Biocontrol Genomics of *Microctonus* Wasps

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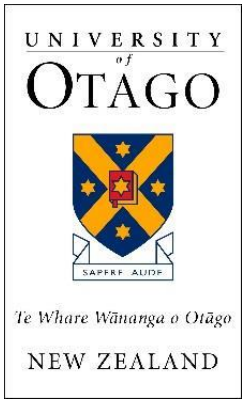

Abstract

The Argentine stem weevil (*Listronotus bonariensis*) caused considerable damage to New Zealand pastures before the introduction of the parasitic wasp *Microctonus hyperodae* and ryegrass endophytes. *Microctonus hyperodae* biocontrol is starting to fail, as is indicated by lower parasitism rates and observed pasture damage. The Argentine stem weevil and *Microctonus hyperodae* have different reproductive modes, with the Argentine stem weevil reproducing sexually and *Microctonus hyperodae* reproducing through parthenogenesis. The Argentine stem weevil may be 'out evolving' *Microctonus hyperodae* as a result.

Although most Hymenoptera, including *Apis mellifera,* use a haploid-diploid method of sex determination, there are many parthenogenic species. Parthenogenesis results from both infection with endosymbionts and through genetic mechanisms. The literature focuses heavily on how parthenogenesis occurs through infection, with little research into genetic or molecular mechanisms.

Sequencing and annotating the genomes of multiple strains of *Microctonus aethiopoides*, which use either sexual or asexual reproductive strategies, and subsequent bioinformatic analysis could allow for the genetic and molecular mechanism through which *Microctonus hyperodae* became asexual to be discovered.

Sequenced and assembled genomes were annotated in order to further study the gene compliment of the *Microctonus aethiopoides* and *Microctonus hyperodae* and analyze how these genes relate to biocontrol fitness in these species.

One group of genes which was assessed in detail is the olfactory gene compliment for the *Microctonus* species as olfactory receptors are fundamental for a range of behavioral responses which are required for effective biocontrol. Variation in the olfactory receptor complement may be due to selective identification of preferred hosts or mate identification in sexual *Microctonus*. Hi-C analysis of the genomes allowed for chromosome level assembly of the "Irish" strain *Microctonus aethiopoides* and *Microctonus hyperodae*, revealing that the number of chromosomes varies in the

Microctonus genus. The Divergence of *Microctonus hyperodae* and *Microctonus aethiopoides* was estimated at 17 MYA; A more ancient divergence date than expected. Kraken2 analysis of sequenced reads failed to reveal endosymbiont infections in the *Microctonus*. Asexuals likely result from genetic mechanisms instead of endosymbiont infections. Genomescope2 analysis revealed low levels of heterozygosity in the genomes of *Microctonus*. Variation in heterozygosity did not correlate with asexual reproduction. *Microctonus* genomes have low repeat levels and have a total genome size of ~120 Mb.

This thesis is a monumental step towards expanding our understanding of the *Microctonus*, host and mate identification processes, and the mechanisms of asexual reproduction in the *Microctonus* asexuals. This thesis should continue to broaden the understanding of the biology of this group of agriculturally significant biocontrol agents.

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Chapter 1: Introduction

The Argentine Stem Weevil

The Argentine stem weevil (*Listronotus bonariensis*) is an economically important pasture pest that caused between $78-251 million worth of damage to pastures in New Zealand before introducing biocontrol measures (Prestidge, Barker and Pottinger, 1991). The Argentine stem weevil is a member of the order Coleoptera which includes other beetle species. *L. bonariensis* was initially identified in New Zealand in 1927 (Marshall, 1937). However, its introduction is likely to have been earlier in the 1900s (Kuschel, 1972). Multiple introductions of *L. bonariensis* likely occurred (Kuschel, 1972). *L. bonariensis* causes damage to pastures through its larva, a stem borer in grasses, corn and cereals (Barker, 2013).

Listronotus bonariensis is very effective at dispersal (Goldson *et al.*, 1999). Its likely region of origin in South America's Andes are similar to alpine grasslands, herbfields and cushion bogs in New Zealand (Wardle *et al.*, 2001). Grass species in the malline grasslands of South America are in the same genera as some of New Zealand's introduced Gramineae, including common cereals and pasture grasses (Morrison, 1938; Jacques, 1940; Kain and Barker, 1966). *Listronotus bonariensis* can reach much higher densities in ryegrass pastures in New Zealand than its proposed native range in South America (Barker and Addison, 1993). New Zealand pastures are primarily composed of species from the northern hemisphere. The lack of native invertebrates, preferably colonizing these pasture systems, results in a lack of species likely to control pests (like *L. bonariensis*) or compete for resources (Goldson *et al.*, 2014). A combination of these factors likely contributed to the resulting abundance of *L. bonariensis*, making it a long-recognized pasture pest (Kelsey, 1958).

Classical biocontrol relies on the introduction of predators or parasites specializing in suppressing a target species in its ecosystem of origin, aiming to irreversibly reduce the number and impact of the target pest species. When it works, classical biocontrol can be described as being 'elegant, self-sustaining, non-polluting and inexpensive' (Gurr, Wratten and Barbosa, 2000).

Pasture losses in New Zealand due to *L. bonariensis* have been significantly curtailed by introducing the parasitic wasp *Microctonus hyperodae* and adopting endophytic ryegrass cultivars, which provide resistance to *L. bonariensis* (Figure 1; Goldson *et al.*, 1993; Popay and Wyatt, 1995). Unfortunately, although initially successful, biocontrol of *L. bonariensis* is now failing for reasons which are yet to be determined (Popay *et al.*, 2011; Goldson *et al.*, 2014).

After considerable investigation, it has been concluded that the mining habit of *Listronotus bonariensis* larvae and the unpredictable flight of adults result in insecticides not being ideal for this pest's control (Figure 1; Goldson *et al.*, 1990). Therefore, it has become apparent that alternative strategies are required for the biocontrol of *L. bonariensis*. As the introduction of new biocontrol agents is unlikely due to difficulties in identifying alternative biocontrol agents and regulatory challenges of introducing novel biocontrol agents to New Zealand (Dymock, 1987), improving the biocontrol effectiveness of *Microctonus hyperodae* is the most attractive option.

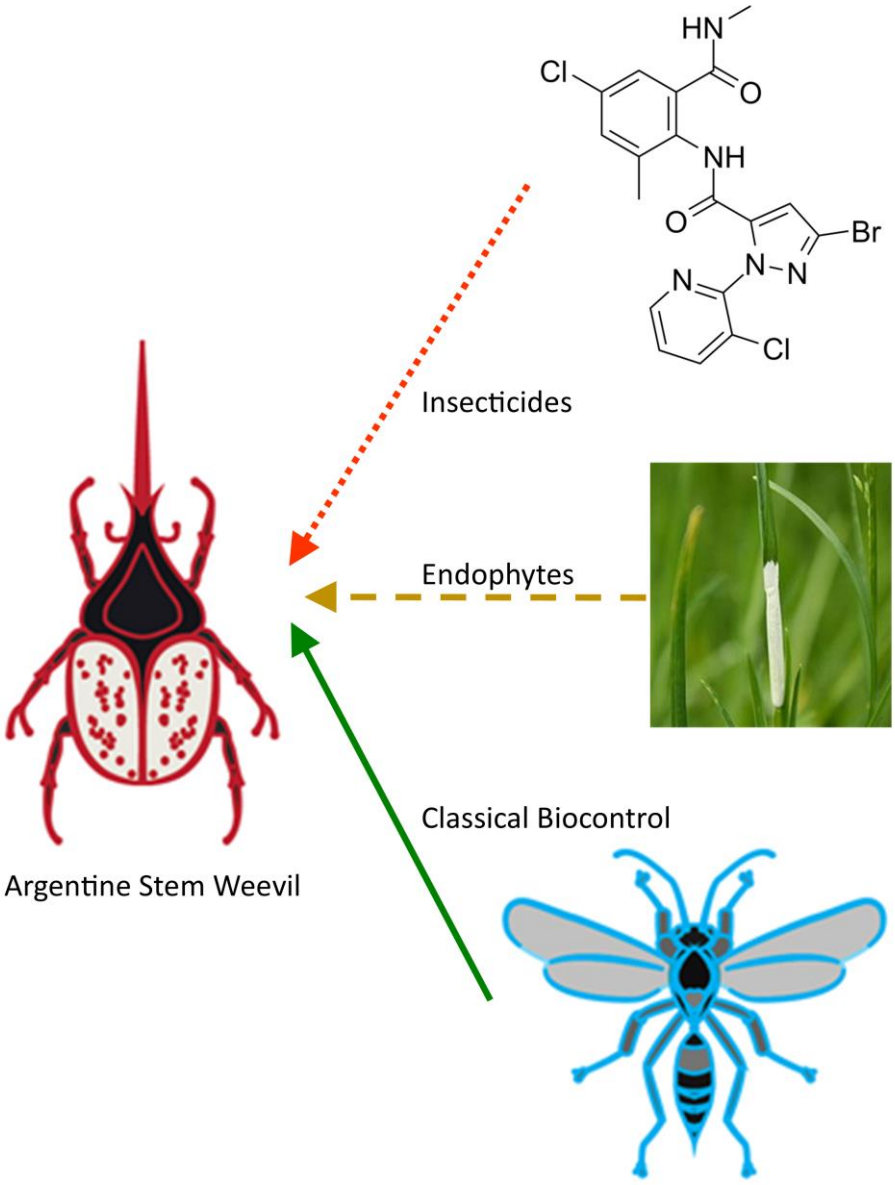

Figure 1) Biocontrol techniques used for the Argentine Stem Weevil. The colored arrows indicate the comparative effectiveness of the biocontrol strategies. The green arrow indicates effectiveness, orange indicates lower effectiveness whereas the red arrow indicates low effectiveness.

Microctonus

Microctonus is in the subfamily Euphorinae, a group of parasitoid wasps that mainly parasitize adult holometabolous insects, including Coleoptera, Hemiptera, Psocoptera and Hymenoptera. *Microctonus* is in the family Braconidae, which contains 13000 described species, most of which are endoparasitoids, all of which are parasites of other insects (Shaw and Huddleston, 1991). Braconidae are generally koinobionts, which means the host is alive and functional whilst being parasitized; hence the parasitoid has to avoid its host's defenses (Shaw, 1994; Pennacchio and Strand, 2005). Some Braconidae subfamilies are gregarious, meaning they lay multiple offspring per host (Pennacchio and Strand, 2005). Braconidae range in size from 2-6mm in length (Shaw and Huddleston, 1991) and are generally very host specialized (this is controlled through numerous intrinsic and extrinsic factors)(reviewed in Pennacchio & Strand (2005)).

The *Microctonus* genus contains koinobiont endoparasitoids of Coleoptera (Shaw, 1985, 1988). *Microctonus* are primarily solitary (Loan and Holdaway, 1961; Loan and Lloyd, 1974; Morales and Hower, 1981; Goldson *et al.*, 1990, 1992) with some gregarious species (Loan, 1967; Luff, 1976; Rieske, Hunt and Raffa, 1989; Gerard *et al.*, 2007). Female hosts of *Microctonus* are rendered sterile soon after the egg is laid, and the host generally dies when the parasitoid emerges or earlier in its development (Jackson, 1928; Loan and Holdaway, 1961).

Microctonus hyperodae

Microctonus hyperodae is the most widespread and best-known species of the genus in South America (Loan and Lloyd, 1974). In New Zealand, *Microctonus hyperodae* reproduces asexually. However, there are sexual morphs that occur in its native South American range (Goldson *et al.*, 1990).

Microctonus hyperodae was initially identified and collected in multiple regions in South America (Concepción (Chile), Pôrto Alegre (Brazil), Colonia (Uruguay), Ascasubi, General Roca, Mendoza and Bariloche (Argentina)) by Goldson *et al.* (1990), introduced into hosts and subsequently released into pastures in multiple parts of New Zealand (Auckland, Hamilton and Bay of Plenty). *Microctonus hyperodae* was subsequently distributed throughout New Zealand in numerous research and commercial releases (McNeill *et al.*, 2002).

Although the clear majority of *Microctonus hyperodae* imported into New Zealand were female, four were identified as impotent males (Goldson *et al.*, 1990). Upon testing the host range variation of *Microctonus hyperodae,* Goldson *et al.* (1992) established that there was only limited off-target parasitism, making it a suitable biocontrol agent. As *Microctonus hyperodae* has evolved asexuality in its native range in South America and is commonly found in *L. bonariensis* weevils in the region it is possible that asexual reproduction either provides a fitness advantage or that asexual reproduction sporadically occurs in sexually reproducing lineages of *Microctonus hyperodae*.

In New Zealand, *Microctonus hyperodae* overwinters in *L. bonariensis* as diapausing first-instar larvae. Diapause is usually induced at a photoperiod of 13.6 hours, which occurs in the middle of March (Goldson *et al.*, 1993). *Microctonus hyperodae* development continues in early spring. Two or three generations of *Microctonus hyperodae* occur between the start of summer and the end of autumn (Barker, 2013).

Although initially successful, *Microctonus hyperodae* biocontrol of *L. bonariensis* has decreased since their introduction (Popay *et al.*, 2011; Goldson *et al.*, 2014).

Escape from biocontrol

Multiple possible explanations for the escape of *L. bonariensis* from biocontrol exist. A current hypothesis explaining why escape from biocontrol may have occurred suggests

that the different reproductive strategies used by the host and its parasite has likely decreased biocontrol effectiveness. As the *L. bonariensis* reproduces sexually, it is hypothesized that it can 'out evolve' its asexual parasite, *Microctonus hyperodae*, and avoid biocontrol as a result as is implied by the modeling research undertaken by Goldson and Tomasetto (2016). One possible mechanism is that asynchrony of *Microctonus hyperodae* emergence and the next generation of *L. bonariensis* reduced the ability of *Microctonus hyperodae* to suppress *L. bonariensis* due to the decreased time window available for *Microctonus hyperodae* to parasitize *L. bonariensis*, providing a fitness advantage to weevils which do not emerge at the same time as *Microctonus hyperodae* (Phillips, Proffitt and Goldson, 1998; Barker and Addison, 2006).

Harrop, Le Lec, *et al.* (2020) applied extensive population genetic analysis to the issue of the failing biocontrol of the Argentine Stem Weevil by *Microctonus hyperodae*. Large amounts of genetic diversity were observed in the weevils with little population structure besides that seen between the North and South Islands of New Zealand (Harrop, Le Lec, *et al.*, 2020). The high levels of variability imply numerous introductions of the Argentine Stem Weevil into New Zealand and supports the hypothesis that the Argentine Stem Weevil has the ability to out evolve *Microctonus hyperodae* (Harrop, Le Lec, *et al.*, 2020).

Microctonus aethiopoides, the clover root weevil (Sitona lepidus) and the lucerne weevil (Sitona discoideus)

Two strains of *Microctonus aethiopoides,* "Moroccan" (also possibly Greek) and "Irish" were introduced into New Zealand to control the invasive pests *Sitona lepidus* (clover root weevil) and *Sitona discoideus* (lucerne weevil), respectively (Goldson *et al.*, 2001; Phillips *et al.*, 2002; McNeill *et al.*, 2006). Both the clover root weevil and the lucerne weevil cause considerable damage to New Zealand pastures. The "Greek" strain may also have been introduced into New Zealand as it has previously been introduced into Australia.

Whether the "Greek" strain established a New Zealand population has not been determined (Aeschlimann, 1995). 21

The "Irish" strain only reproduces by thelytoky (McNeill *et al.*, 2006), whereas the other strains reproduce through arrhenotoky (Goldson *et al.*, 2004; Phillips *et al.*, 2008). The reproductive mode and primary hosts of the *Microctonus* strains and species are summarized in Figure 2.

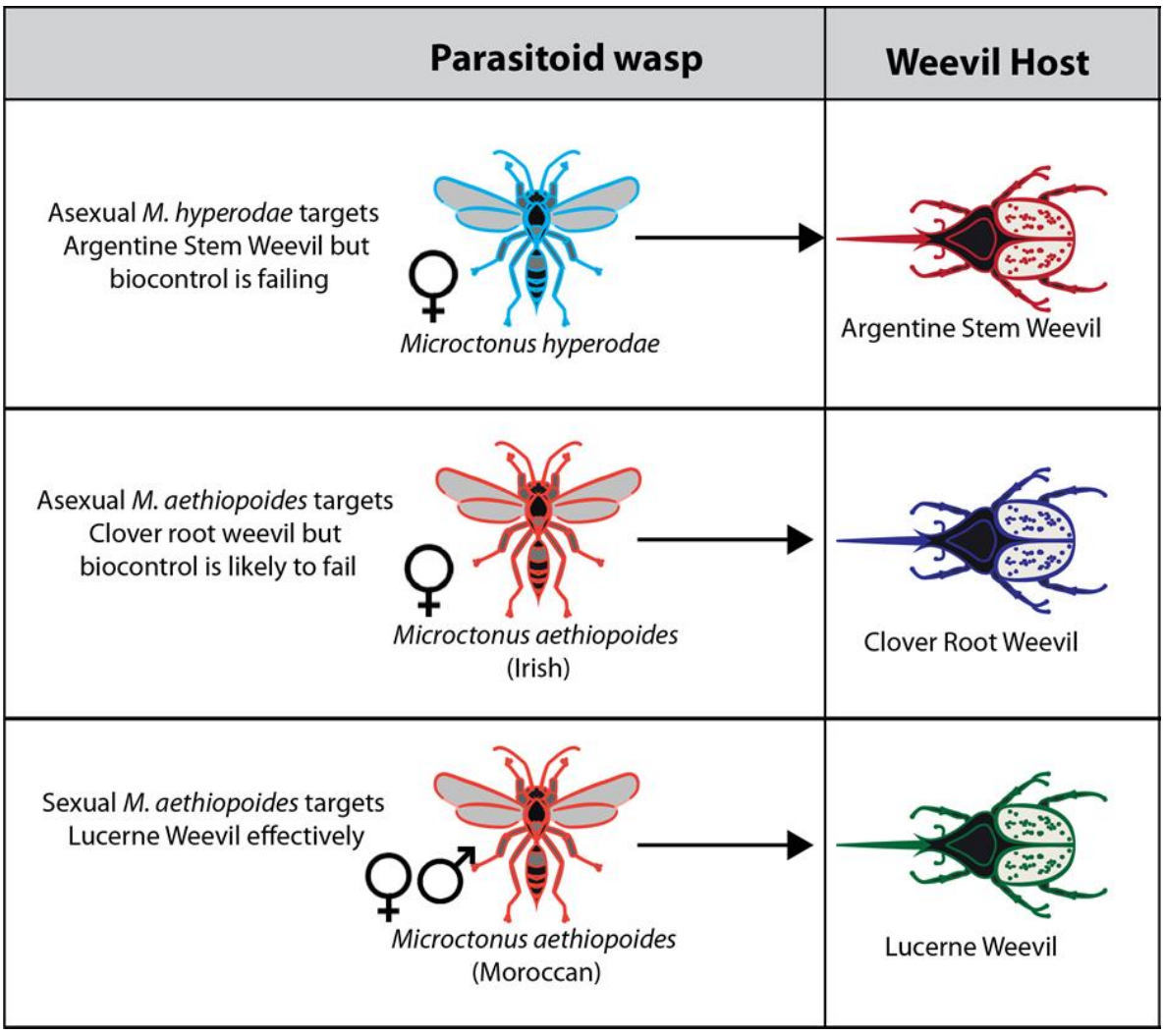

Figure 2) Host range, effectiveness, and reproductive strategy of introduced Microctonus classical biocontrol agents for control of invasive weevil species.

Parasitic wasps are commonly used as classical biocontrol agents globally; hence establishing whether a reproductive strategy is likely to alter a hosts range would be helpful. As altering host range may result in the parasitism of non-target native species as the host ranges of *Microctonus aethiopoides* vary between asexual and sexual strains (Phillips *et al.*, 2008).

A close relative of *Microctonus hyperodae*, *Microctonus wesmael*, is a parasitoid of the cosmopolitan Fuller's rose weevil (*Naupactus cervinus*), which implies that *Microctonus hyperodae* (if sexual) could be selectively bred to control the Fuller's rose weevil as well (Rodriguero *et al.*, 2014).

The utility of *Microctonus* species in New Zealand for repressing pests makes further understanding their biology, especially in failed biocontrol cases, of great importance for ensuring future pasture health.

Sex determination in insects

The mechanisms governing the sexes' determination and development in animals are widely diverged, even on small evolutionary time scales and between related groups (Bull, 1983; Graves, 2008). Insects are known for their rapid turnovers in the function and nature of sex determination genes (Sánchez, 2008; Geuverink and Beukeboom, 2014). The Hymenoptera employ a range of different sex determination strategies, including, but not limited to, parthenogenic arrhenotoky and thelytoky (Figure 3).

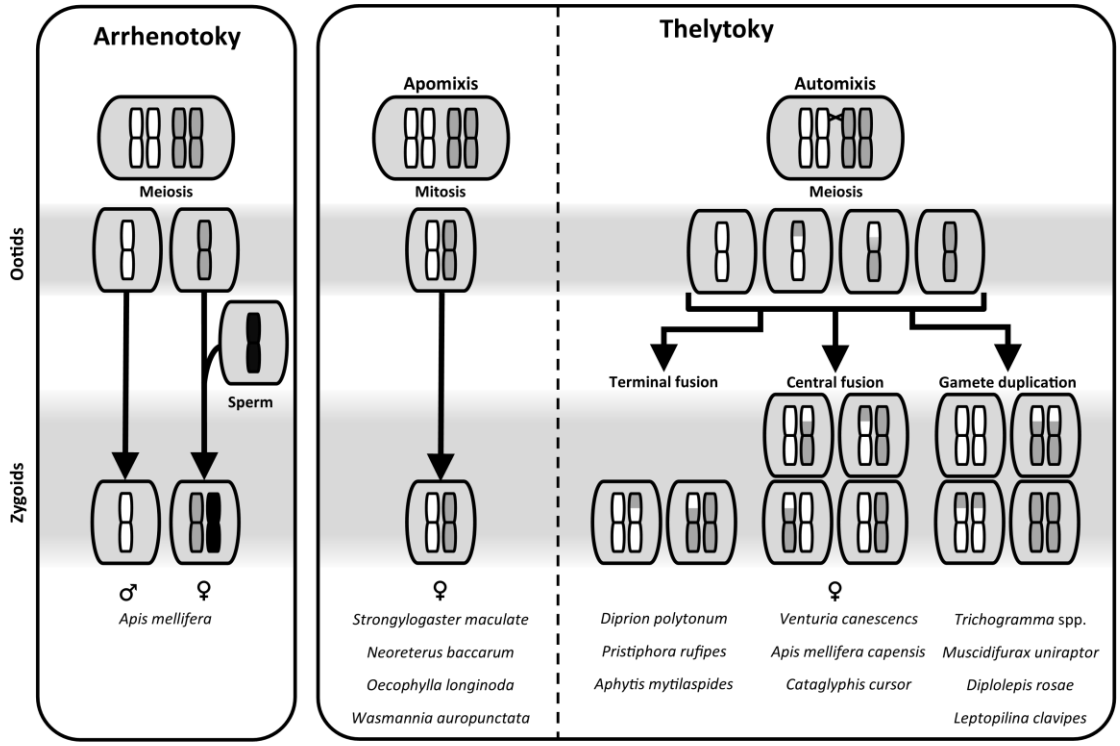

Figure 3) Modes of Hymenoptera reproduction. Adapted from Rabeling and Kronauer (2013). Crossing-over with recombination was used to show the effects of heterozygosity in progeny. Symbols highlight diploid females (♀) and haploid males (♂). Species which utilize each reproductive mode are listed.

Surprisingly parthenogenesis is part of the life cycle of all Hymenoptera, even those which use CSD (Complementary Sex Determination)(Normark, 2003, 2014; Gokhman, 2009). The CSD system is the most common and likely ancestral mechanism for sex determination in the Hymenoptera. The CSD system is defined by fertilized eggs forming diploid females with the unfertilized eggs resulting in the production of haploid males. The CSD system requires variation at the CSD locus in order to produce female offspring (Snell, 1935; Crozier, 1971). CSD is a type of Arrhenotokous parthenogenesis.

Although the genetic features responsible for sex determination differ between various Hymenoptera, the overall system still relies on ploidy (Fujiwara *et al.*, 2004; Hasselmann *et al.*, 2008). CSD may rely on a single locus as is seen in *Apis mellifera* (Beye *et al.*, 2003), or multiple loci which are used to determine sex as observed in the sawfly *Arge nigrinodosa* and the wasp *Cotesia vestalis* (Naito, Ishikawa and Nishimoto, 2000; de Boer *et al.*, 2007).

Some Hymenoptera species, including the model organism *Nasonia vitripennis*, the auto parasitoid wasps *Encarsia pergandiella* and *Trichogramma kaykai,* use paternal genome elimination (Hunter, Nur and Werren, 1993; Beukeboom and Pijnacker, 2000; van Vugt *et al.*, 2003). Paternal genome elimination involves the development of males from fertilized eggs in which the initial diploid loses its paternal chromosome complement in early development (Heimpel and de Boer, 2007).

Some wasp species have cyclic sex determination systems in which they occasionally change between sexual and asexual reproduction like members of the gall wasps (Cynipidae)(Doncaster, 1916; Crozier, 1975; Quicke, 1997). The mechanisms through which this is achieved is still yet to be uncovered.

Some wasp species reproduce asexually but retain the ability to produce male offspring (Plantard *et al.*, 1998; Belshaw *et al.*, 1999), which could be the case for *Microctonus hyperodae* as some 'impotent' males have previously been described (Goldson *et al.*, 1990).

Some thelytoky mechanisms, like gamete duplication, are considered incompatible with single-locus CSD as they can increase homozygosity (Stouthamer and Kazmer, 1994; Werren, 1997). Heterozygosity is essential to single-locus CSD sex determination mechanisms as different versions of the CSD allele are required for ploidy counting and production of diploid females. Homozygosity at the CSD locus results in the production of diploid males which have no reproductive function. This is highlighted by the production of diploid males in *Apis mellifera* in highly inbreed populations which are hastily killed by their nest mates once emerged (Beye *et al.*, 2003).

Meiosis and Mitosis in the Hymenoptera

Meiosis is a type of cell division, which only occurs in germline cells, producing haploid gametes, chromosomal recombination and chromosomal segregation (Loidl, 2016). Meiosis is likely to have evolved once early in the evolution of eukaryotes, which was determined by surveying the conservation of a set of meiosis-specific genes in *Giardia intestinalis* to infer if meiosis is undertaken and also likely sexual reproduction (Ramesh, Malik and Logsdon Jr, 2005). The broad conservation of meiosis in eukaryotes implies that it is likely to have a selective advantage (Barton and Charlesworth, 1998). Canonical meiosis is generally split into two parts Meiosis I and Meiosis II. Cell division occurs during Meiosis I and Meiosis II, whereas duplication of the genome occurs only in Meiosis I (Hoy, 2003).

Mitosis involves producing genetically identical daughter cells by replicating chromosomes, which are subsequently separated into discreet nuclei. Unlike meiosis, mitosis does not generally result in the recombination of chromosomes (Hoy, 2003).

Multiple mechanisms exist through which either meiosis or mitosis can result in gamete production (with a full complement of adult genes).

Forms of Thelytoky

Both sexual and asexual reproductive modes of reproduction have advantages and disadvantages. Sexual reproduction is likely to be advantageous due to its ability to facilitate the purging of deleterious mutations and generate genetic variability in progeny, which could provide resistance to various biotic and abiotic factors (Muller, 1964). Asexual reproduction, on the other hand, has a two-fold reproductive advantage over sexual reproduction, all else being equal, due to each parent providing only half of the genome of its offspring while in asexual taxa resources are not wasted through the production of males (Maynard Smith, 1978).

Thelytoky is a commonly employed reproductive strategy that involves diploid mothers producing diploid daughters. There are two main subtypes of Thelytoky, Automictic and Apomictic. The telling difference between automictic and apomictic parthenogenesis is the lack of crossing over during apomictic thelytoky (Pearcy, Hardy and Aron, 2006)(Figure 3).

Ploidy is restored in apomixis through mitosis (mitotic parthenogenesis), resulting in offspring being genetically identical to their mother (Suomalainen, Saura and Lokki, 1987; Pearcy *et al.*, 2004). Apomixis has been observed in the Hymenopteran species *Strongylogaster maculata* (Peacock, 1939), *Neoreterus baccarum* (Doncaster, 1916; Dodds, 1939), *Oecophylla longinoda* (Ledoux, 1954) and *Wasmannia auropunctata* (Fournier *et al.*, 2005)(Figure 3).

In automictic parthenogenesis, Meiosis occurs, and diploidy is restored by fusion of nuclei (Heimpel and de Boer, 2007). Hence, ploidy is restored through a variety of mechanisms, including Gamete duplication (highlighted by a complete loss of heterozygosity), Central fusion (retained heterozygosity, except regions further from the centromere; also called fusion of non-sister nuclei), Terminal fusion (retains heterozygosity further from the centromere, which is due to the crossing over of the chiasma and telomere; also called fusion of second division sister nuclei) and Random fusion (Pearcy, Hardy and Aron,

2006). Determining the thelytoky mechanism used by *Microctonus hyperodae* is crucial as it affects heterozygosity (Pearcy, Hardy and Aron, 2006; Figure 3).

Gamete duplication has been observed in the Hymenoptera species *Trichogramma* spp. (Stouthamer and Kazmer, 1994), *Muscidifurax uniraptor* (Legner, 1985), *Diplolepis rosae* (Stille and DäVRING, 1980), *Diplolepis spinosissimae* (Plantard *et al.*, 1998) and *Leptopilina clavipes* (Pannebakker *et al.*, 2004)(Figure 3).

Central fusion has been observed in the Hymenoptera species *Venturia canescens* (Speicher, Speicher and Roberts, 1965; Beukeboom and Pijnacker, 2000), *Apis mellifera capensis* (Tucker, 1958; Verma and Ruttner, 1983; Moritz and Haberl, 1994) and *Cataglyphis cursor* (Pearcy *et al.*, 2004)(Figure 3).

Terminal fusion has been observed in the Hymenoptera species *Diprion polytonum* (Smith, 1941), *Pristiphora rufipes* (Comrie, 1938) and *Aphytis mytilaspides* (Rössler Y, 1973)(Figure 3).

Different reproductive modes are found between and within species of *Microctonus*, with different strains of *Microctonus aethiopoides* either reproducing asexually or sexually (Goldson *et al.*, 2004; McNeill *et al.*, 2006; Phillips *et al.*, 2008). Similar patterns of sporadic thelytoky, as is apparent in *Microctonus*, are also seen in other Hymenoptera species (Stouthamer and Kazmer, 1994; Belshaw *et al.*, 1999; Jeong and Stouthamer, 2005; Lattorff *et al.*, 2007). There is limited experimental evidence that shows retention of heterozygosity in *Microctonus hyperodae,* which implies a lack of recombination, which suggests an apomictic parthenogenesis mechanism or intermittent sexual reproduction (Iline and Phillips, 2004). Alternatively, a heterozygosity retaining form of automictic thelytoky may occur in *Microctonus hyperodae* or intermittent sexual reproduction (Rössler Y, 1973; Simon *et al.*, 2003; Iline and Phillips, 2004). Whether other asexual *Microctonus* use the same parthenogenetic mechanism is yet to be determined as *Wolbachia* has been implicated in thelytoky of *Microctonus wesmael* (a close relative of *Microctonus hyperodae*) (Rodriguero *et al.*, 2014). Thelytoky induced by *Wolbachia* is generally through gamete duplication in Hymenoptera resulting in clonal offspring (Stille

and DäVRING, 1980; Stouthamer and Kazmer, 1994; Plantard *et al.*, 1998; Pannebakker *et al.*, 2004). The apparent heterozygosity in *Microctonus hyperodae* suggests gamete duplication and hence *Wolbachia* infection is unlikely to be causing asexual reproduction in this species (Iline and Phillips, 2004).

Improvements in the availability of genome sequencing

Genome sequencing technology is more affordable and accessible than ever before (Shendure and Ji, 2008). The i5k project aims to have the genomes of 5000 insects and arthropod genomes sequenced and has resulted in a rapid increase in the availability of quality arthropod genomes (I5K Consortium, 2013; Richards, Childers and Childers, 2018). The increase in sequencing availability and the increase in computational power has made sequencing the genomes and transcriptomes of non-model organisms a highly informative and cost-effective undertaking (Richards, Childers and Childers, 2018).

