with Phred scores below 20 to ensure high confidence reads for genome polishing and downstream applications.

Raw Oxford Nanopore Technologies (ONT) sequencing data were received in POD5 format for each library. Base-calling was performed using Dorado (v0.5.0) (https://github.com/nanoporetech/dorado) with the simplex super accuracy (sup) model, generating a single BAM file per library. Sample de-multiplexing was conducted using Dorado's demux module, which uses the "alias" column in the sample sheet to assign reads to individual samples, producing unmapped BAM files for each. These unmapped BAM files were converted to FASTQ format using SAMtools fastq (v1.22) (Danecek et al., 2021). For the 8 samples (MaUSA, MaMOR, MaFRN, MaENG, MaSCO, MaWAL, MaIRE &

MaNZI) that were sequenced across two libraries, the corresponding FASTQ files were concatenated to generate a single file per sample for downstream analysis. Quality assessment of ONT reads was carried out using NanoPlot (v1.43) (De Coster & Rademakers, 2023). Read filtering was performed with Chopper (v0.9) (De Coster & Rademakers, 2023), applying a minimum quality score threshold (-q 10), a minimum read length of 500 bp (-l 500), and the --headcrop 10 parameter to remove the first 10 bases from each read, which are typically associated with lower sequencing accuracy.

De novo assembly of ONT reads was performed using Flye (v2.9.1) (Kolmogorov et al., 2019) with the --nano-hq parameter to specify high-quality reads. The estimated genome size was set to 129 Mb using the -g flag, based on previously published genome assemblies of related strains available online (Inwood et al., 2023). Initial polishing of the assembly was carried out using Medaka (v1.11.1) (https://github.com/nanoporetech/medaka), which employed the medaka_consensus module to align ONT reads back to the draft assembly and generate a consensus sequence. To remove redundant haplotigs and improve assembly contiguity, Purge_dups (v1.2.6) (Guan et al., 2020) was used.

Subsequent base-level correction was performed using high-quality Illumina short reads with NextPolish (v0.2.1) (Hu et al., 2020), which aligns Illumina reads to the assembly to further improve consensus accuracy. Further refinement of the polished assemblies was performed using RagTag (v2.1.0) (Alonge et al., 2022) with "scaffold" function to scaffold contigs and improve assembly contiguity.

4.3.4 Quality control

To comprehensively evaluate genome assembly quality, multiple tools were used. Merqury (v1.3) (Rhie et al., 2020) was used to assess assembly accuracy and completeness based on k-mer spectra derived from Illumina short reads. Assembly accuracy and structural completeness were further assessed using QUAST (v5.2.0) (Gurevich et al., 2013), which provides summary statistics such as N50, number of contigs, and genome size estimates. To screen for potential contaminants in the assembled genomes, each *M. aethiopoides* assembly was queried against the NCBI non-redundant nucleotide (nt) database using BLAST (v2.13.0) (McGinnis & Madden, 2004) (blastn -task megablast) with an e-value threshold of 1e^{-3} and output format including taxonomic and descriptive information. Finally, genome completeness was assessed using Compleasm (v0.2.7) (Huang & Li, 2023), a fast and accurate implementation of the BUSCO framework, which identifies the presence of conserved single copy orthologues to evaluate the completeness of the assembly.

4.3.5 Genome structural and functional annotation

To annotate gene models, repetitive elements in the genome were first identified using EDTA (v2.1.0) (Ou et al., 2019), which performs de novo transposable element (TE) discovery and classification. Due to input requirements of EDTA, contig identifiers in the assembly FASTA file were renamed to a simplified sequential format. The resulting TE library was then used to mask the genome using RepeatMasker (v4.1.0) (Chen, 2004), with the -lib and -xsmall options used to apply soft-masking and retain custom repeat annotations.

Gene prediction was done on the repeat-masked genomes using BRAKER3 (v3.0.8) (Gabriel et al., 2023), incorporating both ab initio prediction and protein homology-based evidence. The protein dataset used for homology support was a curated combination of UniProt-SwissProt and OrthoDB Arthropoda sequences. BRAKER3 was executed with "--AUGUSTUS_ab_initio" and "--crf" flags enabled to improve gene model prediction using conditional random fields and AUGUSTUS-based training. The final output included high-quality gff3-formatted gene annotations and corresponding protein sequences.

Functional annotation of predicted proteins was conducted using EggNOG-mapper (v2.1.12) (Cantalapiedra et al., 2021) for orthology-based inference of functional terms, with assigned Gene Ontology (GO) terms, KEGG pathways, COG categories, and PFAM domains integrated into the gff3 file using the --decorate_gff option. Additionally, InterProScan (v5.66) (Jones et al., 2014) was used to annotate the proteins across multiple protein signature databases including PFAM, SMART, PrositeProfiles, Gene3D, SUPERFAMILY, and PANTHER, with GO term and pathway annotations enabled.

4.3.6 Processing NCBI assemblies

To maintain consistency in comparison across datasets, the contig-level NCBI-published genomes (samples MaFRN and MaMOR) were scaffolded using RagTag (v2.1.0). Additionally, the *M. hyperodae* (sample Mhyp) genome was annotated for gene prediction following the BRAKER3 (v3.0.8) pipeline described above.

4.3.7 Genome erosion

To understand genome erosion among the *M. aethiopoides* strains (excluding MaROM, a haploid male from Romania), two SNP-based analyses were performed: (i) heterozygosity using VCFtools (v0.1.15) (Danecek et al., 2011); and (ii) runs of homozygosity (ROH) using PLINK (v1.09) (Purcell et al., 2007). The workflow used short-read sequences from all strains aligned to a single reference genome; here we chose the New Zealand sample (MaNZI) assembly to enable consistent SNP-based comparisons. Paired-end Illumina reads were aligned to the MaNZI reference with BWA-MEM (v0.7.18) (Li, 2013), then converted, sorted, and indexed with SAMtools (v1.22) (Danecek et al., 2021) for use in downstream SNP analyses. VCFtools was used to retain biallelic SNPs (--remove-indels --min-alleles 2 --max-alleles 2) with a 90% missingness filter (--max-missing 0.9), and to estimate individual heterozygosity (--het). For ROH analysis, the VCF file was converted to PLINK format and then ROHs were called using PLINK with --homozyg along with following parameters: --homozyg-window-snp 50, --homozyg-window-het 1, --homozyg-snp 50, --homozyg-kb 50, --homozyg-density 40, --homozyg-gap 100.

4.3.8 Transposable elements

Transposable elements (TEs) were identified and classified in each *M. aethiopoides* strain using RepeatMasker with the EDTA generated species-specific TE library as input. RepeatMasker was run with “rmblast” as the search engine and sensitive mode enabled (-s), excluding low-complexity/simple repeats (-nolow), and alignments were recorded for downstream analyses (-a). To estimate the age distribution of TE insertions, Kimura divergence values were calculated from the RepeatMasker “.align” files using the calcDivergenceFromAlign.pl script (Chen, 2004), with the -s option enabled to generate summary statistics. The divergence outputs were then parsed to produce kimura distance profiles, which serve as a proxy for the relative age of TE insertions, and the resulting data were used to construct TE landscape plots for in R following the approach discussed in the EDTA GitHub repository (https://github.com/oushujun/EDTA/issues/92).

4.3.9 Orthogroups and phylogeny

OrthoFinder (v2.5.2) (Emms & Kelly, 2019) was used to identify orthologous and paralogous gene families for all ten strains. Prior to analysis, proteome files generated from BRAKER3 annotations were pre-processed to retain only the longest isoform per gene. This was achieved using custom bash scripts that parsed the protein FASTA files, compared transcript lengths based on gene and transcript IDs, and retained the longest representative sequence for each gene. OrthoFinder was run in multiple-sequence-alignment mode (-M msa), using MAFFT (v7.505) (Katoh & Standley, 2013) for alignments. For phylogenetic inference, IQ-TREE (v2.2.2.2) (Minh et al., 2020) was executed on the concatenated species-tree alignment built using single copy orthologous sequences in amino-acid mode (-

st AA) with model selection (-m MFP), 1,000 ultrafast bootstrap replicates (-B 1000), SH-aLRT support (--alrt 1000), and branch-length NNI optimisation (-bnni).

4.3.10 Gene family expansion and contraction

CAFE (v5.0) (Mendes et al., 2021) was used to analyse gene family expansions and contractions. Preparation of input files from OrthoFinder was required prior to the analysis. An ultrametric species tree was generated using the chronos() function from the R package ape (v5.7.1) (Paradis et al., 2004), applying a relaxed clock model (model = "correlated") with a smoothing parameter (lambda = 1). The tree was calibrated to absolute time by scaling branch lengths so that the root-to-tip distance corresponded to the estimated divergence time of 17 million years between *M. aethiopoides* and *M. hyperodae* (Inwood et al., 2023). Further, the orthogroup count matrix from OrthoFinder was reformatted by removing the final summary column, adding a description field to the header, and generating a new input file compatible with CAFE requirements. This file was then filtered using associated Python scripts (cafetutorial_clade_and_size_filter.py)(https://github.com/hahnlab/cafe_tutorial/tree/main/python_scripts) to exclude clades with very few gene families and to remove orthogroups with extremely large gene counts, ensuring that the dataset was properly scaled and suitable for reliable estimation of gene family expansions and contractions.

CAFE analyses were performed using the prepared input gene family count table and calibrated ultrametric species tree. A custom script was used to automate the workflow, which included the following steps: (i) run CAFE across a range of birth-death rate parameter values (k = 1-6) with two independent replicates each; (ii) collect and compare log-likelihood values from replicate runs to assess convergence; (iii) select the optimal k value based on the best likelihood score; (iv) extract significant orthogroups from the family results file at a threshold of $P \leq 0.05$. Furthermore, branch-specific significance of gene family change was extracted from the CAFE gamma branch probabilities output file, which reports per-branch probabilities for each orthogroup. For each branch (terminal and internal), the output table was parsed column-wise and a set of gene families with probability ≤ 0.05 was compiled. The resulting per-branch family lists were used to summarise counts of significant expansions/contractions across the phylogeny and identify branch-specific candidate families for downstream GO enrichment analysis. All rapidly expanded and contracted gene families were annotated using EggNOG and InterProScan as described above.

4.3.11 Classification of chemosensory gene families

Chemosensory gene families for all strains were classified from each longest-isoform proteome using a BLAST (v2.13.0) and HMMER (v3.4) (Finn et al., 2011) based workflow with stringent multi-step filtering. Chosen families included odourant/pheromone binding proteins (OBPs/PBPs), odourant receptors (ORs), ionotropic receptors (IRs), chemosensory proteins (CSPs), sensory neuron membrane proteins (SNMPs) and gustatory receptors (GRs).The classification protocol involved: (i) blasting the proteome using BLASTP (v. 2.16.0) against a family-specific Hymenoptera protein set that was chosen from PFAM and NCBI databases; (ii) retaining hits only if they accumulated ≥ 50 total BLAST hits followed by percent identity ≥ 50, bitscore > 100, and e-value ≤ $1e^{-5}$; (iii) validating filtered candidates by HMMER (v3.4) using hmmsearch against the relevant PFAM HMM(s) for that family (PF01395, PBP_GOBP for OBPs/PBPs; PF03392, OS-DN/chemosensory protein for CSPs; PF02949, 7tm_6 for ORs; PF08395, 7tm_7 for GRs; PF01130, CD36 for SNMPs; PF00060, Lig_chan for IRs), keeping only sequences with domain hit i-e-values ≤ $1e^{-3}$; (iv) clustering sequences with CD-HIT (v.4.8.1) (Fu et al., 2012) at 98% identity, and applying length filters (specifically to ORs > 300 aa) to separate likely full-length proteins from short fragments.

4.3.12 GO enrichment analysis

All GO enrichment analyses were performed using the topGO package in R (Alexa A, 2024). Enrichment was assessed separately for the three ontology categories (Biological Process, Molecular Function, Cellular Component) using Fisher’s exact test with the “weight01” algorithm. In the gene family expansion and contraction analysis, significantly enriched terms were visualised as dot plots summarising the top categories based on gene ratio and significance.

4.4 Results

4.4.1 Genome profiling

Prior to assembling the genome of *M. aethiopoides*, genome profiling based on all Illumina data (9.9-16.0 Gb per strain; Table A4.1) resulted in estimated genome sizes across the eight strains of ~121.6 Mb to ~187.8 Mb (Fig. A4.1).

Post-filtering ONT data ranged from 2.4 Gb to 12.2 Gb (Table A4.2). The initial genome assembly contig numbers for each strain ranged from 237 to 1,408 and (Inwood et al., 2023), from 74 to 435 scaffolds post-scaffolding, with final genome sizes ranging from 130.5 to 134.1 Mb (Inwood et al., 2023), and the highest and lowest genome size observed in MaNOR and MaIRE, respectively (Table 4.1). Scaffold N50 values were consistently high across strains (23.7-24.5 Mb), each reaching an L50 of only three scaffolds (Table 4.1) and Nx plots showed that all assemblies had broadly similar contiguity profiles (Fig. A4.2). GC content was highly conserved, averaging ~29.3% across all strains (Table 4.1), while GC distribution curves showed similar unimodal peaks, suggesting no major biases in assembly composition (Fig. A4.3). BUSCO analysis using the hymenoptera_odb10 dataset indicated strong completeness across all assemblies, with high single-copy BUSCO scores between 96.1-96.5%, low duplication (< 0.8%) and fragmentation (< 0.3%), and minimal missing scores (2.6-2.9%) (Fig. 4.2). An extended description of the genome assembly protocol and its interpretation is provided in Appendix 5.

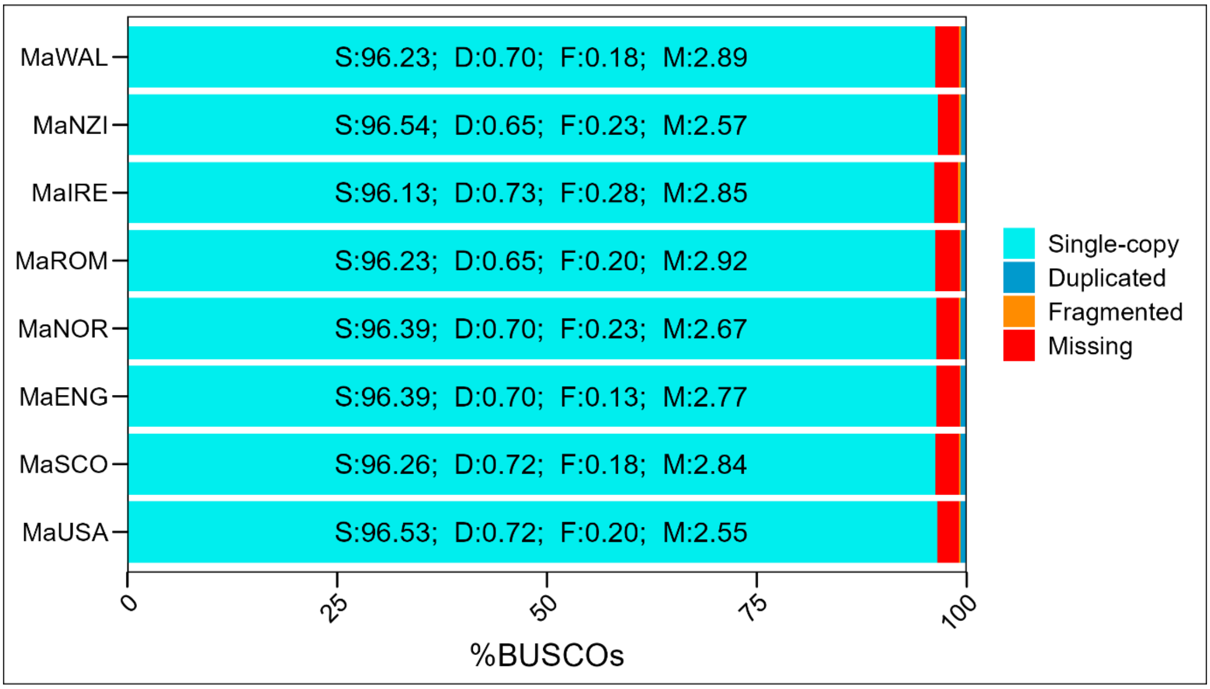

Figure 4.2 BUSCO completeness assessment of *Microctonus aethiopoides* genome assemblies generated in this study. The plot shows the percentage of BUSCOs identified as single-copy, duplicated, fragmented, or missing across all strains.

The previously published assemblies MaFRN and MaMOR contained 1,946 and 2,294 scaffolds, respectively after scaffolding, with GC contents of 29.3% and 29.4% (Table A4.3). Their GC distribution profiles and Nx plots were similar to the other assemblies (Figs. A4.2-A4.3).

Repeat annotation showed that repetitive elements comprised 23.7–25.4% of the genome across all eight *M. aethiopoides* strains assembled in this study, with little variation among assemblies (Table 4.1). However, the repeat content of the published assemblies for MaFRN and MaMOR were each 17.3%, possibly reflecting the inability of short-read assemblies to accurately capture repetitive regions (Cechova, 2020; Inwood et al., 2023). These two strains were thus excluded from TE classification.

Gene prediction identified 15,154-15,975 protein coding genes across strains, and from 13,737-14,430 when retaining only the longest genes (Table 4.1). Additionally, annotating the published genomes including Mhyp, yielded 15,469, 16,060, and 14,321 protein-coding genes (MaFRN, MaMOR, Mhyp, respectively). After retaining only the longest isoform per gene, these counts were 13,980, 14,491, and 12,706, respectively.

Table 4.1 Summary of genome assembly statistics for *Microctonus aethiopoides* strains.

Parameters	**MaUSA**	**MaSCO**	**MaENG**	**MaNOR**	**MaROM**	**MaIRE**	**MaNZI**	**MaWAL**
Number of contigs	736	826	237	395	238	1408	544	770
Longest contig (bp)	5487558	6384305	11650733	5723270	6860472	1627676	8792901	5726698
Contig N50 size (bp)	1498953	2021625	2621563	1972003	3091720	419567	2862459	1626547
Contig L50	26	18	15	23	16	91	16	20
Number of scaffolds	307	339	89	153	74	435	231	314
Genome size (bp)	133349684	131157906	132739510	134153477	132146165	130500875	131930144	130816869
Longest scaffold (bp)	30009381	29707988	30557105	30886853	30916849	29408197	29470290	29450121
Scaffolds N50 size (bp)	24119476	23673627	24453707	24023478	23764779	23707169	23977460	23943118
Scaffolds L50	3	3	3	3	3	3	3	3
GC content	29.37	29.37	29.39	29.4	29.37	29.31	29.36	29.33
Repeat percentage (%)	24.67	23.75	25.14	25.4	24.88	23.69	24.7	24.15
Number of protein coding genes	15,620	15,477	15,210	15,975	15,154	15,298	15,359	15,304
Number of protein coding genes (longest isoforms)	14,122	14,075	13,803	14,430	13,737	13835	13,881	13,852

4.4.2 Genome erosion

Observed heterozygosity varied among *M. aethiopoides* strains (Table 4.2). The highest heterozygosity was detected in MaUSA (Ho = 0.346) and MaMOR (Ho = 0.210), whereas MaIRE (Ho = 0.028) and MaNZI (Ho = 0.027) showed the lowest values, consistent with respect to their asexual reproductive mode (Table 4.2). ROH patterns were consistent with heterozygosity results (Fig. A4.4) in those strains with low heterozygosity exhibited more homozygous segments (longer ROHs; i.e., higher homozygosity), while those with elevated heterozygosity displayed shorter ROHs (Fig. S8) (Table 4.2).

Table 4.2 SNP-based heterozygosity estimates across *Microctonus aethiopoides* strains. The table shows observed homozygous sites (O[HOM]), total sites analysed (N_SITES), observed heterozygous sites (O[HET] = N_SITES- O[HOM]), and observed heterozygosity (Ho= O[HET]/N_SITES)

SampleID	O(HOM)	O(HET)	N_SITES	Ho (Observed Heterozygosity)
MaUSA	1618335	856069	2474404	0.345969777
MaMOR	1955288	519116	2474404	0.209794359
MaFRN	2243919	230485	2474404	0.093147683
MaSCO	2255110	219294	2474404	0.088624978
MaENG	2283945	190459	2474404	0.076971667
MaNOR	2335299	139105	2474404	0.056217578
MaIRE	2404655	69749	2474404	0.028188202
MaNZI	2405979	68425	2474404	0.027653124
MaWAL	2290300	184104	2474404	0.074403371

4.4.3 Transposable elements

Differences in genome size across strains produced in this study were largely attributable to transposable elements (TEs) (Table A4.4). Total TE content ranged from 16.88–19.61% (Table A4.4), which falls within the reported maximum TE proportion of 26.6% for Braconidae genomes (Behm & Sharanowski, 2025). The lowest proportion of TEs was observed in MaIRE (22 Mb) and the highest in MaNOR (26.3 Mb) (Table A4.4). Comparing the Irish-derived MaNZI strain to MaIRE, the former showed almost 1.44% higher TE content, with this increase largely driven by retrotransposons (Table A4.4). When considering reproductive modes, no major difference in total TE content was observed between sexual and asexual strains (Table A4.4).

Kimura-adjusted TE landscapes were broadly left-skewed, with the highest density at low divergence ($K \approx 0$-5), indicating recent TE activity (Fig. A4.5). MaNZI displayed a higher peak in the very low-divergence bins ($K < 5$) compared to MaIRE, driven particularly by LTR elements (Fig. A4.5), consistent with the observed increase in size of retrotransposons in MaNZI (Table A4.4).

4.4.4 Orthogroups and phylogeny

Orthofinder clustered 151,357 of 152,912 predicted proteins (99.0%) into 13,634 gene families, leaving 1,555 proteins (1.0%) unassigned. Among assigned proteins, 8,683 were identified as single-copy orthologous gene families present across all genomes, representing the conserved core gene set. A well-supported (bootstrap values > 95%) phylogeny built using these single copy gene families and rooted with *M. hyperodae* placed MaUSA (collected from *H. postica* host) as a separate lineage from the remaining European and Moroccan strains (all obtained from *Sitona* (hosts) (Fig. 4.3). Within this *Sitona*-host clade, MaMOR paired with MaFRN, consistent with reported inter-strain mating preferences (Goldson et al., 2003; Sundaralingam et al., 2001). MaIRE and MaNZI formed a sister pair of asexual strains, whereas MaSCO, MaENG, and MaWAL clustered together (Fig. 4.3).

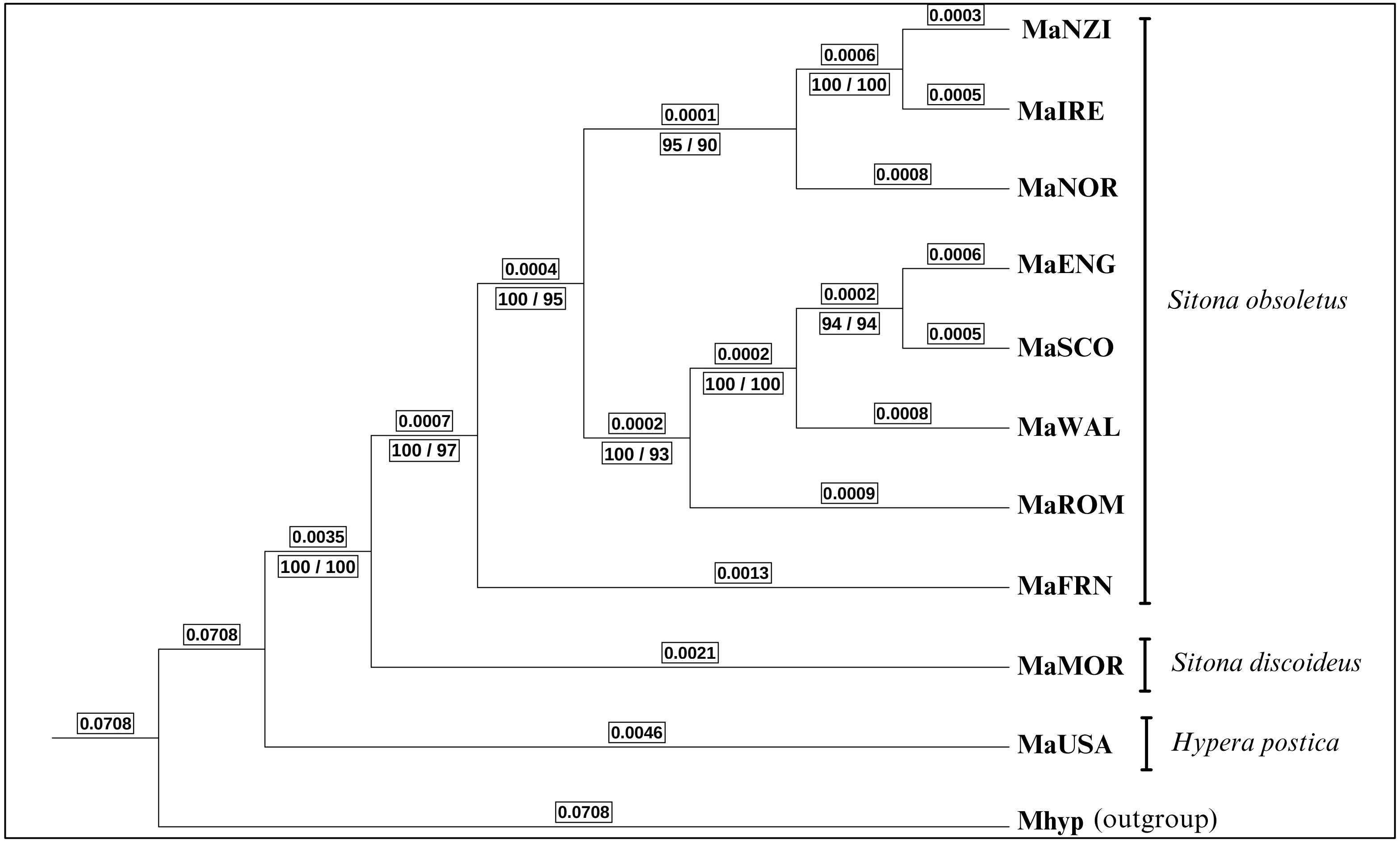

Figure 4.3 Phylogenetic tree built using the maximum-likelihood (ML) method from concatenated alignments of conserved single-copy orthologues, representing relationships among *Microctonus aethiopoides* strains. Node labels represent bootstrap and SH-aLRT support values, and branch lengths indicate substitutions per site. The tree is rooted with *Microctonus hyperodae* (Mhyp) as an outgroup, and the relevant host-associations are indicated on the right-hand side of the figure.

