Species-specific hydrocarbon profiles of South African fig wasp communities (Hymenoptera: Chalcidoidea)

by

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Declaration

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Abstract

Cuticular hydrocarbon (CHC) profiles of insects play roles in behavioural interactions within and between species, encompassing species-, colony- and mate-recognition. CHCs are largely genetically determined and are thus unique to each species, making them useful in chemotaxonomy. However, species exhibit intra-species variation in their CHC profile which can be the result of both intra-species genetic variation as well as environmental influences such as habitat effects, colony effects, diet, host switching, as well as adsorption of CHCs from other insects. Studies have found that the CHC profiles of a specific insect species will often exhibit variations between regions as well as the species of host the insect is associated with. Therefore, an ideal system to investigate the effects of genetic population structure and environment on the CHC profiles of insects is within the fig – fig wasp mutualism. Fig species occur in a wide variety of habitats and host a diverse complement of fig wasp species. We were therefore offered the opportunity to investigate a wide range of potential influences on fig wasp CHC profiles ranging from environmental to genetic effects. Firstly, through GC-MS we found that the CHC profiles of the fig wasps investigated are both species-specific and species-group-specific, with the species *Elisabethiella glumosae*, *Elisabethiella stuckenbergi* and *Ceratosolen capensis*, and two *Otitesella* species-groups (the Uluzi and Sesqui species-groups) separating out significantly. Consensus phylogenies (based on COI, Cytb and EF-1α) showed that within the galling fig wasp genus *Otitesella* there were multiple genetic lineages within a species-group which corresponds to species-level genetic variation, and that each genetic lineage was confined to a single host fig species. The CHC profiles reflected the genetic relationships between the two species-groups, and the CHC profiles within a species group could be differentiated by genetic lineage/host species. This indicated that although genetic lineage was mostly responsible for the observed variation in CHC profiles, factors associated with different host species also had an effect. Strong regional variation overriding both the influence of genetic lineage and factors associated with host species were observed in the CHC profiles of the fig wasps within a species-group. This regional variation in CHC profiles was also observed within two pollinating fig wasp species, *Elisabethiella stuckenbergi* and *Ceratosolen capensis*, which was not supported by population genetic data (COI and Cytb). In fact, very little genetic population structure was found within the pollinating species, even

though the pollinators were collected across South Africa. The lack of genetic structure in pollinating fig wasps can be the result of high gene flow caused by the large dispersal capability of pollinating fig wasps. Our results indicated that fig wasp CHC profiles have the potential to be used in chemotaxonomy and are possibly used as species and mate-recognition cues by the fig wasps. Furthermore, we found both a regional and associated host species effect on the CHC profile. We suggest that the observed regional effect in this study could be attributed to habitat differences and differences in fig wasp community between regions. Moreover, the effect host species had on the CHC profiles may be as a result of dietary differences between galls in different host species. A possible consequence of the observed regional/host species-associated effect on fig wasp CHC profiles is that it could lead to pre-mating isolation within fig wasp species, which could ultimately result in speciation. In addition, our results indicated that the interpretation of the variation in the fig wasp CHC profile was dependent on the scale of the analysis: on a broad, inter-species-level scale, fig wasp CHC profiles were species-specific; on a finer intra-species scale, variation in CHC profiles occurred between fig wasps collected from different regions; and on a within-region scale, variation in CHC profiles within species-groups occurred between genetic lineages/host species. Future studies should look at the application of CHCs in chemotaxonomic studies on the fig wasp phylogeny, as well as the effect of fig wasp community composition on fig wasp CHCs.

Opsomming

Kutikulêre koolwaterstof (KK) profiele van insekte speel rolle in die gedragsinteraksies binne sowel as tussen spesies, en behels die herkenning van spesie- of kolonielidmaatskap asook potensiële maats. Kutikulêre koolwaterstowwe word meestal deur gene bepaal en is dus uniek vir elke spesie, wat dit handig maak vir chemotaksonomie. Spesies vertoon egter soms intraspesie variasie in hul KK profiele wat die gevolg kan wees van beide intraspesie genetiese variasie sowel as omgewingsinvloede soos habitat effekte, kolonie effekte, dieet, tussen-gasheer skuiwings, asook die adsorpsie van ander insekte se kutikulêre koolwaterstowwe. Studies het gevind dat die kutikulêre koolwaterstof profiele van ŉ spesifieke insek spesie op ŉ gereelde basis verskille vertoon tussen streke asook tussen die verskillende gasheer spesies waarmee die insek geassosieer is. Om hierdie redes is die vy – vy-wesp mutualisme ŉ ideale sisteem om die uitwerking van genetiese populasie struktuur en omgewing op die KK profiele van insekte te ondersoek. Vy spesies kom in ŉ wye verskeidenheid van habitatte voor en ondersteun ŉ diverse groep vy-wesp spesies. Dit het ons die geleentheid gebied om ŉ wye reeks moontlike invloede van vy-wesp KK profiele te ondersoek, van omgewings- tot genetiese invloede. Eerstens, deur die gebruik van GC-MS het ons gevind dat die KK profiele van die vy-wespe wat ondersoek was beide spesie-spesifiek en spesie-groep-spesifiek is, met die spesies *Elisabethiella glumosae*, *Elisabethiella stuckenbergi* en *Ceratosolen capensis*, asook twee *Otitesella* spesie-groepe (die Uluzi en Sesqui spesie-groepe) wat betekenisvol onderskei kon word. Konsensus filogenieë (gegrond op COI, Cytb en EF1-1α) het getoon dat daar in die gal-induserende vy-wesp genus *Otitesella* veelvuldige genetiese lyne binne die spesie-groepe voorgekom het ooreenstemmend met tussen-spesie genetiese variasie, en dat elke genetiese lyn beperk was tot ŉ enkele gasheer vy spesie. Die KK profiele het die genetiese verhoudings tussen die twee spesie-groepe weerspieël, en die KK profiele binne ŉ spesie-groep kon onderskei word op grond van hul genetiese lyn/gasheer spesie. Hierdie het getoon dat, alhoewel genetiese lyn meestal verantwoordelik was vir die waargeneemde variasie in KK profiele, faktore wat met verskille in gasheer spesies gepaard gaan ook ŉ effek gehad het. Sterk streeks-verbonde variasie wat beide die invloed van genetiese lyn, én faktore wat met verskille in gasheer spesie gepaard gaan, oortref het, was waargeneem in die KK profiele van die vy-wespe binne ŉ spesie-groep. Hierdie streeks-verbonde variasie in

KK profiele was ook waargeneem in twee bestuiwende vy-wespe, *Elisabethiella stuckenbergi* en *Ceratosolen capensis*, ŉ resultaat wat nie ondersteun was deur die genetiese bevolkingsdata nie (COI en Cytb). In werklikheid was baie min genetiese bevolkings-struktuur opgespoor binne die bestuiwer spesies, selfs as was die bestuiwer spesies regoor Suid-Afrika ingesamel. Die tekort aan genetiese struktuur in die vy-wesp bestuiwers kan die gevolg wees van hoë geenvloei wat veroorsaak word deur die hoë verspreidingskapasiteit van bestuiwende vy-wespe. Die resultate toon aan dat vy-wesp KK profiele die potensiaal besit om in chemotaksonomie gebruik te word, en word moontlik deur vy-wespe gebruik as kenmerke vir die herkenning van spesie en potensiële maats. Verder was daar gevind dat daar beide ŉ streekseffek en ŉ effek geassosieer met gasheer spesie op KK profiele was. Ons stel voor dat die waargeneemde streekseffek in hierdie studie toegeskryf kan word aan verskille tussen habitatte asook streeksverbonde verskille tussen vy-wesp gemeenskappe. Boonop kan die effek wat gasheer spesie op die KK profiele gehad het ŉ gevolg wees van dieetverskille tussen die galle in verskillende gasheer spesies. ŉ Moontlike gevolg van die waargeneemde streeks/gasheer-spesie-geassosieerde effek op vy-wesp KK profiele is dat dit moontlik kon lei tot voor-paring-isolasie binne vy-wesp spesies, wat uiteindelik spesiasie kon veroorsaak het. Daarbenewens wys ons resultate dat die interpretasie van die variasie in die vy-wesp KK profiel was afhanklik van die skaal van die analise: op ŉ breë interspesie vlak was die vy-wesp KK profiele spesie-spesifiek; op ŉ fyner intra-spesie vlak het variasie in KK profiele voorgekom tussen vy-wespe wat in verskillende streke ingesamel was; en op streeksvlak het variasie in die KK profiele binne spesie-groepe voorgekom tussen genetiese lyne/gasheer spesies. Toekomstige studies behoort te kyk na die toepassing van kutikulêre koolwaterstowwe in chemotaksonomiese studies van die vy-wesp filogenie, asook die effek wat vy-wesp gemeenskap samestelling het op vy-wesp kutikulêre koolwaterstowwe.