A bioinformatics method for asexuality mechanism inference

Knowledge about the molecular mechanism may be obtained by searching for core meiotic and mitotic genes. The genomes of 18 diverse hymenopterans were searched for 43 core meiotic and mitotic genes (Tvedte, Forbes and Logsdon John M, 2017). It has been determined that most of these genes are conserved in the Hymenoptera. Therefore, looking for meiosis and mitosis genes in non-model species may provide a method to infer an organism's ability to reproduce sexually (Tvedte, Forbes and Logsdon John M, 2017). However, Tvedte, Forbes and Logsdon Jr (2017) only noted one asexual species (*Diachasma muliebre*) and did not identify any variation in the meiotic or miotic gene set imply a relationship to asexual reproduction when compared to the other Hymenoptera. Nonetheless, this inquiry method may uncover possible variation in copy number or the

presence/absence of these core meiotic and mitotic genes in *Microctonus* species of interest, which may allow for the asexual reproductive mechanism to be inferred.

The meiotic gene set used by Tvedte, Forbes and Logsdon John M (2017) included genes involved in cell cycle control (CYC A, CYC B, CYC B3, CYC D, CYC E, CDK1, CDK2, FZY, CORT, PLK1 and PLK4), initiation and maintenance of chromosome structure (RAD21, REC8, SMC1, SMC3, Separase and TIM2) and meiotic recombination (SPO11, RAD51, RAD51C, RAD51D, XRCC2, XRCC3, HOP2, MND1, RAD54, RAD54B, MLH1, PMS1, MLH3, PMS2, MSH2, MSH4, MSH5, MSH6, RECQ1, RECQ2, RECQ3, RECQ4 and RECQ5). A subset of these are meiosis-specific (CORT, REC8, SPO11, HOP2, MND1, MSH4 and MSH5). The roles of the assayed mitosis and meiosis genes are highlighted in Figure 4. Whether the assayed mitosis and meiosis genes retain function in all the analyzed taxa is uncertain as gene expression analysis would be required to confirm this. Tvedte, Forbes and Logsdon John M (2017) did however filter out sequences which were significantly shorter than the homologs from other Hymenoptera species if they did not contain all of the predetermined functional domains.

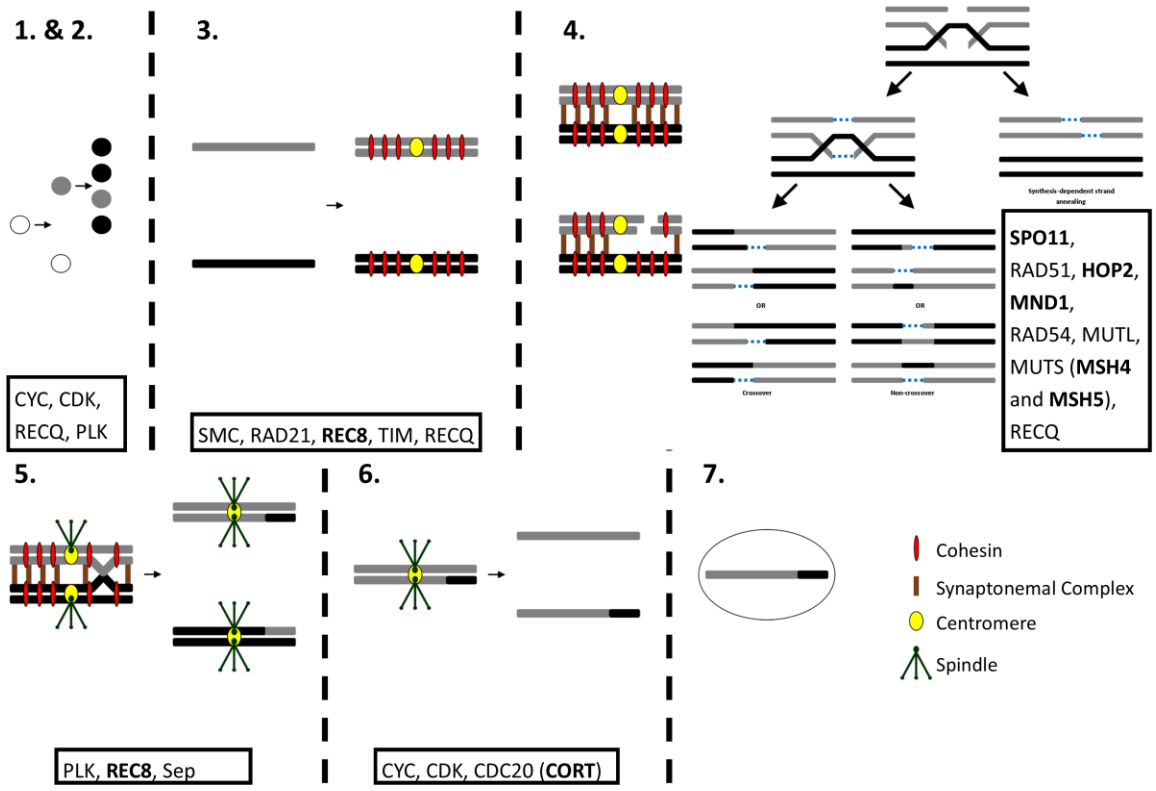

Figure 4) Overview of key processes involved in meiosis. Gamete differentiation during asymmetric cell divisions (1) facilitates entry into meiosis (2). Prior to meiotic entry, chromosomal content is duplicated via DNA replication, followed by the appearance of the centromere and the synaptonemal complex (3). Synapsis and recombination of homologous chromosomes (4) occurs during Prophase I. Synaptonemal complex machinery disassociates to enable segregation of chromosomes during Anaphase I (5). In Meiosis II, sister chromatids separate (6) and the final haploid gamete fully develops (7). Boxes contain meiosis and mitosis genes investigated in this study. Meiosis specific genes are signified in bold. Figure adapted from Hanson *et al.* (2013)**.**

As the thelytoky mechanism of *Microctonus hyperodae* could be apomictic, as suggested by Iline & Phillips (2004), it is most likely that a mitotic gene(s) or the transcription factors responsible for controlling their expression are lost or non-functional. If *Microctonus hyperodae* undertakes intermittent sexual reproduction, then this relationship would be more difficult establish. As the mechanisms through which *Microctonus aethiopoides* undergoes asexual reproduction is not established, even on the cytological level, genes involved in Meiosis should also be exhaustively searched as reproduction could result through automictic mechanisms. Intermittent sexual reproduction could result in the maintenance of mitosis or meiosis genes. It would be beneficial to identify additional mitosis and mitosis regulatory genes so these could also be used to attempt to identify variation between the *Microctonus* species and strains of interest.

Parthenogenesis-inducing endosymbionts

A broad range of insects have their sex ratios skewed or their reproductive strategy altered to parthenogenesis due to the infection with a few microbial species such as *Wolbachia* and *Cardinium* (Stille and DäVRING, 1980; Legner, 1985; Stouthamer and Kazmer, 1994; Iline and Phillips, 2004; Pannebakker *et al.*, 2004). The results of *Wolbachia* infections in Hymenoptera and other Insects vary with a subset of infections resulting in a female skewed sex ratio and occasionally thelytoky. *Wolbachia* are transmitted through the ovaries of female offspring hence altering its hosts reproductive strategy by removing males provides a distinct reproductive advantage to *Wolbachia* through increasing available hosts (Hughes and Rasgon, 2012). Thelytoky caused by *Wolbachia* infection has now been characterized in over 75 species of Hymenoptera (reviewed in Braig *et al.* (2002))

Generally, the causative effect of *Wolbachia* and *Cardinium* in altering host is uncovered through the treatment of infected hosts with antibiotics or exposure to heat (hot enough

to kill the parasite but not the host) (Braig *et al.*, 2002). In addition, many of these experiments have been carried out in other Braconidae species in which reversion to sexual reproduction results (Braig *et al.*, 2002).

Most cases of thelytoky induced by *Wolbachia* is thought to occur through gamete duplication, but this has only been demonstrated in 5 species (Stille and DäVRING, 1980; Legner, 1985; Stouthamer and Kazmer, 1994; Plantard *et al.*, 1998). Other mechanisms through which *Wolbachia* induces thelytoky are covered in O'Neill, Hoffman and Werren, (1997).

Although *Wolbachia* or *Cardinium* infects some Braconidae, there is no evidence to suggest that the thelytoky seen in *Microctonus hyperodae* is due to microbial infection. Treatment of *Microctonus hyperodae* with antibiotics did not produce males suggesting that these endosymbionts are not fundamental to thelytoky in *Microctonus hyperodae* (Goldson correspondence; Phillips, 1995).

In addition to the ability to cause thelytoky, *Wolbachia* infection has also been implied to cause parasitism resistance in Alfalfa weevils to parasitism by *Microctonus aethiopoides* so can provide some benefit to *Microctonus* hosts (Hsaio, 1996).

Comparing parthenogenesis in *Microctonus* asexuals with parthenogenesis caused by *Wolbachia* or *Cardinium* may allow for the mechanism through which thelytoky occurs in *Microctonus hyperodae* to be uncovered. Although, the apomictic mechanism through which *Microctonus hyperodae* appears to reproduce is not characteristic of *Wolbachia* or *Cardinium* infection (which generally occurs through gamete duplication) (Stille and DäVRING, 1980; Legner, 1985; Stouthamer and Kazmer, 1994; Plantard *et al.*, 1998; Iline and Phillips, 2004; Pannebakker *et al.*, 2004). Whether asexual reproduction in the "Irish" strain of *Microctonus aethiopoides* in New Zealand is due to microbial infection may be determined through either antibiotic treatment or assessing raw sequence reads for evidence of infection using the software Kraken2 (Iline and Phillips, 2004).

Identification of genetic variants resulting in thelytokous parthenogenesis

The molecular mechanisms through which genetic thelytoky occurs has proven difficult to establish in insects. The *Drosophila yemanuclein-alpha* meiosis I defective mutation (V478E; *yem^{1}*) is one of the few mutations shown to result in diploid gamete formation, and hence parthenogenesis (Meyer *et al.*, 2010). The mechanisms through which *yem^{1}* is likely to act could be through its effects on kinetochores function in the first meiotic division, resulting in the fusion of two polar bodies and forming diploid gametes (Meyer *et al.*, 2010). Analysis of X chromosome markers of *yem^{1}* mutant progeny did not identify paternal chromosome markers, this suggests development from diploid gametes that underwent gynogenesis (Meyer *et al.*, 2010). Gynogenesis is a type of parthenogenesis that requires fertilization (Meyer *et al.*, 2010).

Unlike other strains of *Apis mellifera,* queen loss in the Cape honeybee results in most workers producing females, from unfertilized eggs, through thelytokous parthenogenesis (Verma and Ruttner, 1983; Oldroyd *et al.*, 2008). The thelytoky observed in the Cape honeybee occurs by fusing the central pair of four haploid meiosis products to form a diploid zygote (Verma and Ruttner, 1983). These findings are consistent with automixis through central fusion (Tucker, 1958; Verma and Ruttner, 1983; Moritz and Haberl, 1994). A locus (*th*) on chromosome 13 has been identified as the possible genetic basis of the thelytokous parthenogenesis in the Cape honeybee, but this is disputed (Lattorff *et al.*, 2007; Chapman *et al.*, 2015; Wallberg *et al.*, 2016). A genetic and molecular mechanism of thelytokous parthenogenesis in the Cape honeybee is yet to be determined (Chapman *et al.*, 2015). Subsequent studies in the Cape honeybee also found no relationship between the locus (*th*) and parthenogenesis. However, they identified several candidates that could be involved in thelytokous parthenogenesis (Wallberg *et al.*, 2016). These candidates include genes with homologues that control the interaction between the centrosome and the meiotic spindle (GB45239), the assembly of the primary cilium

(GB50742) and successful centrosomal binding and maintenance of spindle bipolarity during chromosomes segregation (GB49919) (Wallberg *et al.*, 2016). Further analysis is required to establish which genes are involved in thelytokous parthenogenesis and the underlying molecular mechanism (Wallberg *et al.*, 2016).

In the wasp, *Lysiphlebus fabarum* homozygosity for the 183 alleles at the microsatellite locus *lysi07* results in reproduction through thelytoky (Sandrock and Vorburger, 2011). s In thelytokous *Lysiphlebus fabarum* normal meiosis I is undertaken, but diploidy is restored through a metaphase II fusion of two groups of univalent chromosomes (Sandrock and Vorburger, 2011). This diploidy restoration is equivalent to central fusion automixis (Beukeboom and Pijnacker, 2000; Belshaw and Quicke, 2003). However, although a single locus has been identified the molecular mechanism through which thelytokous parthenogenesis results in *Lysiphlebus fabarum* has not been established (Sandrock and Vorburger, 2011).

As the mechanism through which *Microctonus hyperodae* undertakes thelytokous parthenogenesis is more likely to be apomictic (Iline and Phillips, 2004), the genetic and molecular mechanisms are likely to be different to the automictic mechanisms which have been characterized.

Olfactory receptors

Olfactory receptors play a role in detecting hosts by parasites (Sullivan *et al.*, 2000; Lu *et al.*, 2009; Carey and Carlson, 2011; Smallegange, Verhulst and Takken, 2011). Making it likely that olfactory receptors have crucial roles relating to biocontrol efficiency of *Microctonus* wasps. Olfactory receptors have been identified and characterized in other species of Hymenoptera including *Apis mellifera, Nasonia vitripennis* and *Harpegnathos saltator* (Robertson and Wanner, 2006; Robertson, Gadau and Wanner, 2010; Pask *et al.*, 2017). These olfacotory receptors provide useful comparisons through which olfactory receptors in the *Microctonus* wasps could be catalogued. If there is conserved function in

a clade of olfactory receptors then phylogenetic association pay imply similar roles in these olfactory receptors. Olfactory receptors are used for a broad range of biologically important functions including mate identification, detecting cuticular hydrocarbons and host identification in other insects (Steiner, Hermann and Ruther, 2006; Wei *et al.*, 2007; Pask *et al.*, 2017). Comparing the olfactory receptor families in *Microctonus* species could uncover variation, indicating host preference and biocontrol effectiveness which could have great implications in unlocking and manipulating host range for novel biocontrol targets. An olfactory receptor compliment comparisons between "Irish" and "Moroccan" *Microctonus aethiopoides* strains may reveal why they prefer their respective hosts and could identify genes which could be targeted to manipulate host range. Identification of host-specific variation could allow for the genetic manipulation of host preference, which would allow for more effective host identification, improved host specificity or the genetic manipulation of host range. Enabling a sexually reproducing *Microctonus aethiopoides* strain to target the hosts of asexual strains could allow for the development of more robust biocontrol agents which may benefit from ability to reproduce sexually.

Assessing Reads using Kraken2, Genomescope2 and Smudgeplot

A great deal of useful information about a genome can be obtained by analyzing sequence reads. Kraken2 uses sequence reads as inputs and characterizes reads, allowing for endosymbiont reads to be identified and allowing for an infection with *Wolbachia* to be ruled out or confirmed (Wood and Salzberg, 2014). Genomescope2 uses sequenced reads to estimate genome size and heterozygosity, which are valuable parameters for comparing assemblies and inferring likely mechanisms of asexual reproduction (Ranallo-Benavidez, Jaron and Schatz, 2020). Smudgeplot uses a kmer graph to estimate ploidy. An insect's ploidy level has implications for how easy a genome is to assemble and could allow for hybridization events to be uncovered (Ranallo-Benavidez, Jaron and Schatz, 2020).

Aims

The primary aims of this thesis are to:

1. Improve the biocontrol of the Argentine Stem Weevil by *Microctonus hyperodae*.
2. Assemble and annotate high quality genomes enabling comparative genetic analysis of *Microctonus aethiopoides* and *Microctonus hyperodae*.
3. To improve the understanding of genetic complement of the *Microctonus*.
4. Identify the mechanisms of asexual reproduction in *Microctonus hyperodae* and "Irish" strain *Microctonus aethiopoides* through comparative genetic analysis.
5. Identify genes which could influence the effectiveness of *Microctonus hyperodae* and *Microctonus aethiopoides* as biocontrol agents like their olfactory receptors.

Chapter 2: Kraken2, Genomescope2 and Smudgeplot for the analysis of *Microctonus genomic sequence*

Sequencing the genomes of Microctonus hyperodae and Microctonus aethiopoides

Understanding the genome of *Microctonus hyperodae* provides valuable information regarding its biology, which may be helpful in restoring its biocontrol efficacy. Genomes of *Microctonus hyperodae* and three *Microctonus aethiopoides* strains with interesting differences in reproduction, host preference, behavior, and biocontrol efficacy were sequenced, providing valuable points of comparison. DNA was extracted from "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* provided by AgResearch as adult flies using the TruSeq DNA PCR-Free kit and subsequently sequenced using Illumina HiSeq 2500 250 bp reads with paired-end reads at a coverage of 100X (Bentley *et al.*, 2008; Huptas, Scherer and Wenning, 2016). The resulting sequence reads were of high quality and were subsequently used to assemble genomes as well as Kraken2 (Wood, Lu and Langmead, 2019), Genomscope2, and Smudgeplot analysis of *Microctonus hyperodae* and *Microctonus aethiopoides* " Irish", "French", and "Moroccan" strains (Ranallo-Benavidez, Jaron and Schatz, 2020; For further details see "Sequencing *Microctonus aethiopoides* strains" page 40).

Kraken2 analysis of Microctonus to identify Endosymbionts

Wolbachia can manipulate its hosts' reproduction in multiple ways (Werren, Baldo and Clark, 2008; Weinert *et al.*, 2015). Up to 20% of all insect species are infected with *Wolbachia* (Hilgenboecker *et al.*, 2008). *Wolbachia* is transmitted along the female linage;

hence manipulating a host to produce more female offspring is beneficial like male-killing, feminization of males and parthenogenesis (Hurst and Frost, 2015). The mechanism through which *Wolbachia* induces parthenogenesis in the Hymenoptera, *Trichogramma sp.* and *Leptopilina clavipes* is through disruption of anaphase in the first embryonic division, resulting in the production of a diploid nucleus instead of two haploid nuclei (Stouthamer and Kazmer, 1994; Pannebakker *et al.*, 2004). Wolbachia induces a different mechanism of parthenogenesis in *Muscidifurax uniraptor,* in which a two-cell nuclei fusion occurs after the first mitotic division (Gottlieb *et al.*, 2002).

Kraken2 is an algorithm designed to detect hits from endosymbionts or contaminants in sequenced reads (Wood and Salzberg, 2014; Wood, Lu and Langmead, 2019). If many *Wolbachia* reads are found in the genome sequencing of the *Microctonus aethiopoides* strains or *Microctonus hyperodae,* it would imply that the asexual reproduction observed in the asexual *Microctonus* species is due to a *Wolbachia* infection. A low number or absence of *Wolbachia* reads would imply that a genetic or environmental mechanism of asexual reproduction is more likely to have occurred in asexual *Microctonus*.

Pie charts were produced using Kraken2 output data visualized in the metagenomics analysis web application Pavian allowing for the bacterial compliment of *Microctonus* to be further assesed (Breitwieser and Salzberg, 2016). These pie graphs were used to display the ten bacterial phyla, ten bacterial classes, and ten Alphaproteobacteria genus with the most reads in the *Microctonus hyperodae*. This allowed for the identification of the most common bacterial phyla and classes present in *Microctonus hyperodae* and allowed for comparison with *Microctonus aethiopoides* strains (Breitwieser and Salzberg, 2016). Genera in the Alphaproteobacteria class were also compared between *Microctonus hyperodae* and *Microctonus aethiopoides* strains as these data could provide an indication of the presence of endosymbionts which could result in asexual reproduction manipulation like species in the *Wholbachia* and *Rickettsia* genus.

Microctonus Kraken2 analysis

The “Irish" strain of *Microctonus aethiopoides* has a total of 13702268 reads, of which Kraken2 classified 17.7%. Reads which Kraken2 did not classify likely belong to *Microctonus aethiopoides* as Hymenoptera are not included in the standard Kraken2 database (Wood, Lu and Langmead, 2019). Kraken2 classified 3.301% of the total sequence reads as Bacteria, 0.2797% as Viruses and 0.1478% are classified as Archaea in the “Irish” strain of *Microctonus aethiopoides*. The Archaeal reads are composed of 0.02217% from the Crenarchaeota Phylum and 0.1181% from the Euryarchaeota Phylum. Of the viral species, most were in the family Polydnaviridae (0.1806%), genus Bracovirus (0.1801%) and species *Cotesia congregata bracovirus* (0.1798%; Supplementary S1.5). The “French" strain of *Microctonus aethiopoides* has a total of 8781231 reads, of which Kraken2 classified 17.3%. Reads not classified by Kraken2 likely belong to *Microctonus aethiopoides* as Hymenoptera are not included in the standard Kraken2 database, an unfortunate limitation of this approach (Wood, Lu and Langmead, 2019). Kraken2 classified 3.156% of the total sequence reads as Bacteria, 0.2893% as Viruses and 0.1586% are classified as Archaea in the “French” strain of *Microctonus aethiopoides*. The Archaeal reads are 0.02104% from the Crenarchaeota Phylum and 0.1279% from the Euryarchaeota Phylum. Of the viral species, most were in the family Polydnaviridae (0.1737%), genus Bracovirus (0.1733%) and species *Cotesia congregata bracovirus* (0.1731%). The Virus species *Choristoneura occidentalis granulovirus* also has a notable proportion of reads (0.03464%; Supplementary S1.6). The “Moroccan" strain of *Microctonus aethiopoides* has a total of 9135369 reads, of which Kraken2 classified 17.4%. Reads not classified by Kraken2 likely belong to *Microctonus aethiopoides* as Hymenoptera are not included in the standard Kraken2 database (Wood, Lu and Langmead, 2019). Kraken2 classified 3.204% of the total sequence reads as Bacteria, 0.3038% as Viruses and 0.1448% are classified as Archaea in the “Moroccan” strain of *Microctonus aethiopoides*. The Archaeal reads are 0.0218% from the Crenarchaeota Phylum and 0.1156% from the Euryarchaeota Phylum. Of the viral species, most were

in the family Polydnaviridae (0.1974%), genus Bracovirus (0.1969%) and species *Cotesia congregata bracovirus* (0.1966%). The Virus species *Choristoneura occidentalis granulovirus* also has a notable proportion of reads (0.02564%; Supplementary S1.7). There is likely to be some contamination of all *Microctonus aethiopoides* samples with human DNA as Kraken2 classified ~14% of reads as human in origin. *Microctonus hyperodae* has 130353662 reads, of which Kraken2 classified 7.25%. Reads not classified by Kraken2 likely belong to *Microctonus hyperodae* as Hymenoptera are not included in the standard Kraken2 database (Wood, Lu and Langmead, 2019). Kraken2 classified 1.118% of the total sequence reads as Bacteria, 0.05171% as Viruses and 0.04694% are classified as Archaea in *Microctonus hyperodae*. The Archaeal reads are composed of 0.008548% from the Crenarchaeota Phylum and 0.03656% from the Euryarchaeota Phylum. Of the viral, most were classified as the species Cotesia congregata bracovirus (0.01085%) and *Choristoneura occidentalis granulovirus* (0.00798%; Supplementary S1.8). There is also some contamination present in the *Microctonus hyperodae* samples with ~4.5% of the reads being classified by Kraken2 as human in origin.

Bacterial reads were classified to similar phyla in all *Microctonus* species and strains (Figure 5A). The 10 most common bacterial phyla in the *Microctonus hyperodae* Kraken2 analysis are the Firmicutes, Proteobacteria, Bacteroidetes, Cyanobacteria, Tenericutes, Actinobacteria, Fusobacteria, Spirochaetes, Thermotogae and Deferribacteres (Figure 5A). Of the reads classified as Bacteria by Kraken2 33.02%, 33.46%, 32.61% and 34.51% were classified as Firmicutes in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5A). Of the reads classified as Bacteria by Kraken2 31.56%, 31.28%, 32.75% and 33.06% were classified as Proteobacteria in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5A). Of the reads classified as Bacteria by Kraken2 16.18%, 15.22%, 15.22% and 13.98% were classified as Bacteroidetes in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5A). Of the reads classified as Bacteria by Kraken2 5.10%, 5.12%, 5.04% and 4.95% were classified as Cyanobacteria

in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5A). Of the reads classified as Bacteria by Kraken2 4.10%, 4.38%, 4.35% and 4.07% were classified as Tenericutes in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5A). Of the reads classified as Bacteria by Kraken2 2.47%, 2.50%, 2.73% and 2.28% were classified as Actinobacteria in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctnous hyperodae* respectively (Figure 5A). Of the reads classified as Bacteria by Kraken2 2.12%, 2.30%, 2.00% and 2.18% were classified as Fusobacteria in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5A). Of the reads classified as Bacteria by Kraken2 1.82%, 1.94%, 1.83% and 1.92% were classified as Spirochaetes in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5A). Of the reads classified as Bacteria by Kraken2 0.38%, 0.64%, 0.43% and 0.44% were classified as Thermotogae in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5A). Of the reads classified as Bacteria by Kraken2 0.14%, 0.15%, 0.15% and 0.23% were classified as Deferribacteres in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5A). Of the reads classified as Bacteria by Kraken2 1.82%, 1.94%, 1.83% and 1.92% were classified as not belonging to the ten common phyla ("Other") in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5A).

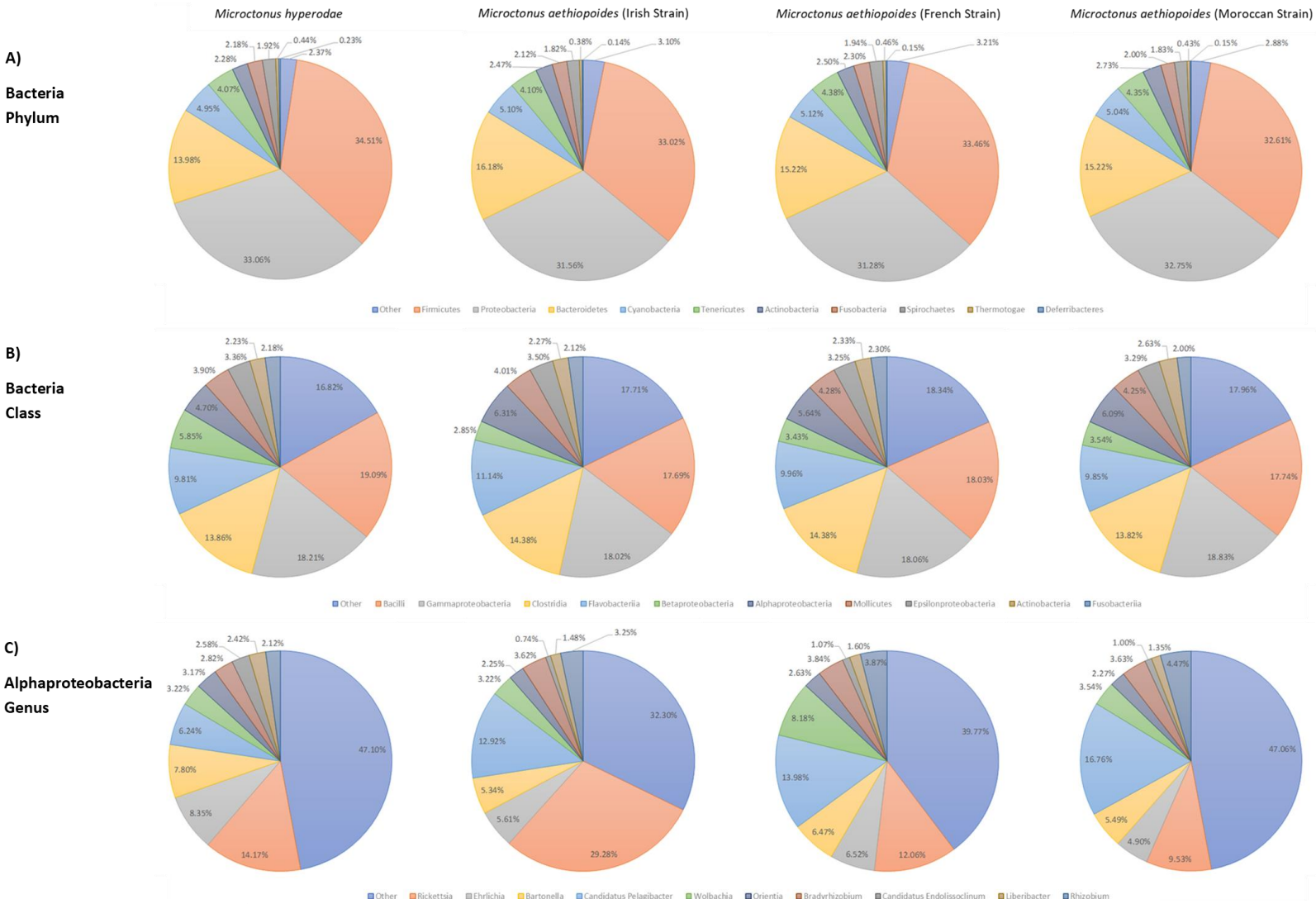

Figure 5) Pie graphs of top 10 Bacterial phyla (A), class (B) and Genus as a percentage of Alphaproteobacteria (C) in Microctonus hyperodae from Kraken2 analysis.

The 10 most common bacterial class in the *Microctronus hyperodae* Kraken2 analysis are the Bacilli, Gammaproteobacteria, Clostridia, Flavobacteriia, Betaproteobacteria, Alphaproteobacteria, Mollicutes, Epsilonproteobacteria, Actinobacteria and Fusobacteriia (Figure 5B). The Class composition of reads classified as bacteria by Kraken2 is broadly similar in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* (Figure 5B). Of the reads classified as Bacteria by Kraken2 17.69%, 18.03%, 17.74% and 19.09% were classified as Bacilli in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5B). Of the reads classified as Bacteria by Kraken2 18.02%, 18.06%, 18.83% and 18.21% were classified as Gammaproteobacteria in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5B). Of the reads classified as Bacteria by Kraken2 14.38%, 14.38%, 13.82% and 13.86% were classified as Clostridia in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5B). Of the reads classified as Bacteria by Kraken2 11.14%, 9.96%, 9.85% and 9.81% were classified as Flavobacteriia in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5B). Of the reads classified as Bacteria by Kraken2 11.14%, 9.96%, 9.85% and 9.81% were classified as Flavobacteriia in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5B). Of the reads classified as Bacteria by Kraken2 17.71%, 18.34%, 17.96% and 16.82% were classified as not belonging to the ten common class ("Other") in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5B).

The 10 most common bacterial Genus in the *Microctonus hyperodae* Alphaproteobacteria Kraken2 analysis are the Rickettsia, *Ehrlichia*, *Bartonella*, *Candidatus Pelagibacter*, *Wolbachia*, *Orientia*, *Bradyrhizobium*, *Candidatus Endolissoclinum*, *Liberibacter* and *Rhizobium* (Figure 5C). The Genus composition of reads classified as Alphaproteobacteria by Kraken2 is broadly similar in the "Irish", "French", and "Moroccan" strains of

Microctonus aethiopoides and *Microctonus hyperodae*, with a few notable exceptions. Of the reads classified as Alphaproteobacteria by Kraken2 29.28%, 12.06%, 9.53% and 14.17% were classified as *Rickettsia* in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5C). Of the reads classified as Alphaproteobacteria by Kraken2 5.61%, 6.52%, 4.90% and 8.35% were classified as *Ehrlichia* in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5C). Of the reads classified as Alphaproteobacteria by Kraken2 5.34%, 6.47%, 5.49% and 7.80% were classified as *Bartonella* in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5C). Of the reads classified as Alphaproteobacteria by Kraken2 12.92%, 13.98%, 16.76% and 6.24% were classified as *Candidatus Pelagibacter* in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5C). Of the reads classified as Alphaproteobacteria by Kraken2 3.22%, 8.18%, 3.54% and 3.22% were classified as *Wholbachia* in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5C). Of the reads classified as Alphaproteobacteria by Kraken2 2.25%, 2.63%, 2.27% and 3.17% were classified as *Orientia* in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctous hyperodae* respectively (Figure 5C). Of the reads classified as Alphaproteobacteria by Kraken2 0.74%, 1.07%, 1.00% and 2.58% were classified as *Candidatus Endolissoclinum* in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5C). Of the reads classified as Alphaproteobacteria by Kraken2 1.48%, 1.60%, 1.35% and 2.42% were classified as *Liberibacter* in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5C). Of the reads classified as Alphaproteobacteria by Kraken2 3.25%, 3.87%, 4.47% and 2.12% were classified as *Rhizobium* in the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5C). Of the reads classified as Alphaproteobacteria by Kraken2 32.30%, 39.77%, 47.06% and 47.10% were classified as not belonging to the ten common Genus ("Other") in the "Irish", "French", and

"Moroccan" strains of *Microctonus aethiopoides* and *Microctonus hyperodae* respectively (Figure 5C).