Examining shared orthogroups across all taxa (*M. aethiopoides* strains and *M. hyperodae*) 9,674 shared orthogroups represented broader *Microctonus* core gene families (Fig 4.4a). In addition, 745 orthogroups were shared by all *M. aethiopoides* strains but absent from *M. hyperodae* (Fig. 4.4a). GO enrichment of these 745 *M. aethiopoides* specific orthogroups highlighted chemosensory and defence functions (e.g., olfactory/sweet taste receptor activity, sensory perception of smell/taste, odourant binding, neuronal cell body), alongside terms linked to intracellular trafficking (trans-Golgi network vesicles, SCF ubiquitin ligase complex) (Table A4.5). A distinct set of 130 orthogroups was shared across *Sitona*-associated *M. aethiopoides* (Fig. 4.4a), with GO enrichment analysis indicating significant terms related to chromatin remodeling and transcriptional regulation (e.g., nucleosome disassembly, regulation of chromatin organisation, transcription elongation by RNA polymerase II, nucleosome binding, and ribonuclease complexes) for these (Table A4.6). MaFRN and MaMOR shared 127 orthogroups, consistent with their phylogenetic closeness (Figs. 4.3, 4.4a). GO enrichment of these clade-specific orthogroups highlighted nucleic-acid and metabolite interactions (thymine binding, uracil binding, dihydropyrimidinase activity), protein–protein interaction capacities (filamin binding, translation-elongation factor binding), enzymatic/cellular features (chitinase activity, transcription export complex 2, retrotransposon nucleocapsid), cytoskeletal assemblies (filamentous actin structures), and membrane-associated compartments (junctional membrane complex, postsynaptic cytosol, plasma membrane) (Table A4.7).

4.4.5 Gene family expansion and contraction

CAFE5 identified a large variable number of gene family expansions and contractions across strains (Fig. 4.4b). The majority of rapidly evolving gene families were annotated to TE-related domains, including reverse transcriptase (RVT_1), exonuclease/endonuclease/phosphatase (Exo_endo_phos_2), helitron-like N-terminal domain (Helitron_like_N), transposases, TE-associated peptidases, endonuclease VII-like domain (Endonuclease_7), thanatos-associated protein domain (THAP), and Myb DNA-binding domain (Myb_D-bind)- collectively pointing to TE-driven activity shaping genome evolution (Tables A4.8-A4.27). In addition, several genes were annotated as viral-derived domains, such as retroviral integrase (rve), baculovirus fusion protein (Baculo_F), YqaJ, and double jelly-roll capsid fold (DJR) (Tables A4.8-A4.27). GO enrichment of these viral-relevant families revealed significant overrepresentation of nucleic acid binding, catalytic activity, DNA helicase activity, DNA integration, DNA repair, and zinc ion binding (Figs. A4.6-A4.7).

Additional rapidly evolving genes families were annotated with immune and olfaction-related domains, such as reprolysin (metalloproteases), serpin (protease inhibitors), Parvo_coat_N (PLA2-like, Phospholipase A2), and 7tm_6 (olfactory receptors) (Tables A4.8-A4.27), for strains including MaENG, MaMOR, MaNOR, MaUSA, and MaWAL. Their corresponding GO terms included arachidonic acid secretion, phospholipase A2 activity, phospholipid metabolism, metallopeptidase activity, sensory perception of smell, odourant binding, and olfactory receptor activity (Figs. A4.6-A4.7).

The asexual Irish-derived clade, MaNZI, displayed 285 rapidly evolving gene families (Fig. 4.4b). Most of these were also associated with TE and viral-derived domains; however, a subset also included families linked to olfaction and immune functions (Tables A4.26-A4.27). GO enrichment in MaNZI highlighted categories related to olfaction (odourant binding, olfactory receptor activity, detection of chemical stimulus) and immune/metabolic processes (phospholipid metabolism, arachidonic acid secretion, phospholipase A2 activity) (Fig. A4.7). Specifically, expansions were observed in the odourant receptor (7tm_6) family, and enrichment of phospholipase A2-like family (Fig. A4.7).

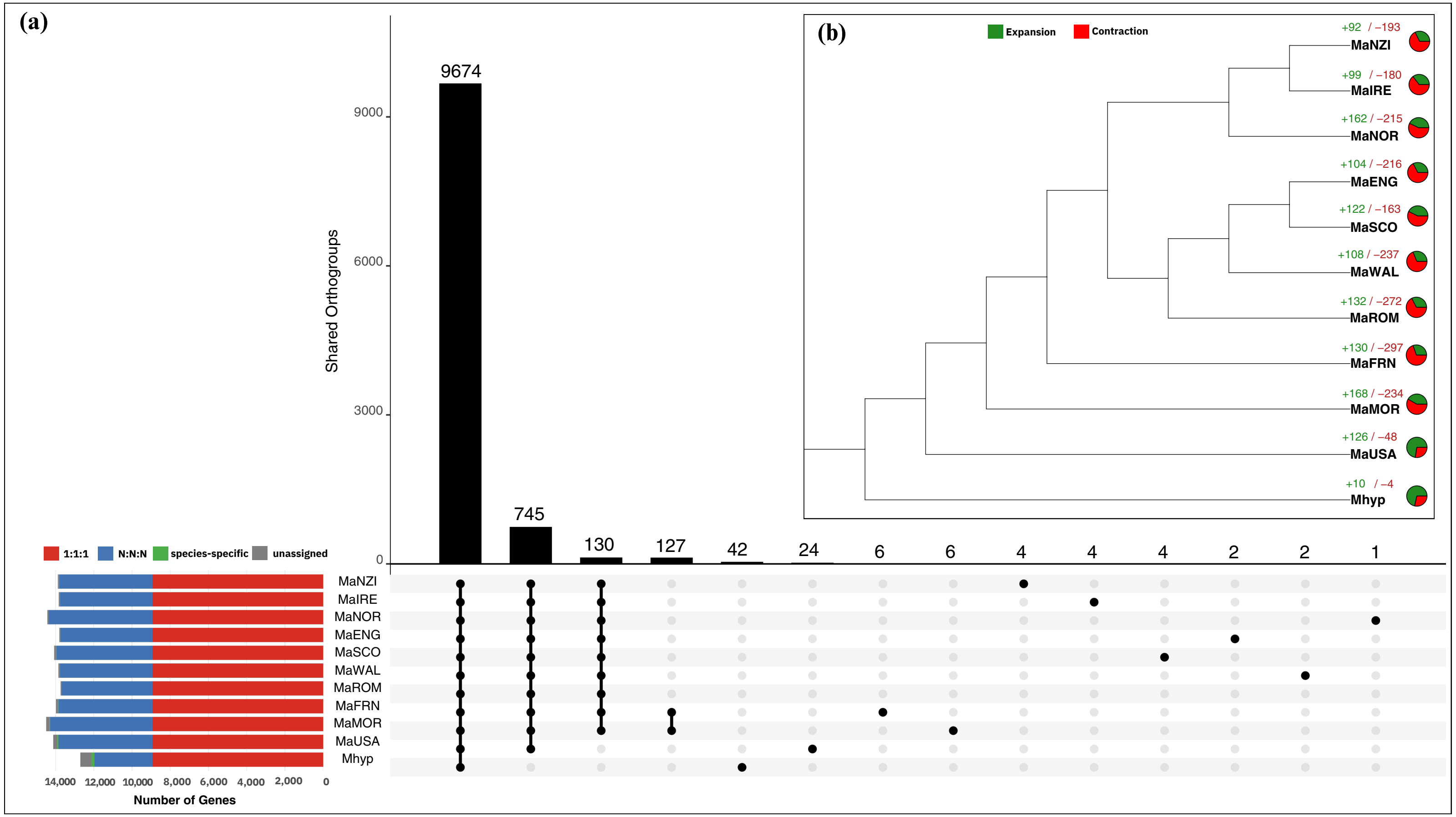

Figure 4.4 Comparative analysis of orthogroups and gene family evolution across *Microctonus aethiopoides* strains. (a) UpSet plot showing the number of shared and unique orthogroups among *M. aethiopoides* strains and the outgroup *M. hyperodae* (Mhyp). Bar plots on the left represent total gene counts per strain categorized as single-copy (1:1:1), multi-copy (N:N:N), species-specific, and unassigned orthogroups; (b) Gene family expansion and contraction analysis inferred using CAFE5. Numbers next to each terminal node indicate the count of expanded (green) and contracted (red) gene families. Pie charts represent the proportion of expansions and contractions for each strain along the phylogeny.

4.4.6 Classification of chemosensory gene families

We classified chemosensory related gene families across all *M. aethiopoides* strains to explore their possible role in strain diversification. The number of OBPs/PBPs, IRs, SNMPs, GRs and CSPs was consistent and conserved across all strains (Table 4.3). In contrast, ORs showed greater variability, ranging from 135 in MaIRE to 162 in MaENG (Table 4.3). OR counts differed substantially between MaIRE and MaNZI, with MaNZI showing a marked expansion of ORs (n = 158, compared to n = 135 for MaIRE), despite a relatively short time period (~17-18 years) since its release.

Table 4.3 Summary of chemosensory gene families identified across *Microctonus aethiopoides* strains. The table presents the number of genes belonging to six major chemosensory gene families: odourant-binding proteins (OBPs/PBPs), odourant receptors (ORs), ionotropic receptors (IRs), chemosensory proteins (CSPs), sensory neuron membrane proteins (SNMPs), and gustatory receptors (GRs).

SampleID	OBPs/PBPs	ORs	IRs	CSPs	SNMPs	GRs
MaUSA	11	161	22	12	3	23
MaMOR	12	138	24	12	3	23
MaFRN	12	138	24	12	3	23
MaSCO	11	160	22	11	3	23
MaENG	12	162	22	12	3	23
MaNOR	11	144	22	12	3	23
MaROM	12	151	22	12	3	23
MaIRE	12	135	22	11	3	23
MaNZI	12	158	23	11	3	23
MaWAL	11	138	22	12	3	23

4.5 Discussion

We generated high-quality genome assemblies of *M. aethiopoides* strains. Our phylogenomic analyses showed divergence patterns primarily based on host associations, with these differences also reflected in heterozygosity patterns. However, differences between strains were limited, with most gene families conserved and rapidly evolving genes largely associated with transposable elements across all strains. Chemosensory gene families were broadly conserved across strains, though odourant receptor copy numbers expansion in the New Zealand-released Irish strain (MaNZI) may reflect adaptation to local host cues post release.

Though we noted a degree of geographic clustering among the strains in this study (e.g., MaSCO, MaENG, and MaWAL grouped together), our species tree primarily pointed to host-based divergence of *M. aethiopoides* strains. We recovered two strongly supported lineages delineated by *Hypera* and *Sitona* host associations. This is consistent with earlier analyses showing a *Hypera*-associated clade (e.g., *H. postica*) in Iran/USA and a *Sitona*-associated clade distributed across Europe and Oceania (Phillips, Vink, et al., 2008; Vink et al., 2003). In this study, we observed a clear split within the *Sitona* clade between the *S. discoideus*-associated strain (MaMOR) and the *S. obsoletus* associated-strains. This supports genetic divergence of MaMOR and may help explain its lower preference for parasitising *S. obsoletus* (Barratt et al., 1997; Phillips et al., 2002).

Reproductive mode plays a central role in shaping patterns of genomic diversity and divergence, as differences in the occurrence of meiosis and recombination between sexual and asexual lineages directly influence heterozygosity, and the rate at which genetic variation is generated or maintained (Otto & Lenormand, 2002; Tvedte et al., 2019). Here, detected heterozygosity patterns across *M. aethiopoides* strains aligned broadly with their evolutionary relationships and reproductive modes. The basal lineages MaUSA and MaMOR exhibited the highest heterozygosity, reflecting the retention of ancestral genetic diversity typical of sexually reproducing populations (Otto & Lenormand, 2002). In contrast, the derived asexual lineages MaIRE and MaNZI, which formed a distinct clade within the *Sitona*-associated group, showed markedly reduced heterozygosity and extensive runs of homozygosity, indicative of limited recombination likely reflecting their asexual reproduction. Intermediate heterozygosity and ROH levels observed in other sexual strains align with their phylogenetic placement between these two extremes. Together, the phylogenetic and genomic diversity patterns highlight the evolutionary transition from sexually reproducing ancestral lineages to derived asexual forms, accompanied by a reduction in genome-wide heterozygosity.

In insects, TEs can shape genome evolution by increasing genome size and duplicating genes, altering gene regulation, introducing novel functions, or disrupting existing genes (Behm & Sharanowski, 2025; Gebrie, 2023). We detected recent or ongoing TE proliferation, along with large sets of rapidly evolving genes across the studied *M. aethiopoides* strains, with many of these associated with TE activity and expansion of TE-associated domains (reverse transcriptase, integrase, endonuclease). These patterns are consistent with genome restructuring and adaptive innovation linked to host and environment related adaptation, as has been commonly reported in parasitoids (Behm & Sharanowski, 2025) and other insects (Bie et al., 2024; Eickbush & Jamburuthugoda, 2008; Petersen et al., 2019). In sexually reproducing species, TEs generally spread between individuals through mating and recombination; however, sex also enhances purifying selection and host defences that remove deleterious insertions, maintaining a dynamic balance between TE proliferation and elimination (Arkhipova & Meselson, 2005; Dolgin & Charlesworth, 2008). In contrast, asexual lineages lack recombination, and theory predicts genome-wide accumulation of TEs, similar to the pattern observed in non-recombining sex chromosomes (Bachtrog, 2013; Chalopin et al., 2015), which can eventually lead to lineage decline due to high transposition rates and their deleterious effects (Arkhipova & Meselson, 2005; Burt & Trivers, 2006; Keightley et al., 2014). However, empirical studies across various taxa, including crustaceans (*Daphnia* water fleas), insects (*Leptopilina* wasps), and mites (Oribatida) (Bast et al., 2016) have reported no evidence for increased TE load in asexual lineages, with some (*Saccharomyces cerevisiae*) (Bast et al., 2019) even showing reduced TE content due to enhanced excision or silencing. Consistent with these findings, our comparison of asexual (MaIRE and MaNZI) and sexual *M. aethiopoides* strains revealed no major differences in TE composition or abundance. This suggests that the asexual lineages may possess mechanisms that suppress TE buildup, or that the transition to asexuality in the Irish population is relatively recent, limiting the time for TE accumulation.

In addition to TEs, we observed some expanded genes that annotated to *Baculovirus* Fusion (Baculo_F), retroviral integrase (rve), and YqaJ nuclease domains. *Bracoviruses* are a subtype of polydnaviruses - large dsDNA viruses endogenous to braconid wasps that enter host cells and act as gene-transfer agents during oviposition, driving expression of virulence genes that manipulate host physiology, facilitating immune suppression and successful larval development (Drezen et al., 2014; Ye et al., 2018). Thus, bracovirus-derived elements may be contributing to genome remodeling in *M. aethiopoides*, as has been documented in other *Braconidae* parasitoids (Herniou et al., 2013). Coupled with viral-derived gene expansions, we observed expansions in immune (e.g., phospholipase A2, proteases) and olfaction-related genes (7tm_6) and enrichments of olfaction and immune/metabolic functions, which together may enhance the capacity of *M. aethiopoides* strains to find hosts and manipulate their physiology to promote egg development and survival (Jin et al., 2024; Parks et al., 2023; Zhu et al., 2025). Interestingly, we found that the asexual clade Ireland-derived New Zealand strain (MaNZI) showed signs of genomic evolution following New Zealand release, with expansion of genes linked to olfaction and immune function likely reflecting adaptive fine-tuning to local hosts and ecological conditions. Such changes could suggest that, even in the absence of sexual recombination, the asexual lineages of *M. aethiopoides* retain the capacity for functional adaptation through gene family diversification.

Because chemosensory gene families play an important role in parasitoid host-finding by detecting host-derived chemical cues and guiding female host-seeking behaviour (Gauthier et al., 2021; Robertson et al., 2010; Wang et al., 2015; Wulff et al., 2024; Yin et al., 2025), we explored this gene family in a focused analysis. We found that *M. aethiopoides* maintained a largely stable repertoire of chemosensory genes across strains but noted modest variation in the number of ORs (MaIRE = 135, while all other strains > 150). Although the classifications in our gene family expansion analyses were solely based on homology-derived protein predictions, which can be affected by genome fragmentation and do not reflect transcriptome-derived annotations commonly used in other parasitoid systems (Gauthier et al., 2021; Wang et al., 2015; Yin et al., 2025), our results should be interpreted as an initial insight. However, ORs in particular may reflect strain-specific modulation linked to host preference and should be investigated further.

As discussed by Hambäck et al., (2024), parasitoid divergence can be explained by two major evolutionary frameworks: the adaptive radiation and oscillation hypotheses. The adaptive radiation hypothesis proposes that diversification follows the emergence of key innovations such as new venom components, domesticated viral genes, or ovipositor modifications that enable parasitoids to exploit new ecological niches. These events are expected to produce clear genomic signatures, including gene family expansions linked to host manipulation or sensory adaptation. In contrast, the oscillation hypothesis suggests that diversification arises from ecological opportunity acting on existing genetic variation, where host shifts and environmental change drive host-associated differentiation without major genomic innovation. Genomically, such processes are expected to produce subtle patterns, such as gene copy number variation or selection on existing gene families, rather than large-scale emergence of entirely new genes.

In our study phylogeny showed host-associated divergence, and heterozygosity differed both with host use and reproductive mode, indicating that ecological specialisation and reproduction jointly influence genomic differentiation in *M. aethiopoides*. However, gene families across the strains were largely conserved, with no major host-associated differences, likely because the compared strains have not diverged over sufficiently deep evolutionary timescales. Therefore, the observed divergence may be driven more by fine-scale genomic variation than large-scale gene family evolution. The limited gene family divergence observed among *M. aethiopoides* strains, coupled with variation largely restricted to transposable element associated genes, suggests that diversification within this species may not have been driven by major genomic innovations. Instead, these patterns align more closely with the oscillation hypothesis, where adaptation arises from ecological opportunity acting on existing genetic variation rather than the emergence of novel gene families.

Further to better understand ecotype-level differentiation, more strain-specific information supported by robust analytical approaches is required, rather than focusing solely on genome-level analysis. For example, the study by Ferguson et al., (2024) comparing the Moroccan and Irish strains of *M. aethiopoides* introduced to New Zealand showed that the Moroccan strain exhibits higher non-target impacts than the Irish strain. The Moroccan strain reproduces sexually (arrhenotokous), which tends to increase genetic diversity (in this study reflected as higher heterozygosity) compared with the asexually reproducing (thelytokous) Irish strain. This raises an intriguing question: does asexuality promote specialisation by limiting genetic diversity and adaptive potential? However, such an interpretation cannot be made straightforwardly, because the same study also reported occasional non-target parasitism by the Irish strain, likely resulting from ecological spillover into natural habitats. This suggests that ecological opportunity and host availability can influence apparent host use beyond reproductive mode alone. Understanding ecotype-level differentiation in *M. aethiopoides* requires linking genetic variation to ecological function, moving beyond descriptive genomics toward mechanistic analyses that connect genotype, phenotype, and environment.

4.6 References

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Chapter 5

Characterisation of the mitochondrial genome of *Microctonus aethiopoides*

Meeran Hussain[1], Rebecca Funari[2], Nicolas Diercksens[3], Elahe Parvizi[1], Mark R. McNeill[4], Ang McGaughran[1]

[1]Te Aka Mātuatua - School of Science, University of Waikato, Private Bag 3105, Hamilton, 3240, New Zealand

[2] Department of Life Sciences, University of Siena, 53100, Siena, Italy

[3] Genomics and Regulatory Systems Unit & Marine Climate Change Unit, Okinawa Institute of Science and Technology Graduate University, Okinawa, Japan

[4] Bioeconomy Science Institute, Tuhiraki, Christchurch, New Zealand

Manuscript for this chapter is undergoing final formatting and will be submitted soon to Mitochondrial DNA Part B.

5.1 Abstract

The mitochondrial genome serves as a powerful marker for evolutionary and phylogenetic studies due to its maternal inheritance and lack of recombination. Here, we report the first complete mitochondrial genome of *Microctonus aethiopoides* (Hymenoptera: Braconidae: Euphorinae), assembled using a hybrid approach combining Oxford Nanopore and Illumina sequencing data.

The circular mitogenome is 33,173 bp in length and contains the typical complement of 37 genes (13 protein-coding genes, 22 tRNAs, and 2 rRNAs) along with a large A+T-rich control region. The genome exhibits a strong AT bias (85.42%) and a unique tRNA gene arrangement, while the protein-coding genes remain conserved across Braconidae. Phylogenetic analysis based on thirteen species from the group Ichneumonidae and Braconidae places *M. aethiopoides* firmly with Euphorinae in the Braconidae subfamily, providing strong support for its taxonomic position.

This newly characterised mitogenome offers a valuable genetic resource for future evolutionary and ecological studies of *M. aethiopoides* and enhances our understanding of mitochondrial genome evolution and gene rearrangement patterns within Braconidae subfamilies.

5.2 Introduction

The mitochondrial genome (mitogenome) plays an important role in phylogenetic reconstruction because its maternal inheritance generally avoids recombination with other lineages, providing a clear marker for tracing evolutionary history (Cameron et al., 2007). The mitogenome is typically circular and contains 37 genes, including 13 protein-coding genes (PCGs), 22 transfer RNAs (tRNAs), and two ribosomal RNAs (rRNAs), along with a non-coding A+T-rich control region. Even with the rise of phylogenomics, where large-scale nuclear datasets are increasingly used to resolve evolutionary relationships, mitogenomes remain a valuable resource (Wu et al., 2020). The widespread use of next-generation sequencing has not only reduced associated costs but also led to a rapid increase in available mitogenome data, both from targeted sequencing and as by-products of genomic studies (Tang et al., 2019; Wu et al., 2020; Yoshizawa et al., 2018).

Insects typically possess mitogenomes ranging from 14 -19 kb in size, however in rare cases there can be unusually large mitogenomes (in the range of 20-28 kb).This increased mitogenome size has been attributed to the expansion of the control region via accumulation of tandem repeats rather than through changes in gene copy number (Morgan et al., 2022; Filipović et al., 2021; Olli et al., 2025; Lin et al., 2021). However, mitogenome size variability has generally remained underexplored because conventional (Sanger- and second-generation) sequencing platforms often fail to assemble repetitive and complex regions like the control region. The limited read length of these platforms (around 1 kb with Sanger sequencing and only 100–300 bp with second-generation methods), results in unreliable assemblies and underestimation of mitogenome lengths and rearrangements (DeSalle et al., 2017; Filipović et al., 2021; Formenti et al., 2021; Monnens et al., 2020; Morgan et al., 2022). Advances in long-read platforms such as Oxford Nanopore Technologies (ONT) now enable accurate resolution of these complex regions, providing new insights into mitogenome size evolution (Filipović et al., 2021; Kinkar et al., 2021; Morgan et al., 2022; Olli et al., 2025).

Gene order in insect mitogenomes is generally conserved, but extensive rearrangements have been reported in several lineages, with Hymenoptera showing particularly high rates of rearrangement (Li et al., 2016; Wei et al., 2010; Zheng et al., 2021). These lineage-specific rearrangements provide important phylogenetic signals that aid the reconstruction of evolutionary histories, the identification of relatedness between species, and the understanding of how gene arrangements change over time - especially at the subfamily and genus levels (Cameron, 2014; Tang et al., 2019). Despite this potential, mitogenome data remain scarce for the parasitoid subfamily Euphorinae (Hymenoptera: Braconidae), limiting insights into their evolutionary history and mitogenome trajectories. Here, we present the complete mitochondrial genome of *Microctonus aethiopoides* Loan, a parasitoid Euphorinae used for biocontrol, providing a new resource for comparative genomics and phylogenetic reconstruction within this ecologically important lineage.