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Chapter 1. **General introduction**

1.1. Introduction

One of the most diverse groups of organisms on Earth are the insects. This diversity is particularly apparent for plant-feeding groups (Mitter *et al.*, 1988), and plant-insect interactions have been extensively investigated using phylogenetic approaches. Since the coevolution concept was first introduced (Ehrlich and Raven, 1964), studies were biased towards the search for strict, pairwise associations under the hypothesis of reciprocal adaptation (Brooks and Ferrao, 2005). The expectation of close congruence between host and host-dependent phylogenies has resulted in widespread testing to corroborate the conserved co-speciation predicted for many host-associate interactions, including the fig – fig wasp mutualism. Thus, numerous attempts have been made to match fig wasp phylogenies with those of their host trees to demonstrate close cospeciation between figs and fig wasps (Molbo *et al.*, 2003; Machado *et al.*, 2005; Marussich and Machado, 2007; Silvieus *et al*., 2007; Jousselin *et al.*, 2008). Instead, these studies indicated substantial variation in phylogenetic correspondence among different fig wasp clades (Marussich and Machado, 2007) that suggested processes other than cospeciation must be taken into account in order to explain fig wasp diversification. Processes such as host switching and extinction are potential explanations for the mismatches between fig wasp and host tree phylogenies (Brooks and Ferrao, 2005; Marussich and Machado, 2007; Hoberg and Brooks, 2008; Nyman, 2010; Warren *et al.*, 2010). Clarifying the specific processes involved in fig – fig wasp evolution has been exacerbated by difficulties in constructing a clear phylogeny, particularly for *Ficus*. Additionally, the systematic relationships among fig wasp groups have still not been fully resolved (Rasplus *et al.*, 1998). Possible reasons for this difficulty are that different species of fig wasps may often have highly similar morphologies (Weiblen, 2002; Cook and Rasplus, 2003), and that genetic studies used to determine taxonomy often don't yield consistent phylogenies (e.g. Rasplus *et al.*, 1998 vs. Weiblen, 2002). An additional method in clarifying phylogenetic relationships is the use of chemotaxonomy (Lockey, 1988; Blomquist and Bagnères, 2010). This method has been used successfully in many different insect groups (reviewed in Blomquist, 2010), and has yet to be applied to fig wasps.

We therefore aim to use a novel approach to investigate interactions amongst diverse species of fig wasps, belonging to one of the most prominent and well-studied plant-insect model systems in the literature, by focusing on chemical profiles of the fig wasp cuticle as well as the population genetic background of individual species.

1.2. Fig and fig wasp background

Trees from the genus *Ficus* of the family Moraceae occur across the world, consisting of over 850 species found in tropical, temperate as well as arid habitats (Janzen, 1979; Compton *et al*., 1994; Cook and Rasplus, 2003). In the form of trees, rock-splitters, shrubs, stranglers, epiphytes and vines (Janzen, 1979; Berg, 1989; Compton *et al*., 1994; Cook and Rasplus, 2003), these plants act as keystone species, providing habitats to numerous insect species as well as highly nutritious food in the form of figs to birds, mammals and insects (Janzen, 1979).

Fig trees are characterised by a distinctive pollination system and high pollinator specificity (Wiebes, 1979) that has evolved to provide these plants with an extremely successful pollination rate regardless of the number of different species of fig occupying a habitat (Janzen, 1979). This involves chemical signalling by the tree as well as the screening of potential pollinators, while restricting the access of other insects to the flowers (Janzen, 1979; Verkerke, 1989; Grison *et al.*, 1999). This system (which will be discussed in more detail later) allows fig trees to successfully cross-pollinate despite potentially long distances between individual trees or low concentrations of a specific fig species in a habitat (Janzen, 1979).

Although the *Ficus* phylogeny is still relatively unresolved, it is believed that Moraceae originated during the mid-Cretaceous period, with *Ficus* speciating during the Tertiary period and dispersing from Eurasia, where the oldest *Ficus* fossils are found, to Africa and the Americas (Zerega *et al.*, 2005). Today there are approximately 112 species of *Ficus* in the Afrotropical region (Africa and Madagascar including surrounding islands) (Berg and Wiebes, 1992; Compton *et al*., 1994; Burrows and Burrows, 2003) which are found in tropical and montane forest, wooded grassland and in transitional habitats (Berg, 1989, Berg and Wiebes, 1992; Burrows and Burrows, 2003). Of these 112 species, 25 are known to occur in South Africa (Berg and Wiebes, 1992; Burrows and Burrows, 2003; van Noort and Rasplus, 2004-

2011). These are mostly found in the humid north-eastern areas of Limpopo, Mpumalanga and Kwa-Zulu Natal and along the coast in the Western and Eastern Cape, although a few species also occur in the more arid regions of the Karoo and Kalahari (Compton and van Noort, 1992; Burrows and Burrows, 2003; Mcleish *et al.*, 2011).

1.2.1. Figs and fig wasp life history

Fig species can be either monoecious, with both male and female flowers borne on the same tree, or gynodioecious, with one type of tree bearing only female flowers and the other bearing both male and female flowers (Janzen, 1979; Verkerke, 1989; Cook and Rasplus, 2003), with most South African species being monoecious (Berg, 1989). The flowers are found inside a closed inflorescence called a fig or syconium. This makes it appear as though fig trees do not produce flowers, and that the "fruit" just magically appears without the need for pollination, causing the Chinese to call the fig the "flowerless fruit". In fact, the fig is pollinated by the females of tiny fig wasp species generally specific to the fig species in question, which crawl through a tiny bract-lined hole [the ostiole, which restricts access to the syconium interior (Verkerke, 1989)] in the base of the syconium with the aid of morphological adaptations, such as an elongated head (Cook and Rasplus, 2003). Once inside, the wasps oviposit by laying their eggs in the ovules of a portion of the female flowers while they pollinate the flowers (Verkerke, 1989; Compton *et al.*, 1994). Flowers that have received eggs develop galls which feed the developing fig wasp larvae instead of developing seeds (Compton and van Noort, 1992; Cook and Rasplus, 2003). Male fig wasps eclose before the females and search for galls containing female fig wasps, which they then chew open to mate with the females (Janzen, 1979; West *et al.*, 1996; Cook and Rasplus, 2003). After this the females will emerge and gather pollen before leaving the syconium through an exit hole that has been chewed by the males (Janzen, 1979; Compton *et al.*, 1994; West *et al.*, 1996; Cook and Rasplus, 2003). Non-pollinating fig wasps (NPFWs) have a similar life-cycle to the pollinators with some species entering the fig in the same way as the pollinators (internal gallers, Compton and van Noort, 1992; Cook and Rasplus, 2003) while others possess longer ovipositors that they use to oviposit through the syconium wall (external gallers, Janzen, 1979; Kerdelhué and Rasplus, 1996). Once the fig wasps have emerged from the syconium

they are attracted to fig-bearing trees of the same species by volatile organic compounds (VOCs) emitted by the tree (Chun *et al.*, 2009; Proffit *et al.*, 2009). After the fig wasps' emergence the figs ripen and the VOC blend of the tree changes to attract all manner of vertebrates who then consume the figs and distribute the seeds (Janzen, 1979; Verkerke, 1989; Compton *et al*., 1994).