Endosymbiont candidates in the *Microctonus*

A high number of *Wolbachia* reads or reads which belong to other known parthenogenesis inducing bacteria could provide the smoking gun for the mechanism of asexual reproduction in the *Microctonus*. The *Wolbachia* genus ranked in the 10 most common read classifications of the Alphaproteobacteria in the *Microctonus* taxa. About 3.5% of Alphaproteobacteria reads were classified as belonging to Wolbachia in the analyzed *Microctonus* by Kraken2 apart from the "French" strain of *Microctonus aethiopoides* which had over twice as many reads classified as *Wolbachia* (Figure 5C). As the "French" strain of *Microctonus aethiopoides* reproduces sexually a higher proportion of *Wolbachia* reads does not appear to correlate with the asexual reproduction phenotype in the *Microctonus*. It is possible that different strains of *Wolbachia* could cause different phenotypic effects and differing rates of *Wolbachia* infections of different strains and species of *Microctonus* is likely. The presence of reads in the *Microctonus* reads does not confirm *Wolbachia* infection in the *Microctonus* and additional experimentation such as antibiotic or heat treatment should be further evaluated to see if possible *Wolbachia* infections can be cured (Phillips, 1995). previous treatment of asexual *Microctonus* with antibiotics to kill Wolbachia endosymbionts makes it more likely that the mechanism of asexual reproduction in the *Microctonus* is not a result of an infection with an endosymbiont (Phillips, 1995). The "Irish" strain *Microctonus aethiopoides* has at least twice the number of reads classified by Kraken2 as genus *Rickettsia* than the other *Microctonus* taxa (Figure 5C); some members of this genus are known to cause parthenogenesis in the wasp *Neochrysocharis formosa* (Hagimori *et al.*, 2006;).

Further examination of *Microctonus hyperodae* and *Microctonus aethiopoides* from New Zealand should be undertaken to rule out endosymbionts as a cause of asexual reproduction. This would provide further insights into the asexual reproduction mechanism in *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" strain as ruling out *Wolbachia* infection narrows down the mechanism of asexual reproduction to a genetic cause. Furthermore, curing *Wolbachia* infections, if they are present, could result in reverting to a sexually reproducing phenotype in asexual *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" strain.

Genomescope2 and Smudgeplot

Genomescope2

Genomescope2 allows for the inference of multiple genome properties from a sequence read input, including genome length, uniqueness, heterozygosity, k coverage and duplicates, which have implications for the biology of the *Microctonus* and ramification for the difficulty of subsequent analysis.

All *Microctonus* genomes were under the 2% error rate limit required for accurate Genomescope2 analysis (Table 1; Vurture et al., 2017). The *Microctonus* read sets had over the 15X coverage required for Genomescope2 analysis and 25X required for Smudgeplot analysis (Table 1; Ranallo-Benavidez et al., 2020).

The *Microctonus hyperodae* assembly length is predicted to be 110 Mbp by genomescope2 (Table 1; Figure 6). The genome length estimates from genomescope2 for the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* is 114 Mbp, 120 Mbp and 118 Mbp, respectively (Table 1; Figure 6).

Table 1) Genomescope2 analysis of Microctonus reads

	Microctonus hyperodae	*M. aethiopoides* "Irish"	*M. aethiopoides* "French"	*M. aethiopoides* "Moroccan"
Length (bp)	110007917	114662719	120167273	118368978
Unique kmers (%)	92.5	91.4	88.4	89.9
Heterozygosity (%)	0.594	0.249	0.336	0.414
Kcoverage (%)	71.8	96.7	65.2	72.8
Error (%)	0.081	0.821	1.05	1.13
Duplicate (%)	2.47	5.18	4.22	4.87

Genomescope2 can also allow for genome uniqueness to be estimated which is very useful as genomes with low uniqueness (large repeat regions) are considerably more difficult to assemble (like the genome of the Argentine stem weevil; Harrop, Le Lec, et al., 2020). The "Unique kmers" for *Microctonus hyperodae*, *Microctonus aethiopoides* "Irish" strain, *Microctonus aethiopoides* "French" strain, and *Microctonus aethiopoides* "Moroccan" strain are 92.5, 91.4, 88.4 and 89.9, respectively (Table 1; Figure 6). The high unique kmer values for the *Microctonus* imply that they have genomes with low levels of repeats and are hence likely easier to assemble.

A kmer profile's shape reflects the complexity of a sequenced genome (Genomescope2 paper: Vurture *et al.*, 2017). The kmer heterozygosity estimated by Genomescope2 for *Microctonus hyperodae* is 0.594% (Table 1; Figure 6). The kmer heterozygosity estimated by Genomescope2 for the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* are 0.249%, 0.336% and 0.414%, respectively (Table 1; Figure 6). These results suggest that *Microctonus hyperodae* has a higher genome heterozygosity level than *Microctonus aethiopoides* (Table 1; Figure 6). There is also variation in heterozygosity in the *Microctonus aethiopoides* strains, with the "Irish" strain having the lowest levels of heterozygosity when compared to the "Moroccan" and "French" strains. Of the *Microctonus aethiopoides,* the "Moroccan" strain has the highest heterozygosity levels (Table 1). The higher levels of heterozygosity in *Microctonus hyperodae* are shown by the kmer coverage plot from Genomescope2 as a second smaller peak to the left of the primary peak (Figure 6). The singular peak observed in the Genomescope2 plots for the *Microctonus aethiopoides* strains shows lower heterozygosity levels (Figure 6).

Jaron *et al.*, (2021) found that high heterozygosity levels characterized only asexuals of hybrid origin. Asexuals that were not of hybrid origin appeared to be largely homozygous, independently of the cellular mechanism underlying asexuality. Asexual reproduction through intraspecific origin generally shows heterozygosity between 0.03% and 0.53% (Ranallo-Benavidez, Jaron and Schatz, 2020; Jaron *et al.*, 2021). Low heterozygosity levels imply an intraspecific origin of asexuality reproduction in "Irish"

strain *Microctonus aethiopoides* and *Microctonus hyperodae* (Table 1; Jaron *et al.*, 2021). The heterozygosity levels observed in the asexual *Microctonus* are much lower than those observed in species with a hybrid origin of asexuality, known to have heterozygosity levels from 1.73% to 8.5% (Table 1; Jaron *et al.*, 2021).

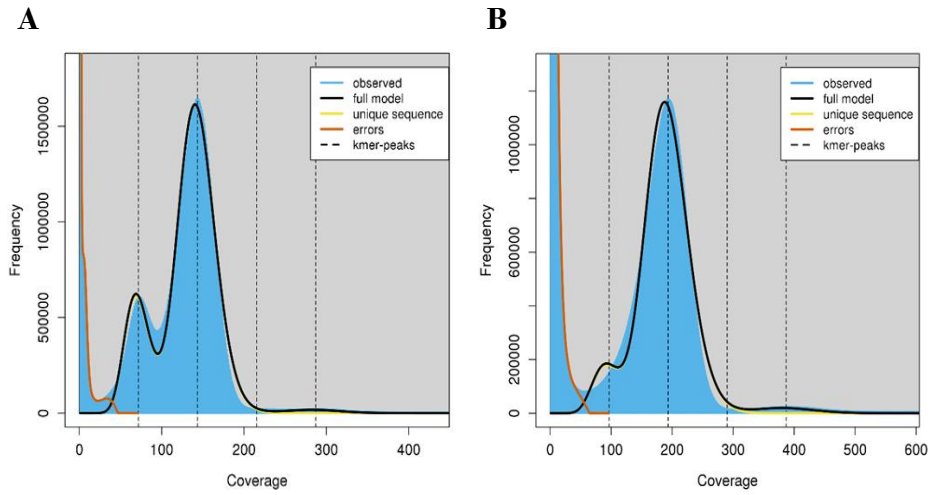

Figure 6) Genomescope2 kmer frequency profile (Kmer frequency VS Coverage) for Microctonus hyperodae (A) and "Irish" strain Microctonus aethiopoides (B). "French" strain Microctonus aethiopoides and "Moroccan" strain Microctonus aethiopoides kmer frequency profiles and log10 frequency profiles are in Supplementary S1.9.

Smudgeplot

Smudgeplot is a companion program to Genomescope2 that uses kmer profiles to infer a genome's ploidy and has been successfully used to differentiate between triploid and tetraploid Root-knot nematodes (Ranallo-Benavidez, Jaron and Schatz, 2020). Little is known about the karyotype of the *Microctonus* clade. There is no previous data on the ploidy levels of the *Microctonus*, and manual karyotype analysis can be challenging and time-consuming, making bioinformatic inference of ploidy lucrative (Gokhman, 2009; Ranallo-Benavidez, Jaron and Schatz, 2020). Previous analysis has shown that Braconids are mostly diploid with the notable exception of *Diplolepis eglanteriae* with is the only known polyploid to date (3n = 27) (Sanderson, 1988; Gokhman, 2009). Smudgeplot analysis should allow for the ploidy of *Microctonus hyperodae* and the *Microctonus aethiopoides* "Irish", "French", and "Moroccan" strains to be inferred.

All of the *Microctonus* seem to be diploids, according to Smudgeplot analysis at a Kmer of 21. The smudgeplots show a clear signal at the AB region of the Smudgeplot for all *Microctonus* with the majority of kmer pairs mapping here (Supplementary S1.10). The Smudgeplot diploid inference is further supported by the AB bin ratio being the highest as this group has the most kmer pairs for all *Microctonus*; this can be observed as the yellow/orange coloration (Supplementary S1.10). The diploid signal is also highlighted in the histograms for both "Total coverage of the kmer pair" (A + B) and the "Normalized minor kmer coverage" (B/(A + B)) through the single major peak in the "Normalized minor kmer coverage" histogram and the two peaks indicated on the "Total coverage of kmer pair" axis (Supplementary S1.10). The AB kmer bin ratio for the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* are 0.86, 0.86 and 0.87, respectively (Supplementary S1.10). *Microctonus hyperodae* has an AB kmer bin ratio of 0.63 (Supplementary S1.10). These high proportions of AB kmer bin ratios also imply the *Microctonus* are diploid (Supplementary S1.10).

Most female Hymenoptera are diploid, and males are haploid due to haplodiploid sex determination, so, unsurprisingly, the *Microctonus* are also diploids; Table 2). Diploids

have two copies of each chromosome, meaning *Microctonus hyperodae* has 12 pairs of chromosomes (2n = 24), whereas *Microctonus aethiopoides* has eight pairs of chromosomes (2n = 16) (Table 2; Figure 7). In addition, the lower genome complexity resulting from a lower ploidy level should make genome assembly easier.

Table 2) Table of proposed ploidy from Smudgeplot analysis of Microctonus taxa Illumina sequence reads (Supplementary S1.10)

	Ploidy
Microctonus hyperodae	Diploid
Microctonus aethiopoides (Irish)	Diploid
Microctonus aethiopoides (French)	Diploid
Microctonus aethiopoides (Moroccan)	Diploid

Chapter 2 conclusions

Kraken2 analysis of sample reads in bacterial phyla and class are similar in the *Microctonus hyperodae* and *Microctonus aethiopoides* strains in the Kraken2 analysis of their sample reads. The *Wolbachia* genus make up at least 3.2% of the Alphaproteobacteria sequences in *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" and "Moroccan" strains. The "French" strain of *Microctonus aethiopoides* has more than twice the proportion of *Wolbachia* genus reads. As The "French" strain of *Microctonus aethiopoides* reproduces sexually and has a much higher proportion of *Wolbachia* reads than the asexual strain of *Microctonus* there appears to be no correlation with having more *Wolbachia* reads and an asexual reproductive strategy. As there are reads classified as belonging to *Wolbachia* in all *Microctonus* strains and species and it is the 5th most common genus classification of sequences classified as Alphaproteobacteria there may be a proportion of *Wolbachia* infected individuals in the *Microctonus* samples. Further analysis is required to confirm *Wolbachia* infections in *Microctonus* strains and species in New Zealand. Most of the Alphaproteobacteria reads were classified as genus *Rickettsia* composing from 9.53% in the "Moroccan" strain of *Microctonus aethiopoides* to 29.28% in the "Irish" strain of *Microctonus aethiopoides*. The "Irish" strain of *Microctonus aethiopoides* has twice the number of reads classified as *Rickettsia* in the Alphaproteobacteria than the other *Microctonus* taxa. As there are such a large proportion of reads classified by Kraken2 as *Rickettsia* it is likely that some *Microctonus* individuals in the samples are infected with *Rickettsia*. A higher proportion of *Rickettsia* reads in the "Irish" strain of *Microctonus aethiopoides* does correlate more strongly with an asexual reproducing phenotype but not in *Microctonus hyperodae*. Further analysis, including antibiotic treatment, of *Rickettsia* in the *Microctonus* is recommended due to its ability to cause parthenogenesis in other species. Genomescope2 analysis implies that Microctonus genomes range from 100 Mbp to 120 Mbp in size. Low heterozygosity levels imply an intraspecific origin of asexuality reproduction in "Irish" strain *Microctonus aethiopoides* and *Microctonus hyperodae*. Allowing for the likely mechanism of asexual

reproduction to be narrowed down. Smudgeplot analysis confirms that the *Microctonus* taxa analyzed are diploids which is consistent with the ploidy observed in other Hymenoptera species. The heterozygosity and ploidy data provides useful insights into the structure of the genome of *Microctonus* which is useful for further genome analysis of these taxa.

Sequencing, Kraken2, Genomescope2 and Smudgeplot methods

Sequencing *Microctonus aethiopoides* strains

AgResearch provided the *Microctonus aethiopoides* samples. 23 adult *Microctonus aethiopoides* "Irish" strain females were provided live and stored at –80 °C until extraction. 4 *Microctonus aethiopoides* "Moroccan" strain and 10 *Microctonus aethiopoides* "French" strain adult females were provided frozen in ethanol.

Tom Harrop processed and sent the samples for sequencing. DNA was extracted from pooled samples using the Qiagen DNA easy blood and tissue kit (QIAGEN, Valencia, CA). Samples subsequently processed using the TruSeq DNA PCR-Free kit (Huptas, Scherer and Wenning, 2016). DNA extraction yields were 189 ng/ml, 103 ng/ml, and 65.7 ng/ml for the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides*, respectively.

Genomic DNA of the "French", "Irish", and "Moroccan" *Microctonus aethiopoides* were extracted from samples and then subsequently sequenced using Illumina HiSeq 2500 250 bp with paired-end reads with 100X coverage by an external sequencing center (Bentley *et al.*, 2008). The "Irish", "French" and "Moroccan" strains of *Microctonus aethiopoides* have a total of 13702268, 8781231 and 9135369 sequenced reads respectively. *Microctonus hyperodae* has 130353662 total sequenced reads.

Kraken2 methods

Kraken2 analysis was run on the raw paired-end reads for the "French", "Irish", and "Moroccan" *Microctonus aethiopoides,* as well as *Microctonus hyperodae* (Wood, Lu and Langmead, 2019). Kraken2 was run using the docker container from Tom Harrop ('shub://TomHarrop/singularity-containers:kraken_2.0.7beta'). Kraken2 was set to run with 24 CPU threads in the paired-end reads configuration with the report flag set. The standard Kraken 2 database, which uses NCBI taxonomic information, complete genomes from RefSeq for bacterial, archaeal, and viral domains, along with the human genome and a collection of known vectors from UniVec_Core, was used for analysis. The analysis used a container constructed by Tom Harrop for reproducible execution of Kraken 2. The resulting Kraken2 report was used as input for the metagenomic data exploration package Pavian to produce a Sankey graph showing the ten phyla, families, genus and species to indicate the microbiome content of the *Microctonus* taxa (Breitwieser and Salzberg, 2016) (Supplementary S1.9). The Kraken report files for *Microctonus* can be found in supplementary files S1.1-1.4.

Genomescope2 and Smudgeplot methods

Kmer counting, Genomescope2 and Smudgeplot analysis was performed for *Microctonus hyperodae* as well as "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides*. The Illumina 250bp reads from each *Microctonus* strain were trimmed and decontaminated with bbmap bbduk and then repaired using bbmap repair.sh (Version 37.57; Bushnell, n.d.). Kmer counting was performed to generate the inputs for genomeplot2 and Smudgeplot using KMC for a kmer length of 21 (Ranallo-Benavidez, Jaron and Schatz, 2020). Subsequently, Kmc_tools transform was used to produce a histogram (v3.1.1; Kokot, Długosz and Deorowicz, 2017). Genomescope analysis was carried out on the provided web-based interface (Ranallo-Benavidez, Jaron and Schatz,

2020). The “smudgeplot.py” “cutoff” script was used to find upper and lower cutoffs for subsequent Smudgeplot analysis (v0.2.2; Ranallo-Benavidez, Jaron and Schatz, 2020). The kmc_tools “transform” “reduce” script was used to extract the region between the cutoffs (v3.1.1; Kokot, Długosz and Deorowicz, 2017). The smudge_pairs script, which is available through installing a specific version of KMC (https://github.com/tbenavi1/KMC), was then used to generate the coverages table output required for Smudgeplot (v3.1.1; Kokot, Długosz and Deorowicz, 2017). Smudgeplot.py plot was used to generate plots from the coverage table (v0.2.2; Ranallo-Benavidez, Jaron and Schatz, 2020). Histograms used for Genomescope2 analysis can be found in supplementary files S1.11-1.14.

Chapter 3: Assembling, Annotating, and estimating divergence in genomes of *Microctonus*

Assembly and Annotation

The genomes of both sexual and asexual *Microctonus aethiopoides* strains were sequenced and assembled to deduce their sequence composition. Genome assembly allows for insights into the biology of these species, including the ability to undertake downstream analysis, including gene prediction and genome annotation, hence inferring the gene complement of the *Microctonus*. Comparing the gene complement of these species may allow for the mechanism of asexual reproduction in *Microctonus* to be inferred. The genome of *Microctonus hyperodae* was previously sequenced and assembled by Tom Harrop, providing insight into how to assemble the other genomes (See supplementary S2.8 for the relevant script). The genomes of *Microctonus hyperodae* and *Microctonus aethiopoides* can therefore be compared. Comparison of the genomes of sexual and asexual *Microctonus* may allow for mechanisms of asexual reproduction to be inferred through the comparison of predicted peptides with defined roles in meiosis and mitosis. The gene complement allows the olfactory receptor complement to be catalogued and compared in homology to those of other Hymenoptera, providing insight into their function in the *Microctonus* which likely has implications for biocontrol efficiency. Sequencing and assembling the genomes of these *Microctonus* species may provide insights into why the biocontrol of the Argentine stem weevil has failed, enabling targeted intervention.

Assembly results for Microctonus aethiopoides strains and Microctonus hyperodae

The final draft assemblies for the *Microctonus aethiopoides* "Irish", "French" and "Moroccan" strains consist of 129188093 bp, 118883977 bp and 120337575 bp, respectively. The assembly for *Microctonus hyperodae* is 106813660 bp. The genomes of *Microctonus aethiopoides* are larger than those of *Microctonus hyperodae*. The Meraculous generated assembly sizes for the *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" strain are 105760060 bp and 128839196 bp.

Hi-C is based on Chromosome Conformation Capture (3C). 3C involves the crosslinking of chromatin with formaldehyde, subsequent digestion, and re-ligation of DNA. Chimeric DNA ligation junctions are generated using biotin (biotin-14-dCTP) labelled nucleotides allowing for selective purification. Selective purification of chimeric sequences uses streptavidin-coated magnetic beads. Chimeric DNA fragmented with restriction enzymes can then be deep sequenced. 3C techniques are used to measure the population-average frequency of physical interaction between two DNA fragments based on the higher likeliness of two sequences to be crosslinked together. The closer two sequences are in physical space, the more likely they will end up being crosslinked. Hi-C can detect interactions within and between chromosomes.

Binning data can adjust the resolution of Hi-C data into fixed genomic intervals. Binning reduces the complexity and number of possible genome-wide interactions, increasing the signal to noise ratio in Hi-C data. The midpoint of a Hi-C restriction fragment is used to assign its bin. Hi-C bins are selected, considering the intended analysis goals. For chromosome number analysis, a bin size of between 1MB-10MB is likely to provide an optimal resolution to infer which scaffolds in a genome sequence correspond to a shared chromosome (Lajoie, Dekker and Kaplan, 2015). Hi-C data is generally visualized using a heat map. Successful Hi-C experiments show a strong diagonal of interactions between proximal genomic regions and an overall exponential decay in interaction signal over

increasing distance. Interactions are hence more common within a chromosome than between different chromosomes, providing a solid and ubiquitous pattern (Lajoie, Dekker and Kaplan, 2015). As a result, Hi-C can generate more contiguous assemblies. Hi-C provides a unique and powerful tool to study nuclear organization and chromosome architecture (Bickhart *et al.*, 2017).

Hi-C increased the total assembly size for the final assemblies of *Microctonus hyperodae* and "Irish" strain *Microctonus aethiopoides* as it identified contigs that have sufficient evidence to assemble into scaffolds (Table 3). The *Microctonus hyperodae* Meraculous and Hi-C assembly's actual length is slightly smaller at 105 Mbp (Table 3), further evidence for the high quality of the *Microctonus hyperodae* genome assembly. The genome length estimates from genomescope2 for the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* is 114 Mbp, 120 Mbp and 118 Mbp, respectively (Table 1). Thus, the "Irish" strain assembly is larger than predicted by Genomescope2. From this, we can infer that we have likely assembled the complete genome for the *Microctonus aethiopoides* "Irish" strain. The Genomescope2 estimates are nearly identical to the assembled genomes for the "French" and "Moroccan" strains of *Microctonus aethiopoides*. The correlation of Genomesope2 estimate with the assembly size implies that the assemblies for the "French" and "Moroccan" strains of *Microctonus aethiopoides* are likely to represent the entire genome even though these assemblies are not as contiguous as those from the "Irish" strain *Microctonus aethiopoides* and *Microctonus hyperodae* (Table 3).

Table 3) Assembly metrics for Microctonus aethiopoides strains and Microctonus hyperodae

	Meraculous & Hi-C		Meraculous			
	M. hyperodae	M. aethiopoides (Irish)	M. hyperodae	M. aethiopoides (Irish)	M. aethiopoides (French)	M. aethiopoides (Moroccan)
Total length (bp)	106813660	129188093	105760060	128839296	118883977	120337575
Number of contigs	3683	2844	14207	6322	10399	13740
N50 (bp)	9364176	23025277	15392	64081	30183	17930
L50	5	3	1758	484	881	1633
GC (%)	29.5	29.38	29.5	29.38	29.3	29.41
Busco (%)	89.5	93.1	86.9	92.6	90.4	88.6

The chromosome number can be determined by the regions of strong interaction in the Hi-C heat map indicated in red. The Hi-C interaction map implies 12 strong regions of interaction for *Microctonus hyperodae* and 8 in *Microctonus aethiopoides* "Irish" strain. As a result, the Hi-C analysis implies *Microctonus* hyperodae and *Microctonus* aethiopoides "Irish" strain likely have differing numbers of chromosomes 12 chromosomes in *Microctonus hyperodae* and eight chromosomes in *Microctonus aethiopoides* (Figure 7).

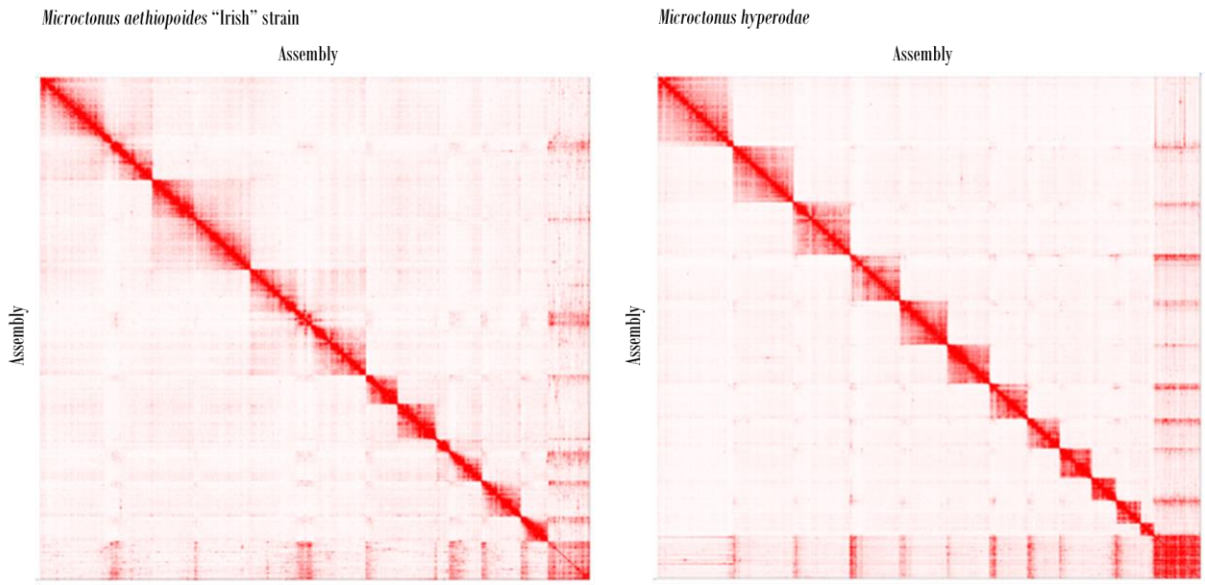

Figure 7) Hi-C interaction map for the genomes of M. hyperodae and the asexual Microctonus aethiopoides "Irish" strain visualized in Juicebox. **The darker read regions indicate intra-chromosomal interactions allowing for chromosome numbers to be inferred.**

The number of contigs composing the Meraculous assemblies of *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" strain is 14207 and 6322. The number of contigs for the final draft assemblies for the "Irish", "French", and "Moroccan" strain *Microctonus aethiopoides* are 2844, 10399 and 13740, respectively. The number of contigs for the final draft assembly for *Microctonus hyperodae* is 3683. The lower number of contigs in the "Irish" strain *Microctonus aethiopoides* and *Microctonus hyperodae* suggests these genomes are the most contiguous. Hi-C decreased the number of contigs for the final draft assemblies of *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" strain suggesting that Hi-C improved the contiguity and assembly quality (Table 3).

The L50 of the Meraculous assemblies of *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" strain are 1758 and 484. The L50 for the *Microctonus aethiopoides* assemblies are 3, 881 and 1633 for the "Irish", "French", and "Moroccan" strains, respectively. The L50 for *M. hyperodae* is 5. These results suggest that the assemblies of *Microctonus aethiopoides* "Irish" strain and *Microctonus hyperodae* are highly contiguous, with 50% of the genome composed of 3 or 5 scaffolds, respectively. Hi-C decreased the L50 value for the final draft assemblies of *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" strain, suggesting that Hi-C improved the contiguity of these assemblies (Table 3).

The largest contigs of the *Microctonus aethiopoides* assemblies are 27171691 bp, 482968 bp and 231661 bp for the "Irish", "French", and "Moroccan" strains, respectively. The largest contig of the *Microctonus hyperodae* assembly is 14794116 bp (Table 3).

The N50 of the Meraculous assemblies of *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" strain are 15392 and 64081. The N50 for the *Microctonus aethiopoides* assemblies are 23025277 bp, 30183 bp and 17930 bp for the "Irish", "French", and "Moroccan" strains, respectively. The N50 for *Microctonus hyperodae* is 9364176 bp (Table 3).

These results imply that the assemblies of *Microctonus aethiopoides* "Irish" strain and *M, hyperodae* are the most contiguous as half the genome of these taxa are contained in contigs of larger than 23025277 bp and 9364176 bp, respectively (Table 3). Hi-C vastly improved contiguity of the *Microctonus hyperodae* and *Microctonus aethiopoides* genome assemblies. Assembly quality correlates with higher quality genetic material input in the initial samples as *Microctonus aethiopoides* "Irish" strain had a higher DNA concentration in initial preparations (Table 3).

The GC percentage of the *Microctonus aethiopoides* assemblies is 29.38 %, 29.3 % and 29.41 % for the "Irish", "French", and "Moroccan" strains, respectively. The GC percentage of the *Microctonus hyperodae* assembly is 29.5 %. The number of "N's" per 100 kbp in the *Microctonus aethiopoides* assemblies are 1431.46, 481.61 and 1915.94 for the "Irish", "French" and "Moroccan" strains, respectively. The number of "N's" per 100 kbp in the *Microctonus hyperodae* assembly is 2667.86 (Table 3).

The number of "N's" per 100 kbp is a metric that assesses the gaps in an assembly. A high number of "N's" per 100 kbp can indicate scaffolds have been joined indiscriminately to improve N50, L50 and contig number metrics. The *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" strain have a higher number of "N's" per 100 kbp as contigs have been joined in the process of Hi-C (Table 3).

BUSCO searches for a specific set of conserved single-copy genetic markers, which can be used as an indication of genome assembly completeness (Simão *et al.*, 2015). The BUSCO scores for the *Microctonus hyperodae* and *Microctonus aethiopoides* Meraculous assemblies are 86.9% and 92.6%, respectively. The BUSCO scores for the *Microctonus aethiopoides* final draft assemblies are 93.1%, 90.4% and 88.6% for the "Irish", "French", and "Moroccan" strains, respectively. The BUSCO score for *Microctonus hyperodae* final draft assembly is 89.5%. All the Microctonus have BUSCO scores of around 90%, suggesting that these genome assemblies are a good representation of a complete genome. BUSCO scores for *Microctonus hyperodae* and *Microctonus aethiopoides* increased slightly after Hi-C, indicating that most of the assayed genes were identifiable in the initial assembly and that none of the assayed genes were lost (Table 3).

The genome assembly of *Nasonia vitripennis* has a BUSCO score of 97.0% which is considered more complete than the genomes of the *Microctonus* spp (Wang *et al.*, 2020). BUSCO scores for *Vespula* wasps has BUSCO completeness between 92.2% and 96.0% which are similar to the BUSCO scores from *Microctonus hyperodae* and the "Irish" strain of *Microctonus aethiopoides* (Harrop, Guhlin, *et al.*, 2020). These results suggest that the *Microctonus* assemblies are relatively complete when compared to some high-quality genome assemblies from other species of Hymenoptera.F

Comparing the genome assemblies of *Microctonus* to model insect genome assemblies

In order to gain insight into the *Microctonus hyperodae* genomes, the assembly quality metrics of the *Microctonus* assemblies were compared to the assembly metrics of insect model reference genomes. The high-quality reference genomes were used as a comparison, including the genome from the Diptera *Drosophila melanogaster* and the Hymenoptera species *Apis mellifera* and *Nasonia vitripennis*. These reference genomes provide a benchmark to compare the *Microctonus* genomes to evaluating how they measure up to some of the highest quality Hymenoptera genomes.

Comparing total genome size of *Microctonus* assemblies to those of model insects *Apis mellifera*, *Drosophila melanogaster* and *Nasonia vitripennis*

All included *Microctonus* have compact genomes, which are smaller than the reference genomes for *Drosophila melanogaster* (143726002 bp; Adams, 2000), *Apis mellifera* (225250884 bp; Wallberg *et al.*, 2019) and *Nasonia vitripennis* (297309692 bp; Dalla Benetta *et al.*, 2020; Figure 8). These data suggest that the *Microctonus* genomes included are very compact.