5.3 Materials and methods

5.3.1 Mitogenome assembly and annotation

DNA from an individual wasp of the New Zealand-released Irish strain of *M. aethiopoides* was extracted as part of a previous comparative genomics study (see Chapter 3) and sequenced using hybrid long-read (ONT) and short-read (Illumina) sequencing technologies. Initial assembly was performed using NOVOPlasty (v4.3.5) (Dierckxsens et al., 2017), with a partial COI sequence of *M. aethiopoides* (NCBI accession: KY845728) provided as the seed. This assembly was then extended using long-read sequencing data and NOVOLoci (v0.4) (Dierckxsens et al., 2025), producing a circular mitochondrial genome.

The assembled mitochondrial genome was subsequently annotated using MITOS2 (v 2.1.9) (Donath et al., 2019), employing the invertebrate mitochondrial genetic code. Further, tRNA predictions were validated using ARWEN (v1.2) (Laslett & Canbäck, 2008) to ensure correct identification and secondary structure. Further, start and stop codons of genes were then manually curated. Finally, a graphical map of the circular mitogenome was generated using the Proksee online system (https://proksee.ca/), providing a visual representation of gene organisation and structural features.

5.3.2 Phylogenetic analysis

Thirteen species were used to infer phylogeny using mitogenomes, including *M. aethiopoides,* nine other Braconidae species were selected, limited to those with available annotations and unique gene orders within their respective subfamilies, and three Ichneumonidae species as outgroups. Mitogenome files were downloaded from GenBank using the following accessions: *Amblyjoppa* sp. (MG923483), *Diadegma semiclausum* (EU871947), *Enicospilus* sp. (FJ478177) for Ichneumonidae; *Aphidius gifuensis* (MT264907); *Asobara japonica* (MN882556); *Cardiochiles fuscipennis* (KF385870);

Cotesia vestalis (NC_014272); *Dinocampus coccinellae* (MG253265); *Habrobracon hebetor* (MN842279); *Psyttalia concolor* (MW279212); *Spathius agrili* (NC_014278); *Zele chlorophthalmus* (NC_039181) for Braconidae.

Amino acid sequences for the 13 identified mitochondrial protein-coding genes (PCGs) were aligned gene-by-gene with MAFFT (v7.505) using the L-INS-i approach (--localpair --maxiterate 1000) (Katoh & Standley, 2013). Poorly aligned regions were removed with trimAl (v1.4.1) (Capella-Gutiérrez et al., 2009) under the -automated1 heuristic. Per-gene protein alignments were then concatenated with AMAS (v1.02) (Borowiec, 2016) in amino-acid mode. Finally, maximum-likelihood inference was conducted with IQ-TREE2 (v2.2.2) (Minh et al., 2020) using a partitioned analysis and ModelFinder (Kalyaanamoorthy et al., 2017) with -m MFP+MERGE, allowing automatic model selection per partition and merging of partitions with similar model/parameter estimates; node support was evaluated with 1,000 ultrafast bootstrap replicates (-B 1000) and 1,000 SH-aLRT replicates (--alrt 1000).

5.4 Results

5.4.1 Mitogenome assembly and annotation

The complete mitochondrial genome of *M. aethiopoides* was found to be circular, with a length of 33,173 bp (Fig. 5.1). It encoded the typical set of 37 mitochondrial genes, including 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), and two ribosomal RNA genes (rRNAs). The overall base composition was 42.00% of A, 43.42% of T, 9.75% of G, and 4.83% of C, resulting in a strong AT bias of 85.42%, which is commonly observed in the other Hymenoptera mitogenomes (Tang et al., 2019).

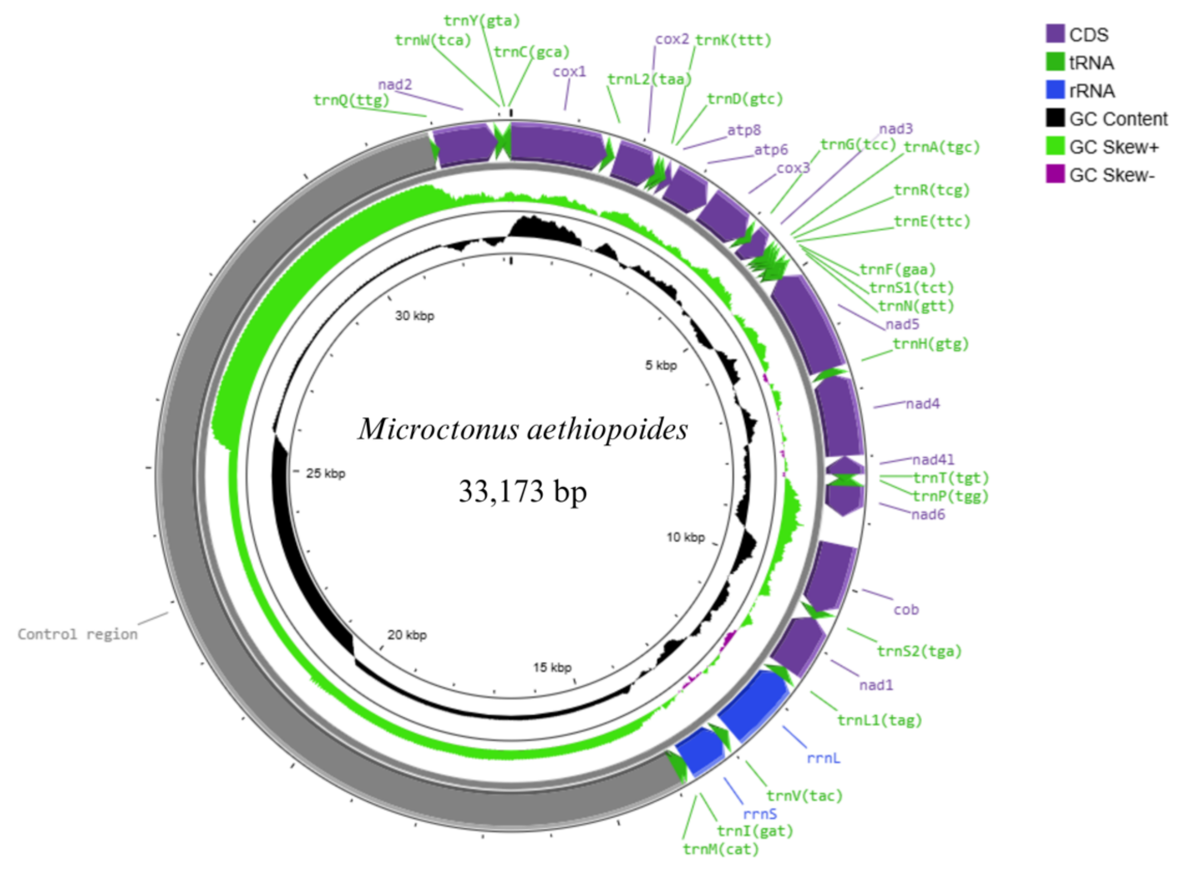

Figure 5.1 Circular map of the *Microctonus aethiopoides* mitochondrial genome. Protein-coding genes (CDS) are shown in purple, transfer RNAs (tRNAs) in dark green, and ribosomal RNA genes (rRNAs) in blue. The control region is indicated in grey. Inner rings display genomic composition metrics, including GC content (black) and GC skew, with positive GC skew shown in light green and negative GC skew in pink.

The cumulative length of the 13 PCGs was 11,001 bp, ranging in size from 150 bp (*atp8*) to 1,668 bp (*nad5*), and accounting for 33.16% of the complete genome (Table 5.1). Among the PCGs, five genes initiated with ATT (Ile), namely *cox1, nad3, nad5, nad6, and nad4l;* four with ATA (Met), *cox2, atp8, nad4, and nad1*; and three with ATG (Met), *atp6, cox3, and cob*. Most PCGs terminated with the complete TAA stop codon, while truncated stop codons (T-) were observed for four PCGs: *cox2, nad4, cob,* and *nad2*. The 22 tRNA genes ranged from 62 to 70 bp in length, with an average size of 65.5 bp, totalling 1,440 bp (4.34% of the genome). The two rRNA genes, *rrnL* (1,227 bp) and *rrnS* (729 bp), together accounted for 1,956 bp, representing 5.90% of the total mitogenome.

Table 5.1 Annotated mitochondrial gene features of *Microctonus aethiopoides* (Irish strain).

Gene	Start	End	Strand	Length	Anticodon	Start Codon	Stop Codon
cox1	1	1542	+	1542		ATT	TAA
trnL2	1544	1609	+	66	TAA		
cox2	1680	2349	+	670		ATA	T (TRUNCATED)
trnK	2350	2417	+	68	TTT		
trnD	2416	2481	+	66	GTC		
atp8	2491	2640	+	150		ATA	TAA
atp6	2631	3299	+	669		ATG	TAA
cox3	3305	4090	+	786		ATG	TAA
trnG	4091	4154	+	64	TCC		
nad3	4155	4505	+	351		ATT	TAA
trnA	4508	4572	+	65	TGC		
trnR	4573	4635	+	63	TCG		
trnE	4636	4700	+	65	TTC		
trnF	4700	4762	-	63	GAA		
trnS1	4763	4830	+	68	TCT		
trnN	4856	4922	+	67	GTT		
nad5	4918	6585	-	1668		ATT	TAA
trnH	6586	6647	-	62	GTG		
nad4	6649	7978	-	1330		ATA	T-
nad4l	7975	8265	-	291		ATT	TAA
trnT	8268	8328	+	62	TGT		
trnP	8329	8393	-	66	TGG		
nad6	8434	8937	+	505		ATT	TAA
cob	9382	10507	+	1127		ATG	T-
trnS2	10508	10574	+	67	TGA		
nad1	10586	11512	-	927		ATA	TAA
trnL1	11553	11618	-	66	TAG		
rrnL	11606	12832	-	1227			
trnV	12918	12979	-	62	TAC		
rrnS	12974	13702	-	729			
trnI	13715	13777	-	63	GAT		
trnM	13777	13846	-	70	CAT		
trnQ	31917	31986	+	70	TTG		
nad2	31990	32974	+	985		ATA	T-
trnW	32975	33043	+	69	TCA		
trnY	33045	33109	-	65	GTA		
trnC	33107	33169	-	63	GCA		

5.4.2 Phylogenetic analysis

The maximum-likelihood consensus phylogeny of 13 mitochondrial PCGs recovered a well-supported topology, with *M. aethiopoides* falling within Euphorinae, and a clade including *Z. chlorophthalmus*, *M. aethiopoides*, and *D. coccinellae* recovered with strong bootstrap support (100%; Fig. 5.2). However, the immediate sister taxon of *M. aethiopoides* (*Z. chlorophthalmus)* showed a lower bootstrap support value, indicating weaker phylogenetic resolution at that node. Ichneumonidae taxa were placed as outgroups to Braconidae, and within Braconidae, Euphorinae formed a sister lineage to the remaining sampled subfamilies with 100 % bootstrap support (Fig. 5.2).

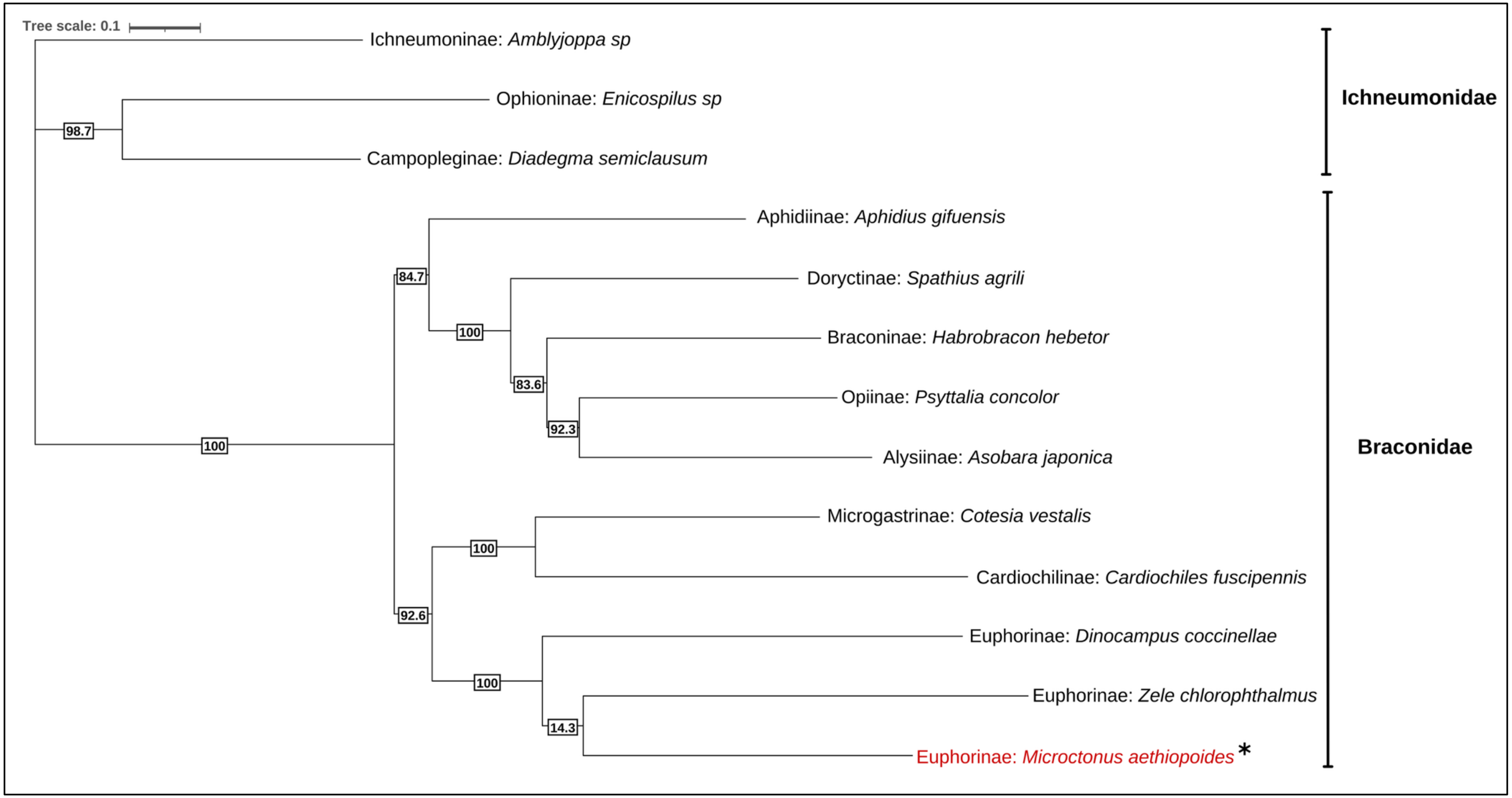

Figure 5.2 Maximum-likelihood phylogenetic tree of selected Hymenopteran species based on concatenated mitochondrial protein-coding genes. Bootstrap values are shown at each node. *Microctonus aethiopoides* (highlighted in red) clusters within the subfamily Euphorinae (family Braconidae).

5.4.3 Gene rearrangements

Gene order rearrangements could be classified into translocations, local inversions (inverted in the local position), gene shuffling (local translocation), and remote inversions (translocated and inverted) (Dowton et al., 2003). When comparing the gene order of *M. aethiopoides* with that of *Drosophila melanogaster*, which follows the canonical Pancrustacea arrangement, we found no rearrangements in protein-coding genes in the mitogenome, whereas several tRNA genes showed rearrangements (Fig. 5.3). Specifically, *M. aethiopoides* exhibited gene shuffling involving *trnC-trnY* and *trnS1-trnN*, remote inversions of *trnI* and *trnM*, and a local inversion of *trnQ*. Across Braconidae species, the *trnI-trnQ-trnM* and *trnW-trnC-trnY* clusters were commonly rearranged relative to *D. melanogaster.*

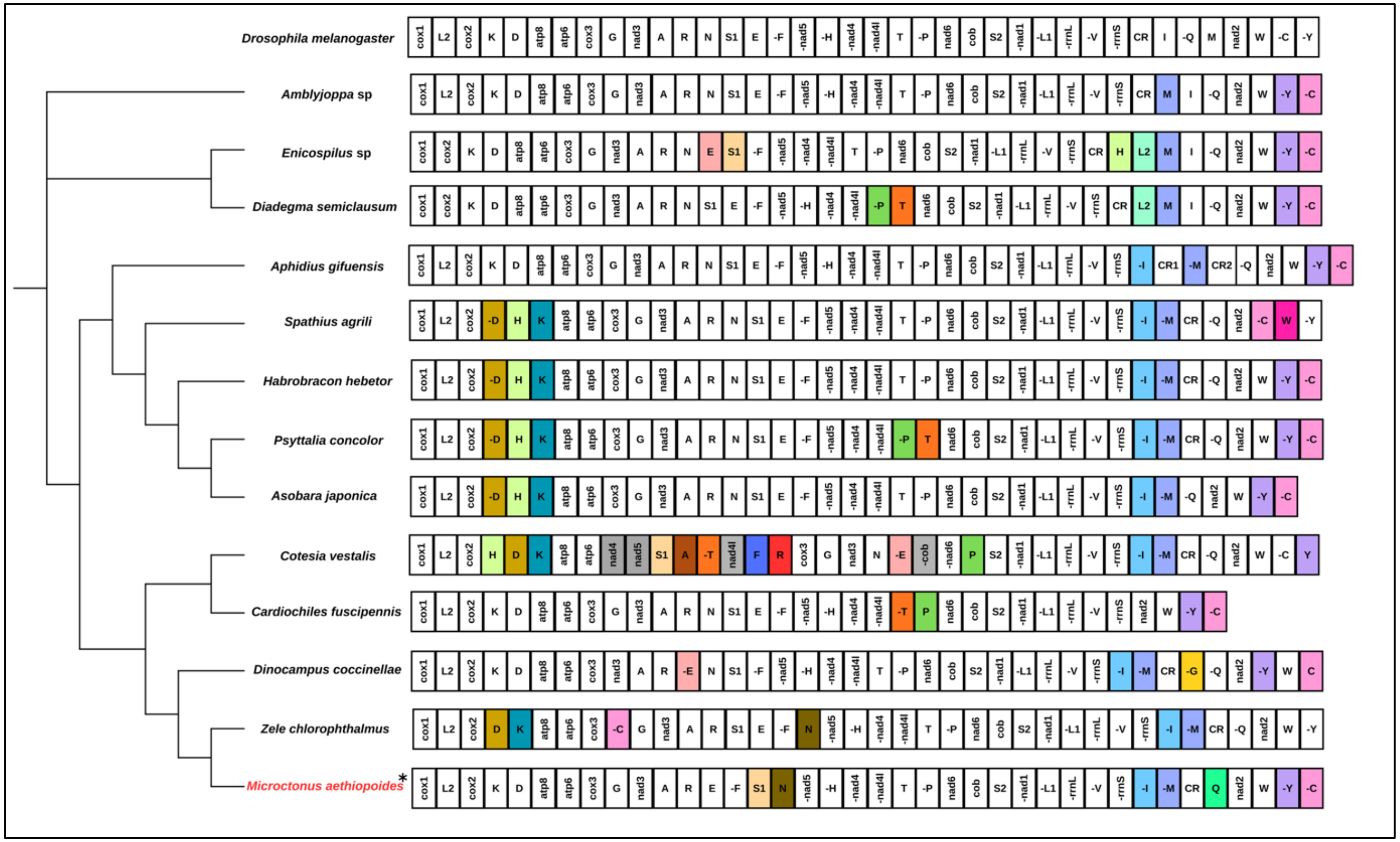

Figure 5.3 Comparison of mitochondrial gene order among selected Hymenopteran species using *Drosophila melanogaster* as the reference. The phylogenetic topology on the left corresponds to the relationships inferred from the mitochondrial phylogenetic analysis. The control region is abbreviated as 'CR', and tRNA genes are indicated by single-letter amino acid codes following the IPUC-IUB convention. Genes preceded by a "–" symbol are encoded on the minority strand. Rearranged protein-coding genes are shaded in grey, while each rearranged tRNA gene is highlighted in a distinct colour. *Microctonus aethiopoides* (shown in red) exhibits lineage-specific rearrangements within the Braconidae.

5.5 Discussion

In this study, we successfully assembled and annotated the complete mitochondrial genome of *M. aethiopoides*, representing the first mitogenome reported for the genus *Microctonus* and a valuable mitogenome resource for the parasitoid subfamily Euphorinae. The mitogenome was relatively large and exhibited a unique gene arrangement, with conserved protein-coding sequences but variable tRNA gene organisation within Braconidae.

The mitogenome assembly for *M. aethiopoides* was approximately 33.17 kb in length, which is larger than the average size typically reported for insect mitogenomes (Cameron, 2014). However, such size variation is not unusual within Braconidae. For instance, *Aleiodes leptofemur* (Hymenoptera: Braconidae) has a mitogenome of 32.28 kb (Broad, 2024), while *Dinocampus coccinellae* (Hymenoptera: Braconidae) and *Zele albiditarsus* (Hymenoptera: Braconidae) possess mitogenomes of 22.88 kb (Barclay & Broad, 2024) and 23.69 kb (Crowley & Broad, 2024), respectively. This larger mitogenome size could be attributed to the complete recovery of the control region, which often contains tandem and inverted repeats (Morgan et al., 2022), made possible by our use of long-read sequencing.

The phylogenetic tree, which included representative species from the families Braconidae and Ichneumonidae, placed *M. aethiopoides* to subfamily Euphorinae within the family Braconidae, with high bootstrap values providing strong evidence for its taxonomic placement. The order of PCGs was relatively conserved across published Braconidae mitogenomes, with exceptions reported for *Stenocorse bruchivora* (Doryctinae), two *Chelonus* spp. (Cheloninae), and two *Cotesia* spp. (Microgastrinae) (Jasso-Martínez et al., 2022; Wei et al., 2010; Yuan et al., 2022), whereas rearrangements of tRNAs are common in Braconidae mitogenomes (Jasso-Martínez et al., 2022; Li et al., 2016). Here, *M. aethiopoides* showed largely conserved PCGs with unique rearrangements only in tRNA genes.

Although the use of long-read sequencing has enabled the complete recovery of the control region, its underlying structural organisation, such as stem-loop motifs, GC-rich blocks, and tandem repeats, remains to be examined in detail. Comparative analyses of these features across closely related species would help determine whether the observed length expansion in *M. aethiopoides* has functional or evolutionary significance (see Jiang et al., (2024), thereby providing valuable insights into mitochondrial genome evolution within Braconidae.

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Chapter 6

Conclusion

6.1 Contextual overview

Parasitoids are among the most ecologically and economically significant insect groups, serving as natural enemies that regulate pest populations in agricultural ecosystems. Within classical biological control (CBC) programmes, the introduction of parasitoids has been an effective and environmentally sustainable method for long-term pest suppression (Heimpel & Cock, 2018; Wyckhuys et al., 2024). CBC relies on the successful establishment and persistence of introduced agents, which in turn depend on their own ability to adapt to novel environments - for example, by maintaining sufficient genetic diversity (Hopper et al., 1993; Leung et al., 2020) and by successfully associating with their host (the target pest).

Microctonus aethiopoides is a parasitoid widely used in CBC to provide control pest species of *Hypera* and *Sitona* where they have become invasive outside their geographic range. In Aotearoa New Zealand, there are two strains of *M. aethiopoides* (Gerard et al., 2006; Stufkens et al., 1987), with these differing in reproductive mode and targeting two different exotic weevil pests: the sexual (arrhenotokous) Moroccan strain for controlling *Sitona discoideus*, and the asexual (thelytokous) Irish strain for controlling *Sitona obsoletus*.

Genetic diversity represents the fundamental basis for adaptation, enabling populations to adapt under changing environmental conditions (Minter et al., 2021). Populations with higher genetic diversity possess a broader range of alleles and genotypes, increasing the likelihood that some individuals will carry traits conferring a selective advantage (Allendorf et al., 2010; Ragauskas et al., 2025). Such individuals are more likely to survive and reproduce successfully, thereby transmitting beneficial alleles to subsequent generations and enhancing the overall adaptive potential of the population (Dlugosch & Parker, 2008; Minter et al., 2021). Among parasitoids, asexual reproduction offers a major advantage to CBC because their female-only reproduction enables rapid population growth and establishment (Heimpel & de Boer, 2008). However, this same asexual nature can be a limitation when interacting with sexually reproducing hosts, as seen in the case of the asexual parasitoid *Microctonus hyperodae*, which has failed to sustain control of the sexually reproducing *Listronotus bonariensis* (Kuschel) in New Zealand, due to a lack of co-evolutionary potential between host and parasitoid (Casanovas et al., 2018). Thus, the long-term evolutionary and adaptive potential of asexual parasitoids remains uncertain given their reduced genetic diversity via restricted recombination and meiosis (Normark et al., 2003). Studies exploring the genetic diversity of asexual parasitoid populations are very limited. The Irish asexual strain of *M. aethiopoides*, which parasitises the sexually reproducing *S. obsoletus*, therefore presents a valuable system for assessing the adaptability and genetic diversity of parasitoids, in addition to the effectiveness of long-term control of its host in a changing environment.