1.2.2. Fig wasp taxonomy

The phylogeny of fig wasps is still uncertain and interpretations of it are constantly changing (Cook and Rasplus, 2003), with various revisions to relationships among groups and within groups within the last 13 years (Rasplus *et al.*, 1998; Marussich and Machado, 2007; Cruaud *et al.*, 2010). Disregarding phylogenetic relationships, fig wasps can be divided into three main behavioural groups: pollinating wasps, parasites and parasitoids, with the latter two grouping together as NPFWs. Both pollinating and parasitic fig wasps are gallers, as they lay their eggs in the flowers inside the syconium which causes a gall to develop, but unlike pollinators, parasitic galling fig wasps do not pollinate the fig in the process (Janzen, 1979; reviewed in Weiblen, 2002). They belong to the Epichrysomellinae, Otitesellinae, Sycoecinae and Sycophaginae (reviewed in Weiblen, 2002). Parasitoids (Sycoryctinae and Eurytomidae) will lay their eggs inside the galls of pollinators and non-pollinating gallers, preying on the larvae of other fig wasps (Janzen, 1979; Compton and van Noort, 1992; Weiblen *et al.*, 2001; Compton *et al.*, 2009; van Noort and Rasplus, 2004-2011). Including all of these behavioural groups, a single crop of figs on an individual tree can contain as many as thirty different fig wasp species (Compton and Hawkins, 1992; Cook and Rasplus, 2003). This means that a syconium can host many different combinations of fig wasps species, the composition of which will depend on many different factors, such as which fig wasps can oviposit in that species of fig, the dispersal ability of those fig wasps, whether fig wasp predators such as ants are present (Weiblen, 2002), as well as chance colonisation events (Hawkins and Compton, 1992) – whether a tree bearing figs are discovered by a specific species of fig wasp.

Studies investigating the origin of the fig – fig wasp mutualism generally find that the mutualism originated at least 60Myr ago (Rønsted *et al.*, 2005), with the oldest group of fig wasps associated with figs being the Agaoninae (Rasplus *et al.*, 1998; reviewed

in Weiblen, 2002). The oldest group of fig wasps colonising the syconium were therefore the pollinators, which were followed by multiple independent colonisations of figs by other Chalcidoidea lineages (which became the galling and parasitoid NPFWs; Rasplus *et al.*, 1998; Cook and Rasplus, 2003), indicating that fig wasps are not a monophyletic group (Rasplus *et al.*, 1998). The Chalcidoidea lineages that came after the pollinators then became the parasites of the figs, and were followed much later by a Chalcidoidea lineage (Sycoryctinae) that developed as parasitoids of the galling lineages (Silvieus *et al*., 2007).

1.2.3. Pollinator and NPFW specificity

A limited measure of coevolution and cospeciation is evident between figs and fig wasps in general (Machado *et al.*, 2001; Cook and Rasplus, 2003), and generally occurs in a more strict fashion between pollinating fig wasps and their host figs than between non-pollinating gallers or parasitoids and their fig hosts (Weiblen and Bush, 2002). Currently it is recognised that co-speciation between figs and fig wasps is much less strict than initially thought (Compton and van Noort, 1992; Mitter and Farrell, 1996; reviewed in Herre *et al.*, 2008), with authors like Lopez-Vaamonde and colleagues (2001) concluding that levels of co-speciation may range from 50% to 64%. The understanding of the evolutionary relationship between figs and fig wasps has evolved from an early assumption of strict co-speciation, 1-to-1 relationships and minimal host switching (Wiebes, 1979), to the current view that while co-speciation does occur between figs and fig wasps in the broader sense, host switching is much more common and host specificity more lax than originally thought (Marussich and Machado, 2007; reviewed in Herre *et al.*, 2008). In general it appears that the pollinators are the most host specific and tend to host switch the least, followed by the non-pollinating gallers, and that parasitoids show the least host specificity and can switch hosts more easily (Jiang *et al.*, 2006; Marussich and Machado, 2007; Silvieus *et al*., 2007). This situation also reflects the broader understanding of host-parasite systems in general, where both co-evolution and host switching plays a role in the evolutionary interactions between groups (reviewed in Hoberg and Brooks, 2008). Although parasitoids tend to be less host-specific than pollinators and other NPFWs, this does not mean that they can host switch indiscriminately, being constrained through niche saturation and competition (Jousselin *et al.*, 2008). The degree of host

specificity that exists in NPFWs may be maintained by the matching of ovipositor length to the thickness of the syconium wall, as well as differences in developmental time of the syconia between fig species (Janzen, 1979).

It is generally agreed that the reason for the closer phylogenetic association between the pollinators and figs is the result of the stricter requirements imposed by the syconium of the host tree on pollinators in order for successful oviposition to occur (Weiblen and Bush, 2002). The fact that a pollinator must have morphology adapted to a specific fig species to enter the syconium, as well as carry the correct pollen in order to fertilise the flowers used in oviposition, ties this group more closely to the correct host than non-pollinating gallers and parasitoids which can oviposit through the syconium wall (Weiblen and Bush, 2002; Silvieus *et al*., 2007). The ability to circumvent the barrier imposed by the syconium (Silvieus *et al*., 2007), as well as the fact that successful oviposition does not necessarily depend on pollination, allows externally galling NPFWs to switch hosts much more easily than the pollinators (Weiblen and Bush, 2002). A recent study on NPFWs have shown that host switching is extensive in the phylogeny of these groups, with most NPFW groups possessing at least one species that occurs on more than one fig species, and the NPFW community of any given fig species presenting a diverse complement of unrelated fig wasp species (Marussich and Machado, 2007).

In parasitoids, multiple host species use might even be necessary to survive, because if a parasitoid species were restricted to a single fig wasp species occurring on a single fig species, its fitness would not only depend on locating the correct fig-bearing species, but also on the probability that the fig wasp species it parasitizes is present in the syconia of the specific tree (Silvieus *et al*., 2007). In this case, being able to parasitize as wide a range of fig wasps as possible greatly increases its chances of successful oviposition.

When host switching occurs, it is probably not through the evolution of novel characteristics, but as a result of plesiomorphic characters allowing it to take advantage of a novel resource, through a process known as ecological fitting (Janzen, 1985; Brooks and Ferrao, 2005; Brooks *et al*., 2006). Since so much convergent adaptation has taken place in diverse Chalcidoidea lineages in order to inhabit the syconium (Cook and Rasplus, 2003), ecological fitting should play an especially large

role in host switching in this system. The relationships among fig wasps and their host should therefore be determined by a combination of diffuse co-evolution, host switching and extinction (Weiblen and Bush, 2002; Brooks *et al.*, 2006; Marussich and Machado, 2007).

1.2.4. Dispersal ability of pollinating fig wasps

It is not clear how far fig wasps can disperse the pollen from a fig since the minute size of the fig wasps make it hard to follow their movements after they have left the syconium. To date the best estimates – based on paternity tests of pollen arriving at receptive trees combined with the density of conspecifics in a habitat – indicated that fig wasps may regularly travel long distances between trees [up to 164.7 km has been recorded (Ahmed *et al.*, 2009)] and that pollen from a single tree can be dispersed across almost 300 square kilometres (Nason *et al.*, 1996, also reviewed in Herre *et al.*, 2008). These data suggest that, given the right conditions, fig trees in a population can maintain very high levels of gene flow. It is suggested that the long dispersal distances are achieved through the wind dispersal of fig wasps (Nason *et al.*, 1996; Ahmed *et al.*, 2009), which is highly likely considering their small size. Zavodna and colleagues (2005) found that high gene flow existed between island and mainland populations of fig wasps separated by 40 kilometres, and that in fact some mainland populations were more isolated than the island population. This indicates that environmental factors other than distance – such as habitat fragmentation and fig wasp life history traits – may play a more important role in the genetic population structure of fig and fig wasp populations (Zavodna *et al.*, 2005). Given the long potential dispersal distances and complex life history of fig wasps, it is important to define what constitutes a fig wasp population. We have defined it here as all the individuals belonging to the same species occurring within a region that fig wasps can potentially disperse across. Thus if two fig wasps of the same species emerging from the syconia of two different fig trees has the potential to arrive at the same tree to oviposit, they are considered to belong to the same population because their offspring will then have the chance to mate. Likewise, a region is then defined as an area that is broadly homogenous in habitat and of a particular size that fig wasps can conceivably disperse across (i.e. home to one fig wasp population).