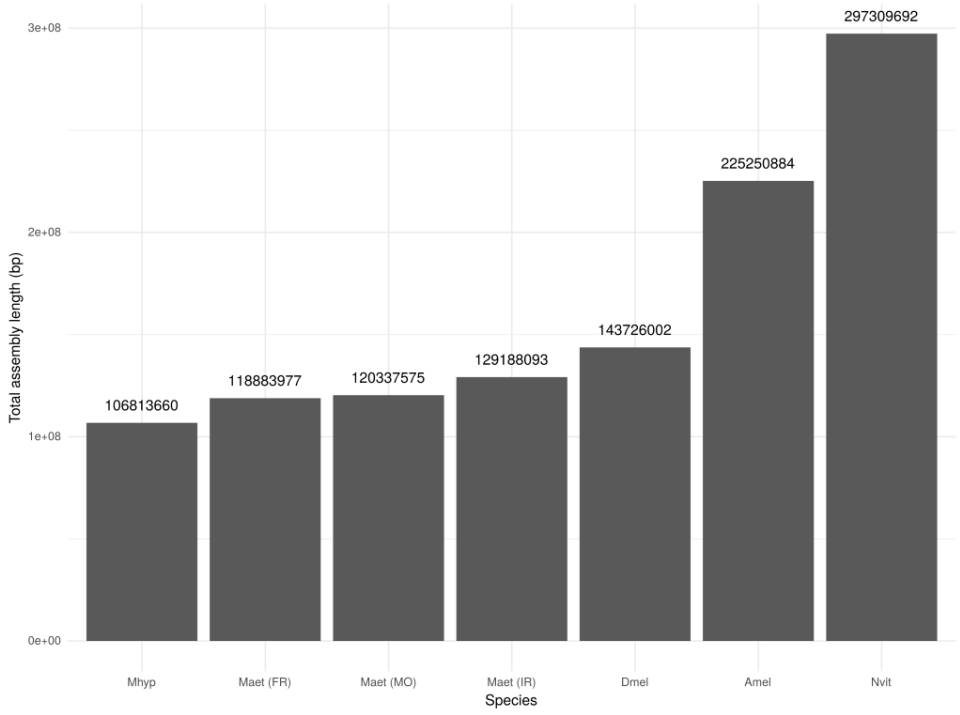

Figure 8) Total size of the Amel (Apis mellifera; Wallberg *et al.*, 2019***), Nvit (Nasonia vitripennis;*** Dalla Benetta *et al.*, 2020***), Dmel (Drosophila melanogaster;*** Adams, 2000***), Maet (IR)(Microctonus aethiopoides "Irish" strain), Maet (MO)(Microctonus aethiopoides "Moroccan" strain) and Maet (FR)(Microctonus aethiopoides "French" strain) genome assemblies.***

Comparing contig number from *Microctonus* assemblies to insect assemblies

In assembled genomes fewer contigs imply a more contiguous genome. All the *Microctonus* assemblies consist of a larger number of contigs than the reference genomes of *Apis mellifera* (which has 177 contigs), *Nasonia vitripennis* (which has 436 contigs) and *Drosophila melanogaster* (which has 1870 contigs; Figure 11). The Hi-C genome assemblies of *Microctonus hyperodae* and "Irish" strain *Microctonus aethiopoides* are much closer to the number of contigs to the reference genomes than the "French" and "Moroccan" strains of *Microctonus aethiopoides* (Figure 9). The *Microctonus* genomes are less contiguous that those from the high-quality reference genomes.

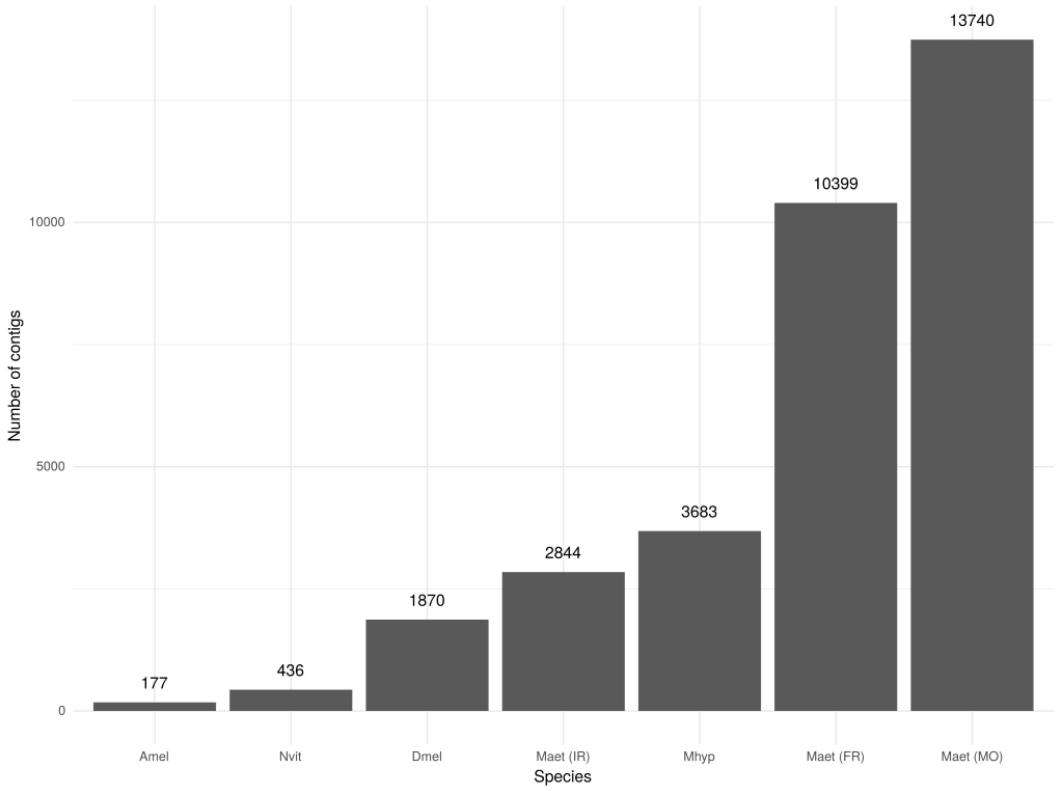

Figure 9) Number of contigs in the genome assemblies of Amel (Apis mellifera), Nvit (Nasonia vitripennis), Dmel (Drosophila melanogaster), Maet (IR)(Microctonus aethiopoides "Irish" strain), Maet (MO)(Microctonus aethiopoides "Moroccan" strain) and Maet (FR)(Microctonus aethiopoides "French" strain).

Comparing the Scaffold L50 from *Microctonus* assemblies to model insect assemblies

The reference genomes for *D. melanogaster*, *Apis mellifera* and *Nasonia vitripennis* have an L50 of 3, 7 and 5, respectively (Figure 10). The L50 values for the Hi-C genomes of *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" of 5 and 3, respectively, are similar to those of the included reference genomes (Figure 10). Suggesting that these genomes are comparable to the reference genomes of these insect model organisms in quality. The comparatively higher L50 values of "French" and "Moroccan" strain *Microctonus aethiopoides* suggest that these are less contiguous than the high-quality insect reference genomes (Figure 10).

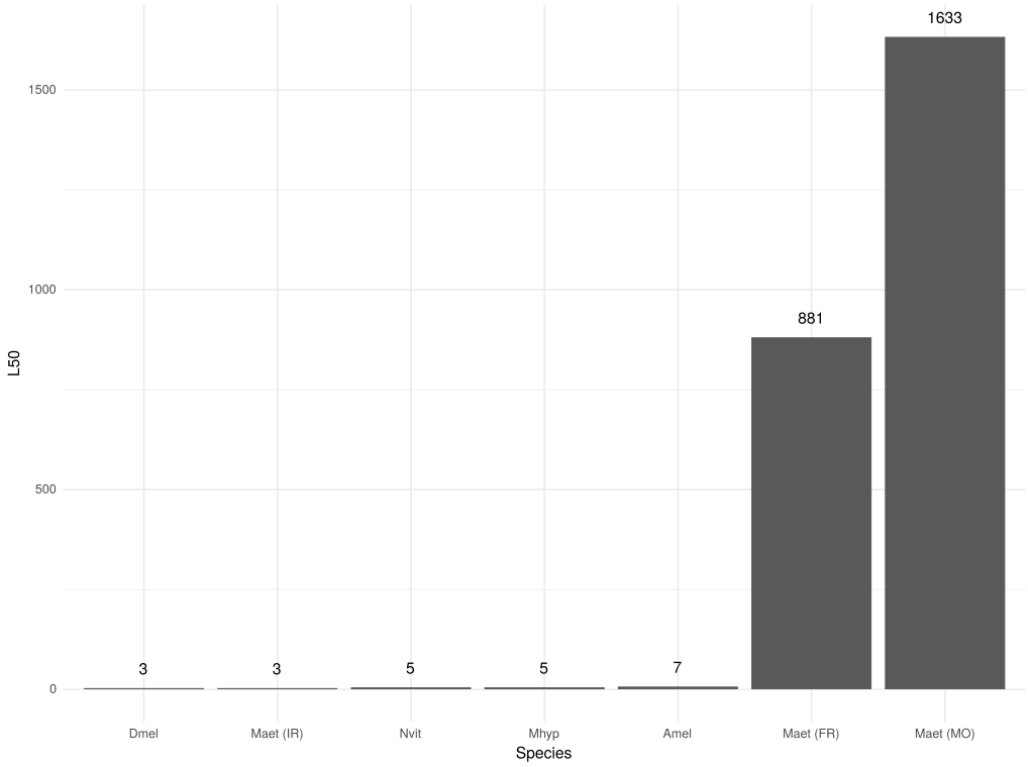

Figure 10) Scaffold L50 of the Amel (Apis mellifera), Nvit (Nasonia vitripennis), Dmel (Drosophila melanogaster), Maet (IR)(Microctonus aethiopoides "Irish" strain), Maet (MO)(Microctonus aethiopoides "Moroccan" strain) and Maet (FR)(Microctonus aethiopoides "French" strain).

Comparing the Scaffold N50 from *Microctonus* assemblies to model insect assemblies

The Hi-C assembly N50 for the "Irish" strain *Microctonus aethiopoides* (23025277 bp) is comparable in size to the high-quality genome assemblies of the parasitic wasp *Nasonia vitripennis* (24760113 bp) and *Drosophila melanogaster* (25286936 bp) and higher than the reference genome of *Apis mellifera* (Figure 11). The comparatively high scaffold N50 values of the *Microctonus hyperodae* and "Irish" strain *Microctonus aethiopoides* implies

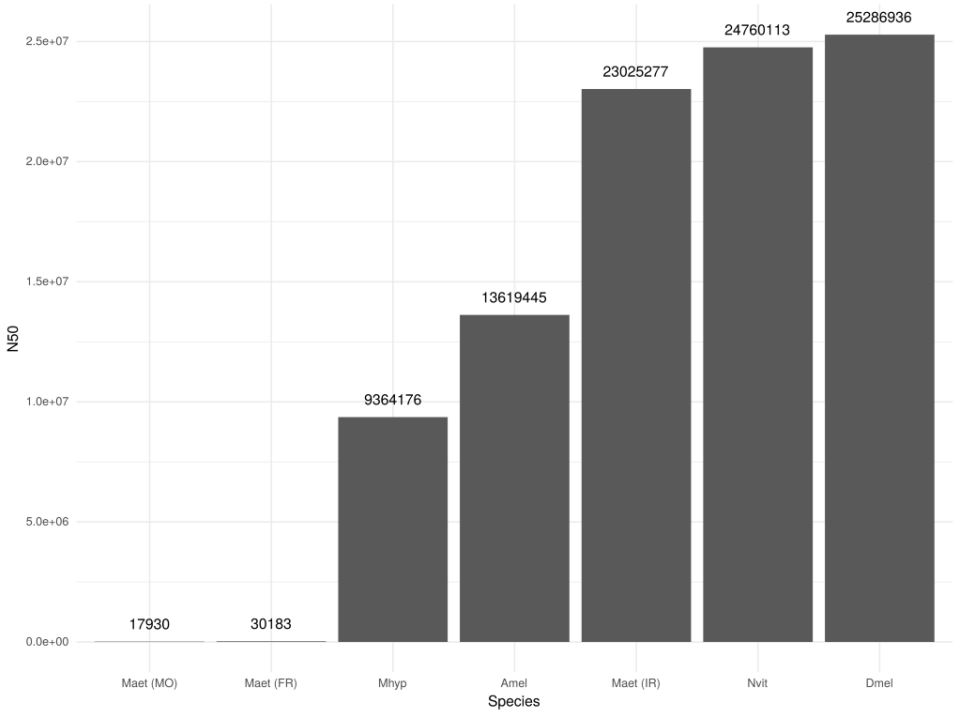

Figure 11) Scaffold N50 of the Amel (Apis mellifera), Nvit (Nasonia vitripennis), Dmel (Drosophila melanogaster), Maet (IR)(Microctonus aethiopoides "Irish" strain), Maet (MO)(Microctonus aethiopoides "Moroccan" strain) and Maet (FR)(Microctonus aethiopoides "French" strain) assemblies.

these genomes are of a high quality and comparable to some high quality Hymenoptera genomes. The lower N50 values of the *Apis mellifera* genome is likely due to the higher chromosome number (n16) (Figure 11). The Hi-C assembly of *Microctonus hyperodae* has a lower N50 than the reference genomes (Figure 11). The "French" and "Moroccan"

strains of *Microctonus aethiopoides* have comparatively low N50 values when compared to the other *Microctonus* assemblies and other insects, which could be a result of the storage conditions, the quality and amount of input DNA sequence (Figure 11).

Microctonus genome assemblies compared to other Hymenoptera species

The *Microctonus aethiopoides* ("Irish") strain Hi-C assembly has the highest scaffold N50 out of the Hymenoptera taxa (http://i5k.github.io/arthropod_genomes_at_ncbi [27/11/2020]). Only 4 Hymenoptera taxa have a scaffold N50 higher than the *Microctonus hyperodae* Hi-C assembly. The Hi-C assembly of *Microctonus hyperodae* has a higher N50 than 96% of Hymenoptera genomes (http://i5k.github.io/arthropod_genomes_at_ncbi [27/11/2020]; Figure 12). Hence, these genome assemblies are more contiguous than the vast majority of Hymenoptera genomes and hence more likely to represent complete and high-quality genome assemblies (Figure 12). The comparative quality of the *Microctonus* genomes makes subsequent annotation and gene analysis more likely to capture the full complement of genes and should result in fewer errors in annotation.

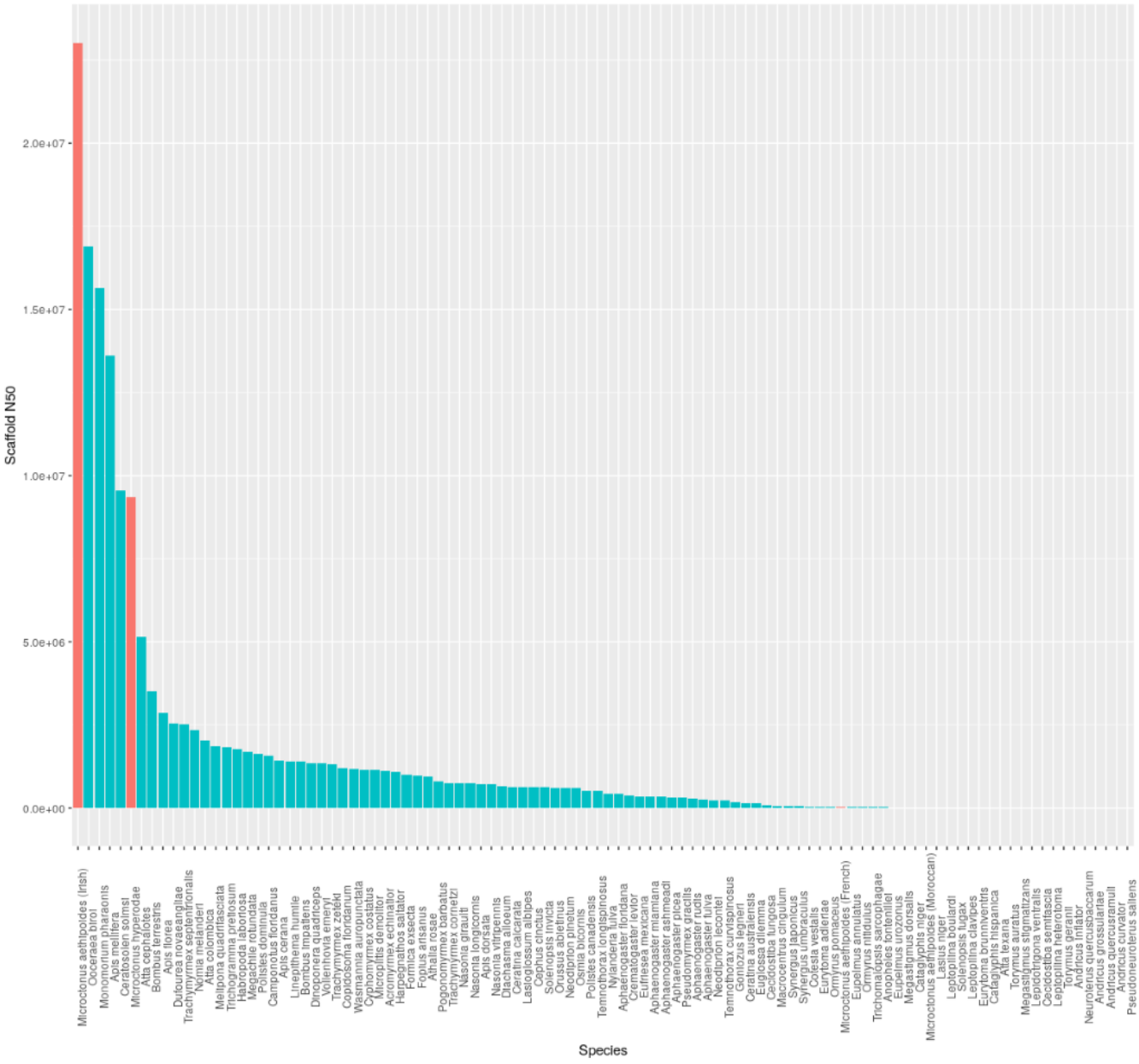

Figure 12) Graph of scaffold N50 from the 96 Hymenoptera taxa from the i5k NCBI database [http://i5k.github.io/arthropod_genomes_at_ncbi [27/11/2020]]

Annotation of Microctonus genomes

Annotation of the genomes of *Microctonus hyperodae,* "Irish" strain *Microctonus aethiopoides*, "French" strain *Microctonus aethiopoides* and "Moroccan" strain *Microctonus aethiopoides* would be invaluable in assessing their biology and evolution. Annotation of the *Microctonus* genomes was undertaken using the Funannotate genome annotation program, which has recently proven effective in annotating three Vespula wasp genomes (Harrop, Guhlin, *et al.*, 2020). The Funannotate software is based on Evidence Modeler, synthesizing multiple gene prediction inputs, and producing consensus models. The gene prediction inputs utilized by Funannotate include Augustus, snap, glimmerHMM, CodingQuarry and GeneMark-ES/ET (Korf, 2004; Majoros, Pertea and Salzberg, 2004; Lomsadze *et al.*, 2005; Haas *et al.*, 2008; Stanke *et al.*, 2008; Testa *et al.*, 2015). Annotations of the *Microctonus* used genomes, RNA sequences and previously annotated peptides as inputs for genome annotation. The number of predicted peptides for *Microctonus hyperodae,* "Irish" strain *Microctonus aethiopoides*, "French" strain *Microctonus aethiopoides* and "Moroccan" strain *Microctonus aethiopoides* are 12982, 13966, 14174 and 14475, respectively (Table 4). Both the asexual *Microctonus, Microctonus hyperodae,* and *Microctonus aethiopoides* have fewer predicted protein sequences from annotation.

Table 4) Microctonus annotation

	M. hyperodae	M. aethiopoides (Irish)	M. aethiopoides (French)	M. aethiopoides (Moroccan)
Predicted peptides	12982	13966	14174	14475
Busco scores (%)	88	94	90	87

The parasitic wasp, *Cotesia congregate*, is also in the same family as the *Microctonus*, Braconidae (Gauthier *et al.*, 2021). The genome annotation of *Cotesia* uncovered 14140 genes similar to the number seen in the *Microctonus* species (Gauthier *et al.*, 2021). The genome annotation of the parasitic wasp *Nasonia vitripennis* has 14086 genes predicted, similar to the number of genes predicted in the *Microctonus* (NCBI *Nasonia vitripennis* Annotation Release 101; Dalla Benetta *et al.*, 2020). It is therefore likely that a majority of the peptides have been predicted in the annotation of *Microctonus hyperodae,* "Irish" strain *Microctonus aethiopoides*, "French" strain *Microctonus aethiopoides* and "Moroccan" strain *Microctonus aethiopoides*.

The BUSCO scores for the genome annotations from *Microctonus hyperodae,* "Irish" strain *Microctonus aethiopoides*, "French" strain *Microctonus aethiopoides* and "Moroccan" strain *Microctonus aethiopoides* are 88%, 94%, 90% and 87%, respectively. Suggesting the peptide predictions for the *Microctonus* are relatively complete compared to a conserved gene set in the Hymenoptera (Table 4).

The high BUSCO scores (Table 4) and the number of gene predictions which are similar to those of other parasitic wasps (Table 4; Gauthier et al., 2021; NCBI *Nasonia vitripennis* Annotation Release 100; Dalla Benetta *et al.*, 2020), suggests that a set of high-quality annotations has been successfully produced for the *Microctonus*. The high-quality *Microctonus* annotations will be invaluable for further bioinformatic enquiries into the meiosis and mitosis gene set as well as the olfactory receptor complement.

Divergence estimates

Divergence estimates were used to get a clearer understanding of the relatedness of *Microctonus hyperodae* and the three strains of *Microctonus aethiopoides*. Current evidence suggests that *Microctonus hyperodae* and *Microctonus aethiopoides* are genetically and morphologically similar (Vink *et al.*, 2003). Whole-genome peptide prediction based on Orthofinder analysis suggests significantly more genetic diversity than has been previously suggested (Vink *et al.*, 2003). How closely related *Microctonus hyperodae* is to *Microctonus aethiopoides* has implications for reverting *Microctonus hyperodae* to sexual reproduction. Suppose Microctonus hyperodae is not closely related to the asexual *Microctonus aethiopoides*. There are more likely to be multiple mutations resulting in asexual reproduction, making causative mutations harder to identify and reversion to an asexual form of reproduction less likely.

Divergence estimates Results and Discussion

It can be inferred from the divergence estimate tree that the "French" and "Moroccan" strains of *Microctonus aethiopoides* are more closely related than either of them is to the "Irish" strain (Figure 13). The estimated time of divergence between *Microctonus hyperodae* and *Microctonus aethiopoides* is calculated as 17 MYA (Figure 13). The asexual "Irish" strain of *Microctonus aethiopoides* diverged from the sexually reproducing "French" and "Moroccan" strains of *Microctonus aethiopoides* about 2 MYA (Figure 13). The "French" and "Moroccan" strains of *Microctonus aethiopoides* diverged in about 1 MYA (Figure 13). The common wasp *Vespula vulgaris* diverged from other members of its genus, *Vespula pensylvanica* and *Vespula germanica*, about 6 MYA (Figure 13). The Hymenoptera diverged from *Drosophila melanogaster* about 480 MYA (Figure 13).

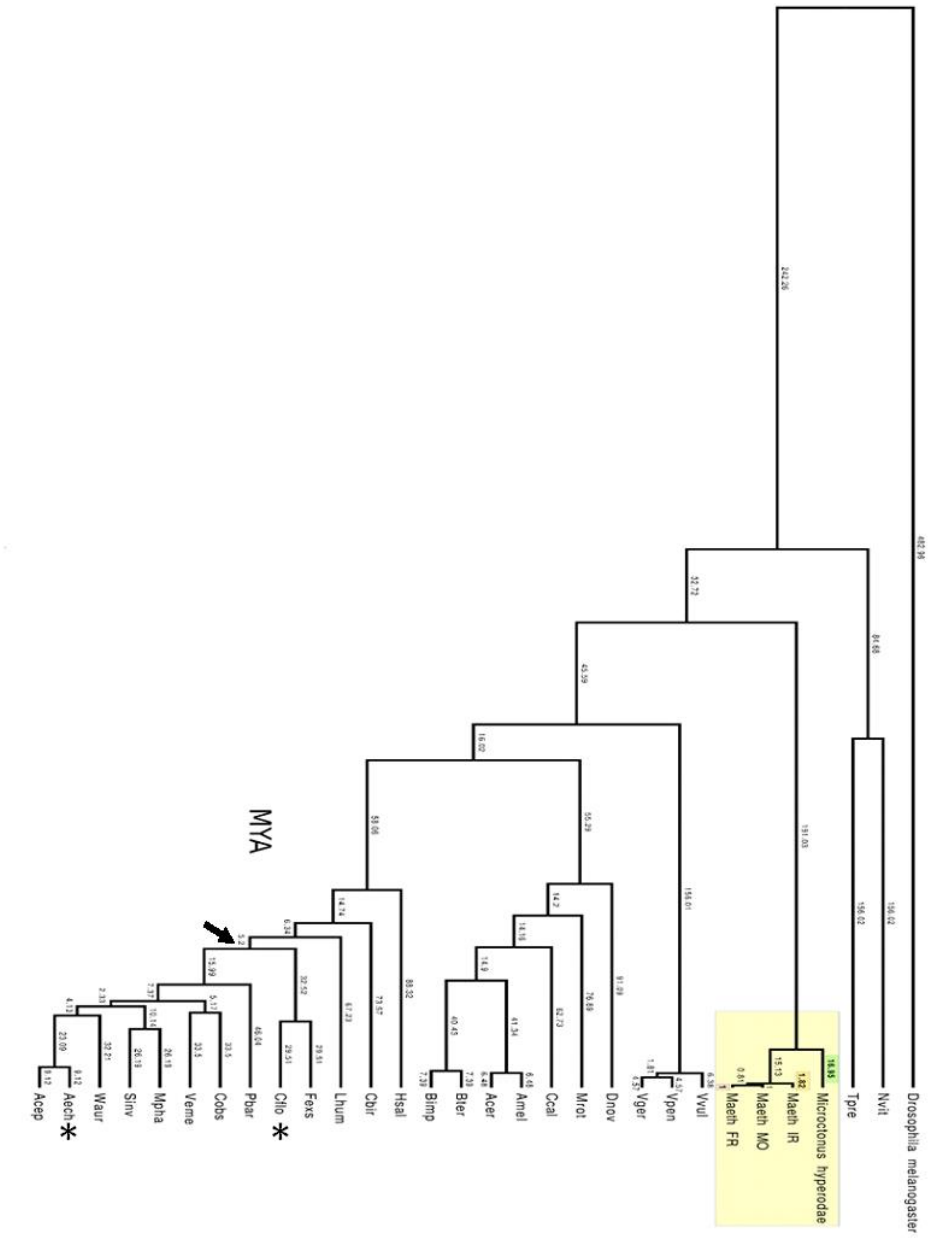

Figure 13) *Rudimentary estimation of Microctonus evolutionary origin based on an ultrametric Orthofinder species tree. A branch length scaling factor of 563.12. The Microctonus clade is highlighted in yellow. The "Irish", "French" and "Moroccan" strains of Microctonus* aethiopoides *are Maeth IR, Maeth FR and Maeth MO respectively. Drosophila melanogaster is the outgroup. The divergence of Acromyrmex echinatior(*) and Camponotus floridanus (*) from Peters et al. (2017) was used to calibrate the molecular clock and is indicated by an arrow. The Hymenopteptera abbreviations are as follows: Nvit (Nasonia vitripennis), Tpre (Trichogramma pretiosum), Vvul (Vespula vulgaris), Vpen (Vespula pensylvanica), Vger (Vespula germanica), Dnov (Dufourea novaeangliae), Mrot (Megachile rotundata), Ccal (Ceratina calcarata), Amel (Apis mellifera), Acer (Apis cerana), Bter (Bombus terrestris), Bimp (Bombus impatiens), Hsal (Harpegnathos saltator), Cbir (Cerapachys biroi), Lhum (Linepithema humile), Fexs (Formica exsecta), Cflo (Camponotus floridanus), Pbar (Pogonomyrmex barbatus), Cobs (Cardiocondyla obscurior), Veme (Vollenhovia emeryi), Mpha (Monomorium pharaonic), Sinv (Solenopsis invicta), Waur (Wasmannia auropunctata), Aech (Acromyrmex echinator) and Acep (Atta cephalotes).*

The divergence estimate of 17 MYA is much larger than would be suggested by previous morphological and genetic analysis (Vink et al., 2003). Previous genetic comparisons only utilize a small number of genetic elements, whereas the analysis undertaken here leverages the proteomes of multiple *Microctonus aethiopoides* strains and *Microctonus hyperodae*. *Microctonus hyperodae* and *Microctonus aethiopoides* are more distantly related than wasps in the *Vespula* genus, in which *Vespula vulgaris* diverged from other members of its genus, *Vespula pensylvanica* and *Vespula germanica*, about 6 MYA. A similar divergence estimate for the divergence of *Vespula* was found by Harrop et al. (2020). The divergence estimates for *Drosophila melanogaster* and the Hymenoptera are consistent with previous estimates (Zhang et al., 2018). Either the proposed *Microctonus hyperodae* and *Microctonus aethiopoides* divergence estimate implies that they are not as closely related as assumed, or *Microctonus hyperodae's* reproductive mode resulted in artifacts skewing the divergence estimate, or rapid protein-coding gene evolution occurred in *Microctonus hyperodae*, or rapid protein-coding gene evolution occurred in *Microctonus aethiopoides*. If asexual reproduction resulted in skewed *Microctonus hyperodae* and *Microctonus aethiopoides* divergence estimates, then the same skewing would be expected in asexual "Irish" strain *Microctonus aethiopoides*. Unless differing mechanism of asexual reproduction is used in this strain, or divergence from the sexual *Microctonus aethiopoides* is much more recent, or there is cryptic sex resulting in gene flow between sexual and asexual *Microctonus aethiopoides*. If the *Microctonus* species are as diverged as these estimates suggest, then identifying gene presence or absence, which resulted in the shift to an asexual reproductive mode, would be more challenging due to the increased likelihood of non-functional variation. It also implies that the mechanism thought which "Irish" strain *Microctonus aethiopoides* and *Microctonus hyperodae* switched reproductive modes to asexual reproduction is less likely to be the same. *Microctonus hyperodae* and *Microctonus aethiopoides* relatedness has significant implications regarding the downstream analysis.

There may be variation in chromosome numbers in the *Microctonus* with 12 in *Microctonus hyperodae* Hi-C analysis and 8 in the *Microctonus aethiopoides* Hi-C (Figure 7), which implies that either the chromosomes have been combined in the genome of *Microctonus aethiopoides* "Irish" strain or fragmented in *Microctonus hyperodae* since they diverged 17 MYA.

Chapter 3 conclusions

Meraculous assembly of *Microctonus* genomes resulted in high quality genomes which were further improved using Hi-C analysis on the genomes of *Microctonus hyperdoae* and the "Irish" strain of *Microctonus* aethiopoides. High genome quality is supported by BUSCO scores of ~90%, as well as comparatively good L50 and N50 values. The *Microctonus* have relatively compact genomes ranging in size from 106 Mbp to 129Mbp in size. There may be variation in chromosome numbers in the *Microctonus* with 12 in *Microctonus hyperodae* Hi-C analysis and 8 in the *Microctonus aethiopoides* Hi-C. The *Microctonus* have between 12982 and 14475 annotated protein coding genes. Annotations also have promising BUSCO scores of ~90%. *Microctonus hyperodae* and *Microctonus aethiopoides* appear to have diverged about 17 MYA. The "Irish" strain of *Microctonus aethiopoides* diverged from the "French" and "Moroccan strains about 2 MYA. High quality *Microctonus* genomes and annotations allow for comparative analysis of genes involved in determining evolutionary fitness and biocontrol efficacy. These genomes provide a powerful tool for further research into the gene composition of the *Microctonus*, cataloguing genes involved in mitosis and meiosis and identifying olfactory receptors, which could help us understand more about the biology of these essential biocontrol agents.

Assembly of Microctonus genomes methods

Processing reads for assembly

The Illumina 250bp reads from each *Microctonus* strain were trimmed and decontaminated with bbmap bbduk and then repaired using bbmap repair.sh (Version 37.57; Bushnell, n.d.). The reads were trimmed to a kmer of 23, with a min kmer set at 11 and a hdist of 1. Read decontamination used "phix174_ill.ref.fa.gz". The reads were subsequently normalized using bbmap bbnorm (Version 37.57; Bushnell, n.d.) with a maximum target coverage of 50 and a minimum of 5. The read processing for *Microctonus hyperodae* was performed by Tom Harrop whereas the read processing for the *Microctonus aethiopoides* species was performed by me.

Meraculous assembly of *Microctonus genomes*

Meraculous was selected as the genome assembly program of choice as it has previously been used to successfully assemble the genome of *Microctonus hyperodae* by Tom Harrop (results presented within). Meraculous relies on the traversal of a subgraph of the de Bruijn graph of oligonucleotides. This subgraph has unique and high-quality extensions in the dataset (Version 2.2.5; Chapman et al., 2011). Meraculous does not use an explicit error correction step and instead uses base quality scores, allowing for the assembly of diploid genomes by Meraculous (Version 2.2.5; Chapman et al., 2011). Meraculous also efficiently uses computer memory by incorporating a novel low-memory hash structure to access the deBruijin graph (Version 2.2.5; Chapman et al., 2011).

The Meraculous assembly algorithm is composed of initial processing and four Perl modules. Pre-processing of reads requires selecting a kmer set before being processed by the Meraculous modules Meraculous.pl, blastMap.pl, oNo.pl and Marauder.pl.