Parasitoids are one of the most ecologically diverse insect groups, with divergence primarily centred around host-associated specialisation (Funk, 2010; Knolhoff & Heckel, 2014; Michielini et al., 2024). Here, different hosts act as potent selective agents, creating strong divergent selection pressures to adapt to the host's specific characteristics (e.g., its immune system) (Antolin et al., 2006; Mackauer et al., 1996). This process can be facilitated by early learning of host cues (through olfaction) during development, leading to strong preferences in adulthood. Such host-driven pressures can impose genetic-level changes among parasitoid populations, leading to evolutionary divergence that may involve the emergence of novel genes and functions associated with new hosts (radiation hypothesis), or selection acting on existing genes and standing variation as populations oscillate between different host-use strategies (Hambäck et al., 2024). This process can also operate at finer scales within species, where genetically distinct lineages adapt to different hosts and exhibit variation in host preference.

6.2 Thesis synthesis and key findings

Genomic research on parasitoids used in CBC is rapidly expanding, offering valuable insights into the molecular mechanisms underlying key adaptive traits. However, substantial knowledge gaps remain, particularly in understanding how the reproductive mode, especially asexual reproduction, shapes genetic diversity and adaptability to environmental change, and how host association influences genomic variation within parasitoid populations.

In **Chapter 2**, I carried out a comprehensive review on parasitoids, focusing on understanding the key traits that influence their long-term effectiveness in CBC programmes. I identified four key adaptive traits: reproductive mode, olfaction, thermal tolerance, and microbiome (symbionts). I discussed each of these traits in detail by synthesising findings from behavioural, physiological, and molecular studies across different Hymenopteran parasitoids, describing their roles and how these traits strongly influence host specificity, persistence, adaptability, and success after their field release. Next, I evaluated examples of some failed biocontrol programmes, providing an overview of the historical context and outcomes of CBC in New Zealand. I also highlighted the importance of parasitoids of *Microctonus* species for biological control and discussed their potential outcomes in terms of efficacy, failures, and non-target impacts. Finally, I elucidated the potential research gaps that need addressing to improve efficacy and persistence in biocontrol programmes, presenting future research ideas that could be undertaken using genomic technologies. Ultimately, this review provided the foundation for the subsequent chapters of my thesis, which focused on two major gaps: understanding how (i) reproductive mode, particularly asexuality, shapes genetic diversity and adaptability to environmental change; and (ii) host association influences genomic variation within parasitoid populations.

One of the major research gaps I discussed in **Chapter 2** was understanding the influence of the asexual reproductive mode on adaptation and genetic diversity. I then addressed this in **Chapter 3** by examining the Irish asexual *M. aethiopoides*, focusing on spatial and temporal differences through a comparison of four contemporary New Zealand regional populations (two from the North Island and two from the South Island) compared with a historic Irish population. Population genomics provides a powerful framework to explore this by examining patterns of genetic variation, genetic differentiation, and linkage disequilibrium across the genome (Bourgeois & Warren, 2021). Using genome-wide data, it becomes possible to understand population structure, detect signatures of selection, and infer recombination, which helps to clarify the reproductive mode that shapes genetic diversity within and among populations (Allendorf et al., 2010; Mozhaitseva et al., 2023). First, I investigated population structure, revealing two distinct clusters that mostly represented two genetic lineages within the contemporary populations rather than geographic isolation. This pattern was also supported by the population-level phylogeny. Interestingly, this finding aligned well with the two distinct genetic lines originally released in New Zealand (Phillips et al., 2006; Vink, 2012), suggesting the clusters observed in my results could represent those same genetic lines. Notably, both genetic lines were well represented in the North Island populations, whereas the South Island populations were dominated by a single lineage. This could indicate the successful dispersal of the single genetic lineage in the South Island. However, since my samples in the South Island included only two sites, further sampling is needed to confirm this pattern. Exploring diversity metrics, I found very low nucleotide diversity within each population, consistent with patterns typically observed in asexual populations (Mozhaitseva et al., 2023), but the contemporary populations displayed significant post-release expansion compared to the historic Irish population, suggesting adaptation despite low diversity. Lastly, I examined potential signs of recombination in these asexual populations. The results of high heterozygosity, fewer homozygous segments, and rapid linkage disequilibrium decay, may be reflective of partial meiosis (automixis) or facultative sexuality (where populations predominantly reproduce asexually but have potential for sexual reproduction), which is consistent with previous studies reporting active meiosis genes (Inwood et al., 2023). Overall, the findings from this study supports the suggestion that asexual populations are not always entirely clonal in *M. aethiopoides*, with mechanisms such as automixis or facultatively sexual behaviour potentially helping to remove deleterious mutations, retain genetic diversity, and contribute to adaptation in Irish *M. aethiopoides*. Additionally, this study provides insight into the dispersal pattern of genetic lineages of the Irish *M. aethiopoides* in New Zealand, and a valuable new resource of population-level genomic data for asexual populations.

Parasitoids rely on the host body for their survival, so important traits include olfaction (for locating the host) and immune responses (for successful development and evasion of host defence). This evolutionary arms race between parasitoid and host creates opportunities for divergence in the parasitoid, that can ultimately lead to speciation on different hosts. Such divergence can also occur within the same species when adaptive pressures vary through host or geography to resulting in differentiated ecotypes (strains) (Goldson et al., 2003, 2005; Häner et al., 2024; Koppik et al., 2015; Zaviezo et al., 2021). In **Chapter 4,** I had the opportunity to explore such divergence at the genomic level within the *M. aethiopoides* strains that parasitise *Hypera* and *Sitona* weevils. I began by producing high-quality genome assemblies using hybrid long-short read sequencing for eight *M. aethiopoides* strains representing different geographic origins and host associations. These, together with two existing public assemblies, were annotated using a uniform pipeline to ensure consistency for downstream comparative analyses. I first constructed a phylogeny using single-copy orthologues, which showed that divergence among *M. aethiopoides* strains is primarily driven by host association, consistent with previous studies (Phillips et al., 2008; Vink et al., 2003). Two major lineages emerged, corresponding to host differentiation of parasitoids on *Hypera* and *Sitona* hosts. Within these lineages, the USA (from *Hypera postica*) and Moroccan (from *Sitona discoideus*) strains formed the most ancestral groups. SNP-based heterozygosity estimates further showed that these two sexually reproducing strains possessed the highest levels of heterozygosity, whereas the asexual Irish-derived strains showed the lowest heterozygosity, likely reflecting the nature of asexual reproductive modes. The combined patterns of phylogeny and heterozygosity could suggest that the USA and Moroccan strains have retained a large proportion of their ancestral genetic diversity, making them potentially more evolutionarily resilient and capable of broader host-use flexibility, which is consistent with the Moroccan strain's documented high non-target impact (Ferguson et al., 2024). This interpretation also lies within evolutionary theory showing that populations with high standing genetic variation have greater adaptive potential (Barrett & Schluter, 2008), and with empirical work demonstrating that host-associated divergence and host shifts in parasitoids are often facilitated by broad underlying genetic variability (Forbes et al., 2017; Henry et al., 2010; Stireman et al., 2006). Although asexual lineages are often expected to accumulate transposable elements (TEs) due to reduced recombination and weakened selection (Bachtrog, 2013; Chalopin et al., 2015), I found no major differences in TE composition between sexual and asexual strains. This may be because of TE-suppression mechanisms in asexual lineages, as observed in *Saccharomyces cerevisiae* (Bast et al., 2019) or due to the relatively recent origin of asexuality in the Irish lineage. I found that most gene families were conserved across strains; however, the rapidly evolving gene families I identified included TE-associated genes, viral-derived domains, and immune-related genes, suggesting ongoing genome restructuring that may facilitate adaptation to host physiology. Chemosensory genes play a central role in host location (discussed in detail in **Chapter 2)**; I thus classified these gene families across all strains and found broad patterns of gene conservation, with only modest variation in odourant receptor copy number. This variation may reflect biologically meaningful differences in response to host-associated cues, though sequencing or annotation biases cannot be entirely ruled out. Overall, my findings in **Chapter 4** highlighted host association as a major driver of genomic divergence in *M. aethiopoides*. However, the observed divergence appears to be largely restricted to genome-wide variation rather than substantial changes in gene family composition, which is consistent with the oscillation hypothesis discussed by Hambäck et al. (2024), proposing that adaptation differences often arise through selection on standing genetic variation rather than the evolution of entirely novel genes.

Apart from the research-based findings above, my thesis also provided valuable genomic resources, including the high-quality genome assemblies of eight *M. aethiopoides* strains and the first complete mitochondrial genome for *M. aethiopoides*. In **Chapter 5**, I characterised the mitogenome of *M. aethiopoides*, and with the use of long-read sequencing, it was possible to assemble the entire circular mitogenome. This assembly revealed an unusually long control region and a unique gene order within Hymenoptera, both of which contribute important insights to evolutionary genomics.

6.3 Considerations

In **Chapter 3**, the primary aim was to determine whether spatial or temporal differences exist within the Irish strain of *M. aethiopoides* populations across New Zealand. While no clear spatial or temporal differentiation was detected, the population structure analyses suggested the presence of two distinct genetic lineages, with one lineage more dominant in the South Island. However, complete interpretation was constrained by the sampling design, with only four regional populations limiting the ability to make strong inferences about fine-scale dispersal patterns or broader population connectivity across the country. A more comprehensive sampling framework will be required to confidently resolve movement dynamics and lineage spread following the original introduction in future. Another consideration relates to interpreting the reproductive mode of the Irish strain. Although the data in **Chapter 3** hinted at potential facultative sexuality, this could not be robustly addressed with the available dataset because no sexually reproducing representative was available for direct comparison. Future work incorporating a verified sexual population will be essential to clarify the extent to which facultative sex occurs.

In **Chapter 4**, I included two publicly available short-read-based assemblies alongside the long-read genome assemblies I generated. Short-read assemblies typically underestimate repetitive regions, particularly transposable elements, due to fragmented contigs and difficulties in resolving repetitive sequences (Cechova, 2020). As a result, transposable element content in these short-read assemblies is likely underrepresented compared with the long-read assemblies, which provide substantially higher continuity and more accurate repeat resolution. This discrepancy means that direct comparisons of TE abundance, TE composition, or TE-driven genome expansion across new and existing strains must be interpreted cautiously. Differences attributed to biological variation may instead reflect methodological biases caused by sequencing technology rather than true genomic divergence. A further consideration relates to the potential biases introduced during gene annotation. Although consistent pipelines and parameters were applied across assemblies, annotation accuracy is inherently influenced by assembly completeness and contiguity. Long-read assemblies generally yield more complete gene models, while short-read assemblies may fragment genes or fail to recover certain loci entirely. Such factors may affect the comparisons of gene family expansions, contractions, or functional categories across strains that may partially reflect annotation artefacts rather than true biological differences.

6.4 Future aspects

My thesis provides a genomic foundation for understanding the evolutionary processes shaping patterns of host-associated divergence among *M. aethiopoides* strains with different host ranges. While this body of work offers new insights into genetic diversity, population structure, reproductive signatures, and strain-level genomic differences, there remains considerable potential for further research. These opportunities are discussed in detail in the Discussions of **Chapters 3, 4, and 5**, and in the broader review presented in **Chapter 2**. Thus, I provide a brief description of potential future research directions as concise snippets below.

The potential for sexual reproduction in the Irish strain *M. aethiopoides* remains an open question and resolving this will require direct comparison with its conspecific sexual populations. Hybridisation experiments and machine-learning-based genomic approaches (Kuhn et al., 2021; Levine & Booth, 2025; Sun et al., 2023) offer strong methodological frameworks for detecting cryptic sex (Freitas et al., 2023; Wachi et al., 2021) and for characterising the underlying reproductive system. Simulation-based studies using tools, such as SLiM4 (Haller & Messer, 2023), could also be applied to test how different asexual mechanisms, such as automixis with central or terminal fusion, gamete duplication, or apomixis, shape long-term adaptive potential under host-parasitoid coevolution. Understanding the consequences of reproductive mode for biocontrol is especially important in systems where sexual and asexual strains may co-occur. In New Zealand, the Moroccan strain has occasionally been observed alongside the Irish strain (McNeill et al., 2002), raising the possibility of gene flow. Occasional sexual reproduction in the Irish strain could, in theory, increase genetic diversity and improve adaptability (Halkett et al., 2008; Li et al., 2015), but laboratory studies show that hybridisation can reduce host specificity and performance (Goldson et al., 2003). Clarifying the frequency, direction, and consequences of gene flow will therefore be vital for future biocontrol decisions.

As ectothermic organisms whose body temperature closely tracks environmental conditions, insects are extremely sensitive to temperature fluctuations (Aguilar et al., 2015;

Popa-Báez et al., 2021), with research showing that their physiological processes, particularly metabolic rate, can increase dramatically, often doubling with a 10 °C temperature rise (Dukes et al., 2009). Climate change, particularly temperature changes, represents another important axis for future work. Studies on other parasitoids show that sexual and asexual strains can differ markedly in thermal responsiveness and fecundity (Amat et al., 2006, 2017; Tenguri et al., 2016; Wang & Smith, 1996), suggesting that reproductive mode may influence ecological resilience. Also, current work on parasitoids is heavily biased toward heat shock proteins as indicators of thermal tolerance (Ding et al., 2021; Liu et al., 2020; Song et al., 2020), leaving much of the broader genetic architecture of stress adaptation uncharacterised. Thus, integrating molecular approaches, such as gene expression profiling or RNA-Seq, would allow researchers to determine how sexual and asexual lineages differ in their physiological response during stress exposure (Ding et al., 2021; Liu et al., 2020; Xiong et al., 2024) and uncover complex regulatory networks that respond to stress (Ashraf et al., 2022; Xiong et al., 2024). Such work would provide a mechanistic basis for predicting how *M. aethiopoides* populations may respond to environmental change.

Despite the central importance of olfaction for parasitoid host location, research on olfactory mechanisms within New Zealand's biocontrol systems remains limited (Saunders et al., 2024). Earlier work on *M. aethiopoides* (Fusco & Hower, 1974; Loan & Holdaway, 1961) provided useful biological context, yet the molecular and behavioural mechanisms of host detection, particularly in relation to volatile cues, remain largely unexplored (but see Steele et al., (2006). With climate change driving increasingly unpredictable environmental conditions in New Zealand's pastures (Chapman et al., 2024), olfaction-based behaviours may be altered (Pinto-Zevallos & Blande, 2024). Future research should therefore further investigate olfactory-related genes and pathways, as well as behavioural conditioning approaches where parasitoids are exposed to pest-specific odourants to enhance host-finding efficiency (Kruidhof et al., 2019). Identifying the specific plant volatiles or pheromones that guide parasitoid foraging may also support the development of synthetic odour blends to attract parasitoids into pest infested areas (Uefune et al., 2021). Such advances could significantly refine biocontrol programmes while improving selectivity and reducing non-target impacts.

Microbiome research on *Microctonus* parasitoids is also still in its early stage. Recent studies have uncovered *Microctonus hyperodae filamentous virus* (MhFV), which is actively transmitted to *Listronotus bonariensis* and may contribute to premature host mortality

(Inwood et al., 2023, 2024). Future work should clarify the functional role of MhFV, assess its presence across hosts and non-hosts, and investigate whether similar viruses occur across other *Microctonus* species in New Zealand. Additionally, the microbiome of *S. obsoletus* showed the presence of defensive endosymbionts, such as *Wolbachia* and *Rickettsia*, which may influence parasitism success (e.g., by moderating sex ratios) (McNeill et al., 2000, 2014; White et al., 2015). Determining whether these endosymbionts are shared between host and parasitoid via horizontal transmission remains an important question. Future work should combine 16S rRNA amplicon sequencing with functional symbiont assays, such as antibiotic treatments to remove particular symbionts, and with volatile compound assays to test symbiont-mediated effects on parasitoid attraction. These approaches will not only enhance understanding of host-parasitoid microbiome interactions but may also provide new tools for improving biocontrol effectiveness.

Overall, integrating behavioural ecology, genomics, transcriptomics, and microbiome analyses into future research will provide a more comprehensive understanding of the evolutionary and ecological factors shaping *M. aethiopoides* populations in New Zealand. Such work will be essential for refining biocontrol strategies, improving strain selection, and ensuring the long-term success and safety of parasitoid introductions in complex agricultural ecosystems.

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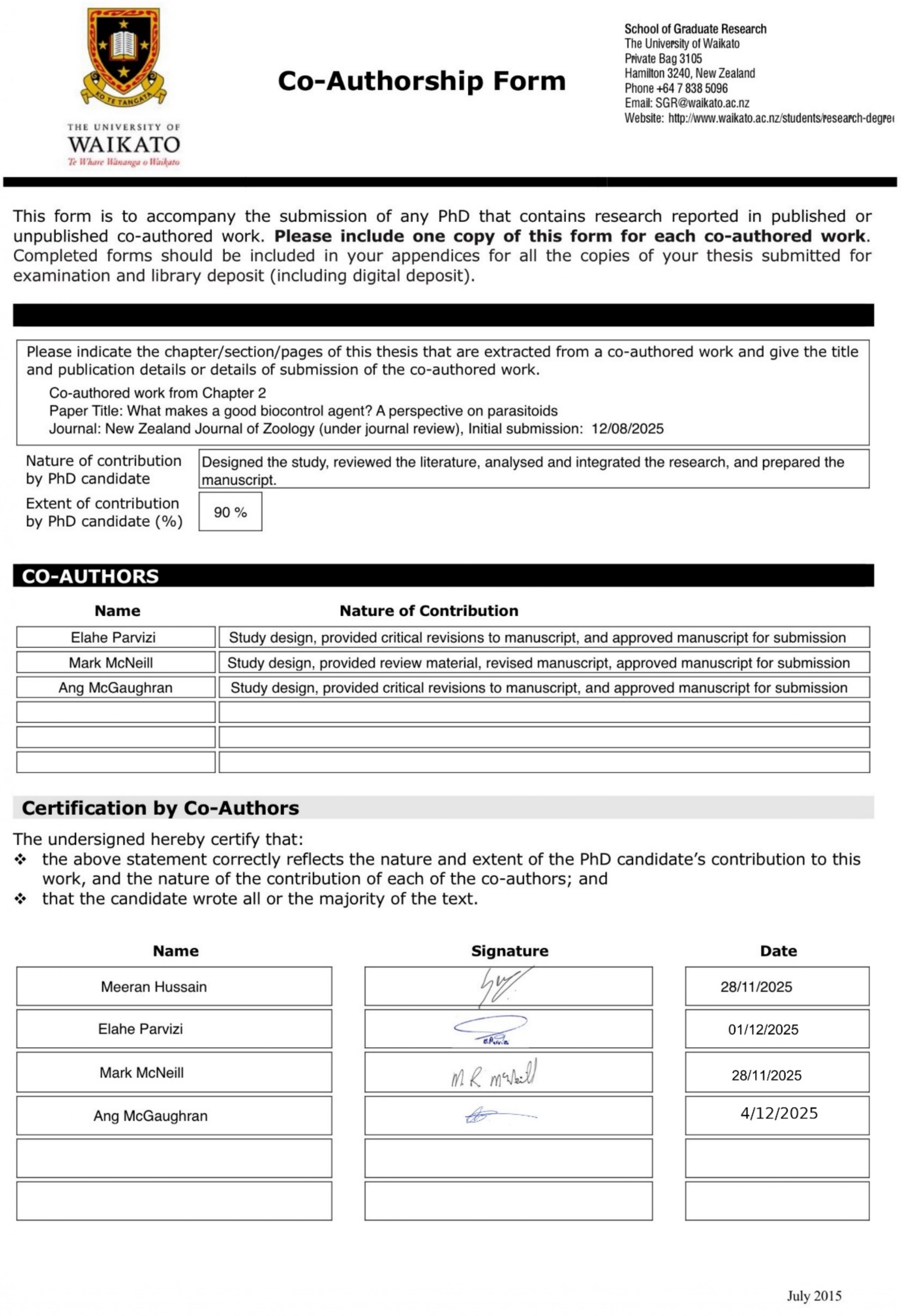
APPENDICES

A1. Co-authorship forms

A1.1 Chapter 2 co-authorship form



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Te Whare Wānanga o Waikato

School of Graduate Research
The University of Waikato
Private Bag 3105
Hamilton 3240, New Zealand
Phone +64 7 838 5096
Email: SGR@waikato.ac.nz
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Co-authored work from Chapter 2
Paper Title: What makes a good biocontrol agent? A perspective on parasitoids
Journal: New Zealand Journal of Zoology (under journal review), Initial submission: 12/08/2025

Nature of contribution by PhD candidate	Designed the study, reviewed the literature, analysed and integrated the research, and prepared the manuscript.
Extent of contribution by PhD candidate (%)	90 %

CO-AUTHORS

Name	Nature of Contribution
Elahe Parvizi	Study design, provided critical revisions to manuscript, and approved manuscript for submission
Mark McNeill	Study design, provided review material, revised manuscript, approved manuscript for submission
Ang McGaughran	Study design, provided critical revisions to manuscript, and approved manuscript for submission

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Meeran Hussain		28/11/2025
Elahe Parvizi		01/12/2025
Mark McNeill	M R McNeill	28/11/2025
Ang McGaughran		4/12/2025

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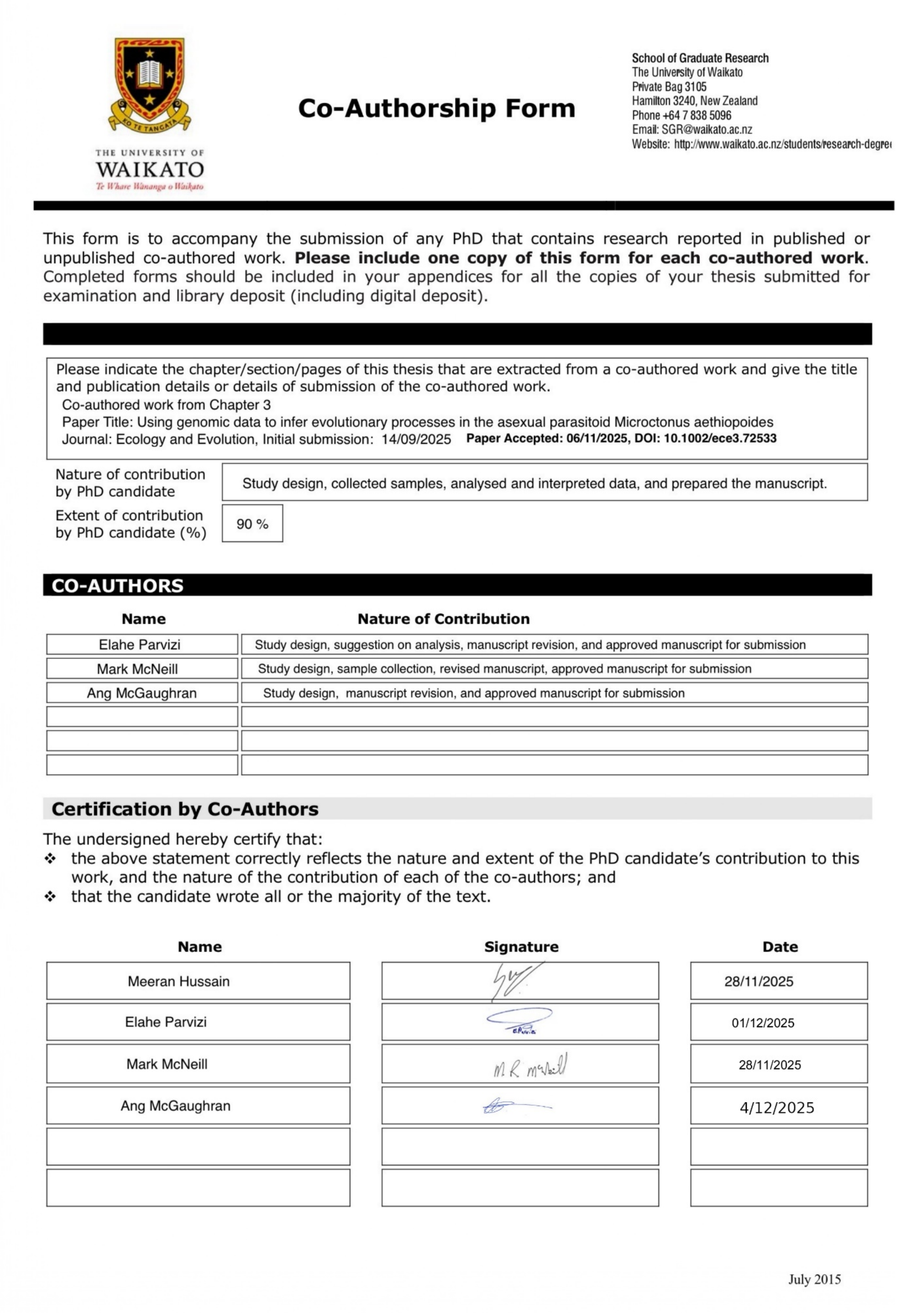
A1.2 Chapter 3 co-authorship form



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Co-authored work from Chapter 3
Paper Title: Using genomic data to infer evolutionary processes in the asexual parasitoid Microctonus aethiopoides
Journal: Ecology and Evolution, Initial submission: 14/09/2025 **Paper Accepted: 06/11/2025, DOI: 10.1002/ece3.72533**