1.3. Chemical ecology and cuticular hydrocarbons

1.3.1. Insect hydrocarbon background

Hydrocarbons (HCs) are chemicals produced by insects that consist of n-alkanes, alkenes and methylalkanes (Lockey, 1988; Lockey, 1991; Gibbs and Pomonis, 1995; Nation, 2002). When these chemicals occur on the insect's cuticle they are known as cuticular hydrocarbons (CHCs) and are often accompanied by ketones, esters, chemical waxes, alcohols, fatty acids, glycerides, sterols and aldehydes (Lockey, 1988; Gibbs and Pomonis, 1995; Nation, 2002; Leonhardt *et al.*, 2009; reviewed in Juárez and Fernández, 2007; Gołebiowski *et al.*, 2010), as well as other chemicals such as carboxylic acids and terpenoids (Leonhardt *et al.*, 2009). Collectively an insect's cuticular chemical complement is known as its CHC profile, and can be explored using gas chromatography to separate compounds and measure their relative intensity. Identification of these separated compounds can be achieved by the use of mass spectrometry.

In insects, HCs can be produced *de novo* in the oenocytes and transported to the cuticle via lipophorins through the pore canals (Diehl, 1975; Lockey, 1988), or obtained from environmental sources (such as diet). The main function of cuticular lipids is to prevent desiccation by water loss through the cuticle (Lockey, 1988; Gibbs and Pomonis, 1995; Blomquist *et al.*, 1998), but can also serve as pheromones (Nation, 2002) that assist in recognition of conspecifics (Lockey, 1988; Lockey, 1991; Blomquist *et al.*, 1998; Howard and Liang, 1993; Howard and Liang, 1993; Dapporto, 2007), nest-mates (Lorenzi *et al.*, 1996; Howard and Liang, 1993) or sex (Lockey, 1991; Howard and Liang, 1993; Lorenzi *et al.*, 1996; Dapporto, 2007; reviewed in Howard and Blomquist, 2005). Since cuticular lipids are non-volatile, direct contact needs to take place between individuals to assess CHCs (Lorenzi *et al.*, 1996).

1.3.2. CHCs in insect taxonomy

Cuticular hydrocarbons are already being used as chemical characters to identify and describe insect species and to help determine taxonomy (Jacob, 1979; Lockey, 1988; Copren *et al.*, 2005; Baracchi *et al.*, 2010; reviewed in Gołebiowski *et al.*, 2010). This is possible since many species investigated possess unique CHC profiles (Lockey, 1991; Lorenzi *et al.*, 1996; reviewed in Howard and Blomquist, 2005), and even

mirrors phylogeny in that closely related species have more similar CHC profiles than species that are more distantly related (Lockey, 1988). Examples include ants (Antonialli Jr. *et al.*, 2008), parasitoids such as *Roptrocerus xylophagorum* (Espelie *et al.*, 1996), *Muscidifurax* species (Geden *et al.*, 1998) and *Anagrus* species (Floreani *et al.*, 2006), termites (Haverty *et al.*, 1997; Copren *et al.*, 2005), cockroaches (Everaerts *et al.*, 1997), stingless bees (Leonhardt *et al.*, 2009), moths (Lavine and Carlson, 1991) and hover wasps (Baracchi *et al.*, 2010). CHCs are a simple way to differentiate between cryptic species (Lavine and Carlson, 1991; Haverty *et al.*, 1997). However, when using CHC profiles as a character in taxonomy, care must be taken because although a CHC profile is unique to a species this does not mean that there is no intraspecies variation (Gibbs and Pomonis, 1995).

1.3.3. Influences on CHCs

As with many other characteristics, various factors can influence the CHC profile of an individual, and in many cases it will be determined by a combination of genetic and environmental factors (Lorenzi et al., 1996; Dapporto et al., 2004). However, it appears that CHCs are often synthesised by the insect itself and in that instance composition should therefore be largely determined by genetics (Lockey, 1988; Blomquist et al., 1998). CHC variation has often been linked with genetic variation (Page et al., 2002; Dronnet et al., 2006), and it has been found that CHC profiles can differ between populations or colonies within a species (Howard and Liang, 1993; Wagner et al., 2001; Leonhardt et al., 2009). In some species however, environmental factors seem to play a large role in the CHC composition – however, the extent to which the CHC profiles are influenced by an insect's environment seems to differ from species to species (Chapman et al., 1995; Liang and Silverman, 2000), and thus no assumptions of environmental influence should be made without thorough investigation. Adsorption of hydrocarbons from the environment has also been cited as the cause of variation in the CHC profile in some ants (Nowbahari *et al.*, 1990; Nielsen *et al.*, 1999). Other species that have been found to have CHC profiles influenced by the environment include stingless bees (Ferreira-Caliman *et al.*, 2010) and hover wasps (Baracchi *et al.*, 2010). Contact between insects in close proximity can also transfer CHCs between species [e.g. between termite species (Vauchot *et al.*, 1996; Vauchot *et al.*, 1998) and between cockroach species (Everaerts *et al.*, 1997)],

resulting in an altered CHC profile. If the species are separated, they lose CHCs acquired from the other species and regain their unique CHC profile, indicating that such acquisition of hydrocarbons is temporary. Finally, CHCs can also be influenced by diet (Lockey, 1988; Etges and Ahrens, 2001). Ants specifically appear to be able to assimilate prey hydrocarbons into their own CHC profile (Liang and Silverman, 2000; Richard *et al.*, 2004; Buczkowski *et al.*, 2005), but phytophagous insect CHCs are also affected by which species of plant they eat – for example *Drosophila mojavensis* and *Drosophila arizonae* raised on different species of cactus exhibit significantly different CHC profiles (Stennett and Etges, 1997). The ability to assimilate dietary hydrocarbons is not common to all species, with diet not influencing CHC profiles in grasshoppers (Chapman *et al.*, 1995). Pollinating and non-pollinating galler fig wasp larvae feed off the endosperm of the fig seed or a fig ovary that has formed a gall (Verkerke, 1989; Compton and van Noort, 1992; Weiblen, 2002), while parasitoid fig wasps can also potentially feed on pollinator and non-pollinating galler fig wasp larvae as well as plant tissue (Weiblen, 2002). In the ant *Acromyrmex subterraneus subterraneus* groups fed on different plants developed significant differences in CHC profile (Richard *et al.*, 2004), which makes it possible that fig wasp CHCs may be affected by the plant tissue they feed on, which in turn depends on the host species. These environmental influences on the cuticular chemicals of insects (adsorption and diet) means that there will always be some potential for intraspecies variation despite the distinct species-specificity of CHC profiles found in many insects.

1.4. Motivation

Being a model mutualism, figs and fig wasps have been intensively studied, and the literature on this system is extremely extensive (Janzen, 1979; Wiebes, 1979; Verkerke, 1989; Compton and van Noort, 1992; Weiblen, 2002; Cook and Rasplus, 2003; Silvieus *et al*., 2007, Herre *et al*. 2008). Despite this, information on fig wasp CHCs is lacking, making this an ideal opportunity to investigate the CHCs of both pollinating and non-pollinating galler fig wasps. Knowledge gained from this study has the potential to aid taxonomists to clarify fig wasp taxonomy through the use of chemical characteristics. Additionally, CHC variation in fig wasps could be used to infer finer-scale interactions between the fig wasp and its environment. From other insect studies, we predict that fig wasps will also possess species-specific CHC

profiles (Lockey, 1988; Lorenzi *et al.*, 1996; Gołębiowski *et al.*, 2010). However, from the literature on CHCs in insects that has been reviewed here, it is clear that various ecological factors can influence the CHC profiles of insects, making it highly likely that intraspecies variation may exist in fig wasps was well. Consequently, we not only investigated inter-species CHC differences but also how environment, fig wasp host species and population genetic structure may influence intra-species CHC variation.

1.5. Objectives

Our main objectives are to determine species-specificity of fig wasp CHCs, as well as exploring the variation in CHC profiles within and between fig wasp species and species-groups. We have made use of three pollinator fig wasp species and two NPFW species-groups. The pollinator species include *Elisabethiella stuckenbergi* (host tree *Ficus burkei*), *Elisabethiella glumosae* (host tree *Ficus glumosa*) and *Ceratosolen capensis* (host tree *Ficus sur*). The two NPFW species-groups occur within the genus *Otitesella*; these are the *Otitesella sesquianellata* species-group (host trees *Ficus glumosa*, *Ficus burtt-davyi*, *Ficus burkei*, *Ficus polita* and *Ficus lutea*, hereafter referred to as the Sesqui species-group) and the *Otitesella uluzi* species-group (host trees *Ficus glumosa*, *Ficus burtt-davyi* and *Ficus burkei*, hereafter referred to as the Uluzi species-group).