Meraculous.pl produces a set of maximal linear sub-paths of the de Bruijn graph (Version 2.2.5; Chapman et al., 2011). blastMap.pl aligns reads back to the assembly to identify read-pair information used to link contig strings together into scaffolds. oNo.pl uses paired reads and splinting singletons from blastMap to produce a scaffold by ordering and orientating a set of contigs. Marauder.pl closes gaps within scaffolds using reads established to fall within the gap established using their mate reads (Version 2.2.5; Chapman et al., 2011).

Initial Meraculous assemblies were carried out at kmers of 31, 71 and 127. The outputs of these assemblies were analyzed and informed future Meraculous assemblies. Assemblies were undertaken on the trimmed and decontaminated or trimmed, decontaminated, and normalized read sets. Assemblies used either diploid or haploid modes (Version 2.2.5; Chapman et al., 2011).

As the assemblies at a kmer value of 71 had the best assembly statistics (N50, L50, total genome size in MB, number of scaffolds and BUSCO score (Version 3.0.2; Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015), kmer values of 63, 67, 75 and 79 were subsequently used as input parameters for assemblies on trimmed and decontaminated reads and trimmed, decontaminated, and normalized reads. The assemblies used either normalized or non-normalized reads.

Best assemblies were selected based on the N50, L50, total genome size in MB, the number of scaffolds, and BUSCO score metrics.

The Meraculous assembly selected for the "Irish" strain of *Microctonus aethiopoides* had a kmer value of 79, was from non-normalized, trimmed, and decontaminated data assembled as a haploid. The Meraculous assembly selected for the "French" strain of *Microctonus aethiopoides* had a kmer value of 79, normalized, trimmed, and decontaminated data assembled as a diploid. The Meraculous assembly selected for the "Moroccan" strain of *Microctonus aethiopoides* had a kmer value of 71, was from normalized, trimmed and decontaminated data assembled as a haploid.

The evaluate Meraculous run script output for each of the assemblies can be found in Supplamentary S2.7 and S2.8. All of the assembly steps were carried out by myself.

Hi-C

Hi-C was carried out for the "Irish" strain *Microctonus aethiopoides* and *Microctonus hyperodae* (10 individual adult flies of each). Samples were prepared for Hi-C using the recommended sample preparation protocol provided by Phase Genomics. Samples were chopped into small pieces and resuspended in 1% formaldehyde in PBS. Samples were incubated at room temperature for 20 minutes, vortexing at 5 and 10 minutes. Glycine was added till a 1/100mL concentration was reached. Samples were then incubated at room temperature for 15 minutes, vortexing for 5-to-10-minute time intervals. The samples were spun down using a microcentrifuge, rinsed with PBS, and spun down again. The supernatant was then removed, and the sample was subsequently frozen at -4 °C. Hi-C sample processing and analysis was performed by Phase Genomics using the *Microctonus hyperodae* and "Irish" strain *Microctonus hyperodae* genome assemblies (Lieberman-Aiden *et al.*, 2009).

RNA sequencing

RNA was extracted from *Microctonus aethiopoides* "Moroccan" and *Microctonus aethiopoides* "Irish" strains and Microctonus hyperodae whole adult flies using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was subsequently sequenced using Illumina 1.9 with ~250 bp paired-end reads with TruSeq Stranded mRNA (Bentley *et al.*, 2008). These sequences were extracted and processed by Peter Dearden.

Annotation of *Microctonus* genomes

The *Microctonus* genomes were annotated with Funannotate; annotations have been included for both the Meraculous assembly and Meraculous and Hi-C assembly. The Meraculous annotation was used for the Mitosis and Meiosis gene cataloguing analysis (see chapter 4), and the Meraculous and Hi-C annotation was used for the Olfactory receptor analysis (see chapter 5). For the number of protein predictions, see Table 4. For further details about the Meraculous assembly annotations for "Irish" strain *Microctonus aethiopoides* and *Microctonus hyperodae,* see Supplementary S2.1-SS2.5.

Repeat modeler was used to model the genome repeats for the *Microctonus* genomes using the NCBI database (open-1.0.11; Smit, Hubley and Green, 2015b). The Funannotate software is based on Evidence Modeler, synthesizing multiple gene prediction inputs and producing consensus models. The gene prediction inputs utilized by Funannotate include Augustus, snap, glimmerHMM, CodingQuarry and GeneMark-ES/ET (Version v1.5.0-12dd8c7; Love *et al.*, 2018). Funannotate was selected as it was highly effective in predicting genes in the Genomes of Vespula with similar evidence, and genomes which were also assembled using Hi-C and Meraculous (Harrop, Guhlin, *et al.*, 2020).

In order to more efficiently annotate the genomes of the *Microctonus,* the input genomes were sorted using Funannotate sort and subsequently masked using RepeatMasker using the NCBI engine (Version v1.5.0-12dd8c7; Love *et al.*, 2018; Version 4.0.0; Smit, Hubley and Green, 2015a). The Funannotate model was trained using Funannotate train on the masked genome assembly using *Microctonus hyperodae* or *Microctonus aethiopoides* sequences depending on the species of interest. Augustus was iteratively trained (Version: 3.3.1; Stanke, Diekhans, Baertsch, & Haussler, 2008) and used as input for Funannotate predict to predict genes (Version v1.5.0-12dd8c7; Love *et al.*, 2018), using the Hymenoptera BUSCO and optimized Augustus settings. Funannotate update was subsequently used to upgrade the annotation (Version v1.5.0-12dd8c7; Love *et al.*, 2018). Annotations and genomes can be found in the Supplementary folder S2.1-S2.5.

An earlier annotation version was used to analyze mitosis and meiosis genes in the *Microctonus,* which have similar BUSCO scores and the number of peptide predictions but was carried out on the Meraculous genome assemblies for *Microctonus hyperodae* and "Irish" strain *Microctonus aethiopoides* (See Supplementary S2.1-S2.6).

I annotated all of the *Microctonus* genomes with the guidance of Joseph Guhlin.

Estimating divergence of *Microctonus*

Orthofinder was run on a compute server using 36 CPU threads in multiple sequence alignment mode using the diamond search engine and maft on the genomes of numerous *Hymenoptera* species and *Drosophila melanogaster* to produce a species tree from peptide databases (Version 2.3.12; Emms and Kelly, 2019). The sum of the branch lengths of *Acromyrmex echinatior* and *Camponotus floridanus* was 0.1101 (0.0524 + 0.0577) in the Orthofinder ultrametric species tree. Dividing the 62 MYA divergence estimate by the summed branch lengths of 0.1101 results in a scaling factor of 563.12, which will allow for the divergence of the *Microctonus hyperodae* and *Microctonus aethiopoides* to be estimated using the divergence of *Acromyrmex echinatior* and *Camponotus floridanus* as a reference as this node was shared by previous research which establishes evolutionary divergence in the Hymenoptera supported by fossil evidence (Peters *et al.*, 2017). Scaling the ultrametric tree by the scaling factor of 563.12 in Figtree resulted in a phylogenetic tree with inferred divergence estimates for the included taxa (Figure 13). I performed the Orthofinder analysis, and the divergence estimates for the *Microctonus* and other assorted Hymenoptera species.

Chapter 4: Conservation of mitosis and meiosis genes in sexual and asexual *Microctonus*

Mitosis and meiosis are responsible for genome duplication. In eukaryotes, mitosis usually results in diploid cell production—meiosis results in recombination and segregation and the production of haploid gametes (Loidl, 2016).

Meiosis in the Hymenoptera is generally poorly characterized so assessing meiosis and mitosis genes for presence and absence would provide some insight into which genes in these systems may have conserved functions to those characterized in other insect taxa.

Identifying candidate cell division genes that are catalogued in other species of Hymenoptera may provide insights into the mechanism of asexual reproduction employed by *Microctonus hyperodae* as well as asexual *Microctonus aethiopoides*. The "meiosis detection toolkit" previously employed by Schurko & Logsdon (2008) was used to search for orthologs in the peptide predictions of asexual and sexual *Microctonus*. The conservation of 38 mitosis and meiosis genes, including 7 of which are meiosis-specific, were catalogued in *Microctonus*.

The 'meiosis detection toolkit' (Schurko and Logsdon, 2008) was subsequently used to explore possible mechanisms of asexual reproduction in the Hymenoptera by cataloguing the presence and absence of genes known to have roles in mitosis or meiosis in other insects. Cataloguing the mitosis and meiosis genes was achieved by searching for their presence in respective Hymenoptera protein databases using a custom blast script. Where orthologs were not identified, genomes were searched and identified orthologs annotated using previously identified orthologs from other Hymenoptera species for prediction (Tvedte, Forbes and Logsdon John M, 2017). Although the 'meiosis detection toolkit' method was unable to establish a precise molecular mechanism for asexual reproduction in *D. muliebre,* it has been useful in establishing the presence and absence of these genes in the Hymenoptera, including absences in sexually reproducing taxa highlighting

variation in the mechanisms through which cell division occurs in these taxa (Tvedte, Forbes and Logsdon John M, 2017).

Although most Hymenoptera reproduce sexually (employing a haplodiploid system), asexually reproducing taxa are common (Van Wilgenburg, Driessen and Beukeboom, 2006). Both meiotic (automixis) and mitotic (apomixis) mechanisms of asexual reproduction have been observed in the Hymenoptera (Lamb and Willey, 1987). I aimed to infer the mechanism of asexual reproduction in asexual *Microctonus* as it could have implications for genome heterozygosity and biocontrol effectiveness.

Loss of any of the characterized genes in the "meiosis detection toolkit" commonly results in a defective meiosis phenotype, so absences or duplications could provide insight into mechanisms of asexual reproduction and which genes are involved in cell division in asexual *Microctonus hyperodae* and *Microctonus aethiopoides* (Villeneuve and Hillers, 2001; Ramesh, Malik and Logsdon Jr, 2005).

Very little is known about the mechanisms of mitosis and meiosis in the Hymenoptera. Identification of conserved gene markers for meiosis in the Hymenoptera would be of great utility for identifying genes involved in mitosis and meiosis in this group and the mechanism of asexual reproduction employed by some Hymenoptera. Gene expression of meiosis marker genes which are broadly conserved in the Hymenoptera, including MSH4/MSH5 and HOP2/MND1, could allow for mitosis or meiosis based asexual mechanisms of reproduction to be inferred and verify their conserved roles in these processes.

Hybridization chain reactions were used to explore the expression of the Meiosis genes in the ovaries of adult *Microctonus aethiopoides* "Irish" strain. The meiosis genes MSH4, MSH5, HOP2 and MND1 were targeted but only MND1 expression was observed due to sample acquisition limitation due to the global pandemic. The expression of these genes in the ovaries implies a function of these meiosis genes and implies a meiosis-based mechanism of asexual reproduction.

Catalogue of Meiosis and Mitosis genes and conservation in the Microctonus

Cyclins (CYC A, CYC B, CYC B3, CYC D and CYC E) and Cyclin-dependent kinases (CDK1 and CDK2)

The five cyclins in the 'meiosis detection toolkit', CYC A, CYC B, CYC B3, CYC D and CYC E, were identified in *Microctonus*. The cyclins are conserved in all included insect taxa implying conserved and essential functions in insect mitosis and or meiosis (Figure 14). The Cyclin-dependent kinases CDK1 and CDK2 are also conserved in the included insect taxa (Tvedte, Forbes and Logsdon John M, 2017). Cyclin-dependent kinases form complexes with cyclins to regulate mitosis and meiosis (Murray, 2004). The CYCA-CDK1 complex controls entry into mitosis in *Drosophila* (Jacobs, Keidel and Lehner, 2001). The CYCB-CDK1 complex (also referred to as mitosis promoting factor complex) induces the start of mitosis (Morla *et al.*, 1989). The subsequent degradation of CYCB and the resulting inactivation of CDK1 degradation allows for exit from mitosis (Hershko, 1999). In *Drosophila*, the CYCB3-CDK1 complex has been exhibited to co-operate with other Cdk1 complexes to promote the G2/M transition (Yuan and O'Farrell, 2015). Another complex, CYCD-CDK4, has been demonstrated to promote cell growth in *Drosophila* (Datar *et al.*, 2000; Meyer *et al.*, 2000). In comparison, the cyclin CYCE-CDK2 complex has been proven to be essential for entry into the S phase in *Drosophila* (Ekholm and Reed, 2000). The conservation of the cyclins and CDKs in searched insect taxa and *Microctonus* suggests a conserved role in *Microctonus* and the mitosis and meiosis in the insects (Figure 14).

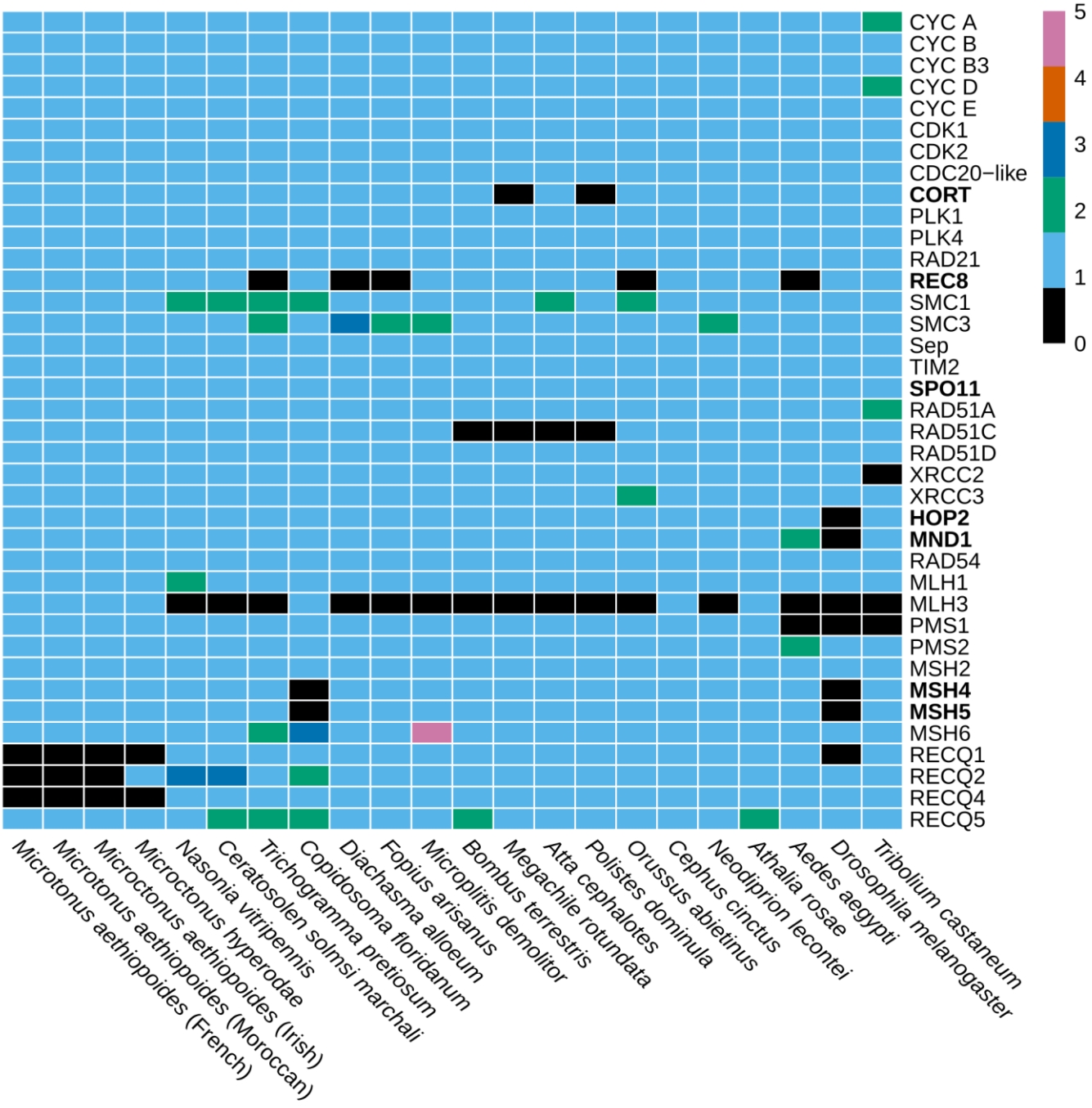

Figure 14) Presence absence heatmap of meiosis detection tool kit proteins in Microctonus, other Hymenoptera, Diptera (Aedes aegypti and Drosophila melanogaster) and Coleoptera (Tribolium). The colour key notates the gene copy number. Expanded from Tvedte et al. (2017).

CDC20 homologs (CORT and FZY)

In *Drosophila melanogaster* females CORT and FZY function together to promote anaphase progression by destroying cyclin A/B/B3 (Swan and Schüpbach, 2007). FZY and Cort are broadly conserved in the insect taxa assayed except for *Polistes dominula* (which may be an annotation or assembly artifact) (Figure 14; Tvedte et al., 2017). Conservation of CORT and FZY in the *Microctonus* and most other insect taxa suggests conserved roles for these proteins in meiosis and mitosis progression (Figure 14).

Polo-like kinases (PLK1 and PLK4)

PLK1 functions in mitotic entry and G2/M checkpoint, coordination of the centrosome and cell cycle, regulates spindle assembly and chromosome segregation, is involved in cytokinesis, exerts multiple functions at the spindle midzone during abscission, facilitates DNA replication and is involved in meiosis (review: Schmucker & Sumara, 2014). Considering its myriad of roles in mitosis and meiosis, it is unsurprising that PLK1 is conserved in insects, including *Microctonus* (Figure 1), suggesting a conserved role of PLK1 in *Microctonus* mitosis and meiosis. PLK4 has a crucial role in centriole biogenesis in the Eukaryotes and is also conserved in *Microctonus* (Zitouni, Nabais, Jana, Guerrero, & Bettencourt-Dias, 2014; Figure 14).

MutS (MSH2, MSH4, MSH5 and MSH6) and MutL (MLH1, MLH3, PMS1 and PMS2) homologs

MutS and MutL are involved in DNA mismatch repair, which is essential in maintaining genomic fidelity (review: Manhart & Alani, 2016). The homologs of MutL, MLH1, MLH3

and PMS2, form heterodimers involved in mismatch repair. The MLH1-MLH3 complex is also involved in crossover formation in meiosis (Kadyrov *et al.*, 2006, 2007; Ranjha, Anand and Cejka, 2014). The knockout of PMS2 in mice results in meiotic defects through an unknown mechanism (Baker *et al.*, 1995).

The MutS homologs also form heterodimers, MSH2-MSH6 and MSH2-MSH3, involved in mismatch repair. The MSH2-MSH6 complex is involved in repairing small mismatches (Onrat *et al.*, 2011). In contrast, The MSH2-MSH3 is involved in recognizing larger indels (Manhart and Alani, 2016). In mammals, MSH4 and MSH5 are required for correct chromosome pairing during meiotic prophase, and both are associated with recombination intermediates destined to form both crossovers and non-crossovers (Kneitz *et al.*, 2000; Santucci-Darmanin *et al.*, 2000).

Both MutS and MutL homologs are conserved in *Microctonus* including MLH3, which appears to be absent in a large subset of the Hymenoptera (Figure 14), which may be due to annotation or assembly artifacts in the genomes and assemblies used in the analysis undertaken by Tvedte, Forbes and Logsdon Jr (2017), or that MLH3 is dispensable for sexual reproduction in these Hymenoptera taxa.

Cohesin complex; Structural maintenance of the chromosomes orthologs (SMC1, SMC2, SMC3 and SMC4), RAD21 and REC8

Sister chromatids that arise during S phase must be segregated to discreet poles before cell division (Nasmyth and Haering, 2009). Chromatid segregation requires the Cohesin complex, composed of SMC1, SMC2 and RAD21 in mitosis in yeast (Nasmyth and Haering, 2009). In meiosis, REC8 is the functional replacement for RAD21. During the removal of cohesion and segregation of chromatids to opposing poles of the dividing cell, the RAD21 or REC8 subunit is cleaved from the cohesion complex (Buonomo *et al.*, 2000; Uhlmann *et al.*, 2000).

The DNA repair protein RAD21 is broadly conserved in all the insect taxa assayed, including sexual and asexual *Microctonus* (Figure 14; Tvedte, Forbes, and Logsdon Jr 2017). The broad conservation of RAD21 in the insect taxa implies a conserved and essential function in insect mitosis (Figure 14). REC8 is conserved in the *Microctonus* taxa implying conserved function in meiosis (Figure 14). The absence of REC8 in numerous sexually reproducing Hymenoptera implies that it is dispensable in the meiosis of some Hymenoptera (Figure 14).

Double-stranded breaks and strand invasion: RAD51 orthologs (RAD51A, RAD51C, RAD51D, XRCC2 and XRCC3), SPO11, RAD54 and RAD54B

Double strand breaks are severe DNA legions that occur through DNA damaging agents and normal cellular processes, which can pose a significant threat to the integrity of a genome. Eukaryotic cells repair double-stranded breaks using the homologous recombinational repair mechanism (reviewed in Dudáš & Chovanec, 2004).

SPO11 is a transesterase that creates DNA double-strand breaks required for recombination and DNA replication (reviewed in Keeney, 2008). SPO11 is conserved in all *Microctonus* and all insect species analyzed (Tvedte, Forbes and Logsdon John M, 2017). The broad conservation of SPO11 in the *Microctonus* and other insects implies a conserved and essential function in these species.

HOP2 forms a complex with MND1 to ensure accurate and efficient homology searching during pachytene of meiotic prophase I (Tsubouchi and Roeder, 2002). Both are conserved in *Microctonus hyperodae* and both sexual and asexual *Microctonus aethiopoides* (Figure 15), as well as all Hymenoptera species, assessed (Tvedte, Forbes and Logsdon John M, 2017).

RAD51 is a recombinase that plays an integral part in homologous recombination (review: Krejci, Altmannova, Spirek, & Zhao, 2012). Homologous recombination is

required for meiotic chromosomal segregation, DNA replication and DNA damage repair (review: Li & Heyer, 2008). RAD51A and RAD54 are required for DNA pairing and strand invasion steps that allow a broken DNA molecule to access an undamaged DNA template (review: Krejci, Altmannova, Spirek, & Zhao, 2012). RAD51 is broadly conserved in Eukaryotes, including the *Microctonus* (Figure 14; Tvedte et al., 2017). DMC1 and RAD54B are required for homologous recombination in meiosis (Shinohara and Shinohara, 2004). DMC1 appears to be absent in *Drosophila melanogaster,* and the majority of the Hymenoptera species assayed, which implies that DMC1 is dispensable in Meiosis in these species (Tvedte et al. 2017).

The RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) and the heterodimers MND1-HOP2 are associated with the stabilization of nucleoprotein filaments in Eukaryotic cells (Masson *et al.*, 2001; Sigurdsson *et al.*, 2001). Knockout of any of the RAD51 paralogs in a chicken B-lymphocyte DT40 cell-line impairs homologous recombination and results in spontaneous chromosomal aberrations (Takata *et al.*, 2001). The RAD51B-RAD51C-RAD51D-XRCC2 and RAD51C-XRCC3 complexes maintain genomic integrity through recombinational repair and Holliday junction resolution in mammalian cells (Yokoyama *et al.*, 2004; Liu *et al.*, 2007). The broad conservation of the RAD51 paralogs suggests that they have a conserved and essential role in the Hymenoptera (Figure 14; Tvedte et al., 2017).

RECQ

The RECQ group of proteins are DNA helicases, which unwind double-stranded DNA required for DNA repair, recombination, and transcription. Some of the RECQ orthologs are required to protect the genome against deleterious mutations (review: Rezazadeh, 2012).

There is variation in the presence and absence of RECQ genes in the *Microctonus*. RECQ1 and RECQ4 were not identified in the peptide databases of all *Microctonus*. RECQ2

appears to be absent only in the *Microctonus aethiopoides* strains. A RECQ5 ortholog was identified in all the *Microctonus*. The RECQ homologs are also broadly conserved in the insects, including other species of Hymenoptera. The absence of some of the RECQ orthologs appears to be novel in the *Microctonus* (Figure 14).

RECQ1 interacts with members of the MMR pathway, which utilizes MSH2/6, MLH1/PMS2 during recombination in humans (Doherty *et al.*, 2005). The other proteins associated with the MMR pathway MSH2/MSH6, MLH1/PMS2 are conserved in the *Microctonus* (Figure 14). Conservation of MSH2, MSH6, MLH1 and PMS2 in the absence of RECQ1 suggests roles that are discrete from its association with RECQ1; alternatively, RECQ1 is dispensable in *Microctonus*. The absence of RECQ1 in the *Microctonus* may result in the low levels of recombination observed in *Microctonus hyperodae* (Iline and Phillips, 2004)

RECQ2 is a negative regulator of RAD51 nucleoprotein filament assembly and can prevent D-loop formation (Wu *et al.*, 2001; Bugreev *et al.*, 2007). RECQ2 mutants have suppressed non-crossover recombinants, indicating that this protein has a role in mediating recombination product formation in other Eukaryotes (S. Cerevisiae) (De Muyt *et al.*, 2012). The RECQ2 absence could alter the heterozygosity of the *Microctonus aethiopoides* strains, which will need to be evaluated by assessing the heterozygosity of the *Microctonus* genomes.

RECQ3 binds specifically to DNA secondary structures at the replication fork and Holliday junctions (Compton *et al.*, 2008). RECQ3 is essential in mitigating the effects of replication stress as RECQ3 mutant *Drosophila* embryos have a decreased hatching frequency, and larvae are more susceptible to replication fork stalling (Bolterstein *et al.*, 2014).

The colocalization and complex formation of RECQ4 with Rad51 after the induction of DNA double-strand breaks imply that it could have a role in DNA double-strand breakthrough homologous recombination (Petkovic *et al.*, 2005). RECQ4 also shows a

very high affinity for branched DNA substrates, with Holliday junctions having the highest affinity (Sedlackova *et al.*, 2015).

RECQ5 disrupts RAD51 filament formation to regulate recombination events and is broadly conserved in the included insect taxa and *Microctonus* (Hu et al., 2007; Figure 14).

Although RECQ genes are absent in some *Microctonus* taxa, there does not appear to be any apparent segregation of presence and absence with the thelytoky phenotype for either the asexual *Microctonus aethiopoides* ("Irish" strain) or *Microctonus hyperodae* (Figure 14).

Mitosis and Meiosis gene catalogue in the Microctonus Summary

Hymenoptera meiosis and mitosis genes are broadly conserved in both asexual and sexual *Microctonus*. The Cyclins, CYC A, CYC B, CYC B3, CYC D and CYC E are present in the *Microctonus* (Figure 14). The Cyclin-dependent kinases CDK1 and CDK2 are present in the *Microctonus* (Figure 14). The Polo-like kinases PLK1 and PLK4 are present in the *Microctonus* (Figure 14). The MutS orthologs, MSH2, MSH4, MSH5 and MSH6, are present in the *Microctonus* (Figure 14 & Figure 17). The MutL orthologs, MLH1, MLH3, PMS1 and PMS2, are present in the *Microctonus* (Figure 14). CDC20-like and Cort are present in the *Microctonus* (Figure 14). RAD21 and REC8 are present in the *Microctonus* (Figure 14). Structural maintenance of the chromosomes orthologs, SMC1, SMC2, SMC3 and SMC4, are present in the *Microctonus* (Figure 14). RAD51 orthologs, RAD51A, RAD51C, RAD51D, XRCC2 and XRCC3, are present in the *Microctonus* (Figure 14). RAD54 and RAD54B are present in the *Microctonus* (Figure 14). The only orthologs that are not conserved are RECQ1 and RECQ4 in all Microctonus and RECQ2 in all *Microctonus aethiopoides* strains (Figure 14).

Phylogenetics of Meiosis specific proteins

The proteins HOP2, MND1, MSH4 and MSH5 are all exclusively involved in meiosis in insects. Phylogenetic analysis was undertaken in order to assess homology and infer if these genes have retained their functions in meiosis in the *Microctonus* taxa.

HOP2 does not have long branch lengths suggestive of rapid evolution (Figure 15). The positioning of the *Microctonus* clade in the HOP2 phylogeny tree is consistent with the expected tree topology. As the *Microctonus* clade groups out with the HOP2 orthologs from the other wasp species *Microplitis demolitor,* it is more likely that HOP2 has a related function in this wasp (Figure 15).

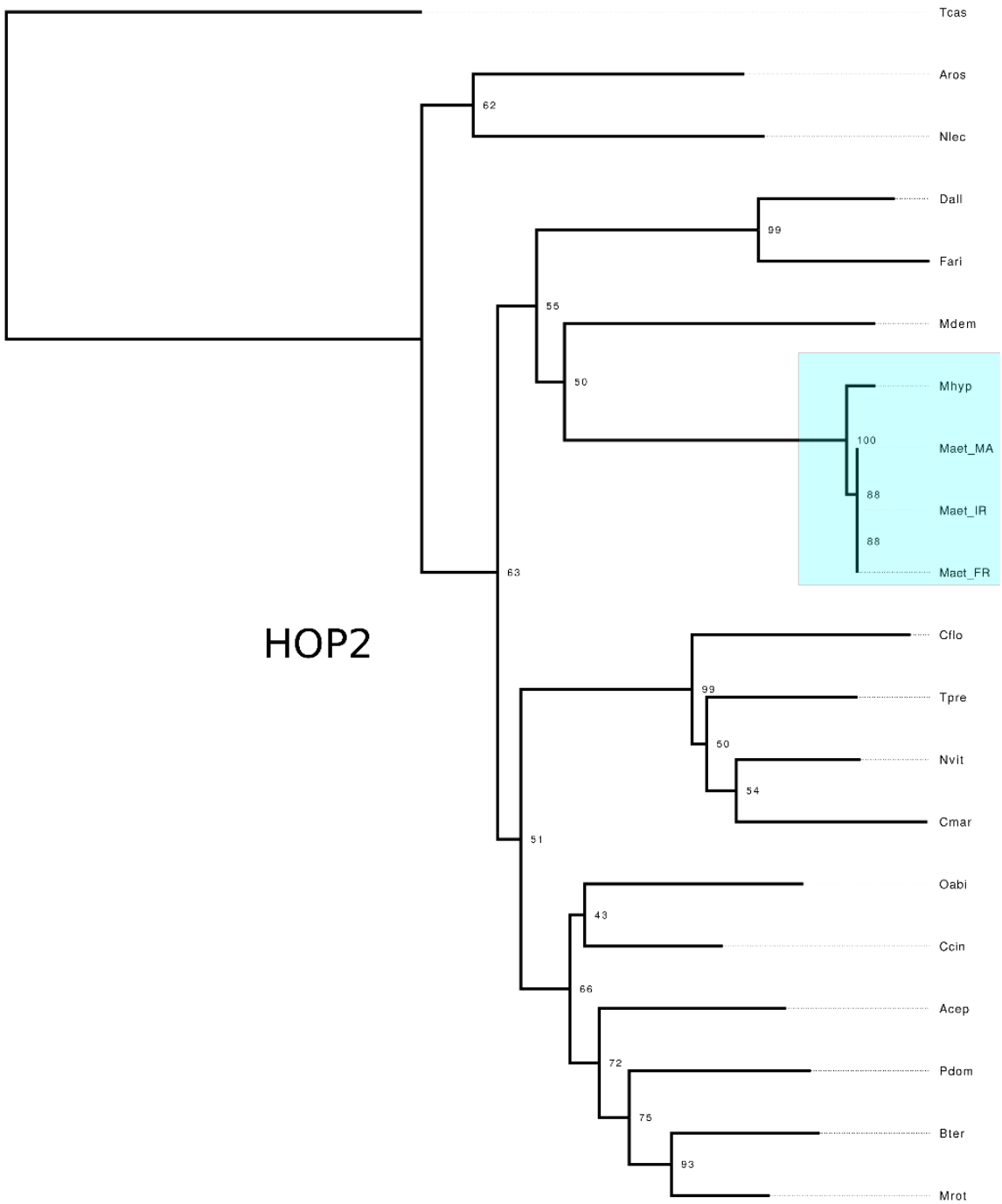

Figure 15) HOP2 neighbour-joining phylogenetic tree constructed from muscle alignments of HOP2 orthologs from Tvedte et al. (2017) and the hmmsearch hits for the included Microctonus taxa. Species name abbreviations are as follows: Tcas (Tribolium castaneum), Aros (Athalia rosae), Nlec (Neodiprion lecontei), Dall (Diachasma alloeum), Fari (Fopius arisanus), Mdem (Microplitis demolitor), Mhyp (Microctonus hyperodae), Maet_MA (Microctonus aethiopoides "Moroccan" strain), Maet_IR (Microctonus aethiopoides "Irish" strain), Maet_FR (Microctonus aethiopoides "French" strain), Cflo (Copidosoma floridanum), Tpre (Trichogramma pretiosum), Nvit (Nasonia vitripennis), Cmar (Ceratosolen solmsi marchali), Oabi (Orussus abietinus), Ccin (Cephus cinctus), Acep (Atta cephalotes), Pdom (Polistes dominula), Bter (Bombus terrestris), and Mrot (Megachile rotundata).