Nature of contribution by PhD candidate	Study design, collected samples, analysed and interpreted data, and prepared the manuscript.
Extent of contribution by PhD candidate (%)	90 %

CO-AUTHORS

Name	Nature of Contribution
Elahe Parvizi	Study design, suggestion on analysis, manuscript revision, and approved manuscript for submission
Mark McNeill	Study design, sample collection, revised manuscript, approved manuscript for submission
Ang McGaughran	Study design, manuscript revision, and approved manuscript for submission

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Meeran Hussain		28/11/2025
Elahe Parvizi		01/12/2025
Mark McNeill	M R McNeill	28/11/2025
Ang McGaughran		4/12/2025

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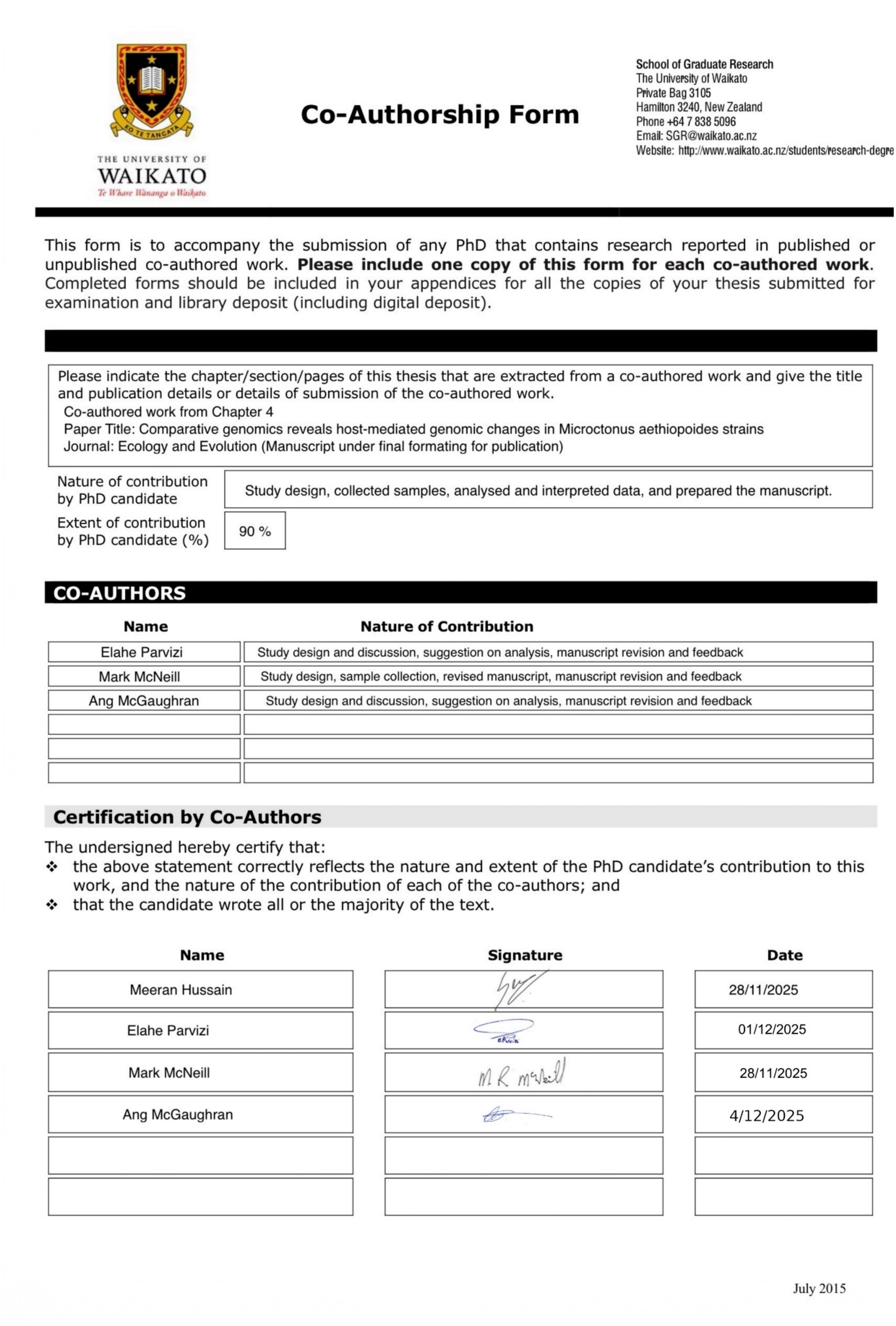
A1.3 Chapter 4 co-authorship form



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The University of Waikato
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Co-authored work from Chapter 4
Paper Title: Comparative genomics reveals host-mediated genomic changes in Microctonus aethiopoides strains
Journal: Ecology and Evolution (Manuscript under final formating for publication)

Nature of contribution by PhD candidate	Study design, collected samples, analysed and interpreted data, and prepared the manuscript.
Extent of contribution by PhD candidate (%)	90 %

CO-AUTHORS

Name	Nature of Contribution
Elahe Parvizi	Study design and discussion, suggestion on analysis, manuscript revision and feedback
Mark McNeill	Study design, sample collection, revised manuscript, manuscript revision and feedback
Ang McGaughran	Study design and discussion, suggestion on analysis, manuscript revision and feedback

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Meeran Hussain		28/11/2025
Elahe Parvizi		01/12/2025
Mark McNeill	M R McNeill	28/11/2025
Ang McGaughran		4/12/2025

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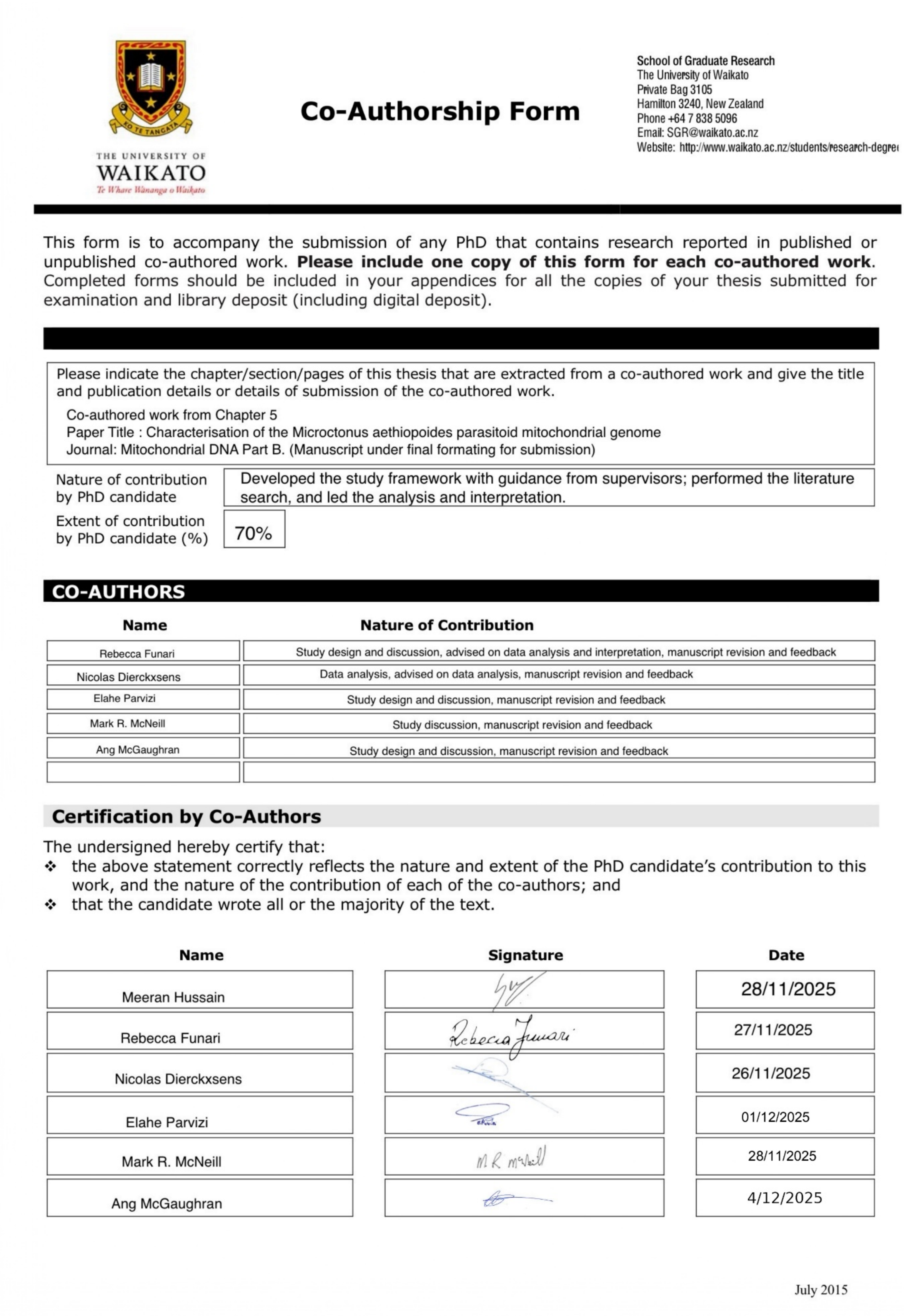
A1.4 Chapter 5 co-authorship form



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Co-Authorship Form

School of Graduate Research
The University of Waikato
Private Bag 3105
Hamilton 3240, New Zealand
Phone +64 7 838 5096
Email: SGR@waikato.ac.nz
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Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Co-authored work from Chapter 5
Paper Title : Characterisation of the Microctonus aethiopoides parasitoid mitochondrial genome
Journal: Mitochondrial DNA Part B. (Manuscript under final formating for submission)

Nature of contribution by PhD candidate	Developed the study framework with guidance from supervisors; performed the literature search, and led the analysis and interpretation.
Extent of contribution by PhD candidate (%)	70%

CO-AUTHORS

Name	Nature of Contribution
Rebecca Funari	Study design and discussion, advised on data analysis and interpretation, manuscript revision and feedback
Nicolas Dierckxsens	Data analysis, advised on data analysis, manuscript revision and feedback
Elahe Parvizi	Study design and discussion, manuscript revision and feedback
Mark R. McNeill	Study discussion, manuscript revision and feedback
Ang McGaughran	Study design and discussion manuscript revision and feedback

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

Name	Signature	Date
Meeran Hussain		28/11/2025
Rebecca Funari	Rebecca Funari	27/11/2025
Nicolas Dierckxsens		26/11/2025
Elahe Parvizi		01/12/2025
Mark R. McNeill	M R McNeill	28/11/2025
Ang McGaughran		4/12/2025

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A2. Parasitoid collection protocol

A2.1 Field work

To obtain *Microctonus aethiopoides*, the most effective approach is to collect its parasitised host, *Sitona obsoletus*. Weevils were collected from pastures containing white clover (*Trifolium repens*) using a commercial leaf-sucking blower-vacuum machine (Fig. A2.1a). The device was fitted with a fine gauze or mesh collection pouch positioned behind the inlet nozzle (Fig. A2.1b). This mesh bag acts as a capture chamber, retaining invertebrates and pasture debris while allowing air to pass through.

During sampling, the blower-vac was passed systematically over the pasture surface to suction invertebrates, litter, and loose soil from the sward. Because the gauze bag fills quickly with organic litter, it was emptied every few minutes into labelled collection containers. All the collected containers in the field were transported to the laboratory for sorting on the same day.

Figure A2.1 Field collection setup for obtaining parasitised *Sitona* weevils. (a) Blower-vacuum used for suction sampling in white clover pastures, alongside the collection container used to transfer debris to the laboratory; (b) Mesh collection pouch fitted that is fit to blower-vacuum nozzle for trapping weevils and pasture debris.

A2.2 Laboratory sorting of weevils

In the laboratory, the pasture debris collected from each field site was spread evenly over large trays, beneath which a heat source or overhead warm light was applied (Fig. A2.2a). As the litter settled and the temperature increased, adult weevils began to move across the tray surface, making them easier to detect. Individual weevils were then hand-collected using sterile forceps/tweezers and transferred into ventilated holding containers (Fig. A2.2b). This sorting method ensured minimal handling stress and allowed rapid separation of weevils from the substrate while keeping field sites distinct for downstream analyses.

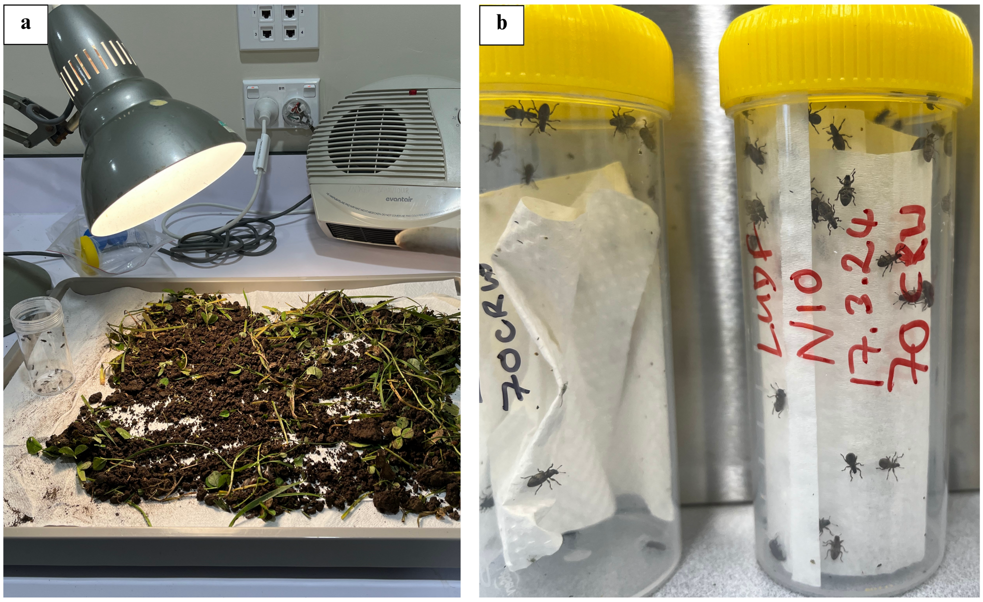

Figure A2.2 Laboratory sorting and temporary holding of *Sitona* weevils. **(a)** Pasture debris spread on white trays under a warm light source to encourage movement of adult weevils for easier detection and manual collection; **(b)** Temporarily housed *Sitona* weevils in labelled ventilated containers prior to transfer into climate-controlled rearing cages.

A2.3 Rearing of weevils in climate cabinets

Following sorting, weevils from each collection site were maintained in separate two-chambered rearing cages to ensure traceability of parasitoid origin (Fig. A2.3a). Each cage consisted of an upper mesh-floored chamber, where live weevils were housed, fed (freshly collected white clover leaves), and watered using cotton rolls, and a lower chamber lined with clean tissue paper to catch emerging parasitoid larvae. Weevils were maintained at 20 °C under a 14:10 h light:dark photoperiod for at least four weeks to allow any developing parasitoid larvae to complete their life cycle.

Fresh white clover bouquets were prepared from *T. repens* plants grown in the greenhouse (Fig. A2.3b & A2.3c), and two moist cotton wicks were supplied every day to provide water and maintain high relative humidity. This controlled environment provided conditions conducive to both weevil survival and parasitoid emergence.

As parasitoid larvae completed their development, they exited the host weevils and dropped through the mesh floor of the upper chamber (Fig. A2.3d). The larvae then pupated beneath the tissue lining the lower chamber of the rearing cage. Tissues containing newly formed pupae were carefully removed, the outer cocoon was gently peeled away, and the larvae were transferred to Eppendorf tubes and frozen until DNA extraction.

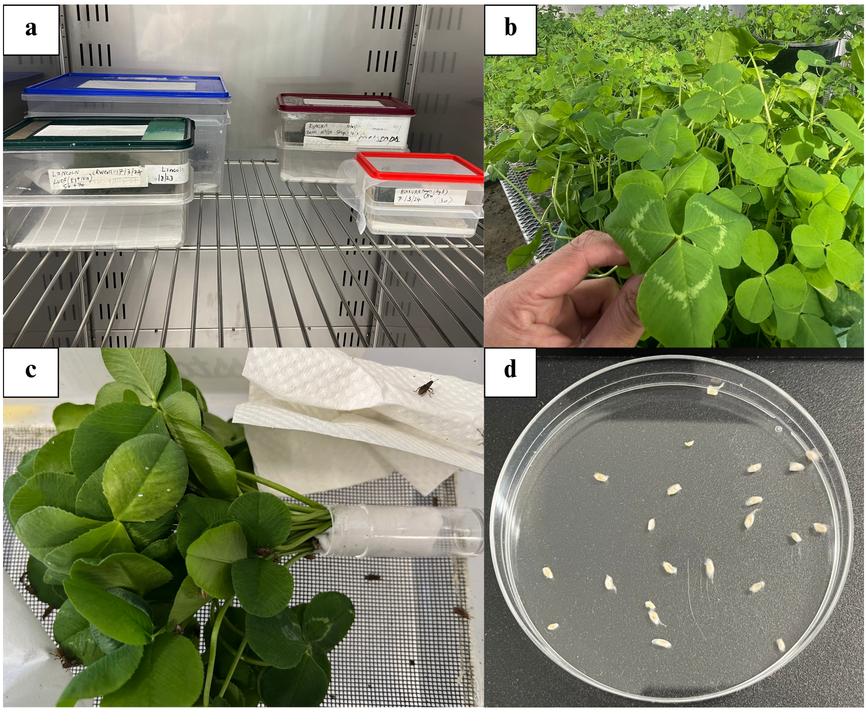

Figure A2.3 Rearing of *Sitona obsoletus* weevils and collection of developing *Microctonus aethiopoides* larvae. **(a)** Two-chambered rearing cages maintained in a climate-controlled cabinet (20 °C, 14:10 h L:D) for parasitoid emergence; **(b)** Greenhouse-grown *T. repens* plants used as a continuous source of fresh white clover bouquets for weevil feeding;**(c)** Fresh white clover bouquet placed in the upper chamber of the rearing cage; **(d)** Parasitoid larvae recovered from the lower chamber after emerging from parasitised weevils.

A3. Supplementary material for Chapter 3

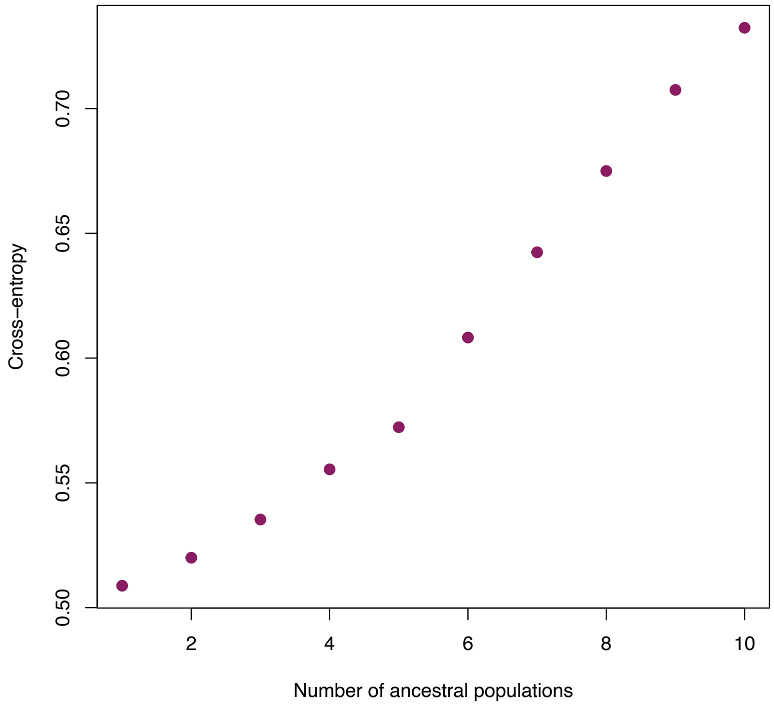

Figure A3.1 Cross-entropy validation for inference of the optimal number of ancestral populations of *Microctonus aethiopoides* (K).

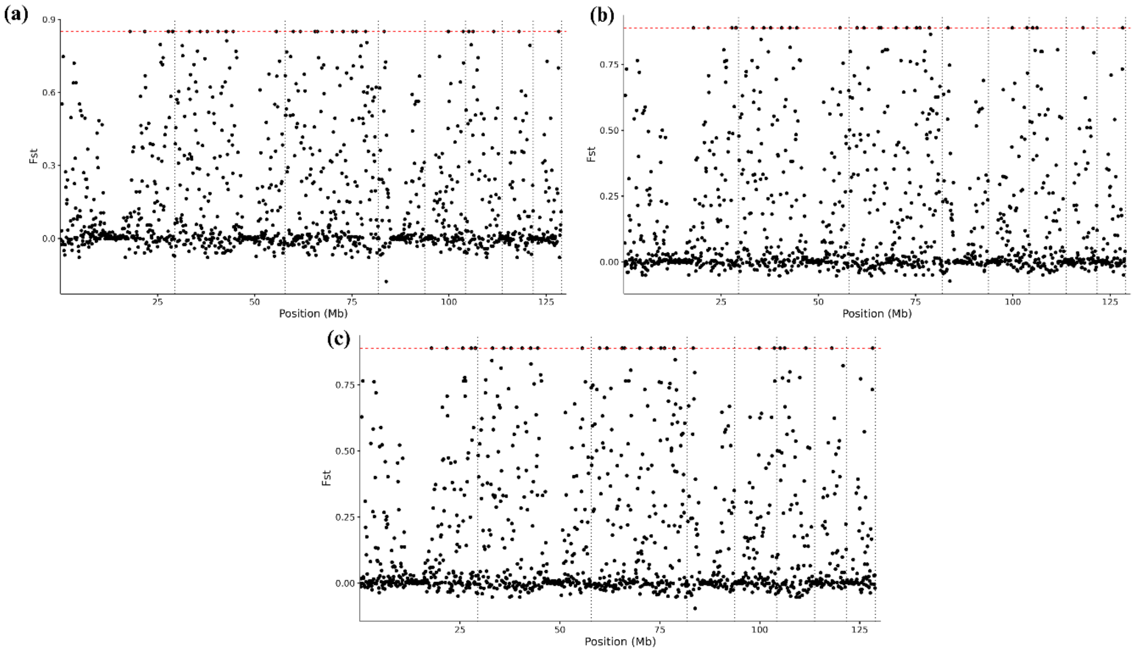

Figure A3.2 Genome-wide distribution of pairwise F_{ST} values for comparisons involving the Hamilton (HAM) population of *Microctonus aethiopoides*. Each panel represents a sliding window analysis of F$_{ST}$ across the genome for the following comparisons: **(a)** Hamilton vs. Mangōnui **(b)** Hamilton vs. Lincoln; and **(c)** Hamilton vs. Dunedin. The red horizontal dotted line indicates the 99th percentile threshold of F$_{ST}$ values, representing the top 1% outliers, and the vertical dotted line represents scaffold boundaries.

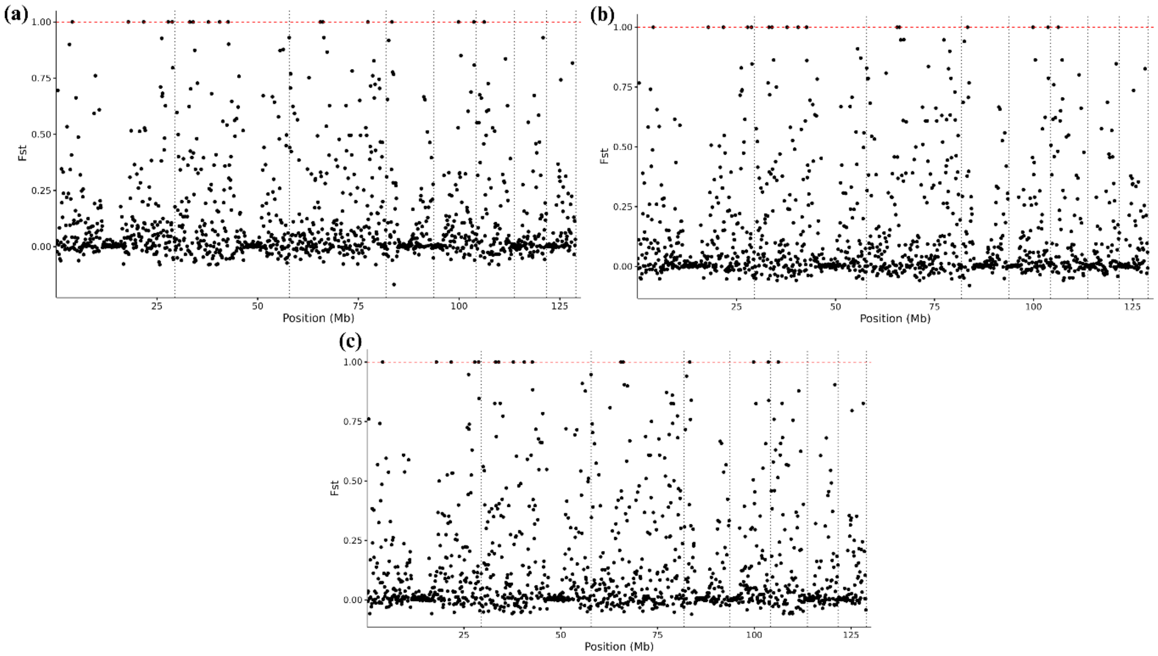

Figure A3.3 Genome-wide distribution of pairwise F$_{ST}$ values for comparisons involving the historic Ireland (IRE) *Microctonus aethiopoides* population compared to the released New Zealand populations. Each panel represents a sliding window analysis of F$_{ST}$ across the genome for the following comparisons: **(a)** Ireland vs. Mangōnui; **(b)** Ireland vs. Lincoln; and **(c)** Ireland vs. Dunedin. The red horizontal dotted line indicates the 99th percentile threshold of F$_{ST}$ values, representing the top 1% outliers, and the vertical dotted line represents scaffold boundaries.