The CHCs of these five wasp groups will be examined to determine:

a) Whether fig wasps possess species-specific or species-group-specific CHC profiles by comparing the CHC profiles of three fig wasp species and two fig wasp species-groups (Chapter 2), and identifying as many CHCs as possible;
b) How much intra-species variation is caused by:
   i. Host tree species, by comparing the CHC profiles of fig wasps that occur on more than one host tree (Chapter 3), and
   ii. Genetic population structure, geographic distance and habitat, by comparing the intra-species-group variation in two NPFW species-groups (Chapter 3) as well as the intra-species variation in two pollinating fig wasp species distributed from the Western Cape to Kwa-Zulu Natal (Chapter 4).

Lastly, these results will be integrated and interpreted in a general discussion (Chapter 5).

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Chapter 2. **Species- and species-group-specific cuticular hydrocarbon profiles of South African fig wasps.**

2.1 Introduction

Cuticular hydrocarbons have successfully been used to distinguish between species and are considered a reliable trait in chemotaxonomy (Jacob, 1979; Nation, 2002; Blomquist and Bagnères, 2010; Gołębiowski *et al.*, 2010), with chemical investigations of the cuticular hydrocarbons of insect species revealing that cuticular hydrocarbon (CHC) profiles are species-specific (Carlson and Service, 1980; Lockey, 1988; Lavine and Carlson, 1991; Lorenzi *et al*., 1996; Sutton *et al.*, 1996; Everaerts *et al.*, 1997; Haverty *et al.*, 1997; Geden *et al.*, 1998; Singer *et al*., 1998; Howard and Blomquist, 2005; Dapporto, 2007; Juárez and Fernández, 2007; Antonialli Jr. *et al.*, 2008; Thomas and Simmons, 2008; Leonhardt *et al.*, 2009; Baracchi *et al.*, 2010). This is a reflection of the strong genetic basis for the composition of the cuticular lipids (Lockey, 1988; Blomquist *et al*., 1998), with insects synthesising many of the hydrocarbons found on their cuticles (Lockey, 1988; Blomquist and Bagnères, 2010). A species also possess stability in their CHC profile over time, as has been shown for hornets, with recent collections having similar species-specific profiles to 20-year-old museum samples (Martin *et al.*, 2009, but see Richard *et al.*, 2004). As a result of this genetic determinism and stability of an insect's CHC profile, chemotaxonomic studies have found that interspecies variation in CHC profiles tend to match the phylogenetic relationship among species (Bagnères and Wicker-Thomas, 2010), with species that are more closely related having more similar CHC profiles (Lockey, 1988; Sutton *et al.*, 1996; Copren *et al.*, 2005). However, even though CHC profiles are largely conserved within a species, variation does occur in these profiles according to colony membership, geographic distance (regional patterns, as has been observed for social wasps) as well as genetic distance (Lorenzi *et al*., 1996; Singer *et al*., 1998; Dapporto *et al.*, 2004a; Dapporto *et al.*, 2004b; Tannure-Nascimento *et al.*, 2007; Dapporto *et al.*, 2009; Baracchi *et al.*, 2010). Here we examine the CHC profiles of three pollinating fig wasp species (*Elisabethiella stuckenbergi*, *Elisabethiella glumosae* and *Ceratosolen capensis*) as well as the variation in CHC profiles within two NPFW species-groups (*Otitesella*). In a study that investigated the *Otitesella* genus in Africa, Jousselin and colleagues (2006) concluded that the fig wasps from this genus could be

divided into two distinct monophyletic species-groups (the Sesqui species-group and Uluzi species-group). The fig wasps within each of these two species-groups have highly similar phenotypes (Jousselin *et al.*, 2006). Moreover, both the Uluzi and Sesqui species-groups within *Otitesella* usually inhabit the same fig tree species. Jousselin *et al.* (2006) demonstrated that the two species-groups have independently diversified and radiated across their host fig species section *Galoglychia* within the genus *Ficus*, resulting in the presence of two parallel lineages of *Otitesella* species across many of the African fig species. This sets the stage for many interesting chemical studies.

Phylogenetic studies have indicated that the pollinating fig wasps are a monophyletic group, and that the genus *Elisabethiella* within the pollinators is also monophyletic (Cruaud *et al.*, 2010). Likewise, *Otitesella* is also a monophyletic genus, but it is genetically far removed from the pollinators as it forms part of the second wave of Chalcidoidea that began specialising on *Ficus* after the mutualistic relationship with the pollinators was formed (Rasplus *et al.*, 1998). Since CHC profiles potentially correspond to phylogenetic relationships among species, we predict that the CHC profiles of the three pollinator species will be more similar to each other than to the *Otitesella* genus, and the two pollinators from the *Elisabethiella* genus would probably have CHC profiles that show a high level of convergence due to their congeneric relationship. In contrast, we expect to see less convergence in the CHC profiles of the two *Otitesella* species-groups, since they each include a number of undescribed species (Jousselin *et al.*, 2006, S. van Noort, personal communication). Having said this however, we do predict that the CHC profiles of species within these two species-groups would be more similar to each other than to the pollinator CHC profiles.

Chemical information has the potential to complement information obtained from genetic analysis (Copren *et al.*, 2005). The genetic investigation of fig wasp groups has been extensive (Compton and van Noort, 1992; Rasplus *et al.*, 1998; Machado *et al.*, 2001; Weiblen, 2002; Cook and Rasplus, 2003; Marussich and Machado, 2007; Cruaud *et al.*, 2010), however, concomitant chemical investigations are lacking. Therefore, investigations of the chemical composition of fig wasp cuticles are long overdue, and have the potential to be used in taxonomic studies incorporating both

genetic and chemical approaches. Thus we investigated the composition of fig wasp cuticular hydrocarbons and tested the hypothesis that fig wasps possess species-specific CHC profiles. Further, we aimed to identify the compounds responsible for the similarity within species and species-groups as well as the dissimilarity between species and species-groups.

2.2 Materials and Methods

2.2.1 Fig wasp collection and study sites

All fig wasps were collected within South Africa. Collection sites included Cape Town, Stellenbosch and Clanwilliam in the Western Cape Province, the Baviaanskloof Nature Reserve in the Eastern Cape Province, and Ithala Game Reserve, Mtunzini and Mabibi Nature Reserve in Kwa-Zulu Natal Province (Figure 2.1; voucher specimens are lodged at the Iziko Museum Cape Town). The pollinators *Ceratosolen capensis*, *Elisabethiella stuckenbergi* and *Elisabethiella glumosae* were collected from their host trees *Ficus sur*, *Ficus burkei* and *Ficus glumosa*, respectively. Non-pollinating galler fig wasps were also collected from both the Uluzi and Sesqui species-groups within the genus *Otitesella*. Only two described species from *Otitesella* formed part of these collections: *Otitesella uluzi*, belonging to the Uluzi species-group and collected from *Ficus burtt-davyi*, and *Otitesella sesquianellata*, belonging to the Sesqui species-group and also collected from *F. burtt-davyi*. Undescribed species belonging to the Uluzi species-group were collected from the host trees *F. glumosa* and *F. burkei*, and undescribed species belonging to the Sesqui species-group were collected from host trees *F. glumosa*, *F. burkei*, *Ficus polita* and *Ficus lutea*. The majority of species within the genus *Otitesella* are undescribed and due to this taxonomic uncertainty (both of the species-groups contain multiple undescribed species, Jousselin *et al.*, 2006; Simon van Noort, personal communication), investigation of CHC profiles was at the species-group level. Consequently all fig wasps from the Uluzi species-group were grouped together (containing the described species *Otitesella uluzi* as well as an unknown number of undescribed species) and all fig wasps from the Sesqui species-group were grouped together (containing the described species *Otitesella sesquianellata* as well as an unknown number of undescribed species). Collection data for all sites are given in Table 6.1a and b (Appendix). Analyses at species level within the two *Otitesella*

species-groups associated with *F. burtt-davyi* and *F. glumosa* are investigated in Chapter 3.