MND1 does not have the long branch lengths suggestive of rapid evolution (Figure 16). The positioning of the *Microctonus* clade in the MND1 phylogeny tree is consistent with expected species relatedness. As the *Microctonus* clade groups out with the MND1 orthologs from the other wasp species *Diachasma alloeum* and *Fopius arisanus,* it is more likely that MND1 has a related function in this wasp (Figure 16).

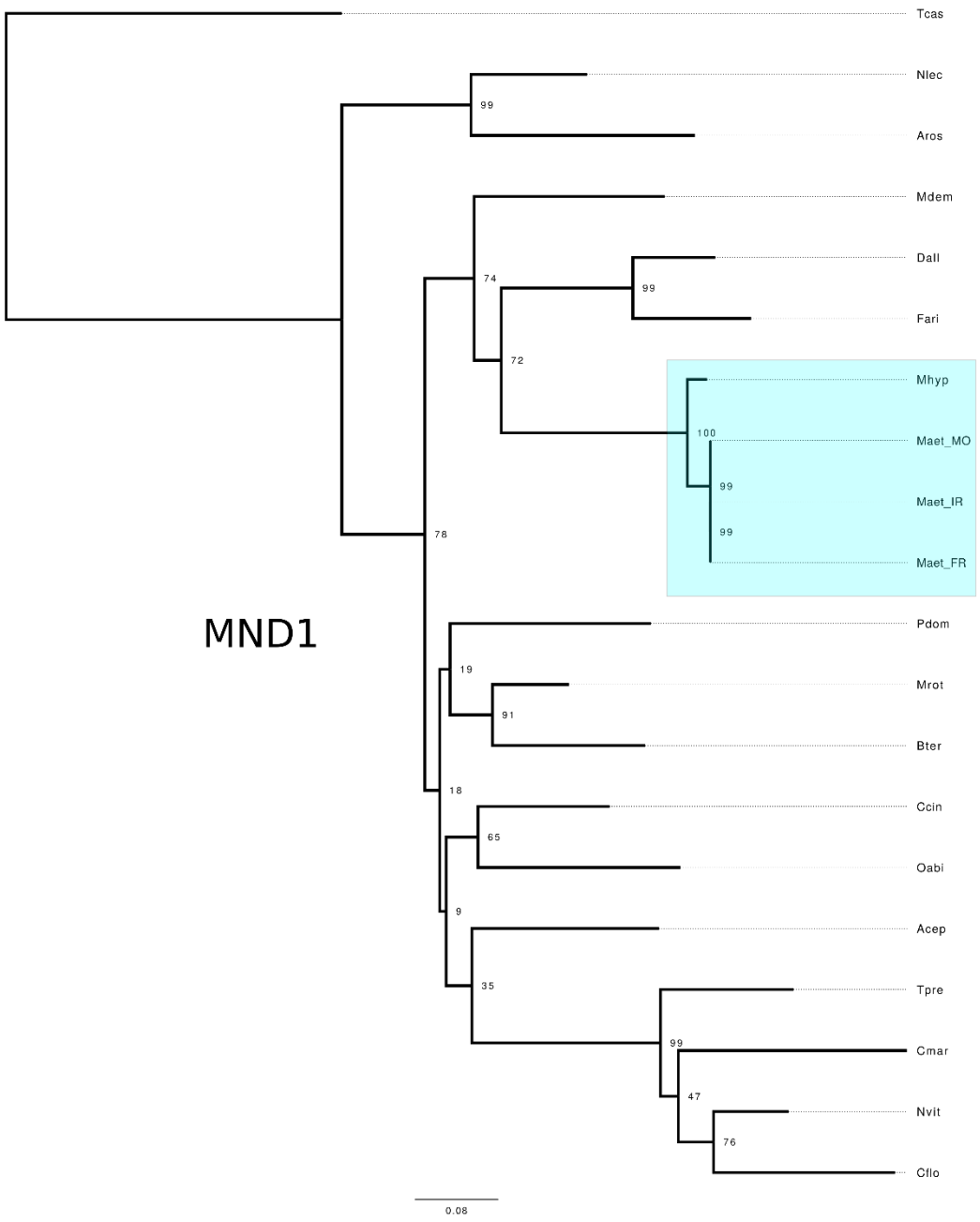

Figure 16) MND1 neighbour-joining phylogenetic tree constructed from muscle alignments of MND1 orthologs from Tvedte et al. (2017) and the hmmsearch hits for the included Microctonus taxa. Species name abbreviations are as follows: Tcas (Tribolium castaneum), Aros (Athalia rosae), Nlec (Neodiprion lecontei), Dall (Diachasma alloeum), Fari (Fopius arisanus), Mdem (Microplitis demolitor), Mhyp (Microctonus hyperodae), Maet_MA (Microctonus aethiopoides "Moroccan" strain), Maet_IR (Microctonus aethiopoides "Irish" strain), Maet_FR (Microctonus aethiopoides "French" strain), Cflo (Copidosoma floridanum), Tpre (Trichogramma pretiosum), Nvit (Nasonia vitripennis), Cmar (Ceratosolen solmsi marchali), Oabi (Orussus abietinus), Ccin (Cephus cinctus), Acep (Atta cephalotes), Pdom (Polistes dominula), Bter (Bombus terrestris), and Mrot (Megachile rotundata).

MSH orthologs are conserved in the *Microctonus* and group out in an evolutionarily consistent topology (Figure 17). The MSH4 and MSH5 clades are monophyletic and have high bootstrap supports of 87 for MSH5 and 94 for MSH4. The Neighbour-joining trees for both pairs of heterodimers, MSH4/MSH5 and HOP2/MND1, suggest homology to orthologs in related Hymenoptera taxa with no evidence of phylogenetic misplacement (Figure 17).

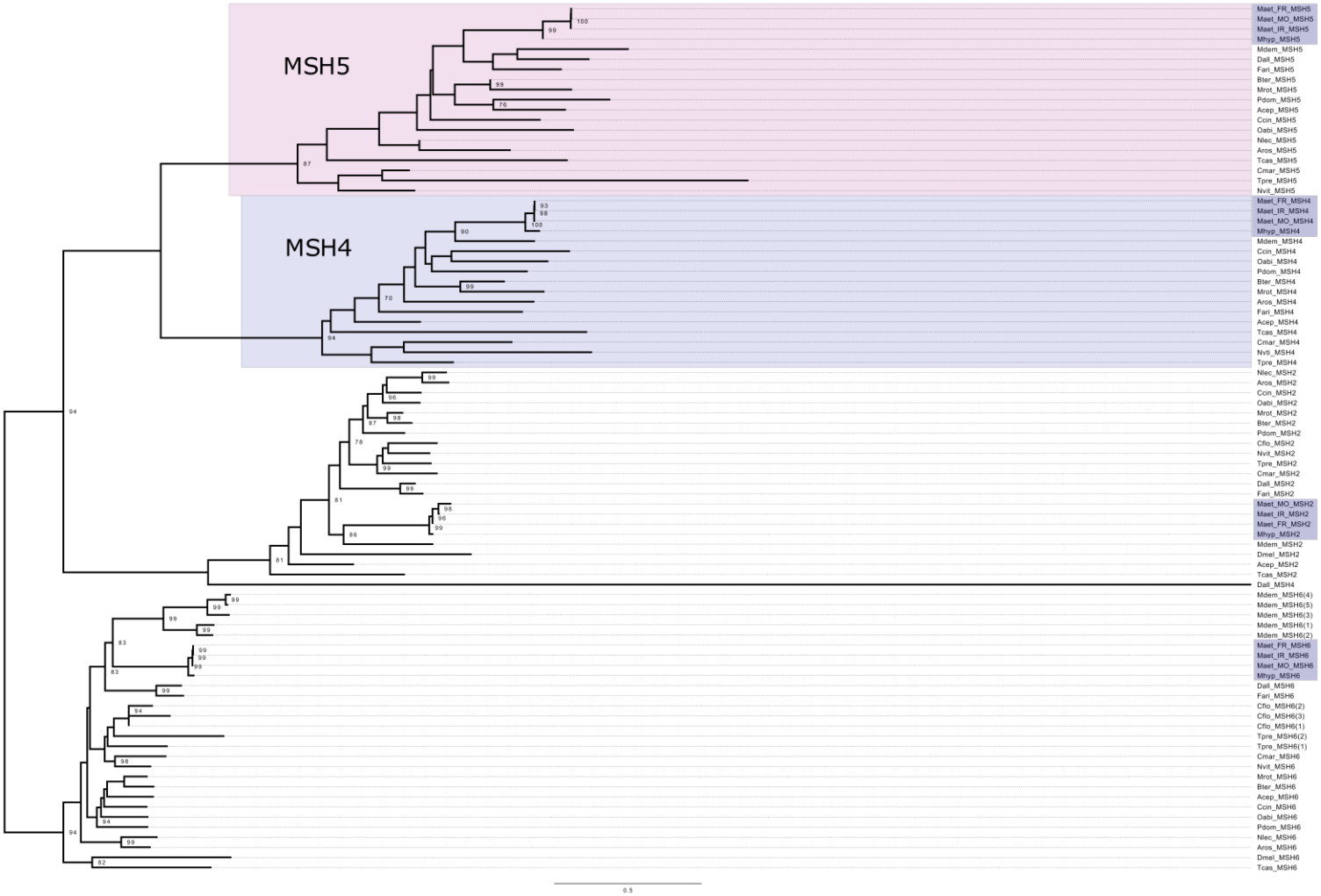

Figure 17) MSH neighbour-joining phylogenetic tree constructed from muscle alignments of MSH orthologs from Tvedte et al. (2017) and the hmmsearch hits for the included Microctonus taxa. Species name abbreviations are as follows: Tcas (Tribolium castaneum), Aros (Athalia rosae), Nlec (Neodiprion lecontei), Dall (Diachasma alloeum), Fari (Fopius arisanus), Mdem (Microplitis demolitor), Mhyp (Microctonus hyperodae), Maet_MA (Microctonus aethiopoides "Moroccan" strain), Maet_IR (Microctonus aethiopoides "Irish" strain), Maet_FR (Microctonus aethiopoides "French" strain), Cflo (Copidosoma floridanum), Tpre (Trichogramma pretiosum), Nvit (Nasonia vitripennis), Cmar (Ceratosolen solmsi marchali), Oabi (Orussus abietinus), Ccin (Cephus cinctus), Acep (Atta cephalotes), Pdom (Polistes dominula), Bter (Bombus terrestris), and Mrot (Megachile rotundata).

Expression of MND1 in the ovaries of Microctonus

As MND1 is required for meiosis in other arthropods its expression in the ovaries of *Microctonus aethiopoides* asexuals provide great insight into whether its asexual reproduction is due to a miosis or mitosis-based mechanism. Expression of MND1 in the ovaries of *Microctonus aethiopoides* asexuals would imply that meiosis is taking place and hence a meiosis-based mechanism of asexual reproduction is likely to be occurring. A pilot study found expression of MND1 in *Microctonus aethiopoides* "Irish" strain ovaries implying the function of these genes in meiosis in this strain (Figure 18). As *Microctonus ovaries* are tiny and delicate, treatment of tissue and staging for Hybridization Chain Reaction (HCR) was challenging. The region of convincing putative MND1 expression in red is highlighted with a dashed box (Figure 18). These experiments need to be repeated in *Microctonus hyperodae* and other *Microctonus aethiopoides* strains, but this falls outside of the scope of this thesis. Additional transcripts should also be analsyzed for expression in the ovaries of *Microctonus* taxa including HOP2, MSH4 and MSH5 as these proteins are also involved in meiosis in other arthropods. HOP2 forms a heterodimer with MND1 so the co-expression of these genes would provide strong evidence in support of *Microctonus aethiopoides* undertaking asexual reproduction based on a meiosis mechanism. HCR probes were designed for HOP2, MSH4 and MSH5 for both *Microctonus hyperodae* and the "Irish" strain *Microctonus aethiopoides*. Unfortunately, the global pandemic made it difficult to acquire samples for *Microctonus hyperodae* and "Irish" strain *Microctonus aethiopoides* HCR experimentation. The validation of HCR in the ovaries of *Microctonus aethiopoides* "Irish" strain makes it likely to work in *Microctonus hyperodae* and the other strains of *Microctonus aethiopoides*. HCR could be used to explore the expression of Meiosis genes in other *Microctonus* as well.

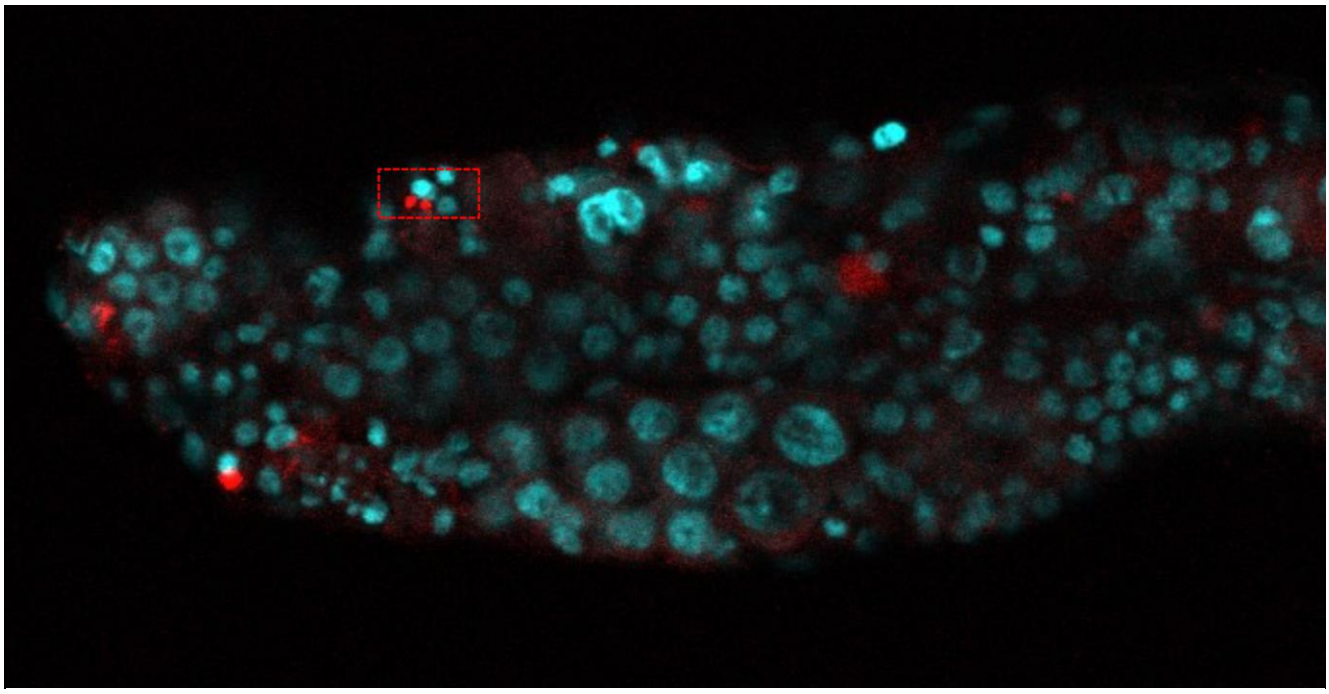

Figure 18) Hybridization Chain Reaction stain of the ovaries of asexual "Irish" Microctonus hyperodae. Dapi (cyan) MND1 (red). Expression was detected with a MND1 probe with the B3 binding protein and the complementary AF488 fluorescent marker. MND1 expression is the red pair of spots highlighted by the red dashed box.

Microctonus thelytoky

It is suggested by Iline & Phillips (2004) that gamete formation in *Microctonus hyperodae* may occur without meiosis. In their paper, vertical polyacrylamide electrophoresis was used to survey 16 enzymes and ten calcium-binding proteins in populations of *Microctonus hyperodae* from either side of the Andes and New Zealand. Two of the included loci showed maintenance of heterozygotes without the occurrence of homozygotes, suggesting that *Microctonus hyperodae* thelytoky is apomictic or an automictic mechanism that maintains heterozygosity. The broad conservation of the meiosis gene set in the *Microctonus* suggests that a mechanism involving meiosis is likely as an accumulation of random mutations in these genes would likely occur without any fitness cost resulting in loss of sequence similarity to orthologs of related Hymenoptera taxa, which does not appear to be the case as homology is phylogenetically supported for the heterodimers MSH4/MSH5 and HOP2/MND1 (Figure 15-17). Expression of MND1 in the ovaries of *Microctonus* was detected with HCR, suggesting that meiosis-based mechanisms of asexual reproduction are being utilized in *Microctonus aethiopoides* "Irish" strain (Figure 18).

Evidence suggests that thelytokous *Venturia canescens* wasp populations frequently arise from arrhenotokous populations (Schneider *et al.*, 2002). The limited evidence for *Wolbachia* infections (see "

Endosymbiont candidates in *the Microctonus*" page 31) in *Microctonus aethiopoides* and high frequency for reproductive mode transition in other wasps suggests a genetic proclivity to becoming asexual. Mitosis and Meiosis gene conservation patterns in the *Microctonus* imply meiosis-based asexual reproduction in the included asexual taxa.

The broad conservation of the MSH4 /MSH5 and HOP2/MND1 heterodimers in included taxa (With the exceptions of MSH4 and MSH5 absence in *C. floridanum* and HOP2/MND1 absence in *Drosophila melanogaster*) suggests that these genes could be good

candidates for detecting meiosis in the ovaries of insects (Figure 14). There are no current markers for meiosis in the Hymenoptera. A physical screen of meiosis using a technique like HCR makes for a valuable addition to a 'meiosis detection toolkit' as screening peptide predictions does not provide insight into the expression of these genes. Expression in the ovaries would provide more rigorous evidence for the occurrence of meiosis in asexually reproducing species.

Chapter 4 conclusions

Hymenoptera meiosis and mitosis genes are broadly conserved in both asexual and sexual *Microctonus*. The only genes not conserved are RECQ1 and RECQ4 in all *Microctonus* and RECQ2 in all *Microctonus aethiopoides* strains. Phylogenetic analysis of meiosis genes HOP2, MND1, MSH4 and MSH5 did not imply that these genes not pseudo genes and they are likely conserved in the *Microctonus*. HCR for the MND1 gene implies it is expressed in the ovaries of the "Irish" strain of *Microctonus aethiopoides* suggesting the conservation of meiosis in asexual *Microctonus*. These results provide great insight into the processes of mitosis and meiosis in the *Microctonus* and will likely aid in uncovering the mechanisms of asexual reproduction in asexual *Microctonus*.

Mitosis and Meiosis analysis Methods

Meiosis and mitosis catalogue, phylogenetics and HCR

Hymenoptera meiosis and mitosis genes were collected from the NCBI database corresponding to the accession numbers used by Tvedte et al. (2017; see supplementary for accessions).

Peptide sequences for mitosis and meiosis genes were collected using the accession numbers provided by Tvedte et al. (2017). Fasta files were made for the genes and ortholog groups the cyclins (CYC A, CYC B, CYC B3, CYC D and CYC E), the cyclin-dependent kinases (CDK1 and CDK2), the Polo-like kinases (PLK1 and PLK4), MutS homologs (MSH2, MSH4, MSH5 and MSH6), CDC20 homologs (CORT and CDC20-like), RAD21 and REC8, structural maintenance of the chromosomes orthologs (SMC1 and SMC3), RAD51 orthologs (RAD51A, RAD51C, RAD51D, XRCC2 and XRCC3), RAD54 and RAD54B, RECQ helicase orthologs (RECQ1, RECQ2, RECQ4 and RECQ5), MutL orthologs (MLH1, MLH3, PMS1 and PMS2), heterodimers HOP2 and MND1, SPO11, TIM2, DMC1, and Separase.

Collected protein sequences were aligned using muscle (3.8.13; Edgar, 2004) and trimmed using TrimAl (Version 1.2; Capella-Gutierrez, Silla-Martinez, & Gabaldon, 2009) using the "strictplus" trimming parameters. These trimmed alignments were used to construct gene-specific hidden Markov models (HMM) using hmmbuild from Hmmer (Version 3.2.1; Eddy, 2011). The aligned ortholog sequences and HMM profiles can be found in Supplementary files S3.11 and S3.12.

The HMMs were used to search the predicted protein databases of *Microctonus hyperodae and* the *Microctonus aethiopoides* strains. The hmmsearch used an E value cutoff of 1e-15 (Version 3.2.1; Eddy, 2011). The top hits were selected and peptides retrieved using esl-sfetch from Hmmer (Version 3.2.1; Eddy, 2011).

The gene hits from *Microctonus* were concatenated with their Hymenoptera orthologs, duplicates were removed using a custom bash script. The peptide sequences for each gene and ortholog groups were aligned using muscle (3.8.13; Edgar, 2004) and trimmed using TrimAL with the "strictplus" parameters (Version 1.2; Capella-Gutierrez, Silla-Martinez, & Gabaldon, 2009).

Phylogenetic trees were constructed for both individual genes and the ortholog groups. Neighbour-joining trees were constructed using rapidnj (2.3.2; Simonsen, Mailund, & Pedersen, n.d.) using a bootstrap parameter of 1000 replicates for genes and 1000000 for ortholog groups. Different bootstraps were used on gene trees to ortholog groups as ortholog groups are harder to resolve due to having more sequences and likely more sequence variation. See supplementary S3.1-3.8 for the scripts used for meiosis and mitosis ortholog discovery, alignment, and phylogenetic reconstruction.

The phylogenetic trees were visually inspected to confirm the identity of gene hits from the hmmsearch in Figtree (1.4.4). Gene presence and absence was catalogued. The gene presence-absence table was used to construct a presence and absence heatmap in R (Team, 2013).

HCR in *Microctonus aethiopoides* "Irish" strain ovaries with MND1-AF488

A transcriptome was constructed for *Microctonus aethiopoides* and *Microctonus hyperodae* using RNA sequence data and Trinotate, providing the transcript sequence required to design hybridization chain reaction probes for MND1(v3.1.1; Bryant et al., 2017). Probes were designed from transcripts for MND1 with an adapter compatible with an AF488 fluorophore by Molecular Technologies (v3.1.1; Bryant et al., 2017).

Microctonus aethiopoides "Irish" strain wasps were reared from Clover root Weevil collected from Timaru, New Zealand. *Microctonus aethiopoides* is the only *Microctonus* wasp to parasitize Clover root Weevil in New Zealand. Ovaries were dissected out of the abdomen of three-day-old *Microctonus aethiopoides* "Irish" strain wasps into PBS. The ovaries were subsequently fixed in a 1:1 mix of 4% formaldehyde: heptane at 4 % in PBS for 5 minutes. The bottom aqueous layer was then removed and replaced with MeOH, and incubated for 5 minutes. Once the tissue had fallen to the bottom of the tube, the upper heptane layer phase was removed. Samples were washed three times with MeOH and incubated for 5 minutes. Samples were stored at -20°C in PBS.

The HCR Insitu protocols were used from the following reference ("https://files.molecularinstruments.com/MI-Protocol-HCRv3-GenericSolution-Rev6.pdf"). As the dissected tissue was delicate, care was taken damage it with the pipette tip or through rough handling. Ovaries were also treated with Dapi at a concentration of 1:1000. Ovaries were mounted to slides in glycerol and covered with coverslips. The embryos were imaged using a confocal microscope imaging DAPI and AF488 channels to detect MND1 HCR probes. See supplementary S3.9 and S3.10 for transcripts used by Molecular Technologies to construct HCR probes.

HCR probes were also designed for HOP2, MSH4 and MSH5 but due to issues with acquiring tissue for experimentation, due to the current global pandemic, these experiments were not undertaken.

Chapter 5; Olfactory receptor evolution in the *Microctonus* reveal host identification candidates

The insect chemoreceptor superfamily was first identified in Drosophila melanogaster and consisted of odorant and gustatory receptors (Clyne et al., 1999). The gustatory receptors date back to the origin of the arthropods (Robertson et al. 2003; Vieira and Rozas 2011). Insect olfactory receptors subsequently evolved from the gustatory receptors (Robertson et al. 2003; Vieira and Rozas 2011). Insect olfactory and gustatory receptors have seven transmembrane domains and function as ion channels different from the olfactory receptors found in vertebrates (Robertson, 2019).

Insect olfactory receptors are heterotetramers composed of two copies of Orco and two copies of an olfactory tuning receptor that bind to a ligand. The olfactory receptor complex forms a ligand-gated ion channel (Benton et al., 2006; Sato et al., 2008; Wicher et al., 2008; Butterwick et al., 2018). Typically there is only one copy of Orco in an insects' olfactory receptor repertoire, which is consistent with what is observed in the Microctonus and *Apis mellifera* and *Nasonia vitripennis* (Zhou et al., 2012; McKenzie and Kronauer, 2018; Opachaloemphan et al., 2018). The number of olfactory receptors, on the other hand, vary significantly between insect species. For example, damselflies only have three, and some ants have as many as 500 olfactory receptors (Zhou et al., 2012; McKenzie and Kronauer, 2018; Opachaloemphan et al., 2018). The olfactory receptors in the Hymenoptera are highly divergent, with the 9-exon gene subfamily being the most extensively amplified (Zhou et al., 2012, 2015; McKenzie et al., 2016; McKenzie and Kronauer, 2018). Olfactory receptors are primarily expressed in olfactory receptor neurons situated within antennae and maxillary palps, where volatile chemicals are perceived (Clyne et al., 1999; Fox et al., 2001).

Olfactory receptors in the honey bee *Apis mellifera* and the Jeweled wasp *Nasonia vitripennis* were previously identified, annotated and catalogued (Robertson and Wanner, 2006; Robertson, Gadau and Wanner, 2010). *Apis mellifera* and *Nasonia vitripennis* are

broadly considered hymenopteran model organisms and should be valuable comparisons for my *Microctonus* data due to their relatedness. Phylogenetic analysis of olfactory receptors in *Apis mellifera* and *Nasonia vitripennis* established clades of olfactory receptors including the 9-exon, L and H clades (Table 5; Robertson and Wanner, 2006; Robertson, Gadau and Wanner, 2010).

Olfactory cues can be used for mate identification, as is seen in the males of *Nasonia vitripennis,* where they respond to the cuticular hydrocarbon cues from females by courting them (Steiner, Hermann and Ruther, 2006). Male *Nasonia vitripennis* also use chemical cues which are attractive to only virgin females (Ruther *et al.*, 2007; Ruther, Steiner and Garbe, 2008; Steiner and Ruther, 2009). Female wasps examine their fly host pupae using their antenna before oviposition, suggesting that olfaction is used to identify a suitable host (Edwards, 1954). Inspection of Argentine stem weevils by *Microctonus hyperodae* preceding oviposition using their antenna suggests olfaction is also used for host identification in this species (McNeill, Goldson and Baird, 1996). We would expect that olfactory receptors are also required to identify suitable hosts and mates in the *Microctonus*. The host range of *Microctonus aethiopoides* varies depending on the strain, with the host range varying significantly more than is seen in *Microctonus hyperodae* (Barratt *et al.*, 1997). *Microctonus hyperodae* has a specific host range which is part of the reason it was selected as a biocontrol agent for the Argentine Stem Weevil in the first place (Goldson *et al.*, 1992; Barratt *et al.*, 1997).

This section aims to identify olfactory receptors in the *Microctonus aethiopoides* "Irish", "French", and "Moroccan" strains as well as *Microctonus hyperodae*. There are considerable differences in the biology of *Microctonus aethiopoides* and *Microctonus hyperodae* and also within the *Microctonus aethiopoides*. There are both sexual and asexual taxa in this selection of *Microctonus,* allowing for comparing the olfactory receptor complements of these two reproductive strategies. There is also variation in the host range of *Microctonus* taxa (see Introductory chapter page 6 for further detail). This research aims to identify receptors that could be of interest for further study into these fundamental biological processes—especially considering the biocontrol implications of

having a more in-depth understanding of these critical processes and establishing targets that could improve biocontrol.

9-exon receptors are used to detect cuticular hydrocarbon pheromones, suppressing worker ovary development in the ant *Harpegnathos saltator* (Pask *et al.*, 2017). We would expect to see fewer 9-exon receptors in asexual *Microctonus* as mate identification is no longer required for reproduction. These receptors may also vary with roles in host identification (if cuticular hydrocarbons are used). Loss or functionally significant mutations in olfactory receptors of asexual *Microctonus* may have implications for identifying mates as these are more likely due to a lack of selection in these species. Variation of olfactory receptor complements in the *Microctonus* strains and species may be related to their differences in host preference and have implications for their effectiveness in host identification.

Some plants release plant volatiles in response to herbivory. These plant volatiles are sensed by wasp species that use these chemical signals to target hosts (Wei et al., 2007). Some of the chemical components in plant volatiles are thought to be perceived by members of the H clade of olfactory receptors, like the detection of linalool by AmOr151 and AmOr152 (Claudianos *et al.*, 2014). Previous experiments with *Microctonus hyperodae* with a novel carrot weevil, *Listronotus oregonensis*, host suggest that they respond to the weevil but not plant volatiles in a y shaped olfactometer experiment (Boivin, 1999). It would be helpful to establish whether *Microctonus* respond to plant volatiles for host identification or not.

Olfactory receptors of interest include the 9-exon clade involved in detecting cuticular hydrocarbons (Pask *et al.*, 2017; Slone *et al.*, 2017), The H clade, which has been inferred to have functions in detecting plant volatiles (Claudianos *et al.*, 2014) and the L clade contains a receptor activated by a component of QMP one of the few hymenopteran olfactory receptors with a defined role is of interest (Wanner *et al.*, 2007). Olfactory receptors, which have homology to receptors that vary depending on sex, are also of interest as these may be required for sex-specific behaviours (Wanner *et al.*, 2007).

Olfactory receptors are known to undergo a birth and death evolution model, which results in numerous species-specific expansions and contractions. The olfactory receptor complements in a given Hymenoptera is thus highly variable (Nei and Rooney, 2005; Zhou *et al.*, 2012, 2015; Engsontia *et al.*, 2015). We also expect to see high levels of variability in the olfactory receptor complement of *Microctonus hyperodae* compared to *Apis mellifera* and *Nasonia vitripennis* receptor compliments. High levels of variability between olfactory receptors are observed in the genus *Apis* when comparing the olfactory receptor complements of the honey bees *Apis mellifera* and *Apis florea* (Karpe *et al.*, 2016).

Quantifying the number of olfactory receptors present in the *Microctonus aethiopoides* strains and *Microctonus hyperodae* may provide insight into the complexity of chemical cues utilized by a given species. A higher number or more diverse range of olfactory receptors may be associated with the requirement to differentiate between a more complex set of chemical cues. The number and variety of chemical cues may vary regarding whether a *Microctonus* taxon is required to identify a mate like the sexual "French" and "Moroccan" strains of *Microctonus aethiopoides* (asexual strains like the "Irish" strain of *Microctonus aethiopoides* and *Microctonus hyperodae* do not need to identify mates). *Microctonus* taxa have different host ranges, so they likely need to differentiate between host-specific chemical cues (possibly in the form of cuticular hydrocarbons) to reproduce successfully. Some *Microctonus* taxa avoid superparasitism chemical signals may be used to differentiate between parasitized and unparasitized hosts (Barker, 2013).

Olfactory receptors in Microctonus

According to my genome annotations, *Microctonus aethiopoides* has 128, 115 and 106 olfactory receptors in the "Moroccan", "French", and "Irish" strains respectively (Table 5). *Microctonus hyperodae* has only 100 olfactory receptors, fewer than any of the *Microctonus aethiopoides* strains (Table 5). Both asexual *Microctonus* (the "Irish" strain

of *Microctonus aethiopoides* and *Microctonus hyperodae*) have fewer olfactory receptors than their sexually reproducing counterparts (Table 5). Fewer olfactory receptors may result from the asexual *Microctonus* being more host-range specialized, requiring a smaller range of olfactory cues to identify a viable host and successfully reproduce. Alternatively, the smaller number of olfactory receptors in the asexual *Microctonus* could result from the loss of olfactory receptors previously required for mate identification.

The *Microctonus aethiopoides* have 12, 15 and 12 gustatory receptors in the "Moroccan", "French", and "Irish" strains respectively. *Microctonus* hyperodae has 12 gustatory receptors. The number of gustatory receptors in the *Microctonus* do not vary significantly, with the "French" strain having two more than the other taxa, which implies that the "French" strain of *Microctonus aethiopoides* has more decerning taste than *Microctonus* from other regions (Table 5).