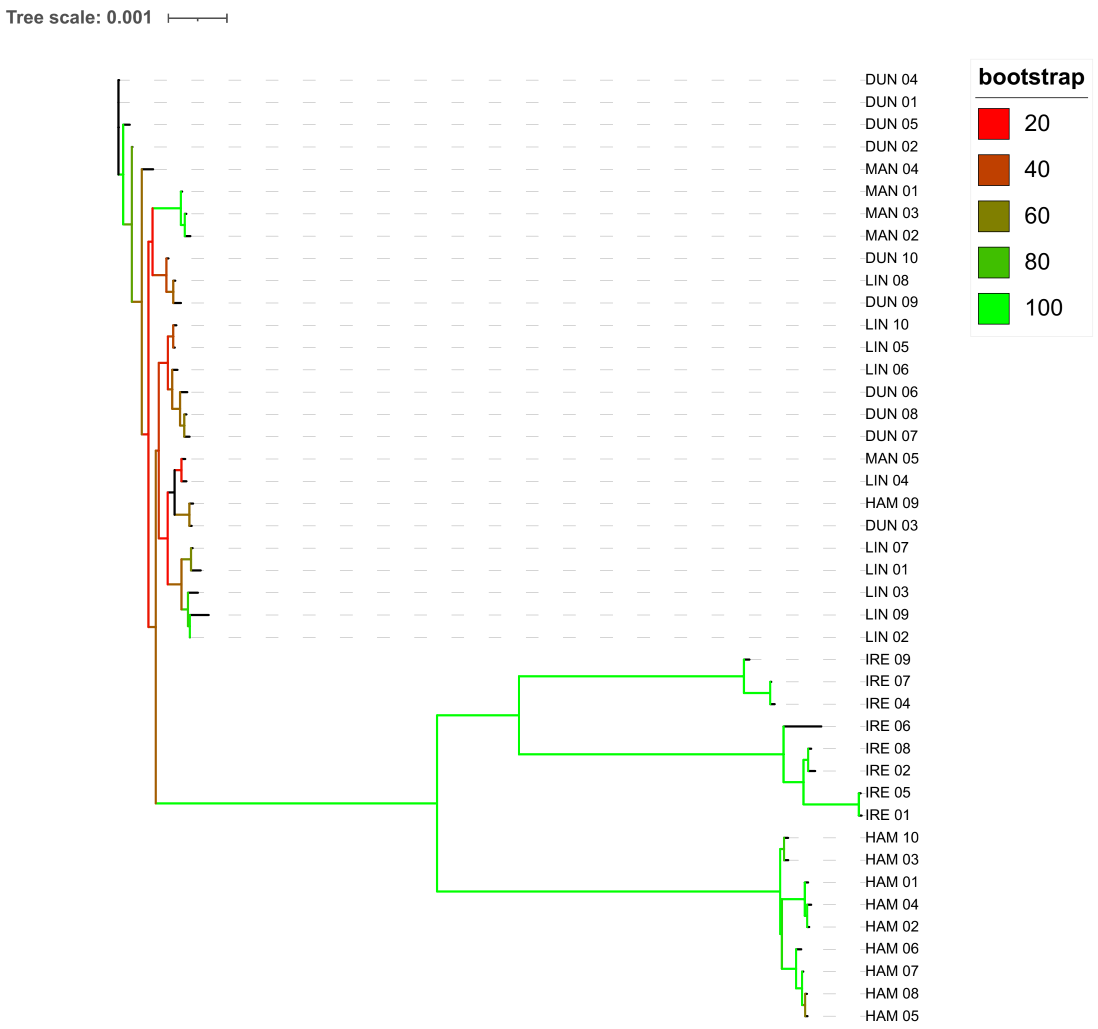

Figure A3.4 Maximum-likelihood phylogenetic tree of *Microctonus aethiopoides* populations from Ireland and New Zealand, based on genome-wide SNP data. Bootstrap support values are indicated by branch colours, ranging from red (low support) to green (high support), as indicated by the provided key.

Table A3.1 Details of all samples used in this study, including collection sites, sample type, and DNA concentration (ng) measured prior to sequencing

Sl. no	Location	Latitude	Longitude	Sample type	Collection date	Sample-ID	Eluted volume (ul)	Qubit (ng/ul)	Qubit Conc (ng)
1	Mangōnui	-35.01041445	173.5992881	Larvae	16/05/2024	MAN-01	55	8	440
2	Mangōnui	-35.01041445	173.5992881	Larvae	16/05/2024	MAN-02	55	5.1	280.5
3	Mangōnui	-35.01041445	173.5992881	Larvae	16/05/2024	MAN-03	55	14.8	814
4	Mangōnui	-35.01041445	173.5992881	Larvae	16/05/2024	MAN-04	55	10.2	561
5	Mangōnui	-35.01041445	173.5992881	Larvae	16/05/2024	MAN-05	55	16.7	918.5
6	Hamilton	-37.77696193	175.3126127	Larvae	11/03/2024	HAM-01	55	5.68	312.4
7	Hamilton	-37.77696193	175.3126127	Larvae	11/03/2024	HAM-02	55	6.44	354.2
8	Hamilton	-37.77696193	175.3126127	Larvae	11/03/2024	HAM-03	55	7.88	433.4
9	Hamilton	-37.77696193	175.3126127	Larvae	11/03/2024	HAM-04	55	9.86	542.3
10	Hamilton	-37.77696193	175.3126127	Larvae	11/03/2024	HAM-05	55	8.86	487.3
11	Hamilton	-37.77696193	175.3126127	Larvae	11/03/2024	HAM-06	55	5.28	290.4
12	Hamilton	-37.77696193	175.3126127	Larvae	11/03/2024	HAM-07	55	8.16	448.8
13	Hamilton	-37.77696193	175.3126127	Larvae	11/03/2024	HAM-08	55	11.5	632.5
14	Hamilton	-37.77696193	175.3126127	Larvae	11/03/2024	HAM-09	55	6.86	377.3
15	Hamilton	-37.77696193	175.3126127	Larvae	11/03/2024	HAM-10	55	5.38	295.9
16	Lincoln	-43.64262001	172.4689808	Larvae	23/08/2023	LIN-01	55	15	825
17	Lincoln	-43.64262001	172.4689808	Larvae	23/08/2023	LIN-02	55	18	990
18	Lincoln	-43.64262001	172.4689808	Larvae	23/08/2023	LIN-03	55	8.8	484
19	Lincoln	-43.64262001	172.4689808	Larvae	23/08/2023	LIN-04	55	24	1320
20	Lincoln	-43.64262001	172.4689808	Larvae	23/08/2023	LIN-05	55	17	935
21	Lincoln	-43.64262001	172.4689808	Larvae	23/08/2023	LIN-06	55	9	495
22	Lincoln	-43.64262001	172.4689808	Larvae	23/08/2023	LIN-07	55	8.56	470.8
23	Lincoln	-43.64262001	172.4689808	Adult	23/08/2023	LIN-08	55	3.56	195.8
24	Lincoln	-43.64262001	172.4689808	Adult	23/08/2023	LIN-09	55	2.34	128.7
25	Lincoln	-43.64262001	172.4689808	Adult	23/08/2023	LIN-10	55	4.26	234.3
26	Dunedin	-45.85701779	170.3915749	Larvae	07/09/2023	DUN_01	55	9.22	507.1
27	Dunedin	-45.85701779	170.3915749	Larvae	07/09/2023	DUN_02	55	5.78	317.9
28	Dunedin	-45.85701779	170.3915749	Larvae	07/09/2023	DUN_03	55	13.7	753.5
29	Dunedin	-45.85701779	170.3915749	Larvae	07/09/2023	DUN_04	55	10.3	566.5

30	Dunedin	-45.85701779	170.3915749	Larvae	07/09/2023	DUN_05	55	4.26	234.3
31	Dunedin	-45.85701779	170.3915749	Larvae	07/09/2023	DUN_06	55	13.6	748
32	Dunedin	-45.85701779	170.3915749	Larvae	07/09/2023	DUN_07	55	13.3	731.5
33	Dunedin	-45.85701779	170.3915749	Larvae	07/09/2023	DUN_08	55	7.3	401.5
34	Dunedin	-45.85701779	170.3915749	Larvae	07/09/2023	DUN_09	55	6.4	352
35	Dunedin	-45.85701779	170.3915749	Larvae	07/09/2023	DUN_10	55	9	495
36	Ireland (Athenry)	53.30119371	-8.746320598	Adult	2002	IRE-01	55	3.16	173.8
37	Ireland (Athenry)	53.30119371	-8.746320598	Adult	2002	IRE-02	55	3.06	168.3
38	Ireland (Athenry)	53.30119371	-8.746320598	Adult	2002	IRE-03	55	3.04	167.2
39	Ireland (Athenry)	53.30119371	-8.746320598	Adult	2002	IRE-04	55	3.02	166.1
40	Ireland (Athenry)	53.30119371	-8.746320598	Adult	2002	IRE-05	55	2.62	144.1
41	Ireland (Athenry)	53.30119371	-8.746320598	Adult	2004	IRE-06	55	2.06	113.3
42	Ireland (Athenry)	53.30119371	-8.746320598	Adult	2004	IRE-07	55	2.78	152.9
43	Ireland (Athenry)	53.30119371	-8.746320598	Adult	2004	IRE-08	55	2.54	139.7
44	Ireland (Athenry)	53.30119371	-8.746320598	Adult	2001	IRE-09	60	2.5	150

Table A3.2 Summary of read mapping statistics for each sample of *Microctonus aethiopoides*. The table includes the sample ID, the total number of reads retained after quality filtering, the number of reads mapped to the reference genome, and the corresponding percentage of mapped reads. MAN = Mangonui, HAM =Hamilton, LIN = Lincoln, DUN = Dunedin, IRE = Ireland.

Sl.no	**Sample**	**Total reads after filtration (150*2)**	**No reads mapped (150*2)**	**Mapping (%)**
1	MAN_01	36,453,952	36,101,707	99.0
2	MAN_02	34,251,174	33,977,958	99.2
3	MAN_03	35,171,518	34,928,188	99.3
4	MAN_04	23,266,006	22,968,128	98.7
5	MAN_05	47,288,376	46,716,325	98.8
6	HAM_01	55,992,644	54,849,757	98.0
7	HAM_02	24,800,414	24,234,959	97.7
8	HAM_03	20,087,704	19,940,912	99.3
9	HAM_04	41,273,268	40,337,496	97.7
10	HAM_05	24,639,488	24,452,818	99.2
11	HAM_06	21,372,098	20,013,901	93.6
12	HAM_07	36,391,030	35,932,084	98.7
13	HAM_08	43,846,808	43,225,397	98.6
14	HAM_09	29,684,160	29,107,958	98.1
15	HAM_10	38,648,384	38,103,580	98.6
16	LIN_01	26,368,138	25,691,336	97.4
17	LIN_02	35,206,002	34,537,238	98.1
18	LIN_03	29,002,534	28,687,208	98.9
19	LIN_04	25,173,044	24,887,899	98.9
20	LIN_05	40,098,562	39,356,289	98.2
21	LIN_06	24,721,446	24,403,279	98.7
22	LIN_07	37,718,142	37,084,014	98.3
23	LIN_08	30,028,108	26,782,060	89.2
24	LIN_09	25,525,434	20,234,417	79.3
25	LIN_10	51,014,722	44,782,596	87.8
26	DUN_01	26,313,144	25,854,306	98.3

27	DUN_02	40,416,140	39,254,919	97.1
28	DUN_03	26,345,776	25,130,862	95.4
29	DUN_04	74,662,184	73,055,045	97.9
30	DUN_05	34,370,342	33,627,857	97.8
31	DUN_06	34,445,638	33,646,636	97.7
32	DUN_07	39,667,840	38,818,325	97.9
33	DUN_08	32,874,472	32,591,054	99.1
34	DUN_09	25,984,624	25,479,018	98.1
35	DUN_10	25,158,904	24,721,560	98.3
36	IRE_01	30,344,284	30,140,784	99.3
37	IRE_02	20,157,824	20,007,931	99.3
38	IRE_04	46,919,472	46,587,448	99.3
39	IRE_05	31,800,998	31,577,560	99.3
40	IRE_06	52,465,230	52,096,465	99.3
41	IRE_07	16,769,832	16,654,113	99.3
42	IRE_08	33,220,002	32,988,126	99.3
43	IRE_09	18,304,984	18,179,108	99.3

A4. Supplementary material for Chapter 4

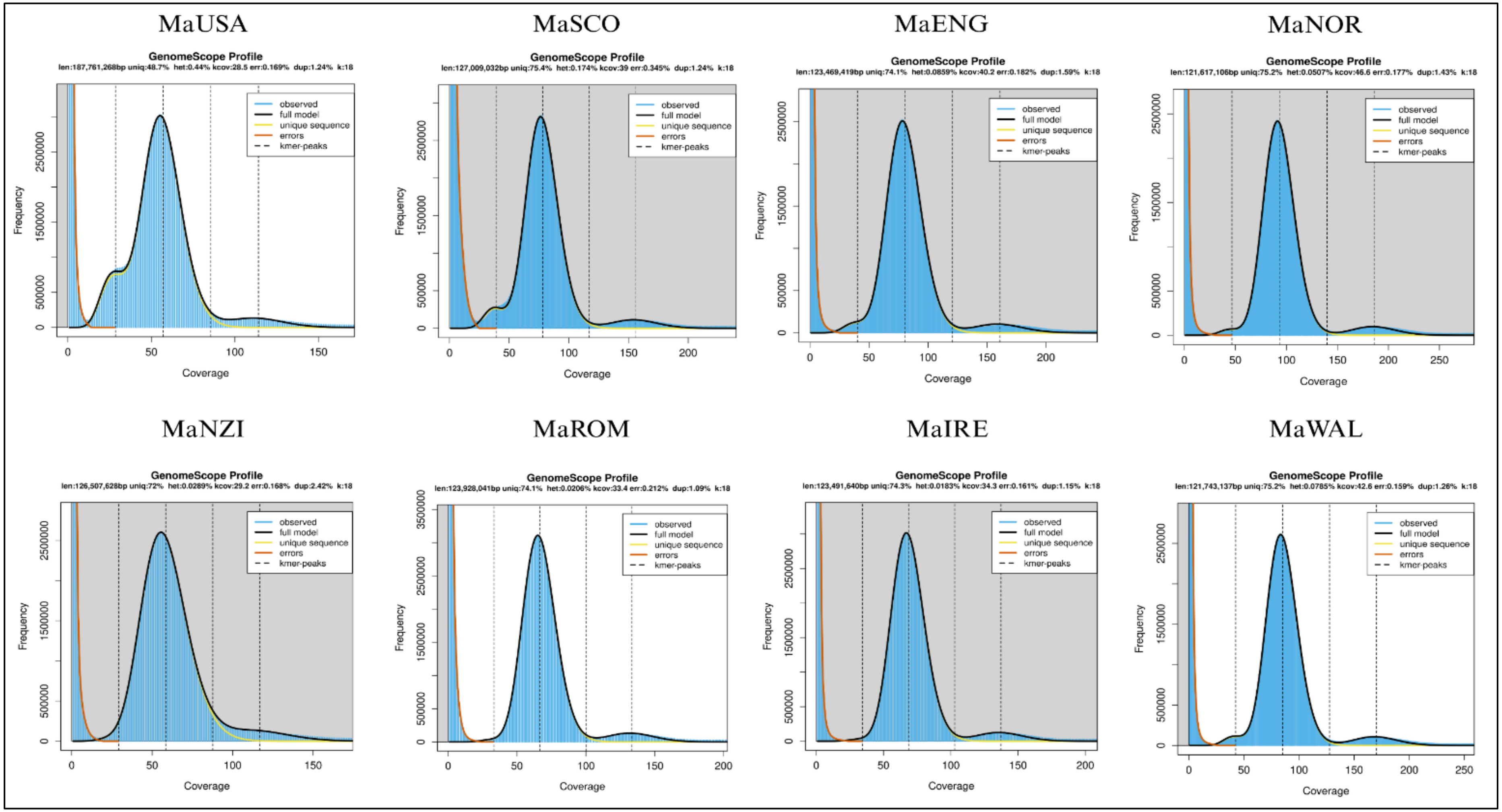

Figure A4.1 Genomescope profiles of newly assembled *Microctonus aethiopoides* genomes

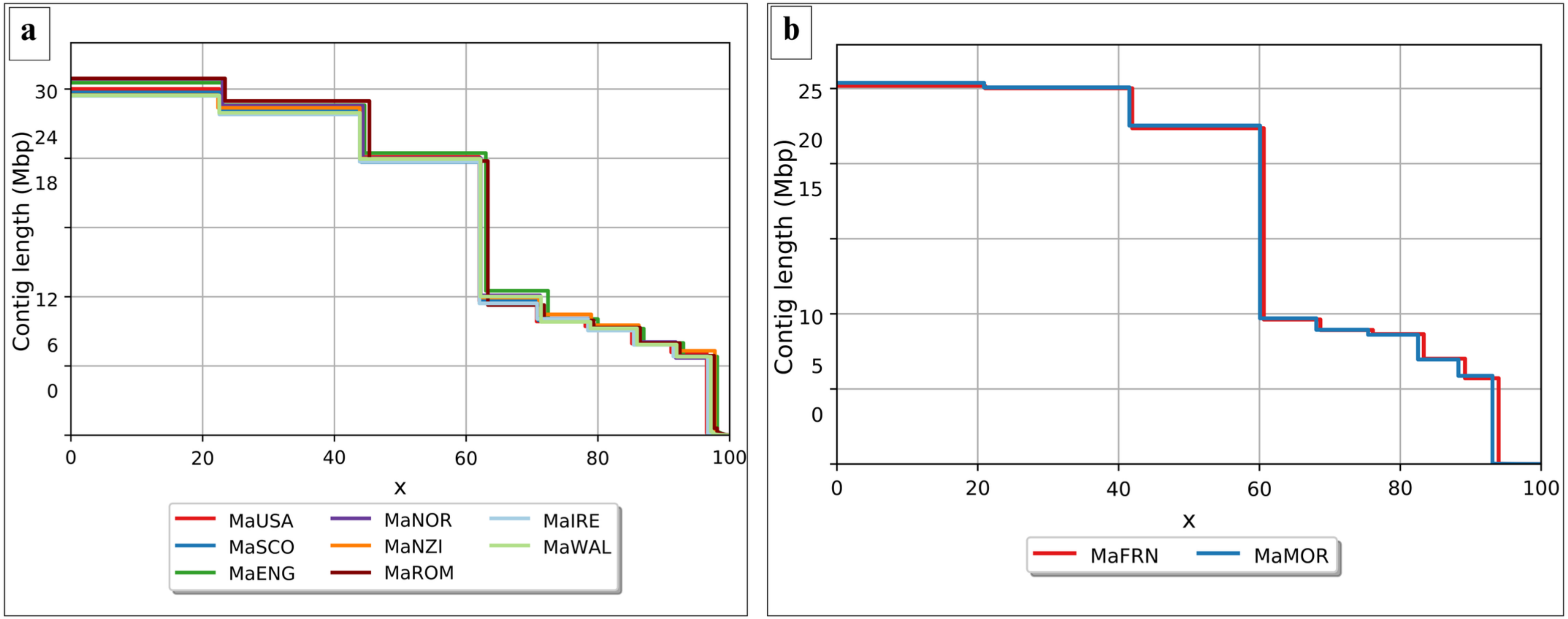

Figure A4.2 Nx plot of *Microctonus aethiopoides* genome assemblies used in this study. The plot shows the contig length (Mbp) at each cumulative proportion (x%) of the genome assemblies.

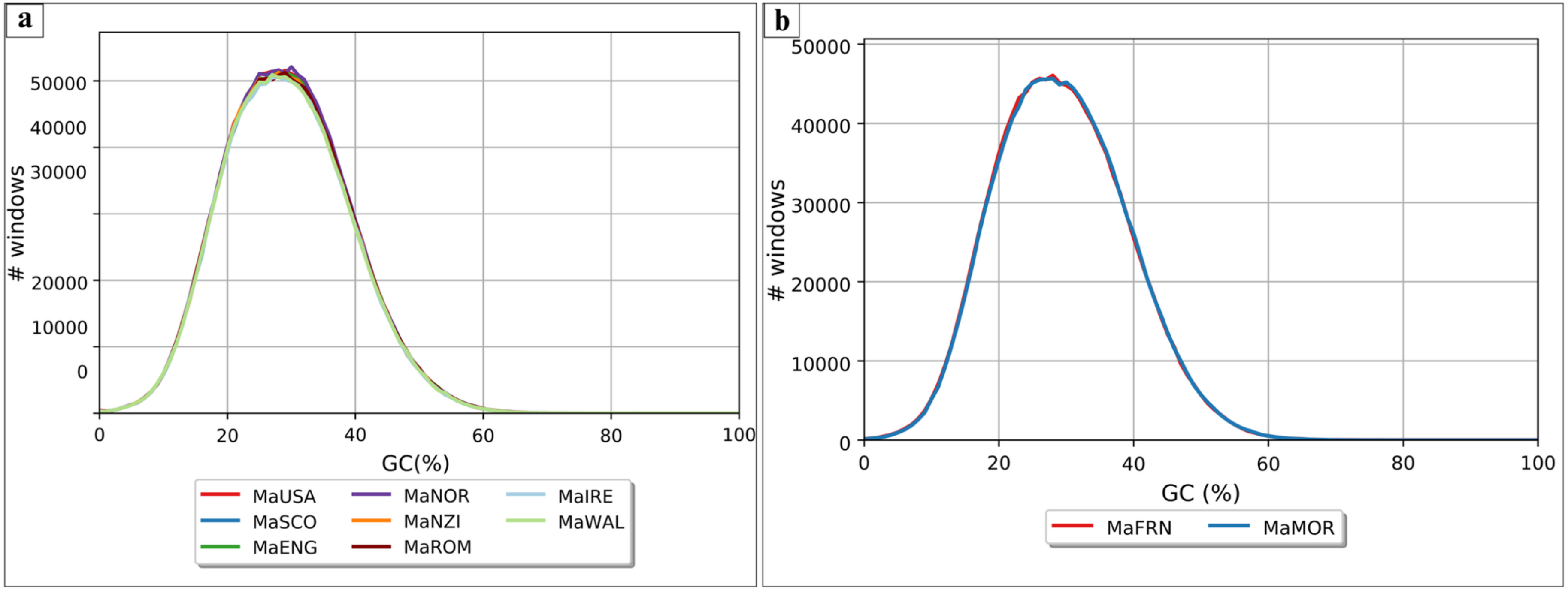

Figure A4.3 GC content distribution across *Microctonus aethiopoides* genome assemblies used in this study.

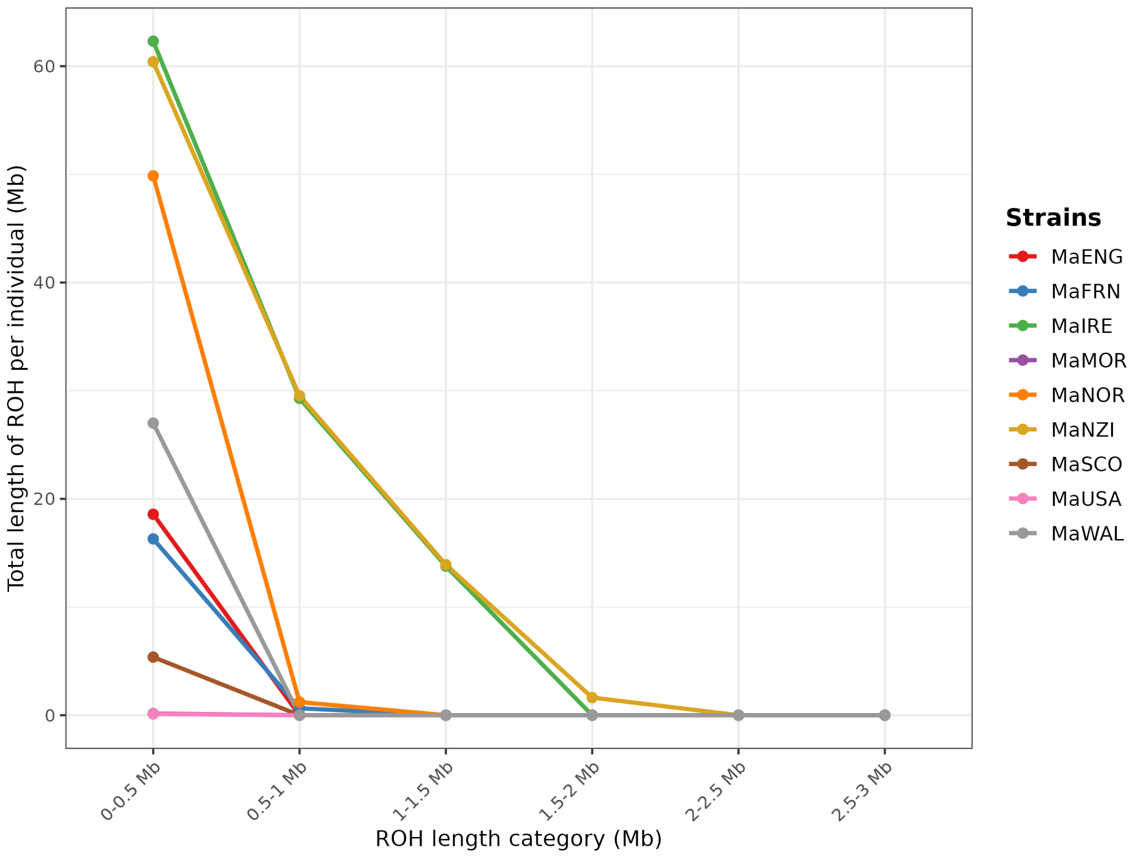

Figure A4.4 Distribution of total runs of homozygosity (ROH) length per individual across *Microctonus aethiopoides* strains. The total ROH length (Mb) per individual is shown across different ROH length categories. The MaROM strain was excluded from this analysis as it represents a male haploid genome.