Collections were carried out from March 2010 to December 2010 and GPS coordinates were recorded for all sample sites (see Figure 2.1). Female fig wasps were collected by picking figs as the fig wasps were starting to emerge from the figs. Figs were placed in a container made from a cardboard cylinder connected to a plastic funnel that lead to a sealed plastic jar which trapped emerging fig wasps. After the fig wasps emerged, jars were placed for 5 minutes in a freezer (-20°C) to slow the wasps down to allow easy handling, after which they were identified under a stereomicroscope (Wild Heerbrugg, Switzerland). *C. capensis*, *E. stuckenbergi*, *E. glumosae*, *O. uluzi* and *O. sesquianellata* were identified to species level, and fig wasps from *F. glumosa*, *F. burkei*, *F. polita* and *F. lutea* that belonged to the genus *Otitesella* were identified to species-group level.

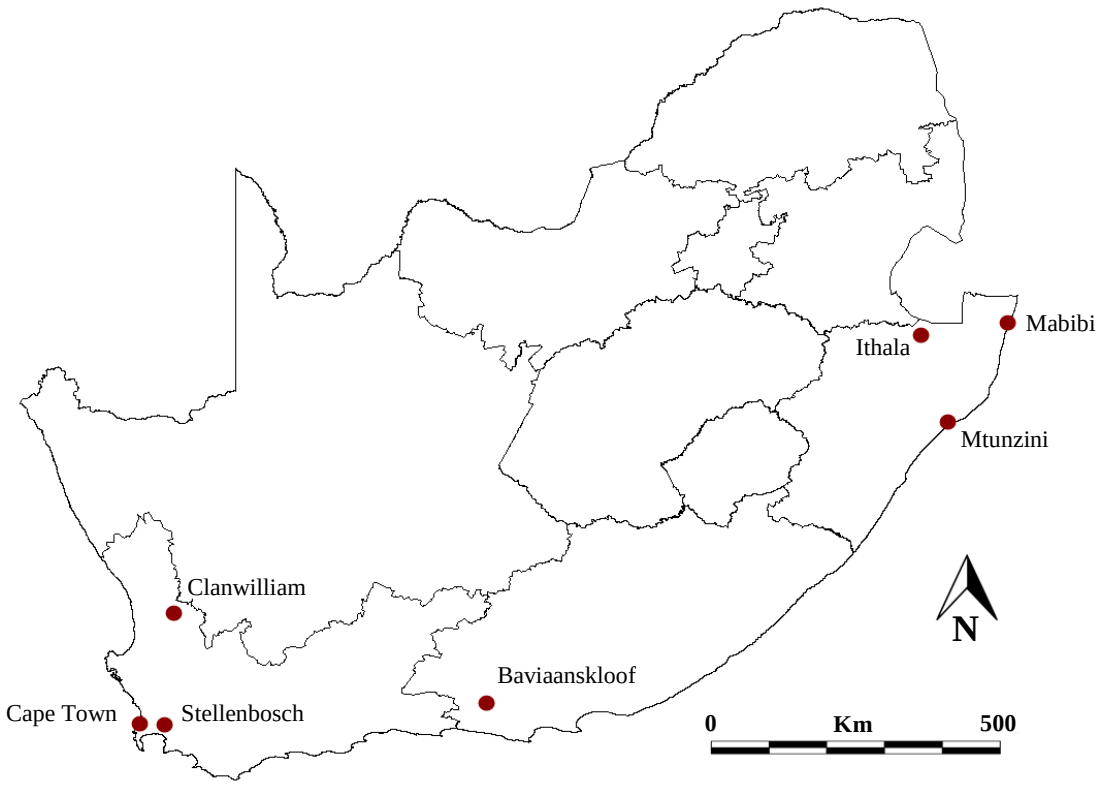


Figure 2.1: A map of South Africa indicating the regions where fig wasps were collected. Cape Town (S33°55.575 E18°25.040), Stellenbosch (S33°55.892 E18°52.391) and Clanwilliam (S32°03.933 E19°04.802) in the Western Cape Province, Baviaanskloof Nature Reserve (S33°32.335; E23°57.813) in the Eastern Cape Province, and Ithala Game Reserve (S27°31.318; E31°13.341), Mabibi Nature Reserve (S27°22.846; E32°42.839) and Mtunzini (S28°57.666; E31°45.312) in Kwa-Zulu Natal Province.

Optimally, a sample consisted of 10 individuals from the same species (except in a few instances where very few fig wasps were available), washed in a glass vial (2ml; Chemetrix [Pty] Ltd.) containing either 100 µl (in the lab) or 200 µl (in the field, to compensate for increased evaporation of the solvent in the field) of hexane for 10 minutes. At the end of 10 minutes vials were briefly agitated before fig wasps were removed to produce a cuticular lipid extract.

2.2.2 Gas Chromatography/Mass Spectrometry (GC/MS) analyses

The samples containing the cuticular lipid extract were concentrated under a stream of pure nitrogen to a volume of 10µl. This was done by measuring the level of 10µl of solvent in a glass insert (200µl; Chemetrix [Pty] Ltd.) and evaporating all samples to the same level. One micro litre of the concentrated sample was injected into an Agilent 6850 Gas Chromatograph to measure the hydrocarbon profiles of the fig wasps. The gas chromatograph was fitted with a splitless inlet, flame-ionisation detection and a HP-1 capillary column (30m x 0.32mm x 0.25µm film thickness, Agilent Technologies, CA). The injection port was set at 290^{o}C and the detector at 320^{o}C. Helium acted as the carrier gas at a flow rate of 60.4ml/min with Nitrogen acting as the make-up gas. The temperature was programmed as follows: 2 min at 80^{o}C, increased to 200^{o}C at a rate of increase of 15^{o}C/min, and then a further increase to 310^{o}C at a rate of increase of 5^{o}C/min, where the temperature remained for 10 min. Gas chromatograms of chemicals were generated using GC ChemStation software (Rev. A.09.03, Agilent Technologies, 1990-2002).

Representative samples of each species were analysed by GC/MS using an Agilent 5975 Mass Spectrometer fitted with a HP-1MS capillary column (30m x 0.25mm x 0.25µm film thickness, Agilent Technologies, CA). An authentic C_7 – C_{40} straight-chain hydrocarbon series (Supelco® Analytical, Bellefonte, USA) was used as a standard to identify n-alkanes and to link the retention times in our dataset with Kováts Indices (I). The Kováts Index of a chemical compound is a measure of its retention time in relation to the retention times of a standard series of straight-chain hydrocarbons [e.g. if a compound elutes between heneicosane (C_{21}, I = 2100) and docosane (C_{22}, I = 2200) its Kováts Index will fall between 2100 and 2200] (McNaught and Wilkinson, 1997). Non-straight-chain hydrocarbons were then identified by first matching the mass spectrum of a peak to library records of mass

spectra [Wiley; NIST (National Institute of Standards and Technology, USA)] and then confirming the match by comparing the library Kováts Index with the actual Kováts range indicated by the retention time of the peak in relation to the retention times of the straight-chain hydrocarbon series.