Identification, annotation, alignment, trimming and subsequent phylogenetic reconstruction using the maximal-likelihood phylogenetic tree construction software raxml-ng resulted in a phylogenetic tree in which *Microctonus* hyperodae olfactory receptor group out with support along with receptors from those previously characterized in *Apis mellifera* and *Nasonia vitripennis*. Clades were identified using homology to olfactory receptors in the clades previously described (Figure 19; Zhou *et al.*, 2012, 2015; Karpe *et al.*, 2016).

Microctonus likely uses multiple different signals to identify hosts or mates, and there are likely multiple olfactory receptors that collectively respond to a chemical cue (not one olfactory receptor one function). The L, H and 9-exon clades are of particular interest in Hymenoptera due to their critical biological roles. Further analysis of receptors in this group from *Microctonus* could provide insights into their potential roles in food identification, mate identification, host localization, host identification and super parasitism avoidance. The use of the 9-exon clade in the perception of cuticular hydrocarbons has been rapidly evolving and utilized as pheromone and reproductive control signals in other species of Hymenoptera. Suppose there is a conserved set of olfactory receptors with conserved roles, then homology may provide insight into

function. Olfactory receptor groups which warranted closer inspection are the X / L clade, the XI / exon-9 clade and the XVIII / H clade (Figure 19).

Table 5) Clade groupings of full length Microctonus olfactory receptors as classified by InsectOR . Adapted from Snehal D. Karpe et al. (2016)***, which obtained subfamily notation from*** Zhou et al. (2015, 2012b)**.**

Clade name	*Hymenopteran subfamily*	*Apis orthologs belonging to the clade*	*Microctonus hyperodae*	*Microctonus aethiopoides "Irish"*	*Microctonus aethiopoides "French"*	*Microctonus aethiopoides "Moroccan"*
Orco	Orco	AmOr2	1	1	1	1
I	A	AmOr168–170	0	2	0	0
II	I	AmOr161	1	1	1	1
III	V	AmOr163–167 and AmOr118	3	3	3	4
IV	U	AmOr121	0	0	0	0
V	Q	AmOr160	4	5	5	6
VI	T	AmOr114–115	1	1	1	1
VII	M	AmOr62	0	1	1	1
VIII	P	AmOr63–67	7	7	10	11
IX	K	AmOr1 and AmOr3	1	0	1	1
X	L-putative pheromone receptors	AmOr4–17, AmOr26–28, AmOr31–34, AmOr36–50, AmOr18–25, AmOr51–61, AmOr29, AmOr30 and AmOr35	7	5	11	12
XI	9 exon-putative CHC receptors	AmOr97–113, AmOr122–138, AmOr140, AmOr159, AmOr162 and AmOr172–177.	11	14	20	21
XII	F	AmOr171	10(1)	10	14	13
XIII	B	AmOr119	3	3	3	3
XIV	C	AmOr116	2	2	2	2
XV	E	AmOr68–73	23(1)	22(1)	20(1)	25(1)
XVI	Z	AmOr141	0	0	0	0
XVII	G	AmOr143–145	1	2	2	2
XVIII	H—putative floral scent receptors	AmOr142 and AmOr146–158.	4	6	3	4
XIX	W	AmOr120	0	0	0	0
XX	Orphan	AmOr117	0	0	0	0
XXI	J—bee expanded clade	AmOr74–96	3(1)	4(1)	1	2(1)
Other			15	15	15	16
Total Olfactory		174	100	106	115	128
Gustatory Receptors		10	12	12	15	12
Total		*184*	*112*	*118*	*130*	*140*

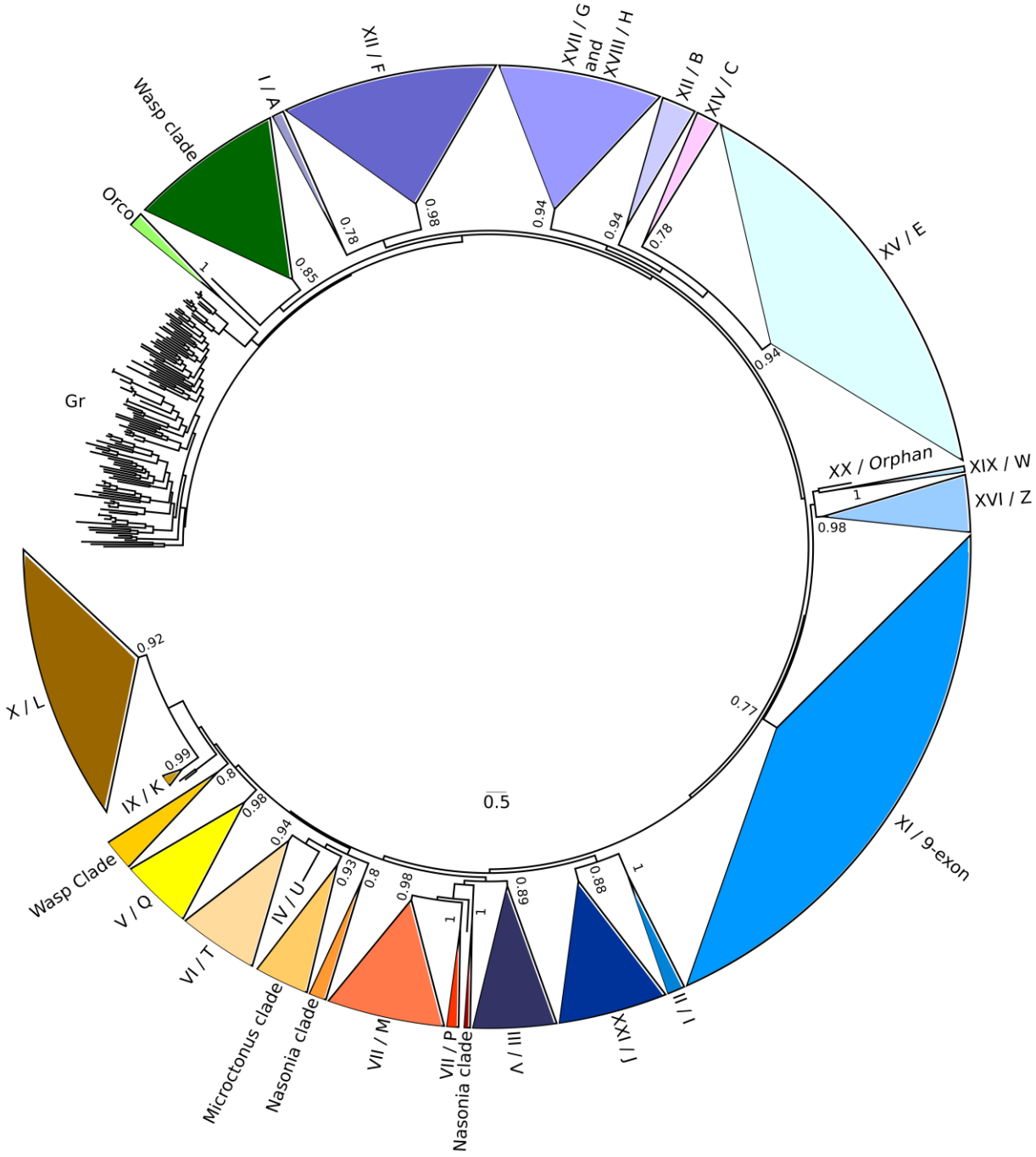

Figure 19) Maximum-likelihood phylogenetic tree of olfactory and gustatory receptors from Apis mellifera, Nasonia vitripennis, Microctonus aethiopoides ("French", "Moroccan" and "Irish" strains) and Microctonus hyperodae. Microctonus olfactory receptors which grouped out strongly with Apis mellifera and Nasonia vitripennis clades were labeled with the clade and Hymenoptera sub species names from Snehal D. Karpe et al. (2016). ***TBE bootstrap support is shown on nodes for each clade. Gr (Gustatory receptor) clade. Nodes with a TBE bootstrap of over 0.7 are consider to be supported. The number of receptors in each clade from Microctonus can be found in Table 6 on the previous page.***

H and G clade

The H olfactory receptor clade consists of three, four and six olfactory receptors belonging to the "Moroccan", "French", and "Irish" strains of *Microctonus aethiopoides* respectively (Figure 20). The H clade contains four olfactory receptors from *Microctonus hyperodae*. The H and G clades group out with orthologs from *Apis mellifera* with strong bootstrap support (0.94) (Figure 19 and 20).

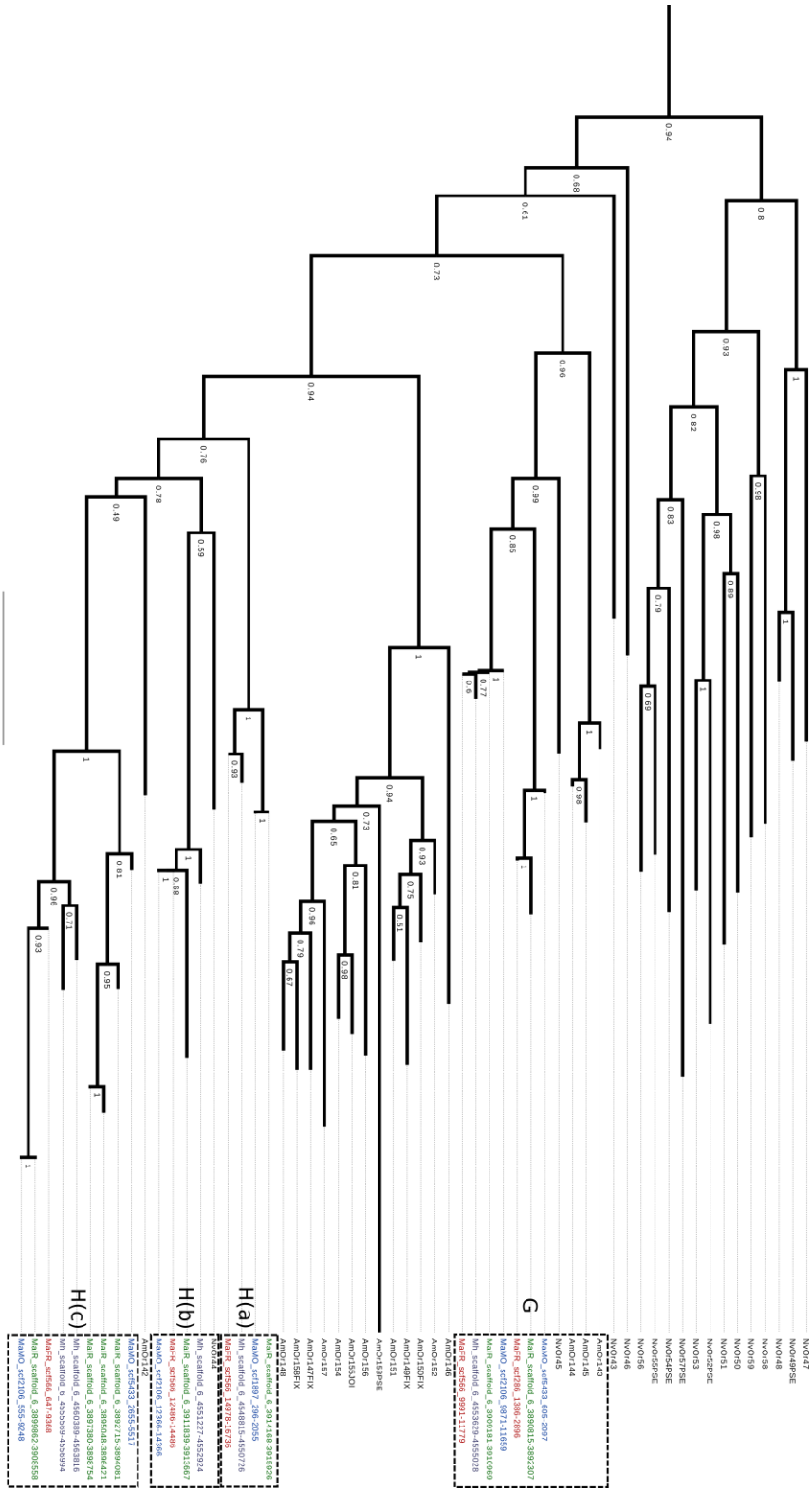

Figure 20) H and G clade subtree Maximum-likelihood phylogenetic tree of olfactory and gustatory receptors from Apis mellifera, Nasonia vitripennis, Microctonus aethiopoides ("French", "Moroccan" and "Irish" strains) and Microctonus hyperodae.

The H subfamily of olfactory receptors includes putative floral scent receptors in *Apis mellifera*. *Microctonus* homologs are also likely to be involved in floral scent detection. Floral scents may be used to locate hosts as volatiles are probably released due to the herbivory of the Weevils. Carbohydrates are the primary energy source for adult parasitoid wasps such as nectar (Jervis et al. 1993). *Microctonus* could also use floral odor molecules to identify sources of nectar. Considering the rapid evolution of most olfactory receptors, if olfactory volatiles were used as a signal from plant volatiles to identify hosts, these would become more specialized to identify the favorite food of the Weevil species of interest. There are three groups of H clade olfactory receptors in the *Microctonus*.

The sister clade of clade H, Clade G, groups out with the AmOr143-145 and NvOr45 and has a high bootstrap support of 0.96 (Figure 20). Clade G consists of two receptor copies for the *Microctonus aethiopoides* and one in *Microctonus hyperodae,* implying either a duplication event in *Microctonus aethiopoides* or species-specific loss in *Microctonus hyperodae* (Figure 20). The duplication event is supported by the proximity of the "Irish" strain homologs on Scaffold 6 (3909181-3910969 and 3890815-3892307) (Figure 20). The "Moroccan" and "French" strain orthologs are not found on the same scaffold, but this is likely due to a less contiguous genome assembly (Figure 20). The first G group consists of MaMO_scf2106_9871-11659, MaIR_scaffold_6_3909181-3910969, MaFR_scf566_9991-11779 and Mh_scaffold_6_4553629-4555028. The second G group consists of MaMO_scf5433_605-2097, MaIR_scaffold_6_3890815-3892307 and MaFR_scf286_1386-2896, has longer branch lengths than the first, implying an increased rate of sequence divergence, possibly due to the easing of selection constraints resulting from a duplication event (Figure 20). Clade G receptors have no known function in other insect species; however, the conservation of this group in *Apis mellifera, Nasonia vitripennis* and *Microctonus* species implies that this group has a conserved function in the Hymenoptera (Figure 20).

Clade H(a) consists of one ortholog from each of the *Microctonus* taxa, and there is no obvious homology to orthologs from *Nasonia* or *Apis*, making the potential role of this clade difficult to characterize as it likely has a function specific to *Microctonus* species

(Figure 20). Clade H(b) consists of one ortholog from each of the *Microctonus* taxa with perhaps some homology to NvOr44, but this is not strongly supported as it only has a bootstrap of 0.59 (Figure 20). Clade H(c) consists of two "Moroccan" strain olfactory orthologs, two *Microctonus hyperodae* olfactory receptor orthologs, one "French" strain olfactory receptor ortholog and four orthologs from the "Irish" strain (Figure 20). All "Irish" strain Clade H4 orthologs are on scaffold 6 of the genome and occur in tandem. The long branches of the "Irish" strain orthologs imply rapid evolution compared to other *Microctonus*. Hence, this clade may be important for the survival and reproduction of the "Irish" strain of *Microctonus aethiopoides;* it may even be involved in detecting the plant-specific volatiles produced by the herbivory of its Clover Root Weevil host or a viable source of nectar (Figure 20). There is bootstrap support of 0.94 for a clade containing H(a), H(b) and H(c) along with AmOr146, AmOr152, AmOr150FIX, AmOr149FIX, AmOr151, AmOr153PSE, AmOr156, AmOr155JOI, AmOr154, AmOr157, AmOr147FIX, AmOr158FIX, AmOr148 and AmOr142 (Figure 20). AmOr151 and AmOr152 respond to floral scents. AmOr151 responds to linalool and nerol (AmOr151), whereas AmOr152 responds to neral, myrcene and 6-methyl-5-heptene-2-one (Claudianos *et al.*, 2014).

Both *Apis mellifera* and *Nasonia vitripennis* have 17 H clade olfactory orthologs, more than double the number of H clade olfactory receptors identified in the *Microctonus* taxa (Table 5). Along with the response to floral odors, there is an increased expression of these receptors in worker honey bees over males. Zhou et al. (2015) inferred that the H clade is likely to be involved in detecting floral odors in bees. Y shaped olfactormeter experiments could see if any H clade orthologs from *Microctonus* respond to plant volatiles as these may be used as a cue to identify hosts as is seen in other wasps (Wei *et al.*, 2007). If this group is involved in detecting plant volatiles, then the "French" and "Moroccan" strains likely need to differentiate between more complex chemical profiles for detecting hosts.

L clade

The L subfamily of olfactory receptors includes the putative pheromone receptors in *Apis mellifera* (Zhou *et al.*, 2012, 2015). *Microctonus* olfactory receptors in the L clade could also detect pheromones. Pheromone detection is vital for mate identification and probably helpful in differentiating between related strains and species by other Hymenoptera species and probably the *Microctonus*.

The L clade is well supported by a bootstrap of 0.92 (Figure 19). The "French", "Moroccan", and "Irish" strains of *Microctonus aethiopoides* have eleven, twelve and five L clade olfactory receptors respectively (Table 5; Figure 21). *Microctonus hyperodae* has seven olfactory receptors, which group out with the L clade.

Figure 21) L clade subtree Maximum-likelihood phylogenetic tree of olfactory and gustatory receptors from Apis mellifera, Nasonia vitripennis, Microctonus aethiopoides ("French", "Moroccan" and "Irish" strains) and Microctonus hyperodae.

The "French" and "Moroccan" strains have more olfactory receptors in the L clade (Table 5; Figure 21). L clade receptors are therefore more numerous in sexually reproducing *Microctonus*, implying that these olfactory receptors may be involved in mate or host identification. None of the identified *Microctonus* olfactory receptors grouped out with the "group a" orthologs from *Apis mellifera*, which is the only clade that contains an olfactory receptor with a putative function (Figure 21; Wanner et al., 2007).

There are two *Microctonus* ortholog groupings in the olfactory receptor L clade. The *Microctonus* clade, which groups out with the "rest" of the L receptors from *Apis mellifera*, is strongly supported by a bootstrap value of 0.98 (Figure 21). Four *Microctonus hyperodae* olfactory receptors (Mh_003897-T1, Mh_003896-T1, Mh_003898-T1 and Mh_003895-T1) group out with a subset of the *Apis mellifera* "group rest" (Figure 21). The "Moroccan" strain of *Microctonus aethiopoides* has two receptors in "group rest" (MaMO_scf1644_8334-15628 and MaMO_scf1644_16480-17889) which occur in tandem on the same genome scaffold "scf1644". The French strain of *Microctonus aethiopoides* has three olfactory receptors in "group rest" (MaFR_scf174_13641-20998, MaFR_scf174_21850-23451 and MaFR_scf174_24303-25939) situated in tandem on the same scaffold "scf174" (Figure 21). The "Irish" strain of *Microctonus aethiopoides* has three receptors in the "group rest" (MaIR_scaffold_3_17679167-17686464, MaIR_scaffold_3_17687316-17688917 and MaIR_scaffold_3_17689769-17691405) which are all positioned in tandem on "scaffold 3" (Figure 21).

The rest of the *Microctonus* L clade receptors are located in a clade with the *Nasonia vitripennis* olfactory receptor NvOr5 (Figure 21). This group seems to have expanded in the *Microctonus*. The *Microctonus aethiopoides* expansion clade has ten, eight and two olfactory receptors from the "Moroccan", "French", and "Irish" strains respectively (Figure 21). *Microctonus hyperodae* has three olfactory receptors in the *Microctonus* expansion clade (Figure 21). This group seems to have undergone radiation after the divergence of the "French" and "Moroccan" strains from the "Irish" strain (Figure 21). Longer branch lengths in this group also imply a more rapid evolution of this clade (Figure 21). Rapid evolution likely resulted from the increased lability due to the

duplication of the olfactory receptors, which then gain a new function or undergo pseudogenization allowing for a competitive advantage. The exceptionally long branch observed for MaFR_010420-T1 is likely to be an annotation artifact (Figure 21).

Both *Microctonus hyperodae* orthologs are positioned on scaffold 2 in tandem. Seven of the ten orthologs from the "Moroccan" strain of aethiopoides were annotated by InsectOR (Figure 21). Two of the InsectOR annotations are situated on scaffold scf1644 (in tandem) and four on scaffold scf255 (in tandem) (Figure 21). Six of the eight orthologs were annotated by InsectOR (Figure 21). Five of these olfactory receptors are situated in tandem with the scaffold "scf174" and one on "scf11" (Figure 21). Both "Irish" strain orthologs are in tandem on scaffold 3 (Figure 21).

The decrease in the number of olfactory receptors possibly involved in pheromone detection in asexual *Microctonus* is consistent with the loss of selective pressure due to no longer needing to identify mates. Further experimentation, like looking at receptor response using an electroantennogram, on L clade receptors to explore its effect on *Microctonus* behavior is of particular importance. If *Microctonus hyperodae* has lost the ability to identify mates producing males through genetic manipulation will not be adequate to instigate reproduction and successful biocontrol.

9-exon family

The 9-exon olfactory receptor family is thought to detect cuticular hydrocarbons in insects (Zhou et al., 2012). Cuticular hydrocarbons are used for various things, including host identification in other Hymenoptera (Zhou et al., 2012). Cuticular hydrocarbons may also be used for host identification in *Microctonus* or as pheromones for mate identification/location (Zhou et al., 2012).

Both sexual strains of *Microctonus aethiopoides* ("French" twenty and "Moroccan" twenty-one) have significantly more annotated orthologs in the 9-exon clade. *Microctonus*

hyperodae and the "Irish" strain of *Microctonus aethiopoides* only have eleven and fourteen 9-exon olfactory receptors. It is not clear as to whether this pattern is due to individual losses in *Microctonus hyperodae* and the "Irish" strain of *Microctonus aethiopoides* after they diverge from the "French" and "Moroccan" strains or if there was an expansion in the sexual "French" and "Moroccan" strains after they diverged from the "Irish" strain (Table 5; Figure 22). 9-exon olfactory receptors absent in both asexual species make for good mate identification candidates.

Figure 22) 9-exon clade subtree Maximum-likelihood phylogenetic tree of olfactory and gustatory receptors from Apis mellifera, Nasonia vitripennis, Microctonus aethiopoides ("French", "Moroccan" and "Irish" strains) and Microctonus hyperodae.

The *Microctonus* 9-exon orthologs show homology to the *Apis mellifera* orthologs AmOr105, AmOr97PSE, AmOr100PSE, AmOr98, AmOr104, AmOr102, AmOr103, AmOr99, AmOr101 and AmOr162PSE by grouping out with strong bootstrap support of 0.99 (Figure 19). The rest of the 9-exon receptors from *Apis mellifera* form a separate clade which groups out with the rest of the 9-exon receptors with a moderate bootstrap value of 0.77. There are numerous subgroups in the 9-exon clade of the *Microctonus* (Figure 22).

9-exon a family

The 9-exon "a" group is highly supported with a bootstrap value of 1 (Figure 22). The 9-exon group "a" contains orthologs from *Microctonus hyperodae* (Mh_scaffold_1_3382978-3384967) and the "Irish" and "Moroccan" *Microctonus aethiopoides* strains (MaIR_003077-T1 and MaMO_scf3475_932-3260), respectively. The absence of a "French" orthologs implies that this ortholog has been lost after the divergence of the "Moroccan" and "French" strains of *Microctonus aethiopoides* (Figure 22). The 9-exon group "a" make for suitable candidates for further study into the roles of olfactory receptors in the *Microctonus*, particularly regarding host or mate identification as there is a "French" strain-specific receptor loss in this group.

9-exon b family

The 9-exon "b" group is highly supported with a bootstrap of 1 (Figure 22). The 9-exon group "b" includes orthologs from *Microctonus hyperodae* (Mh_scaffold_1_3432261-3437129) and "French" and "Moroccan" *Microctonus aethiopoides* strains

(MaFR_scf331_19619-24545 and MaMO_scf257_8386-12181) respectively. The absence of an "Irish" ortholog implies a species-specific loss of this olfactory receptor (Figure 22). The 9-exon group "b" makes for good candidates for further study into the roles of olfactory receptors in the Microctonus, particularly regarding host or mate identification, as there is a "Irish" strain-specific receptor loss in this group.

9-exon c

The 9-exon "c" group is supported with a bootstrap value of 0.87 (Figure 22). The 9-exon group "c" includes one ortholog from each of the four *Microctonus* taxa (Mh_scaffold_1_3437810-3442520, MaIR_003090-T1, MaMO_scf257_14221-19644 and MaFR_scf331_25278-30563 for *Microctonus hyperodae,* "Irish" strain *Microctonus aethiopoides*, "Moroccan" strain of *Microctonus aethiopoides* and "French" strain *Microctonus aethiopoides*) respectively (Figure 22). The conservation of this ortholog in all *Microctonus* implies a conserved function in this genus (Figure 22).

9-exon d

The 9-exon "d" group is highly supported with a bootstrap value of 1 (Figure 22). The 9-exon "d" group contains two orthologs from the *Microctonus aethiopoides* "Moroccan" (MaMO_scf3768_5488-8178 and MaMO_scf257_1384-7012), and "French" (MaFR_006887-T1 and MaFR_scf2589_109-2853) strain and one from the "Irish" strain (MaIR_003087-T1) (Figure 22). There are no *Microctonus hyperodae* orthologs in this group (Figure 22). The retention pattern of these orthologs implies a species-specific loss in *Microctonus hyperodae* or a duplication after the divergence of *Microctonus hyperodae* and *Microctonus aethiopoides*, which is then followed by either a duplication before the divergence of the "French" and "Moroccan" *Microctonus aethiopoides* strain followed by

a strain-specific loss in the "Irish" strain, or a duplication after the divergence of the "Irish" strain (Figure 22). The 9-exon group "d" make for good candidates for further study into the roles of olfactory receptors in the *Microctonus,* particularly regarding host or mate identification as there is variation in this group of receptors with complete loss in *Microctonus hyperodae*.

9-exon e1 and e2

The 9-exon e1 and e-2 groups are highly supported by a bootstrap value of 0.94 (Figure 22). The 9-exon "e1" and "e2" groups contains five orthologs from the "Moroccan" *Microctonus aethiopoides* strain (MaMO_scf3768_2519-4437, MaMO_scf8710_767-2415, MaMO_scf7738_965-2575, MaMO_scf2113_6113-8078 and MaMO_scf2113_333-2019), two from the "Irish" *Microctonus aethiopoides* strain (MaIR_scaffold_2_4551951-4554628 and MaIR_scaffold_7_1014998-1368233), three orthologs from the "French" strain of *Microctonus aethiopoides* (MaFR_scf2589_4230-6145, MaFR_scf687_19393-24056 and MaFR_scf687_12463-17450) and 3 orthologs from *Microctonus hyperodae* (Mh_scaffold_8_520627-526515 and Mh_scaffold_1_3386221-3392935) (Figure 22). Ancestral duplication produced two orthologs for all *Microctonus* taxa followed by an additional gene duplication in all taxa and subsequent loss in *Microctonus aethiopoides* "Irish" strain. Then two subsequent duplications in the *Microctonus aethiopoides* "Moroccan" strain after divergence from the *Microctonus aethiopoides* "French" strain. Long branch lengths imply rapid evolution in this clade. This group of receptors may be used to detect pheromones or host identification specialization as there are significantly more olfactory receptors in this group that belong to sexual *Microctonus* taxa. The 9-exon group e1 and e2 make for good candidates for further study into the roles of olfactory receptors in the *Microctonus,* particularly regarding host or mate identification.

9-exon f

The 9-exon "f" group is highly supported by a bootstrap value of 1 (Figure 22). The 9-exon “f” group has two orthologs for "French” (MaFR_scf163_1091-3001 and MaFR_scf1049_10-1475), “Moroccan” (MaMO_scf126_46480-48079 and MaMO_scf126_43769-45234) and “Irish” (MaIR_scaffold_3_7077468-7079067 and MaIR_scaffold_3_7074757-7076222) *Microctonus aethiopoides* (Figure 22). No orthologs were identified for *Microctonus hyperodae* in this group (Figure 22). The olfactory receptor absence pattern suggests two duplications after the divergence of *Microctonus aethiopoides* from *Microctonus hyperodae* or two separate losses in *Microctonus hyperodae*. The 9-exon group “f” is a good candidate for further experimental analysis as it is a good host identification and specificity related olfactory receptors candidate in the *Microctonus*.

9-exon g

The 9-exon “g” group is highly supported with a bootstrap value of 0.91 (Figure 22). The 9-exon “g” group has one ortholog for the “French” (MaFR_scf687_27794-29853), “Moroccan” (MaIR_scaffold_2_4516430-4522360) and “Irish” (MaIR_scaffold_2_4516430-4522360) strains of *Microctonus aethiopoides*. There were no *Microctonus hyperodae* orthologs in this clade, implying either this clade originated from a duplication after divergence from *Microctonus hyperodae* or a species-specific loss in *Microctonus hyperodae* (Figure 22). The 9-exon group “g” is a good candidate for further experimental analysis as it is a good host identification and specificity related olfactory receptor candidate in the *Microctonus*.

9-exon h

The 9-exon "h" group is supported by a bootstrap value of 0.99 (Figure 22). The 9-exon "h" group has three "French" (MaFR_scf205_79073-80575, MaFR_003986-T1 and MaFR_scf205_75672-77706), one "Moroccan" (MaMO_scf1625_16130-17977) and one "Irish" (MaIR_scaffold_2_10432541-10434216) strain *Microctonus aethiopoides* ortholog(s) (Figure 22). *Microctonus hyperodae* has one 9-exon(8) ortholog (Mh_scaffold_1_8219978-8221642) (Figure 22). The receptor retention pattern implies two "French" strain *Microctonus aethiopoides* specific duplications in this group. The 9-exon "h" group may be involved in host or mate recognition in the "French" strain of *Microctonus aethiopoides* which is supported by the receptor absence in the closely related "Moroccan" strain. The 9-exon group "h" is a good candidate for further experimental analysis.

9-exon i

The 9-exon group "i" is supported with a bootstrap value of 0.85 (Figure 22). The 9-exon "i" group contains two orthologs from the "French" (MaFR_scf687_34565-36468 and MaFR_scf6000_742-2447), two orthologs for the "Moroccan" (MaMO_scf10582_72-1975 and MaMO_scf7394_1252-2957) and one ortholog for the "Irish" (MaIR_scaffold_2_10425600-10428808) strain *Microctonus aethiopoides* (Figure 22). *Microctonus hyperodae* has 2 9-exon "I" orthologs (Mh_scaffold_1_3393559-3400758 and Mh_scaffold_1_8217250-8218969) (Figure 22). The 9-exon "i" group likely underwent an ancestral duplication and a subsequent strain-specific gene loss in the "Irish" strain of *Microctonus aethiopoides*.

9-exon j

The 9-exon "j" group is supported by a bootstrap value of 0.93 (Figure 22). The 9-exon "j" group contains three orthologs from the "Moroccan" strain (MaMO_scf5705_116-1938, MaMO_scf116_9795-11718 and MaMO_scf5705_2740-4705), three orthologs from the "French" strain (MaFR_scf331_116-1914, MaFR_scf14_104932-106839 and MaFR_scf331_2686-4704) and two orthologs from the "Irish" strain (MaIR_scaffold_2_4559845-4562089 and MaIR_scaffold_2_4557178-4559142) *Microctonus aethiopoides* (Figure 22). *Microctonus hyperodae* does not have an ortholog that fits this clade (Figure 22). This group of olfactory receptors likely originated from duplication in *Microctonus aethiopoides*. This group likely underwent a second duplication after the "Irish" strain divergence. The 9-exon group "g" is a good candidate for further experimental analysis as it is a good host identification and specificity related olfactory receptor candidate in the *Microctonus* due to its variation and absence in *Microctonus hyperodae*.

9-exon k

The 9-exon "k" group is highly supported by a bootstrap value of 0.99 (Figure 22). The 9-exon "k" group consists of two orthologs from the "Moroccan" strain (MaMO_scf10898_662-1868 and MaMO_scf10509_126-2034), two orthologs from the "French" strain (MaFR_scf2589_7457-9332 and MaFR_scf331_9494-12367) and two orthologs from the "Irish" strain (MaIR_scaffold_2_4571695-4574497 and MaIR_scaffold_2_4564174-4567125) *Microctonus aethiopoides*. *Microctonus hyperodae* also has two orthologs in the 9-exon group "k" (Mh_000346-T1 and Mh_scaffold_1_3421127-3426144) (Figure 22). Two ancestral duplications resulted in the formation of this group (Figure 22). The subsequent retention implies a conserved function.