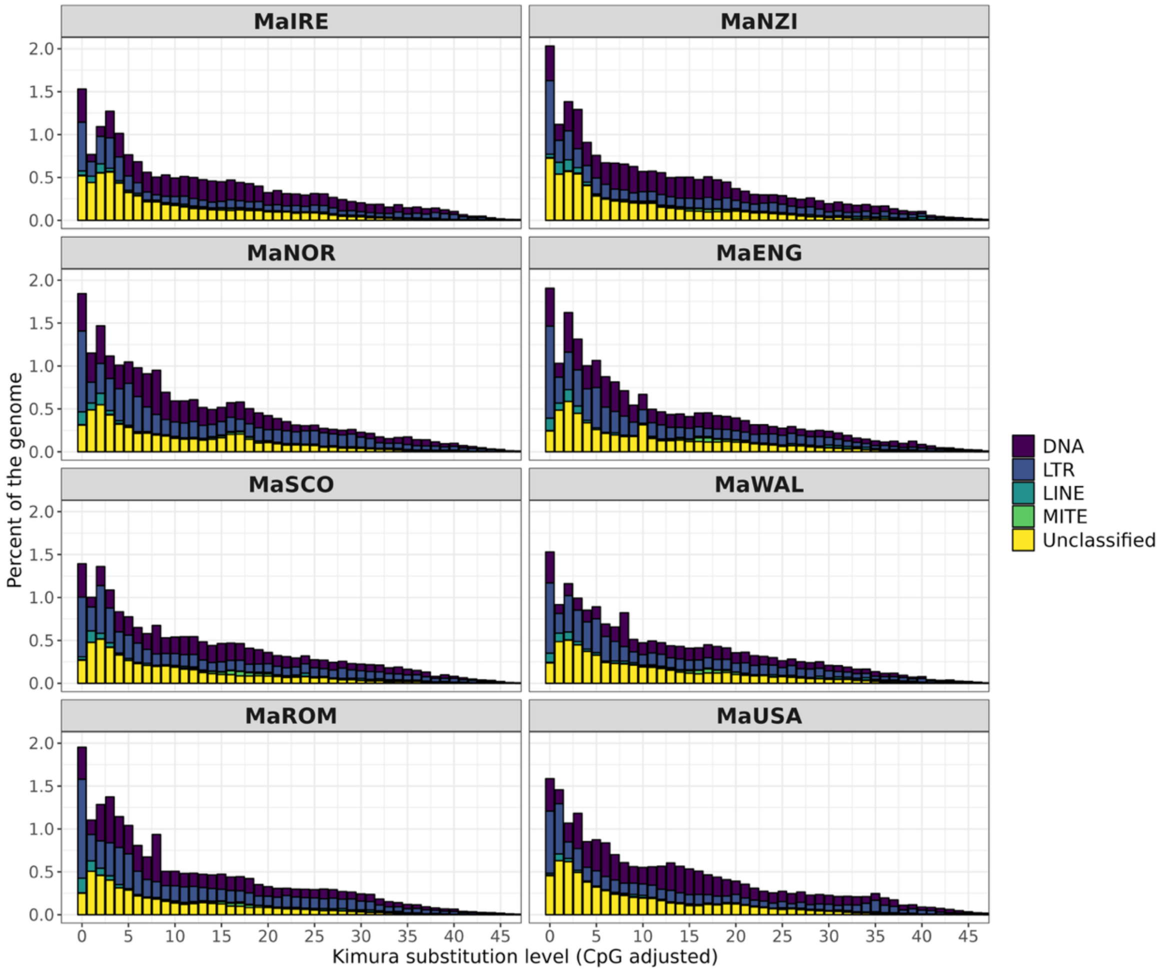

Figure A4.5 Kimura substitution level (CpG-adjusted) profiles of transposable elements (TEs) across *Microctonus aethiopoides* genomes generated in this study. Each panel represents a strain, showing the relative proportion of the genome occupied by major TE classes (DNA, LTR, LINE, MITE, and unclassified elements) plotted against Kimura substitution levels. Peaks at lower divergence values indicate more recent TE activity, whereas the gradual decline toward higher substitution levels reflects older TE insertion.

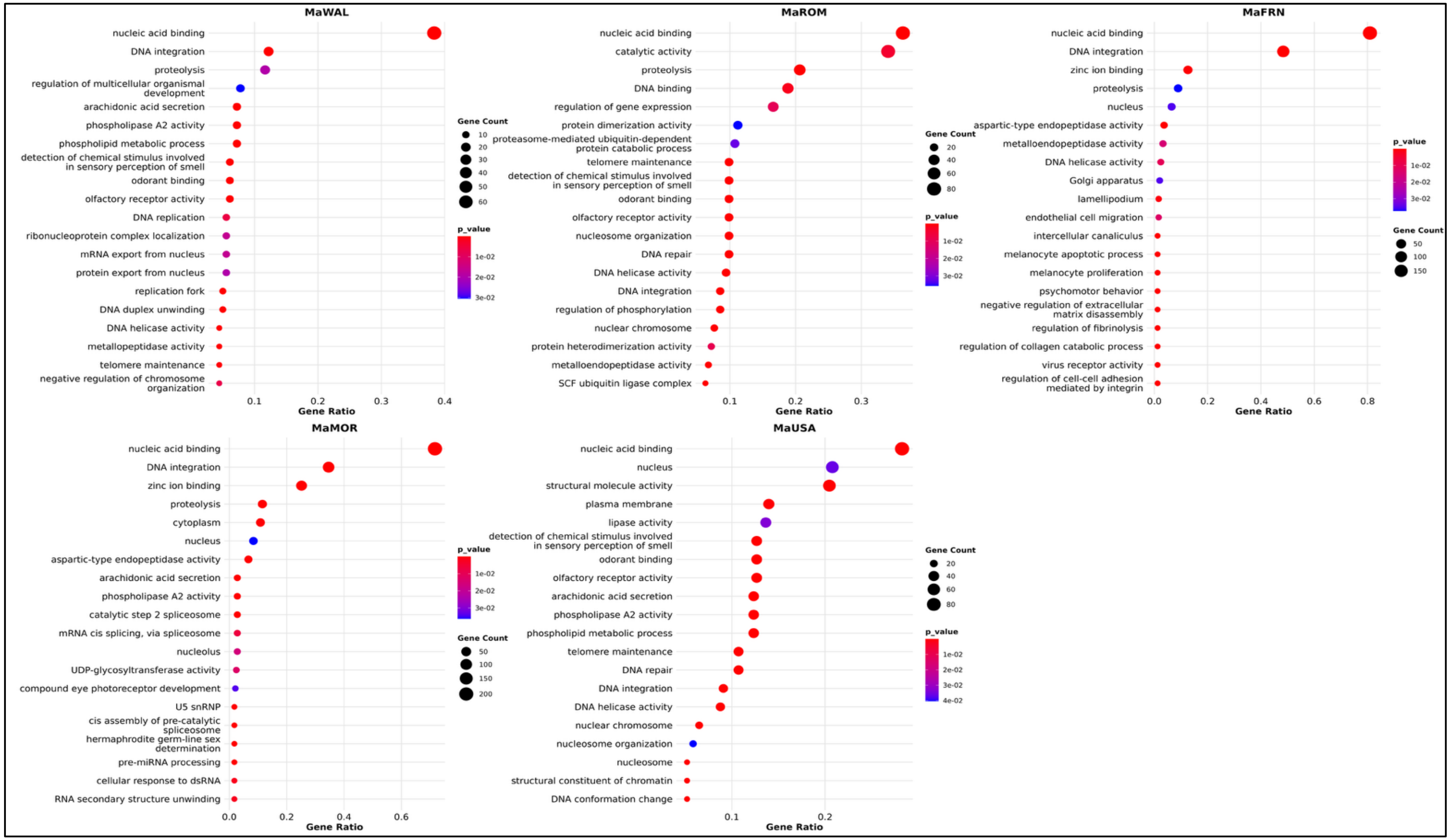

Figure A4.6 Gene Ontology (GO) enrichment of rapidly expanding gene families in *Microctonus aethiopoides* strains MaWAL, MaROM, MaFRN, MaMOR and MaUSA. The bubble plots display significantly enriched GO terms across with gradient colour represents statistical significance, with red indicating highly significant terms and blue indicating lower significance. The size of each bubble corresponds to the number of genes annotated to the respective GO term.

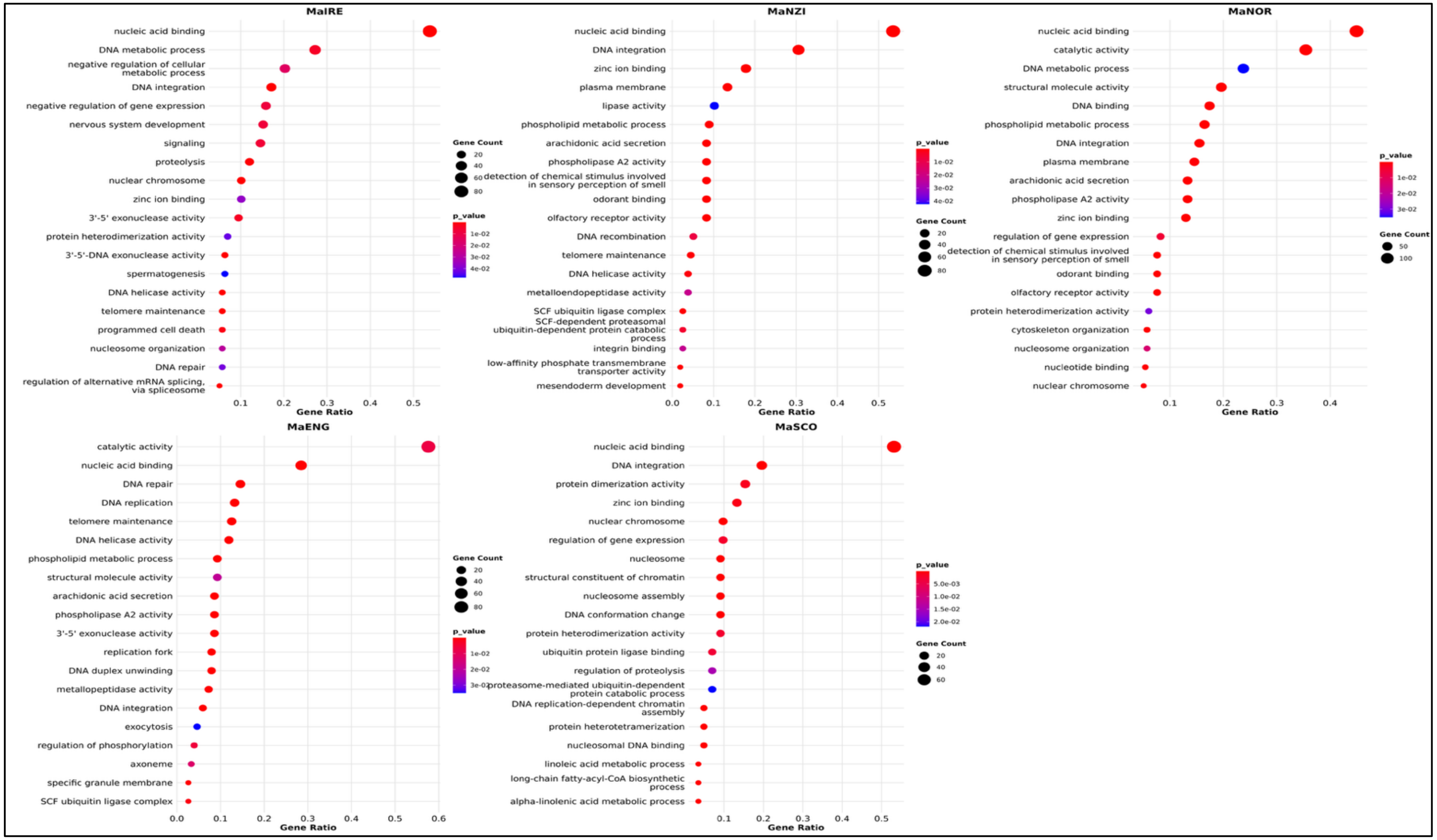

Figure A4.7 Gene Ontology (GO) enrichment of rapidly expanding gene families in *Microctonus aethiopoides* strains MaIRE, MaNZI, MaNOR, MaENG and MaSCO. The bubble plots display significantly enriched GO terms across with gradient colour represents statistical significance, with red indicating highly significant terms and blue indicating lower significance. The size of each bubble corresponds to the number of genes annotated to the respective GO term.

Supplementary tables A4.1-A4.27: The complete set of supplementary tables associated with Chapter 4 is available online at the following link:

https://docs.google.com/spreadsheets/d/1ObG93qVZPKXQwty4NLjBfAGrNChXrxJw/edit?usp=drive_link&ouid=112078435561239605021&rtpof=true&sd=true

A5. Genome assembly protocol - Extended

Note: Software versions and citations are detailed in the Chapter 4 Methods section and are therefore not repeated here.

Genome assemblies were generated for eight *Microctonus aethiopoides* strains using a hybrid long-read/short-read approach. Oxford Nanopore Technologies (ONT) long reads were used to construct the primary assemblies, and Illumina paired-end reads were used for polishing and quality assessment.

Nanopore libraries were prepared using the SQK-NBD114-24 barcoding kit and sequenced in three multiplexed runs (R0119, R0120 and R0149). Nanopore raw data were provided in POD5 format, and all steps were performed on the REANNZ high-performance computing platform (add url). Illumina NovaSeq data were generated for the same strains which is used for short-read-based polishing, k-mer analysis, and assembly validation.

A5.1 Nanopore basecalling and demultiplexing

Raw ONT signals in POD5 format were base called using Dorado with the simplex "sup" (super-accuracy) model run using GPU nodes (A100), producing an unmapped BAM file containing all reads for each library. Demultiplexing of barcoded samples was performed with the Dorado demux module, using the base-called BAM files as input. This step produced separate unmapped BAM files for each ecotype library, named according to the alias in the sample sheet (Tables A5.1). Demultiplexing was run with "--no-classify" to retain reads assigned to specific barcodes and avoid discarding reads that did not match Dorado's internal classification thresholds.

Demultiplexed, unmapped BAM files for each sample were converted to FASTQ using SAMtools "fastq". For libraries R0119 and R0120, individual BAM files were converted into sample-specific FASTQ files. For library R0149, BAM files were similarly converted to FASTQ. Samples from the libraries R0119 and R0120 were concatenated to single FASTQ files, for example, FASTQ files for MaUSA from R0119 and R0120 were combined into a single file, whereas single-run FASTQ files were obtained for MaSCO and MaWAL.

Table A5.1 Demultiplexing sample sheet of R0119, R0120 and R0149 library.

Kit	Experiment_id	Flow_cell_id	Barcode	Alias
SQK-NBD114-24	R0119	PAQ57576	barcode01	MaUSA
SQK-NBD114-24	R0119	PAQ57576	barcode04	MaENG
SQK-NBD114-24	R0119	PAQ57576	barcode05	MaNOR
SQK-NBD114-24	R0119	PAQ57576	barcode06	MaNZI
SQK-NBD114-24	R0119	PAQ57576	barcode07	MaROM
SQK-NBD114-24	R0119	PAQ57576	barcode08	MaIRE
SQK-NBD114-24	R0120	PAQ57576	barcode01	MaUSA
SQK-NBD114-24	R0120	PAQ57576	barcode04	MaENG
SQK-NBD114-24	R0120	PAQ57576	barcode05	MaNOR
SQK-NBD114-24	R0120	PAQ57576	barcode06	MaNZI
SQK-NBD114-24	R0120	PAQ57576	barcode07	MaROM
SQK-NBD114-24	R0120	PAQ57576	barcode08	MaIRE
SQK-NBD114-24	R0149	PAW14881	barcode09	MaSCO
SQK-NBD114-24	R0149	PAW14881	barcode19	MaWAL

A.5.2 Long-read quality control and filtering

Initial quality control of Nanopore reads was performed using Chopper by removing low-quality and very short reads prior to assembly. For each strain, raw concatenated fastq files were processed with a minimum mean quality threshold of Q10 and a minimum read length of 500 bp. In addition, the first 10 bases of each read were trimmed (--headcrop 10) to remove potentially lower quality signal at read starts. Quality check was done using NanoPlot on these filtered fastq files to confirm improved read length and quality distributions.

The density plots illustrate that each sample contained a large proportion of reads of lengths 1–15 kb with Q-scores clustered between 10 and 25, indicating some level of fragmentation across samples (Fig. A5.1).

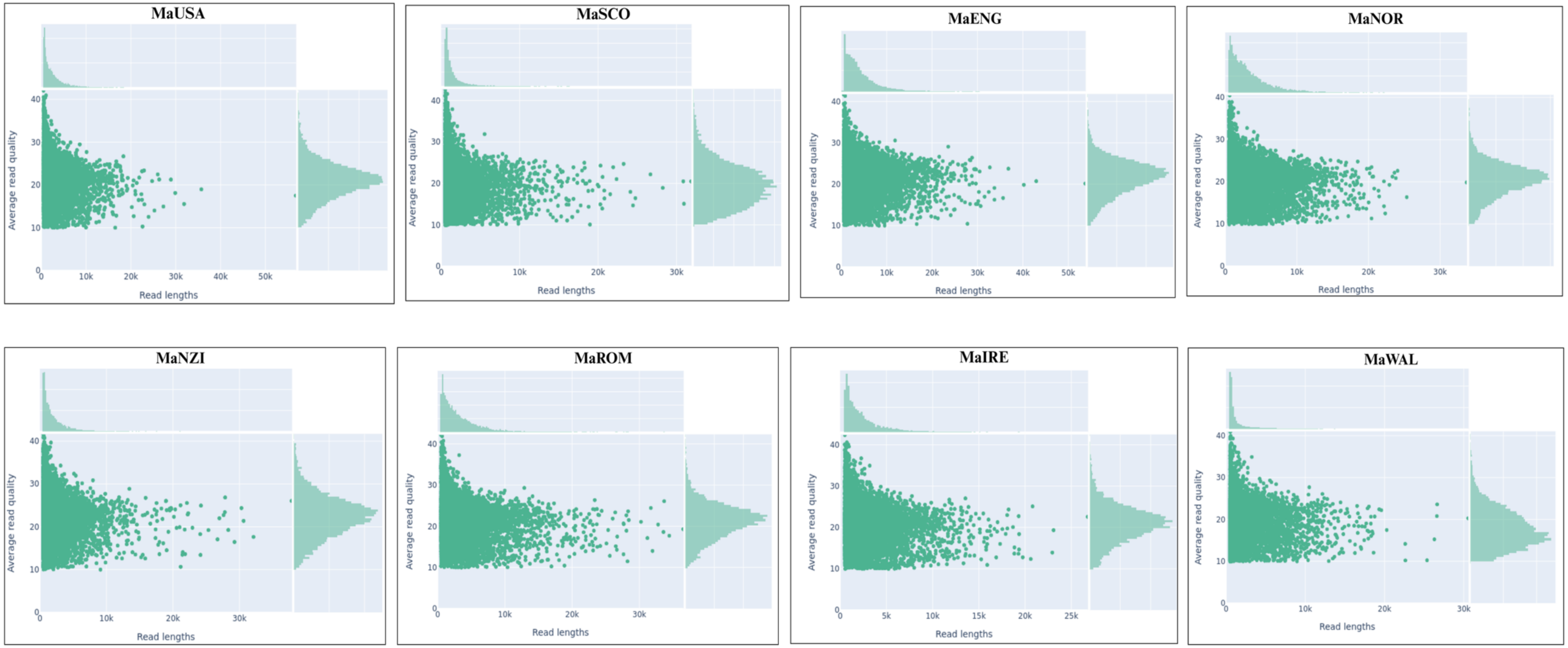

Figure A5.1 Read length versus average read quality for eight *Microctonus aethiopoides* strains. Each panel displays the distribution of long-read nanopore data of each strain, showing the relationship between read length (x-axis) and average per-read quality (y-axis). Density plots along the margins illustrate the overall distribution of read lengths (top) and read qualities (right).

A5.3 Long-read genome assembly and polishing

De novo genome assemblies were generated for each strain using Flye with the filtered Nanopore reads. For each sample, Flye was run in high-quality mode "--nano-hq" with a 129 Mb haploid genome size used (based on previously published *M. aethiopoides*). Flye output provided contig-level assemblies for each ecotype. Further polishing was performed using Medaka, where the draft genome from Flye and the corresponding filtered Nanopore reads were supplied as input, providing polished consensus assemblies for each ecotype. To assess structural assembly quality and to facilitate comparisons among ecotypes, QUAST was used to calculate standard assembly metrics by running comparative mode and including all assemblies simultaneously, enabling direct comparison of assembly statistics across strains.

Assemblies for MaENG, MaROM, and MaNOR exhibited the highest contiguity, reflected by their large N50 values (2.3-2.7 Mb for MaENG/MaROM; ~1.9 Mb for MaNOR) and relatively small numbers of contigs ≥ 1 kb; these samples also had the largest contigs, with MaENG reaching ~11.6 Mb, suggesting high-quality long-read data and effective genome reconstruction (Table A5.2). In contrast, MaIRE displayed the most fragmented assembly, with 2,291 contigs, a very low N50 of 405 kb, and an L50 of 96, indicating that many small contigs were needed to reach half the genome size (Table A5.2). MaUSA, MaSCO, MaNZI, and MaWAL showed intermediate contiguity, with N50 values ranging from ~1.3-2.3 Mb and moderate numbers of contigs (Table A5.2).

Table A5.2 QUAST results table showing Flye-assembled genome metrics for all eight *Microctonus aethiopoides* strains.

Assembly	**MaUSA**	**MaSCO**	**MaENG**	**MaNOR**	**MaNZI**	**MaROM**	**MaIRE**	**MaWAL**
# contigs (>= 0 bp)	1875	1994	684	713	1468	544	2296	1740
# contigs (>= 1000 bp)	1533	1522	581	620	1124	459	1939	1336
# contigs (>= 5000 bp)	646	634	321	476	410	312	1171	565
# contigs (>= 10000 bp)	528	523	262	412	341	276	929	474
# contigs (>= 25000 bp)	376	369	204	299	263	216	594	341
# contigs (>= 50000 bp)	272	234	162	220	200	165	421	240
Total length (>= 0 bp)	143890854	142460225	139513639	138266682	137473960	138893914	134190385	135549999
Total length (>= 1000 bp)	143650873	142135536	139439383	138201322	137236158	138835049	133949410	135278848
Total length (>= 5000 bp)	141317769	139965195	138733879	137796648	135451579	138446968	131929837	133391075
Total length (>= 10000 bp)	140469336	139170366	138316679	137326288	134950175	138180592	130141288	132736417
Total length (>= 25000 bp)	138016243	136601435	137362672	135455572	133713780	137194083	124654891	130579293
Total length (>= 50000 bp)	134381174	131814461	135876008	132646744	131398499	135325114	118394622	126921262
# contigs	1870	1987	683	712	1467	544	2291	1730
Largest contig	5493130	6381050	11645682	5719414	8786625	6855208	1626518	5722894
Total length	143889267	142457847	139513165	138266183	137473948	138893914	134188751	135548273
GC (%)	30.34	30.46	29.56	29.52	29.53	29.58	29.42	29.47
N50	1392930	1904951	2468937	1945017	2349143	2717035	405479	1489815
N90	90108	71794	266175	148688	137032	226610	40643	87897

auN	1720474.4	2384753.1	3248835.7	2040353.4	2948845.8	2865756.4	502158.2	2199767.4
L50	29	20	17	24	17	17	96	21
L90	198	175	81	127	107	79	474	167
# N's per 100 kbp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

A5.4 Haplotig and redundancy removal

To reduce redundancy arising from heterozygosity (e.g., alternative haplotypes assembled as separate contigs), Medaka-polished assemblies were processed with purge_dups. For each strain, the BAM file produced during the Medaka run was converted to SAM and then to PAF format using paftools.js. The PAF file was summarised with pbcstat to generate coverage statistics, and coverage cut-offs were estimated using calcuts. The polished FASTA files (Medaka output) were then split into smaller fragments using split_fa, and self-alignments were produced with minimap2 (-x asm5) to create a compressed self-mapping PAF file. The purge_dups pipeline was then used to identify duplicated and haplotig sequences based on coverage and self-alignment patterns. Regions flagged as duplicates were written to a BED file (dups.bed), and the get_seqs utility was used to partition the assembly into a purged primary assembly (purged.fa) and a set of removed haplotigs (hap.fa). Only the purged assemblies were used in downstream analyses.