2.2.3 Statistical analyses

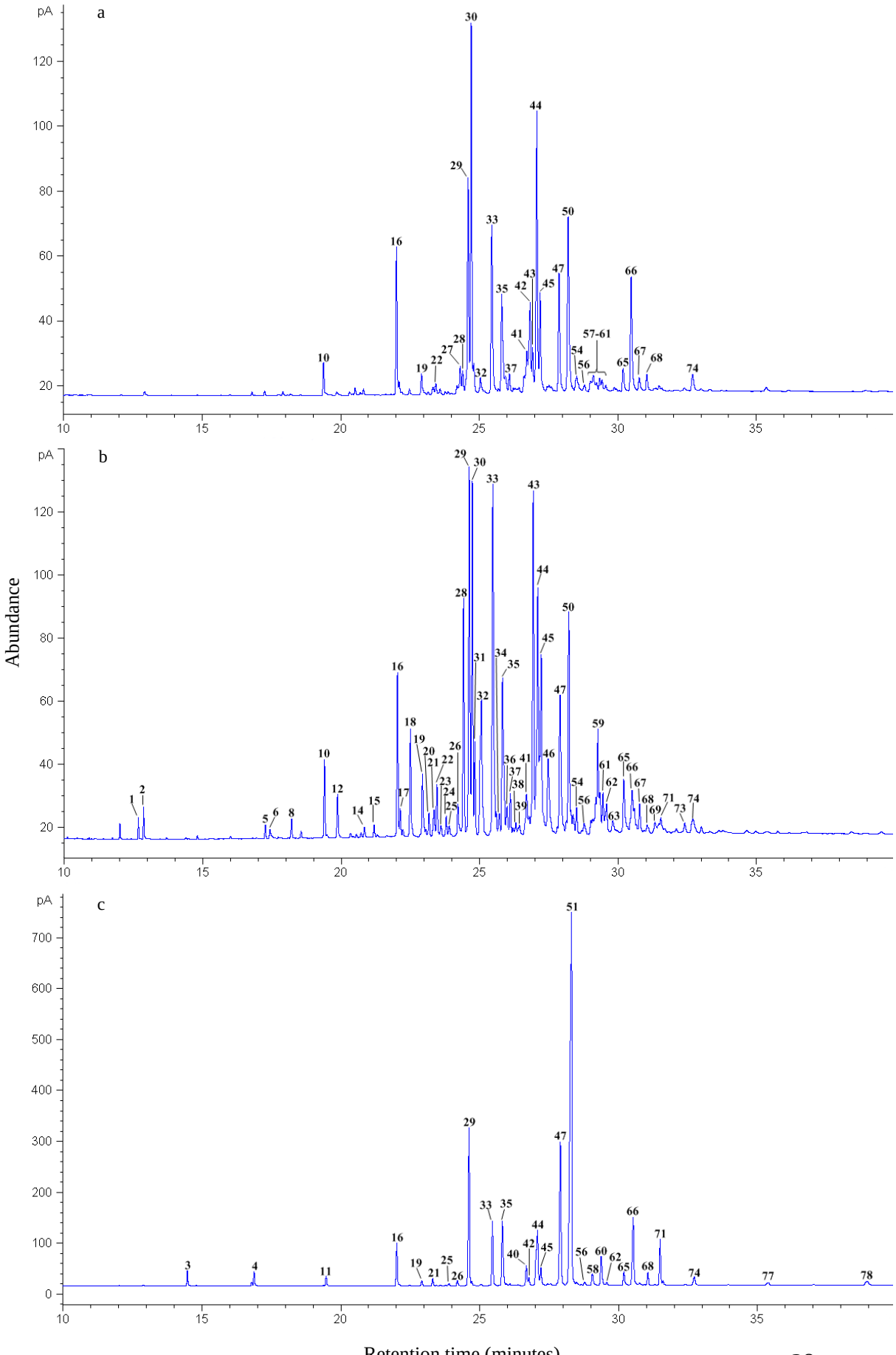
Gas chromatograms as well as mass spectra of individual peaks were examined for contaminants, which were removed from the dataset prior to analyses. For analyses in SPSS (PASW Statistics 18 version 18.0.0, 2009), individual compounds were standardised as the percentage contribution to the total hydrocarbon blend for the sample in question, after which the peak areas were transformed to log contrasts using the Aitchison's formula $Z_{ij} = \ln[Y_{ij}/g(Y_j)]$ (Aitchison, 1986), with Zij being the standardised peak area for i, Yij being the peak area of individual j, and $g(Yj)$ being the geometric mean of all peaks for j. Compounds that occurred in extremely low frequencies across all samples were excluded from analyses. Principal Component Analysis (PCA with varimax rotation) was performed to reduce the number of variables, followed by Canonical Discriminant Analysis (DA).

For analyses in PRIMER (Plymouth Routines in Multivariate Ecological Research, version 5.2.9, 2004: Plymouth Marine Laboratory, UK) individual compounds were standardised as the percentage contribution to the total hydrocarbon blend for the sample in question, and were then transformed using double square root transformations. This was done to ensure that equal weightings were given to compounds that were rare or only occurred in very low concentrations. Compounds that occurred in extremely low frequencies throughout the dataset were excluded from the analyses. Analysis of similarity (ANOSIM, 999 permutations from a random sample of total possible permutations) based on a dissimilarity matrix calculated with Bray-Curtis coefficients (Bray and Curtis, 1957) was used as a measure of the differences between fig wasp species CHCs and to determine the statistical significance of pairwise comparisons. A Global R value (produced by ANOSIM) approaching 1 indicates that groups are more dissimilar, while a Global R approaching 0 indicates that groups are more similar (R values can range from -1 to 1, Chapman and Underwood, 1999). Sequential Bonferroni correction was used to determine significant α levels for multiple pairwise comparisons.

PRIMER's SIMPER (Similarity percentages) was used to identify individual compounds responsible for within-species similarity and between-species dissimilarity. This test also yields the ratio of the mean contribution of a compound to the standard deviation of its contribution, which gives an indication of how consistently a compound contributes to similarity within a group or differences between groups, as the case may be – the higher the ratio, the more consistent the contribution of the compound (Wossler and Crewe, 1999).

2.3 Results

Sixty-four compounds were positively identified using GC/MS. These included straight-chain alkanes (C_{23-38}), branched alkanes, alkenes, esters and alcohols (Table 2.1). In addition to these chemical classes, two triterpenes (α-amyrin and β-amyrin acetate), a cycloalkane (cyclotetracosane), a branched alkene [2,6,10,15,19,23-hexamethyl-(all-E)-2,6,10,14,18,22-tetracosahexaene, aka squalene], two aldehydes (1-octacosanal and hexacosanal) and an amide (Z-9-octadecenamide) were identified. Representative gas chromatograms of each of the five groups of fig wasp investigated are shown in Figure 2.2.



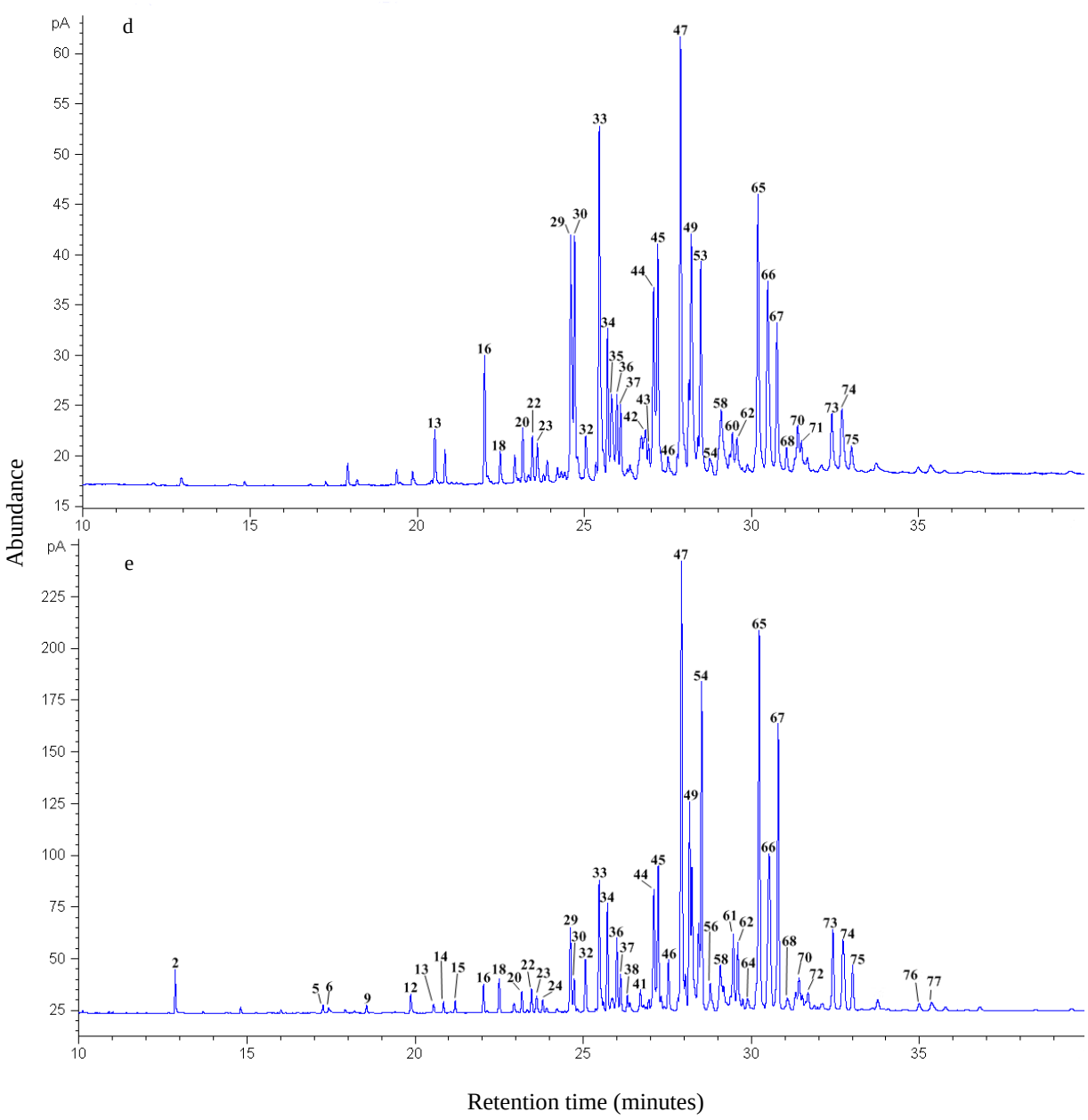


Figure 2.2: Representative gas chromatograms of samples from *E. stuckenbergi* (a), *E. glumosae* (b), *C. capensis* (c), the Uluzi species-group (d, host tree *F. burkei*) and the Sesqui species-group (e, host tree *F. burkei*). Numbered compounds are identified in Table 2.1.