The strain or species-specific patterns of olfactory receptor retention by the *Microctonus* in the 9-exon clade makes this an important group to assess in determining how the host range is established. Of particular interest for further scientific investigation is the a, b, d, e1, e2, f, g, h and j clades due to their high levels of variation (Figure 22).

Chapter 5 conclusions

Perception of volatile chemicals has great implications for biocontrol effectiveness as olfaction is fundamental to certain behaviors and survival in many insects. Similar variations in olfactory receptor complement in *Microctonus aethiopoides* are expected due to the high levels of variability found in the Hymenoptera. High levels of variation in olfactory receptor complement were observed in and between *Microctonus aethiopoides* strains. High levels of variation were also observed between *Microctonus aethiopoides* and *Microctonus hyperodae*. The overall number of olfactory receptors do not vary significantly between the *Microctonus* strains and species. Variation was observed in olfactory receptors with characterized functions in other Hymenoptera species including the 9-exon, H and L olfactory receptor clades. The 9-exon, H and L clade are worthwhile targets for further analysis in the *Microctonus*. As is seen in other Hymenoptera, olfaction is likely to be required for host identification in *Microctonus* taxa. Host identification has obvious biocontrol fitness implications as hosts identification is fundamental for reproduction in the *Microctonus*. Hence further understanding the olfactory compliment in the *Microctonus* may provide insights into how biocontrol of the Argentine Stem Weevil has failed or how it can be restored. The potential of identifying olfactory receptors which have roles in biocontrol and how this could allow the manipulation of host range is a particularly lucrative line of enquiry and could allow for the development of robust *Microctonus* biocontrol wasps for novel hosts.

Methods of olfactory receptor identification, annotation, and phylogenetic analysis

The *Apis mellifera*, *Nasonia vitripennis* and *Drosophila melanogaster* olfactory receptor protein Fasta databases were collected (Robertson and Wanner, 2006; Robertson, Gadau and Wanner, 2010). The olfactory receptor databases were used to make a blast database using blast+ 'makeblastdb' (Camacho *et al.*, 2009). The olfactory receptor databases were used to blast search the protein databases of the "Irish", "French", and "Moroccan" strains of *Microctonus aethiopoides* as well as *Microctonus hyperodae* using blastp (See Supplementary S4.1 Camacho *et al.*, 2009). The blastp search used an E value cut-off of 1e-50. Redundant accessions were removed using a custom bash script, and sequences were subsequently collected using esl-sfetch from the hmmer group of programs (Eddy, 2011). Putative olfactory receptors under 100 amino acids and over 500 amino acids were then filtered out using a custom python script.

Exonerate was used to generate a gff3 file using the genome assemblies of each respective *Microctonus* strain or species and the protein database (Slater, no date) using "--model protein2genome" and "--showtargetgff" parameters as required by InsectOR (Karpe, Tiwari and Ramanathan, 2021). The olfactory receptor blast hits for all *Microctonus* strains and species, the exonerate gff files, and the genome assemblies were used as input to InsectOR. InsectOR is a web-based program specifically used to annotate olfactory receptors (Karpe, Tiwari and Ramanathan, 2021).

The olfactory receptors identified by the initial (this is also the input for InsectOR) blast search which did not overlap the InsectOR annotations, were identified using a custom bash script and combined with the InsectOR annotations to create an olfactory receptor database for each of the "Irish", "French" and "Moroccan" strains of *Microctonus aethiopoides* as well as *Microctonus hyperodae*. See supplementary files S4.1-4.3 for olfactory receptor discovery and filtering Snakemake files.

Phylogenetic analysis of olfactory receptors

The *Microctonus* olfactory receptor Fasta databases were combined with databases from *Apis mellifera* and *Nasonia vitripennis* (Robertson and Wanner, 2006; Robertson, Gadau and Wanner, 2010). The combined olfactory receptor database was aligned using Muscle (v3.8.31; Edgar, 2004). The alignments were subsequently trimmed using TrimAL using the "--gappyout" parameters in the PHYLIP format (Capella-Gutierrez, Silla-Martinez and Gabaldon, 2009).

Phylogeny trees were constructed using Raxml-ng, a phylogenetic tree inference tool that uses the maximum-likelihood method (v1.0.1; Kozlov, Darriba, Flouri, Morel, & Stamatakis, 2019). Analysis was run using 120 threads, JTT model (as this was used in another olfactory receptor phylogenetic maximum-likelihood phylogenetic reconstructions (Guo *et al.*, 2021)), 20 "pars" and "rand" trees were used as seeds, trees were generated with both FBP and TBE bootstrap support with 1000 generations (Kozlov *et al.*, 2019). TBE is better for very large phylogenetic trees than FBP due to the low bootstrap support, resulting from using this technique, primarily in internal nodes when using this metric (Lemoine *et al.*, 2018). The TBE bootstrap was hence used to annotate olfactory receptor phylogeny trees. Trees were processed into figures using Figtree.

Putative *Microctonus* olfactory receptors, which did not group out with TBE bootstrap support in the maximum-likelihood tree, were manually blasted against the NCBI non-redundant protein database to confirm olfactory receptor homology (Camacho *et al.*, 2009). Putative *Microctonus* olfactory receptors, which did not produce convincing blast hits, were removed from the subsequent analysis as these may have been misannotated by InsectOR. Extremely long putative *Microctonus* olfactory receptors (longer than 800 amino acids) were removed, as these have likely been misannotated as most olfactory receptors are between 350 and 450 amino acids long (Karpe, Tiwari and Ramanathan, 2021). Misannotated olfactory receptors could result in artifacts in alignment and subsequent phylogenetic reconstruction.

After the removal of misannotated putative *Microctonus* olfactory receptors, the olfactory receptors were aligned with Muscle again (Edgar, 2004) and subsequently trimmed using TrimAl with the "gappyout" parameter (Capella-Gutierrez, Silla-Martinez and Gabaldon, 2009). A maximum-likelihood phylogenetic tree was once again inferred using raxml-ng using 120 threads, JTT model, 20 "pars" and "rand" trees were used as seeds, trees were generated with both FBP and TBE bootstrap support with 1000 generations (Capella-Gutierrez, Silla-Martinez and Gabaldon, 2009). The JTT model was selected as it was previously used in olfactory receptor phylogenetic tree reconstruction (Guo *et al.*, 2021). Phylogenetic trees were rendered using Figtree, including TBE bootstrap support.

Microctonus putative olfactory receptors, which were grouped out with an Apis mellifera or Nasonia vitripennis olfactory receptor with a bootstrap of over 75, were considered "supported". A bootstrap over 80 was considered "well supported", whereas a bootstrap value over 90 was considered "strongly supported". See supplementary S4.4-4.9 for scripts on phylogenetic reconstruction, unstylized olfactory receptor phylogeny trees and olfactory receptor sequence Fasta files.

Chapter 6: General Discussion and Conclusion

Summary of analysis

The genomes of *Microctonus hyperodae* and three strains of *Microctonus aethiopoides* were sequenced to a high level of coverage using Illumina sequencing. Kraken2 used sequenced reads to assay the *Microctonus* microbiota and ruled out bacterial infection as a likely mechanism of asexual reproduction (Wood, Lu and Langmead, 2019). The ploidy of the *Microctonus* genomes was established as diploid through analysis with Smudgeplot and Genomescope2. Genomescope2 also allowed for genome heterozygosity in the *Microctonus* to be calculated (Vurture *et al.*, 2017; Ranallo-Benavidez, Jaron and Schatz, 2020). Sequenced reads were also used to assemble genomes for *Microctonus hyperodae* and the three strains of *Microctonus aethiopoides* using the Meraculous assembler (Chapman *et al.*, 2011). High-quality assemblies were achieved for *Microctonus hyperodae* and *Microctonus aethiopoides* "Irish" strain. In comparison, suitable quality assemblies were produced for *Microctonus aethiopoides* "French" and "Moroccan" strains. The *Microctonus* genomes were annotated using Funannotate with RNA sequence and homologous peptide databases from other Hymenoptera species to produce quality genome annotations (Love *et al.*, 2018). Orthofinder was used to produce a Hymenoptera species tree using *Microctonus* peptide databases and a diverse range of Hymenoptera species for comparison. The Orthofinder species tree was converted to ultrametric and calibrated to provide divergence estimates for the *Microctonus* (Emms and Kelly, 2019). A cataloguing approach was used to establish the presence and absence of previously established mitosis and meiosis genes, from other Hymenoptera species, in the *Microctonus* using a custom script that utilizes HMMs and Hmmer to search the *Microctonus* peptide databases (Eddy, 2011). Phylogenetic analysis of meiosis genes MND1, HOP2, MSH4 and MSH5 were compared to orthologs from other species using a custom phylogenetics script using blast, TrimAL, MUSCLE and rapid-nj (Simonsen, Mailund and Pedersen, no date; Edgar,

2004; Camacho *et al.*, 2009; Capella-Gutierrez, Silla-Martinez and Gabaldon, 2009). *Microctonus* and other Hymenoptera were used to compare MND1, HOP2 and the MSH group, which was undertaken using a custom script to produce neighbour-joining trees. The hybridization chain reaction was used to show MND1 expression in the ovaries of the "Irish" strain *Microctonus aethiopoides*. Olfactory receptors were annotated using a targeted approach using InsectOR (Karpe, Tiwari and Ramanathan, 2021). The *Microctonus* olfactory receptor complement was compared to orthologs from *Apis mellifera* and *Nasonia vitripennis* using a custom analysis and phylogenetics pipeline, allowing for likely biological functions to be inferred (see "Chapter 5 conclusions

Perception of volatile chemicals has great implications for biocontrol effectiveness as olfaction is fundamental to certain behaviors and survival in many insects. Similar variations in olfactory receptor complement in *Microctonus aethiopoides* are expected due to the high levels of variability found in the Hymenoptera. High levels of variation in olfactory receptor complement were observed in and between *Microctonus aethiopoides* strains. High levels of variation were also observed between *Microctonus aethiopoides* and *Microctonus hyperodae*. The overall number of olfactory receptors do not vary significantly between the *Microctonus* strains and species. Variation was observed in olfactory receptors with characterized functions in other Hymenoptera species including the 9-exon, H and L olfactory receptor clades. The 9-exon, H and L clade are worthwhile targets for further analysis in the *Microctonus*. As is seen in other Hymenoptera, olfaction is likely to be required for host identification in *Microctonus* taxa. Host identification has obvious biocontrol fitness implications as hosts identification is fundamental for reproduction in the *Microctonus*. Hence further understanding the olfactory compliment in the *Microctonus* may provide insights into how biocontrol of the Argentine Stem Weevil has failed or how it can be restored. The potential of identifying olfactory receptors which have roles in biocontrol and how this could allow the manipulation of host range is a particularly lucrative line of enquiry and could allow for the development of robust *Microctonus* biocontrol wasps for novel hosts.

Methods of olfactory receptor identification, annotation, and phylogenetic analysis" page 116). This broad range of analysis has provided and will continue to provide significant insights into the biology of the *Microctonus*.

General characteristics of Microctonus genomes

Well assembled and annotated genomes have become essential to understanding the biology of *Microctonus* and provides a fundamental tool to further understand these agriculturally important classical biocontrol agents. Furthermore, genome sequencing, sequencing analysis, annotation and downstream analysis can provide beneficial insights into genome size, complexity, relatedness, and chromosome number.

Microctonus hyperodae likely diverged from *Microctonus aethiopoides* about 17 MYA, which is more distantly related than assumed based on phenotype alone, giving us insights into the relatedness of *Microctonus hyperodae* and *Microctonus aethiopoides* (Figure 13). The more ancient divergence makes it less likely that identical mechanisms of asexual reproduction are identified in *Microctonus hyperodae* and *Microctonus aethiopoides*. A more accurate divergence estimate could be obtained by comparing other closely related species in the Braconidae like the recently sequenced and assembled *Cotesia* wasps or *Microplitis demolitor*, unfortunately these genomes have either only recently become available (regarding *Cotesia* wasps) or lower quality (*Microplitis demolitor*) (Gauthier et al., 2021; Burke et al., 2018).

Microctonus have genomes that are ~120 Mb in size (Table 1, 3 and Figure 8). This genome size is supported by both the consistent assembly sizes between the *Microctonus* strains and species as well as the Genomescope2 estimate (Table 1, 3 and Figure 8). Although the *Microctonus* have similar genome sizes *Microctonus* hyperodae, and *Microctonus* aethiopoides "Irish" strain likely have differing numbers of chromosomes, which were identified through the chromosome level assemblies using Hi-C revealing 12 chromosomes in *Microctonus hyperodae* and eight chromosomes in *Microctonus aethiopoides* (Figure 7).

The Braconidae are known to have a range of three to twenty-three chromosomes and the number of chromosomes in both *Microctonus hyperodae* and *Microctonus aethiopoides* fall within this range, making the chromosome number predicted in the *Microctonus* more likely to be correct (Gokhman, 2009). Braconidae *Cotesia congregate,* and *Microplitis demolitor* have ten chromosomes similar to the chromosome number found in the *Microctonus* (Gauthier et al., 2021; Burke et al., 2015). The chromosomes number variation in *Microctonus* could be further supported through the karyotype analysis of other genus members. Karyotype analysis is time-consuming, technical, and expensive making further genome sequencing and Hi-C analysis of the "French" and "Moroccan" strains the preferred method through which their chromosome number may be inferred (Gokhman and Quicke, 1995; Belton *et al.*, 2012). As the "Irish" strain *Microctonus aethiopoides* diverged from the other *Microctonus aethiopoides* strains recently (~2 MYA), the chromosome copy number in these strains is likely also to be 12, although the number of chromosomes is known to vary on the species level in parasitic Hymenoptera (Figure 7; Gokhman, 2009). The ancestral chromosome number in the *Microctonus* is likely to be 12 as the 14-17 chromosome copy number range is considered the initial chromosome number of parasitic wasps (Gokhman, 2009). It is most likely that the *Microctonus aethiopoides* karyotype resulted from multiple chromosomal fusion events after it diverged from *Microctonus hyperodae*. A preliminary Satsuma synteny plot analysis implies that "Irish" strain *Microctonus aethiopoides* chromosomes 1 and 8 were fused to form chromosome 1 from "Irish" strain *Microctonus hyperodae*. "Irish" strain *Microctonus aethiopoides* chromosomes 2 and 5 were fused to form chromosome 2 from "Irish" strain *Microctonus hyperodae*. "Irish" strain *Microctonus aethiopoides* chromosomes 3 and 4 were fused to form chromosome 3 from *Microctonus hyperodae,* and "Irish" strain *Microctonus aethiopoides* chromosomes 11 and 12 were fused to form chromosome 5 for *Microctonus hyperodae* (Supplementary S5.1).

Smudgeplot analysis revealed that the *Microctonus* are diploids (Table 2) which is consistent with the ploidy observed in the vast majority of the parasitic Hymenoptera

with a few notable exceptions like the thelytokous gall wasp *Diplolepis eglanteriae,* which is the only known polyploid to date (3n = 27) (Sanderson, 1988; Gokhman, 2009).

Searching for mechanisms of asexuality in Microctonus

Some *Microctonus* reproduce through thelytokous parthenogenesis like the "Irish" strain of *Microctonus aethiopoides* and *Microctonus hyperodae* (Loan and Lloyd, 1974; Iline and Phillips, 2004; Goldson *et al*., 2005).

It is essential to rule out *Wolbachia* as a mechanism of asexual reproduction as it is a common cause of asexual reproduction in the Hymenoptera (Braig *et al*., 2002). Over 3% of Alphaproteobacterial reads were characterized as belonging to *Wolbachia* in the *Microctonus;* hence an endosymbiont mechanism of asexual reproduction is possible in the *Microctonus* species. Higher proportion of *Microctonus* reads do not correlate with the parthenogenesis phenotype (Figure 5C). *Wolbachia* or *Rickettsia* could be causing asexual reproduction in *Microctonus,* so heat or antibiotic treatment should be evaluated as a possible mechanism to revert *Microctonus* asexuals to a sexual reproductive strategy (Phillips, 1995, 1996).

In the mechanism of asexual reproduction could be genetic implying it could be uncovered by looking at variation in genes involved in mitosis and meiosis in other insects (Tvedte, Forbes and Logsdon John M, 2017). A clear genetic mechanism of asexual reproduction was not apparent when cataloguing identified mitosis and meiosis genes in *Microctonus* (Figure 14). However, the broad conservation of Meiosis genes in the *Microctonus* implies that these have a conserved function in *Microctonus* asexuals (Figure 15). The meiosis genes MSH4, MSH5, MND1 and HOP2, do not appear phylogenetically misplaced when compared to orthologs from other Hymenoptera, implying function and conservation. This implies that meiosis is conserved in *Microctonus* (Figure 15-17). If meiosis is conserved in the *Microctonus*, an automatic asexual mechanism is more likely. The expression of the meiosis gene MND1 in the ovaries of the asexual "Irish" strain

Microctonus aethiopoides is further evidence for an automictic mechanism of asexual reproduction in *Microctonus* (Figure 18).

Genes involved in mitosis in *Microctonus* are broadly conserved except for RECQ1 and RECQ4 (Figure 14). Loss of RECQ4 in human mutant cells showed defective S-phase arrest in response to UV damage or hydroxyurea treatment (Park *et al.*, 2006). RECQ2 (also known as BLM) was not identified in the *Microctonus aethiopoides* strains (Figure 14). The absence of RECQ2 does not imply a clear mechanism of asexual reproduction but may cause an increase in genome rearrangements and genomic instability (Garcia *et al.*, 2011).

Neither higher nor lower heterozygosity estimates from Genomescope2 segregate with asexual reproduction in the *Microctonus* (asexuals have the highest and lowest heterozygosity) (Table 1). The presence of some heterozygosity in the genomes of asexual *Microctonus* suggests that the mechanism of asexual reproduction is not clonal and more likely to be a reproductive mechanism such as automixis seen in the clonal raider ant *Cerapachys biroi* (Oxley *et al.*, 2014).

Microctonus have about 14000 protein-coding genes, which are similar to the number of protein-coding genes identified in the related *Cotesia* species and the parasitic wasp *Nasonia vitripennis,* which have 14140 and 13141 protein-coding genes, respectively (Table 4; NCBI *Nasonia vitripennis* Annotation Release 101; Gauthier et al., 2021). Suggesting that most if not all of the protein-coding genes in the *Microctonus* have been annotated, making any apparent gene absences more likely to be authentic as opposed to annotation or assembly artifacts. The high BUSCO scores for all *Microctonus* annotations are further evidence that genome annotations are mostly complete (Table 4). The production of this high-quality set of annotations will be invaluable in future research into the *Microctonus*.

Host preference in Microctonus

Microctonus hyperodae and *Microctonus aethiopoides* vary in host range and preference (Barratt *et al.*, 1997; Vink *et al.*, 2003). Host range must be specified in the genome of *Microctonus* through presence, absence and specification of olfactory receptors as they have been shown to mediate host preference in other species (Suh, Bohbot and Zwiebel, 2014). There is variation in the olfactory receptor complement between the *Microctonus* taxa (Table 5 and Figure 20) H and G clade subtree Maximum-likelihood phylogenetic tree of olfactory and gustatory receptors from Apis mellifera, Nasonia vitripennis, Microctonus aethiopoides ("French", "Moroccan" and "Irish" strains) and Microctonus hyperodae.), which suggest that the olfactory receptors in the *Microctonus* are under selective pressure (especially receptors with longer branch lengths) (Figure 22). The 9-exon *Microctonus* hyperodae olfactory receptor complement in the *Microctonus* may vary due to host preference. Genetic manipulation and screening of 9-exon olfactory receptors that vary in the *Microctonus* may allow host range manipulation. Altering the 9-exon olfactory receptors in a sexual strain of *Microctonus* aethiopoides may allow for the selective targeting of the Argentine stem weevil and provide a system in which selective breeding for biocontrol effectiveness can be undertaken. Some variations in the 9-exon clade of *Microctonus* could be due to this group of receptors being involved in mate identification. If *Microctonus hyperodae* has lost or the olfactory receptor responsible for mate identification has been too extensively altered, mate identification may be less effective or entirely absent if *Microctonus hyperodae* is reverted to sexual reproduction.

Future prospects

Further bioinformatic analysis may be more successful in identifying the exact mechanism through which *Microctonus* became asexual as more recently released high quality Braconidae genomes, like those from *Cotesia* species, would provide additional points of comparison (Gauthier et al., 2021).

Although the Clover Root Weevil biocontrol by the "Irish" strain *Microctonus aethiopoides* is still currently effective, its asexual reproductive strategy leaves it vulnerable to losing effectiveness in the future (Casanovas, Goldson and Tylianakis, 2018). The failing biocontrol of *Microctonus hyperodae* provides essential insight into how biocontrol agents fail and could inform the selection of more suitable biocontrol agents that are less likely to be out evolved in the future.

If a mechanism of asexual reproduction is identified in the *Microctonus*, genetic manipulation methods will need to be developed. As all *Microctonus* species are non-model organisms, genetic manipulation tools are limited. Genetic manipulation in other hymenopteran non-model organisms has been achieved through molecular treatment or RNAi (Dearden, Duncan and Wilson, 2009; Duncan, Hyink and Dearden, 2016).

RNAi uses double-stranded DNA molecules to manipulate gene expression (Hannon, 2002). RNAi has been used to manipulate gene expression in *Apis mellifera* and other insects (Lynch and Desplan, 2006; Dearden, Duncan and Wilson, 2009). The effective use of RNAi in other insects may make it useable in *Microctonus* gene expression manipulation.

Suggested targets include MND1, HOP2, MSH4, and MSH5 as these likely have conserved roles in meiosis in the Hymenoptera allowing for a meiotic or mitotic mechanisms of asexual reproduction to be uncovered. Which would enable narrowing the search further for plausible genetic mechanisms of asexual reproduction in the *Microctonus*.

Small molecular inhibitors are small organic compounds that can target and disrupt molecular interactions. Small molecular inhibitors have successfully manipulated reproduction in *Apis mellifera* (Duncan, Hyink and Dearden, 2016). By treating newly emerged *Apis mellifera* with DAPT (a gamma-secretase inhibitor), Duncan, Hyink and Dearden, (2016) used DAPT to repress the ovary activation inhibitory effects of queen mandibular pheromone, resulting in worker ovary activation and implicating Notch signaling as the molecular mechanism responsible for the control of ovary activation. Given the successful manipulation of reproduction in *Apis mellifera,* a range of small molecular inhibitors including DAPT could be used to manipulate molecular targets in *Microctonus hyperodae* or *Microctonus aethiopoides* to assess the function of Notch receptor signaling in these wasps.

Conclusion

In conclusion, the research in this thesis has transformed our knowledge about the genetics of the *Microctonus*. Knowledge about the genetics of the *Microctonus* has gone from relatively little genetic information to having High-quality genome assemblies for *Microctonus aethiopoides* "Irish" strain and *Microctonus* hyperodae. Good quality genomes for the "French" and "Moroccan" strain of *Microctonus* aethiopoides. Also, high-quality genome annotations were produced for the *Microctonus* hyperodae, "Irish" strain *Microctonus aethiopoides*, "French" strain *Microctonus aethiopoides* and "Moroccan" strain *Microctonus aethiopoides*. These annotations enabled the analysis of the mitosis and meiosis genes in these groups and the olfactory receptor complement. These have implications for reproductive mechanisms and how the *Microctonus* perceive their surroundings, which have important implications for biocontrol and strategies used to ensure biocontrol efficiency is maintained. The phylogenetic relationship of the *Microctonus* to other Hymenoptera was inferred, and a 17 MYA divergence of *Microctonus* hyperodae and *Microctonus* aethiopoides was identified, a more ancient origin than

phenotypic characteristics alone would imply. We better understand the genome structure with different chromosome numbers implied in *Microctonus* hyperodae and the "Irish" strain of *Microctonus aethiopoides* (8 and 12, respectively). Genomescope2 analysis suggests little variation in heterozygosity and low repeat numbers in the genomes of the *Microctonus*, making this a comparatively easy species to undertake further research. Smudgeplot analysis implies that the *Microctonus* have diploid genomes, which have positive implications for genome assembly and annotation.

The failing biocontrol of the Argentine stem weevil could cause considerable damage to the health of pastures in New Zealand (Prestidge, Barker and Pottinger, 1991; Popay *et al.*, 2011; Goldson *et al.*, 2014). Improving the biocontrol efficiency of *Microctonus hyperodae* may be the key to mitigating damage, as its initial introduction improved pastoral health (Goldson *et al.*, 1993; Popay and Wyatt, 1995). Comparative analysis of the genomes of *Microctonus hyperodae* and *Microctonus aethiopoides* could allow for the genetic basis of parthenogenesis to be established. Once the genetic cause of parthenogenesis is established, genetic manipulation could allow for reversion to sexual reproduction, facilitating selective breeding. Selective breeding of *Microctonus hyperodae* would allow for more effective biocontrol agents to be produced. This approach not only has implications in this specific case of failed biocontrol but could rescue or prevent the failure of other classical biocontrol systems, including ones that use asexual *Microctonus aethiopoides* in New Zealand (Goldson *et al.*, 2014). This thesis presents essential tools for *further studying Microctonus hyperodae and could help find a remedy for its failing biocontrol*.

This thesis aims to improve the biocontrol of the Argentine Stem Weevil by *Microctonus hyperodae*. High quality genomes and annotations were generated allowing for future analysis and comparison. Future analysis and comparisons with other Hymenoptera will further aid our understanding of the biology of the *Microctonus* and likely enable the restoration and retainment of biocontrol efficacy in this genus. This thesis has narrowed the search for mechanisms of asexual reproduction in the *Microctonus* and provided additional tools for studying mitosis and meiosis in the *Microctonus* and likely other

Hymenoptera species. This thesis has endeavored to identify and assess variation in fundamental olfactory receptors which have great utility for furthering the understanding of biology in the *Microctonus* but also allow for a better understanding of host sensing and biocontrol efficacy.

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Supplementary

See the table below for a description of the included supplementary materials. Supplementary files can be found at the following google drive link: https://drive.google.com/drive/folders/125KdtQtrTWQeDQcrePAI45lN2ITmYMCC?usp=sharing

S1: S1_Kraken2_Genomescope2_Smudgeplot

Supplementary	Description	Filename
S1.1	"Irish" strain *Microctonus aethiopoides* Kraken2 report	S1-1_MaIR_kraken_reports.txt
S1.2	"French" strain *Microctonus aethiopoides* Kraken2 report	S1-2_MaFR_kraken_reports.txt
S1.3	"Moroccan" strain *Microctonus aethiopoides* Kraken2 report	S1-3_MaMO_kraken_reports.txt
S1.4	*Microctonus hyperodae* Kraken2 report	S1-4_Mh_kraken_reports.txt
S1.5	"Irish" strain *Microctonus aethiopoides* Kraken2 Pavian Sankey Plot	S1-5_MaIR_kraken_pavian_sankey.png
S1.6	"French" strain *Microctonus aethiopoides* Kraken2 Pavian Sankey Plot	S1-6_MaFR_kraken_pavian_sankey.png

S1.7	"Moroccan" strain *Microctonus aethiopoides* Kraken2 Pavian Sankey Plot	S1-7_MaMO_kraken_pavian_sankey.png
S1.8	*Microctonus hyperodae* Kraken2 Pavian Sankey Plot	S1-8_Mh_kraken_pavian_sankey.png
S1.9	*Microctonus* Genomescope2 kmer frequency profiles	S1-9_genomescope2_profile.pdf
S1.10	"French" and "Moroccan" strain *Microctonus aethiopoides* Smudgeplots	S1-10_smudgeplots.pdf
S1.11	"Irish" strain *Microctonus aethiopoides* histogram for Genomescope2 and Smudgeplot analysis	S1-11_MaIR_reads_genomescope2.histo
S1.12	"French" strain *Microctonus aethiopoides* histogram for Genomescope2 and Smudgeplot analysis	S1-12_MaFR_reads_genomescope2.histo
S1.13	"Moroccan" strain *Microctonus aethiopoides* histogram for Genomescope2 and Smudgeplot analysis	S1-13_MaMO_reads_genomescope2.histo

S1.14	*Microctonus hyperodae* histogram for Genomescope2 and Smudgeplot analysis	S1-14_Mh_reads_genomescope2.histo

S2: S2_Assemblies_Annotations

Supplementary	Description	Filename
S2.1	The directory containing Funannotate annotation for "Irish" strain *Microctonus aethiopoides*	S2-1_MaIR_meraculous_annotation_funannotate
S2.2	The directory containing Funannotate annotation for "French" strain *Microctonus aethiopoides*	S2-2_MaFR_meraculous_annotation_funannotate
S2.3	The directory containing Funannotate annotation for "Moroccan" strain *Microctonus aethiopoides*	S2-3_MaMO_meraculous_annotation_funannotate
S2.4	Directory	S2-4_Mh_meraculous_annotation_funannotate

	containing Funannotate annotation for *Microctonus hyperodae*	
S2.5	The directory containing Hi-C assembly Funannotate annotation for "Irish" strain *Microctonus aethiopoides*	S2-5_MaIR_HIC_annotation_funannotate
S2.6	The directory containing Hi-C assembly Funannotate annotation for *Microctonus hyperodae*	S2-6_Mh_HIC_annotation_funannotate
S2.7	Snakemake script for genome assembly of *Microctonus aethiopoides*	S2-7_Snakefile_Ma_assembly
S2.8	Snakemake script for genome assembly of *Microctonus*	S2-8_Snakefile_Mh_assembly_Harrop

	hyperodae	

S3: S3_Meiosis_Mitosis_Analysis

Supplementary	Description	Filename
S3.1	HOP2 ortholog alignment	S3-1_HOP2.aln
S3.2	HOP2 abbreviated accessions	S3-2_HOP2_rename.txt
S3.3	MND1 ortholog alignment	S3-3_MND1.aln
S3.4	MND1 abbreviated accessions	S3-4_MND1_rename.txt
S3.5	MSH ortholog alignment	S3-5_MSH.aln
S3.6	MSH abbreviated accessions	S3-6_MSH_rename.txt
S3.7	*Microctonus* meiosis and mitosis accessions	S3-7_Mitosis_Meiosis_gene_present.csv
S3.8	Snakemake script for the discovery of *Microctonus* mitosis and meiosis orthologs	S3-8_Snakefile_Meiosis
S3.9	"Irish" strain *Microctonus aethiopoides* HCR probe transcripts	S3-9_MaIR_hcr_transcripts.fa
S3.10	*Microctonus hyperodae* HCR probe transcripts	S3-10_Mh_hcr_transcripts.fa
S3.11	The directory containing all aligned and trimmed orthologs for hmm searching *Microctonus* peptide databases for	S3-11_meiosis_align_trim_hmm_input

	meiosis and mitosis orthologs	
S3.12	The directory containing all hmm profiles for searching *Microctonus* peptide databases for meiosis and mitosis orthologs	S3-12_meiosis_hmm

S4: S4_Olfactory_Analysis

Supplementary	Description	Filename
S4.1	Snakemake script for initial olfactory receptor blast database and discovery of initial hints	S4-1_Snakefile_Olfactory_blast
S4.2	Snakemake script for processing inputs for InsectOR olfactory receptor search	S4-2_Snakefile_iOR_inputs
S4.3	Snakemake script for filtering InsectOR results for phylogenetic analysis	S4-3_Snakefile_iOR_filter
S4.4	Olfactory receptor alignment and trimming script	S4-4_Olfactory_align_raxml.sh
S4.5	Script for running raxml-ng phylogenetic tree	S4-5_raxml-ng_Olfactory.sh
S4.6	Olfactory receptor sequences from *Apis mellifera*, *Nasonia vitripennis* and *Microctonus*	S4-6_OR.fa
S4.7	Olfactory receptor sequences abbreviation database *Apis mellifera*, *Nasonia vitripennis* and *Microctonus*	S4-7_OR.rename.txt
S4.8	Aligned and trimmed PHYLIP format input file for raxml-ng	S4-8_OR.trimmed.phy
S4.9	Stylized phylogenetic tree of olfactory receptors from *Apis mellifera*, *Nasonia vitripennis* and *Microctonus*	S4-9_OR.tre.pdf

S5: S5_Satsumu

Supplementary	Description	Filename
S5.1	Satsuma synteny plot from "Irish" strain *Microctonus* Hi-C *assembly* and *Microctonus hyperodae* Hi-C assembly	S5-1_Satsuma_Synteny_Plot.pdf

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