After purging duplicated haplotigs and redundant sequences, all assemblies showed a substantial reduction in contig number, indicating that the purging process successfully removed duplicated or alternative haplotype sequences. Assemblies for MaENG, MaROM, and MaNOR remained the most contiguous after purging, with low contig counts (237-395) and high N50 values (2.0-3.1 Mb), demonstrating strong primary assemblies with minimal fragmentation (Table A5.3). Assemblies for MaUSA, MaSCO, MaNZI, and MaWAL showed intermediate contiguity, with contig numbers ranging from ~544 to 828 and N50 values around 1.5-2.8 Mb, indicating moderate fragmentation but still high-quality assemblies (Table A5.3). MaIRE remained the least contiguous even after purging, with 1,408 contigs and a low N50 of 419 kb (Table A5.3).

Table A5.3. QUAST results table showing haplotig-removed assembled genome metrics for all eight *Microctonus aethiopoides* strains.

Assembly	**MaUSA**	**MaSCO**	**MaENG**	**MaNOR**	**MaNZI**	**MaROM**	**MaIRE**	**MaWAL**
# contigs (>= 0 bp)	736	828	237	395	545	238	1409	777
# contigs (>= 1000 bp)	713	751	215	384	483	214	1382	696
# contigs (>= 5000 bp)	470	464	181	355	297	194	1021	431
# contigs (>= 10000 bp)	425	422	171	328	266	180	847	391
# contigs (>= 25000 bp)	329	314	145	259	216	159	564	295
# contigs (>= 50000 bp)	253	212	126	198	168	129	410	222
Total length (>= 0 bp)	133465123	136552793	132701466	134080610	132108931	132266686	130348973	130775715
Total length (>= 1000 bp)	133447586	136500228	132686441	134073190	132064663	132249932	130330100	130725216
Total length (>= 5000 bp)	132735283	135713330	132585106	133975523	131542014	132179879	129236911	129986366
Total length (>= 10000 bp)	132403396	135401073	132504140	133772133	131305600	132069089	127942988	129700355
Total length (>= 25000 bp)	130786103	133576793	132033166	132576873	130484646	131728963	123237212	128120411
Total length (>= 50000 bp)	128158652	129945253	131349442	130417237	128757587	130642561	117619707	125510941
# contigs	736	826	237	395	544	238	1408	770
Largest contig	5491093	6382190	11646367	5720216	8789163	6856451	1626675	5724264
Total length	133465123	136552713	132701466	134080610	132108919	132266686	130348964	130774611
GC (%)	29.39	30.35	29.41	29.42	29.38	29.41	29.33	29.34
N50	1499488	2020577	2621023	1970957	2861055	3090758	419415	1625988
N90	122839	122103	394330	198685	188366	385230	52614	142179

auN	1759952.1	2486493.4	3408784.5	2102168.1	3067088.0	3001119.5	516235.7	2279846.7
L50	26	18	15	23	16	16	91	20
L90	152	122	63	105	78	60	405	128
# N's per 100 kbp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

A5.5 Illumina short-read processing

Illumina NovaSeq data was processed to filter low-quality bases and adapter contamination, using Trim Galore in paired-end mode. B ases with Phred scores below 20 were trimmed from read ends. Trimmed reads were then evaluated with FastQC/MultiQC, which showed high quality reads (Fig. A5.2) suitable for both polishing and k-mer-based assembly assessment.

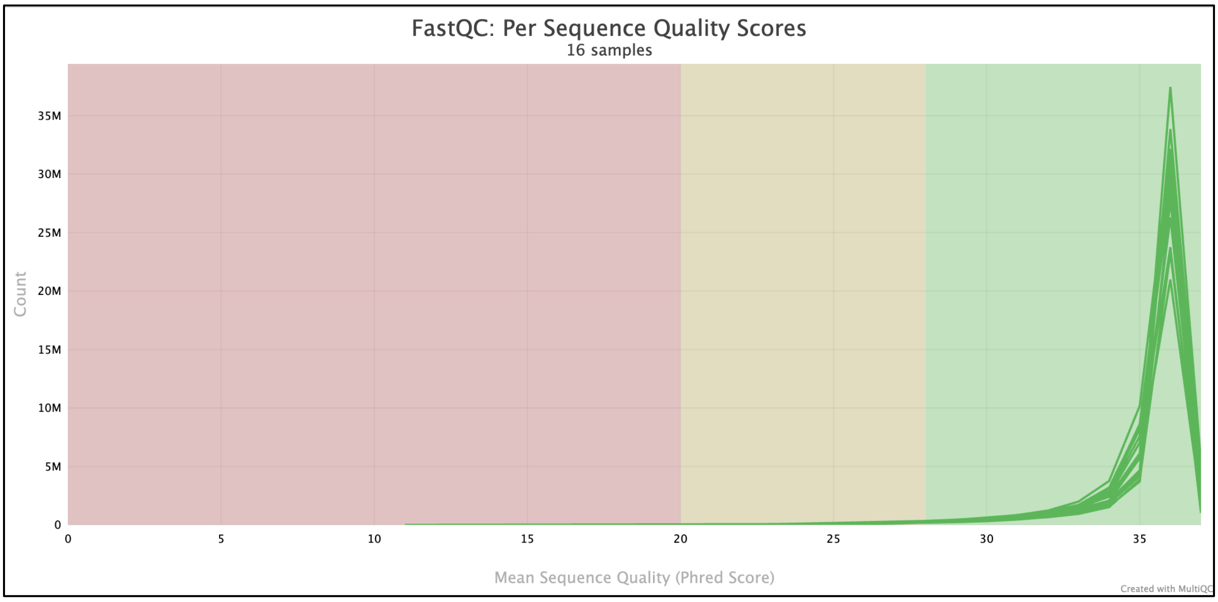

Figure A5.2. FastQC per-sequence quality score distribution for eight Illumina samples (total 16 R1 and R2 files). The plot shows the distribution of mean Phred scores across all reads, with most sequences falling within the high-quality range (Q30-Q38, green region).

A5.6 K-mer-based genome profiling using Genomescope

To characterise genome size, heterozygosity, and repeat content, and to evaluate assembly quality using read k-mers, k-mer databases were constructed from filtered Illumina reads using meryl. For each strain, meryl was used to count k-mers (e.g., k = 18) from high-quality paired-end reads. K-mer histograms were then generated from the meryl databases and used as input to GenomeScope (See Chapter 4 for results).

A5.7 Short-read polishing with NextPolish

Short-read polishing of the purged Nanopore assemblies was performed using NextPolish, which integrates BWA-MEM and SAMtools for read mapping and alignment processing. For each strain, the purged assemblies were indexed with BWA and filtered paired-end Illumina reads were aligned to the assembly using BWA-MEM. The resulting alignments were processed with SAMtools to filter unmapped reads, fix mate information, sort the BAM file, and mark duplicates.

NextPolish was run iteratively in two rounds. In the first round, the purged assembly was polished using the aligned Illumina reads, producing an intermediate polished genome (genome.polishtemp.fa). In the second round, the intermediate genome was re-indexed and re-aligned to the Illumina reads, and a second polishing step was performed, resulting in the final polished assembly (genome.nextpolish.fa) for each strain. This polishing primarily improves base-level accuracy with minimal impact on broader assembly metrics. These NextPolish-corrected assemblies were used for subsequent scaffolding and downstream analyses.

A5.8 Reference-guided scaffolding with RagTag

To improve long-range assembly contiguity and chromosomal organisation, each polished contig-level assembly was scaffolded against a high-quality *M. aethiopoides* reference genome (Irish strain, GCA_030347275.1) using RagTag. For each strain, RagTag was run in scaffold mode with the reference assembly as the guiding genome and the NextPolish-corrected assembly as the query. Under the hood, RagTag uses whole-genome aligners such as minimap2 to map query contigs to the reference and then orders, orients, and joins them into scaffolds based on reference genome that is provided. The output for each strain was a scaffolded genome (ragtag.scaffold.fasta) with improved contiguity across all *M. aethiopoides* strains.

Following scaffolding, contig numbers were reduced by approximately half compared with the purged assemblies, with most genomes now represented by 110-426 scaffolds (Table A5.4, Fig. A5.3). All assemblies, including MaIRE, now exhibited extremely high N50 values between 23.7-24.5 Mb, indicating that only three scaffolds (L50 = 3) are required to cover half of each genome (Table A5.4, Fig. A5.3). This reflects successful ordering and orienting of contigs into larger, chromosome-level scaffolds based on the reference genome.

Table A5.4. QUAST results table showing Ragtag scaffolded genome metrics for all eight *Microctonus aethiopoides* strains.

Assembly	**MaUSA**	**MaSCO**	**MaENG**	**MaNOR**	**MaNZI**	**MaROM**	**MaIRE**	**MaWAL**
# contigs (>= 0 bp)	329	428	110	164	295	108	456	382
# contigs (>= 1000 bp)	314	363	89	153	238	84	435	314
# contigs (>= 5000 bp)	139	154	58	129	99	65	212	124
# contigs (>= 10000 bp)	112	130	50	107	78	56	149	96
# contigs (>= 25000 bp)	63	67	32	62	46	44	47	48
# contigs (>= 50000 bp)	36	24	21	25	24	23	13	19
Total length (>= 0 bp)	13344558	13663020	132753909	13416089	13218728	13234215	13051530	130858127
Total length (>= 1000 bp)	13343446	13658626	132739510	13415347	13214684	13232540	13050087	130816869
Total length (>= 5000 bp)	13293231	13602306	132647713	13407710	13175510	13226034	12984461	130303124
Total length (>= 10000 bp)	13273909	13585613	132581517	13391439	13159829	13218985	12939036	130107042
Total length (>= 25000 bp)	13193188	13479772	132240397	13315491	13105277	13199848	12772437	129311374
Total length (>= 50000 bp)	13099500	13328212	131859929	13188842	13027383	13123011	12659000	128317880
# contigs	329	426	110	164	294	108	455	375
Largest contig	30009381	29707988	30557105	30886853	29470290	30916849	29408197	29450121
Total length	133445586	136630122	132753909	134160896	132187272	132342159	130515297	130857023
GC (%)	29.39	30.34	29.39	29.40	29.37	29.39	29.31	29.33
N50	24119476	23673627	24453707	24023478	23977460	23764779	23707169	23943118
N90	7942825	6864352	7989359	8077256	7912293	7982948	7835318	7855452

auN	20477291.7	19664097.2	21118168.0	20830941.6	20481894.2	21036909.9	20095517.9	20298440.7
L50	3	3	3	3	3	3	3	3
L90	7	8	7	7	7	7	7	7
# N's per 100 kbp	30.50	29.28	9.57	17.22	18.91	9.82	73.02	30.19

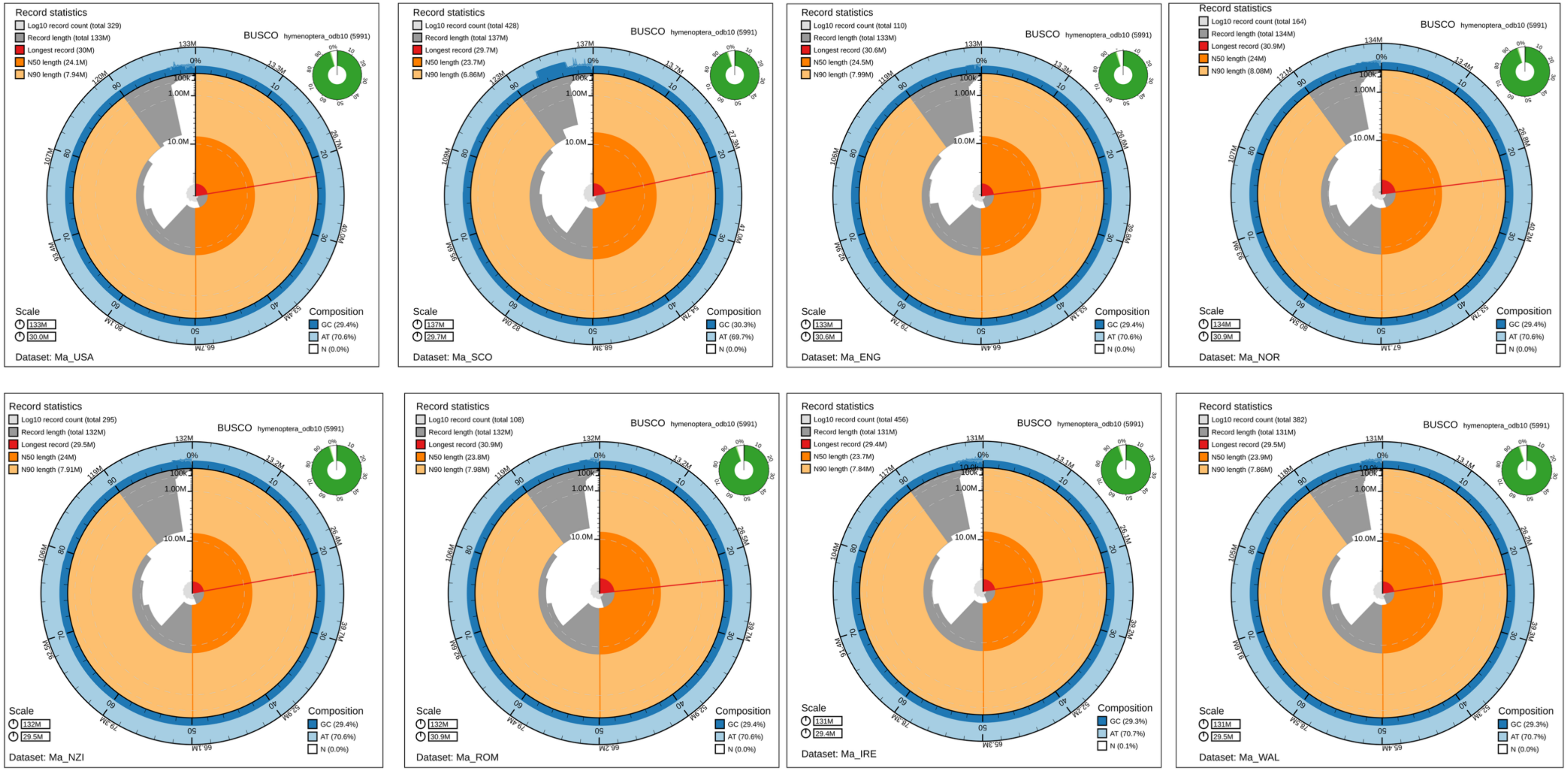

Figure A5.3 Snail plots representing scaffolded genome statistics and BUSCO completeness for each *Microctonus aethiopoides* genome assembly.

A5.9 Evaluation of assembly completeness with Merqury

Assembly completeness and consensus quality were further assessed using Merqury. For each strain, the meryl database and the scaffolded assembly were supplied to Merqury, which computes k-mer concordance between reads and assemblies to measure completeness metrics. The resulting plot is used to understand the distribution of shared and unique k-mers between reads and assemblies, which helps identify potential under- or over-representation of genomic content, residual contamination, or uncorrected sequencing errors.

Each sample exhibits a strong single dominant peak corresponding to the expected haploid k-mer multiplicity, with only a minor shoulder at higher multiplicities, suggesting minimal residual haplotigs after purging (Fig. 5.4). The very small proportion of "read-only" k-mers, visible as a narrow peak at very low multiplicities, indicates that most sequencing k-mers are represented in the final assemblies, reflecting high completeness (Fig. 5.4). For example, MaUSA shows a well-defined primary k-mer peak and a smaller secondary peak which is much lower in magnitude and only partially overlaps with the tail of the primary peak, suggesting that a limited number of duplicated or repetitive k-mers remain in the assembly.

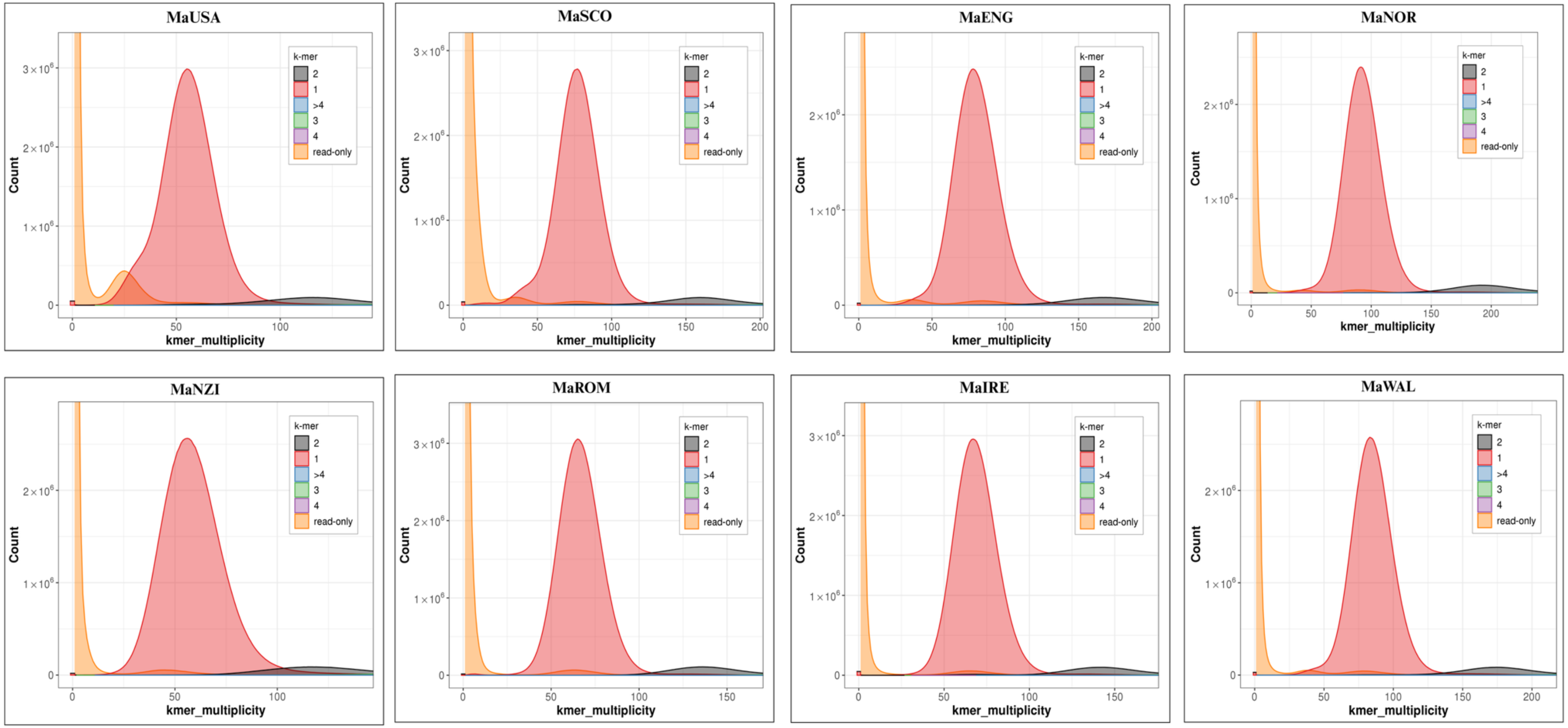

Figure A5.4 K-mer multiplicity distributions for all eight *Microctonus aethiopoides* strains generated using both short reads and assemblies.

A5.10 Contamination check and filtration

Potential contamination in scaffolded assemblies was assessed by blasting against nucleotide database using BLASTn (task megablast) with a tabular output format that included sequence IDs, taxonomic IDs, bit scores, scientific names, kingdoms, and hit descriptions. For faster run times, the scaffolded FASTA files were first split into smaller chunks, and BLASTn was run in parallel on each chunk, then the results were concatenated.

The contamination plots show that the genomes are dominated by Arthropoda-derived sequences, with cumulative lengths matching the expected genome size (~130 Mb). Minimal bacterial (Proteobacteria) and fungi (Ascomycota) reads were observed in some assemblies (MaUSA, MaSCO, MaNZI and MaROM); this could reflect either low-level environmental contamination or traces of endosymbiont DNA (Fig. A5.5). However, these contamination scaffolds were extremely small (<6 Mb total in all samples) and were removed based on the contig ids using a custom shell script.

After removing contaminant scaffolds, the assemblies were reassessed using QUAST and Compleasm for BUSCO scores (see Chapter 4 for results). Both evaluations confirmed that all genome assemblies exhibited high contiguity and strong completeness, with BUSCO complete scores exceeding 95%. Overall, the produced assemblies in this study are clean, free of major non-arthropod contaminants, and have high contiguity suitable for comparative genomics analyses.

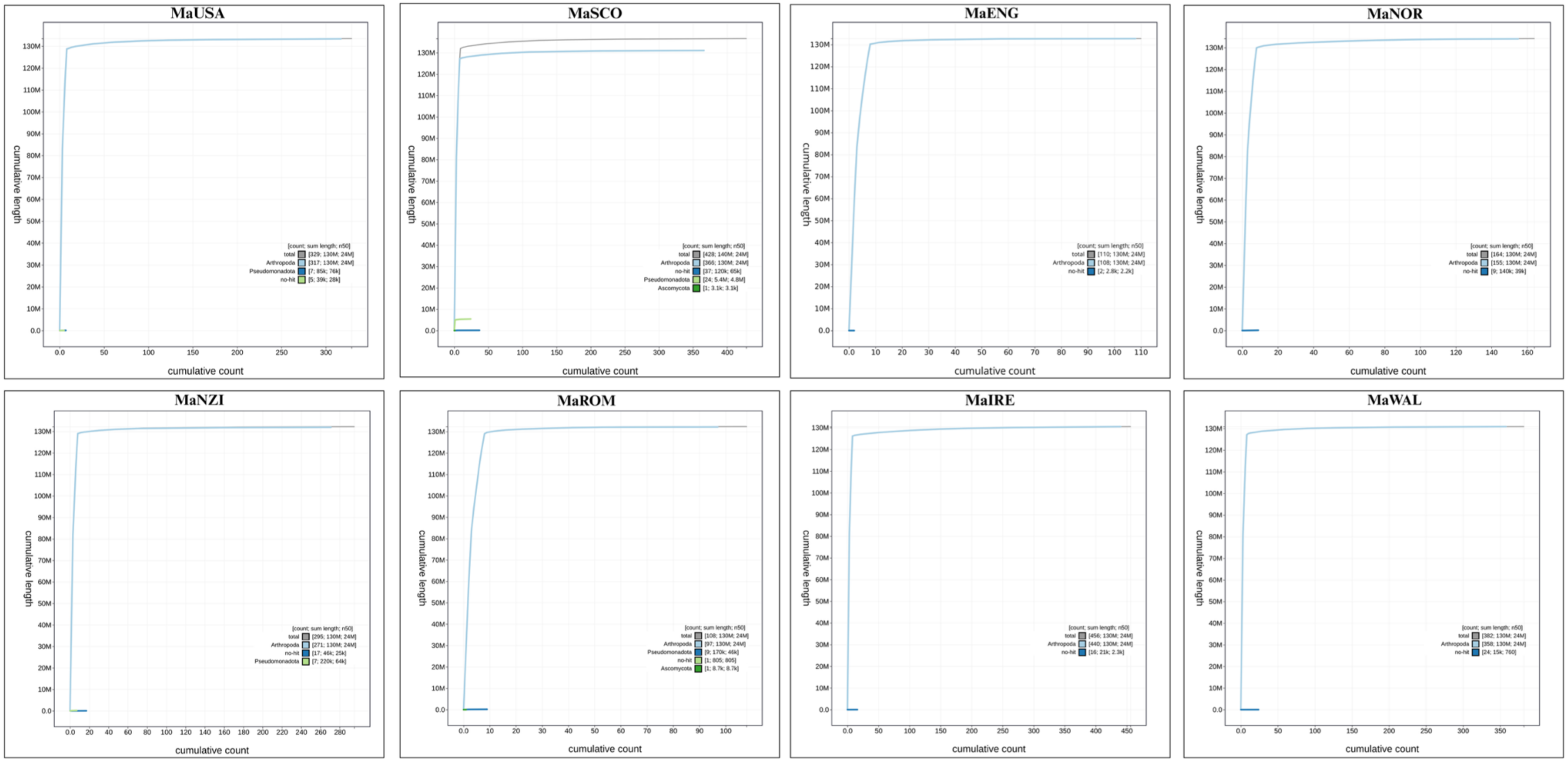

Figure A5.5 Plot showing the taxonomic composition of the contigs in eight *Microctonus aethiopoides* genome assemblies.