Table 2.1: The 64 cuticular compounds identified and their percentage contribution (Mean and SE), in the cuticular extract of *E. stuckenbergi*, *E. glumosae*, *C. capensis*, the Uluzi species-group and Sesqui species-group. Fourteen chemicals that could not be identified (most often due to low quality matches of mass spectra in the mass spectra libraries) are also included and are given as either "unknown" or "branched alkane" (if they could be identified to chemical class). Superscripts are used to differentiate between different unknown chemicals and different unknown branched alkanes. Peak numbers correspond to numbered peaks in Figure 2.2.

Peak	Compound	*E. stuckenbergi*			*E. glumosae*			*C. capensis*			Uluzi species-group			Sesqui species-group		
		Mean	±	SE	Mean	±	SE	Mean	±	SE	Mean	±	SE	Mean	±	SE
1	unknown[1]		–		0.03	±	0.02	0.07	±	0.01		–			–	
2	dibutyl-1,2-benzene dicarboxylate	0.10	±	0.05	1.39	±	0.10	0.06	±	0.02	1.03	±	0.15	1.30	±	0.16
3	10-heneicosene		–			–		4.68	±	0.64		–			–	
4	Z-9-tricosene		–			–		1.24	±	0.17		–		0.22	±	0.05
5	tricosane	0.05	±	0.02	0.25	±	0.04	0.07	±	0.02	0.08	±	0.02	0.17	±	0.05
6	Z-9-octadecenamide	0.10	±	0.05	0.59	±	0.07	0.15	±	0.03	0.60	±	0.12	0.49	±	0.07
7	2,4-dimethyldocosane		–			–		0.03	±	0.01		–		0.04	±	0.01
8	1 or 9-tetracosene		–		0.12	±	0.03	0.17	±	0.05	0.18	±	0.12	0.15	±	0.09
9	tetracosane	0.02	±	0.01	0.19	±	0.03	0.02	±	0.01	0.09	±	0.02	0.15	±	0.08
10	11-butyldocosane	0.05	±	0.02	1.02	±	0.08	0.14	±	0.02	0.74	±	0.11	0.32	±	0.07
11	Z-12-pentacosene		–			–		0.29	±	0.05		–		0.35	±	0.16
12	pentacosane	0.05	±	0.02	0.97	±	0.02	0.25	±	0.06	0.58	±	0.08	0.82	±	0.10
13	5-butyldocosane		–			–		0.01	±	0.00	0.02	±	0.01	0.12	±	0.04
14	3-ethyltetracosane	0.01	±	0.01	0.04	±	0.02	0.01	±	0.01	0.13	±	0.03	0.35	±	0.07
15	hexacosane	0.03	±	0.01	0.35	±	0.04	0.02	±	0.01	0.25	±	0.04	0.38	±	0.07
16	1-heptacosene	5.33	±	0.41	2.27	±	0.14	1.86	±	0.06	0.76	±	0.12	0.69	±	0.11
17	9-heptacosene	0.06	±	0.02	0.48	±	0.04	0.32	±	0.10	0.19	±	0.04	0.54	±	0.17
18	heptacosane	0.07	±	0.03	1.93	±	0.08	0.10	±	0.01	0.78	±	0.10	1.06	±	0.08
19	7-hexyldocosane	0.08	±	0.04	0.94	±	0.11	0.21	±	0.03	0.21	±	0.05	0.26	±	0.05
20	unknown[2]		–		0.38	±	0.03		–		0.10	±	0.04	0.17	±	0.04
21	unknown[3]	0.55	±	0.08	0.34	±	0.05	0.27	±	0.03	0.05	±	0.02	0.10	±	0.02
22	n-octacosene	0.07	±	0.03	0.80	±	0.05	0.16	±	0.04	0.28	±	0.04	0.93	±	0.10
23	9-octyleicosane		–		0.26	±	0.03	0.03	±	0.01	0.07	±	0.04	0.04	±	0.01

Peak	Compound	*E. stuckenbergi*			*E. glumosae*			*C. capensis*			Uluzi species-group			Sesqui species-group		
		Mean	±	SE	Mean	±	SE	Mean	±	SE	Mean	±	SE	Mean	±	SE
24	octacosane	0.11	±	0.04	0.61	±	0.05	0.18	±	0.03	0.30	±	0.04	0.44	±	0.05
25	squalene	0.01	±	0.01	0.35	±	0.08	0.44	±	0.06	0.40	±	0.09	1.19	±	0.43
26	3,7-dimethylheptacosane	0.99	±	0.11	0.47	±	0.06	0.10	±	0.01	0.07	±	0.02	0.09	±	0.03
27	1-pentacosanol	0.78	±	0.08		–			–			–		0.05	±	0.02
28	hexacosanal	4.38	±	0.81	3.96	±	0.37		–		1.13	±	0.30	0.94	±	0.31
29	Z-9-nonacosene	15.77	±	1.00	6.34	±	0.50	7.51	±	1.04	3.68	±	0.23	1.14	±	0.25
30	1-nonacosene	13.01	±	1.28	0.69	±	0.48	7.37	±	0.75		–		0.92	±	0.42
31	cyclotetracosane	0.10	±	0.04	1.80	±	0.09		–		0.05	±	0.02	1.21	±	0.57
32	nonacosane	0.16	±	0.07	4.68	±	0.10	0.33	±	0.05	1.64	±	0.15	1.94	±	0.12
33	Z-14-nonacosene	0.58	±	0.25	6.29	±	0.39	1.57	±	0.16	1.62	±	0.19	1.69	±	0.26
34	branched hexacosane	6.84	±	0.35	0.72	±	0.02	2.22	±	0.32	0.37	±	0.09	0.94	±	0.20
35	n-methylnonacosane	1.39	±	0.13	2.92	±	0.15	2.32	±	0.19	1.03	±	0.09	0.52	±	0.12
36	n-dimethylnonacosane	0.99	±	0.06	1.24	±	0.06	0.84	±	0.12	1.28	±	0.16	1.03	±	0.10
37	n-triacontene	0.05	±	0.02	2.05	±	0.16	0.39	±	0.05	0.46	±	0.07	0.44	±	0.10
38	triacontane	0.06	±	0.02	0.60	±	0.07	0.14	±	0.01	0.36	±	0.05	0.43	±	0.06
39	11-decylheneicosane	0.01	±	0.00	0.66	±	0.06	0.11	±	0.02	0.54	±	0.12	0.15	±	0.04
40	1-heptacosanol	2.63	±	0.27	0.29	±	0.11	0.61	±	0.09	0.00	±	0.00		–	
41	9-hentriacontene	3.42	±	0.68	0.94	±	0.12	1.24	±	0.06	0.31	±	0.04	0.21	±	0.05
42	1-octacosanal	4.84	±	0.48	0.84	±	0.05	0.03	±	0.02	0.27	±	0.04	0.32	±	0.07
43	branched alkane[1]	5.72	±	1.08	6.02	±	0.54	0.01	±	0.01	1.27	±	0.25	2.64	±	0.90
44	branched alkane[2]	10.66	±	0.44	10.06	±	0.49	5.64	±	0.29	10.34	±	0.76	1.81	±	0.34
45	n-hentriacontene	0.51	±	0.21	5.18	±	0.16	1.25	±	0.14	3.07	±	0.40	4.00	±	0.62
46	hentriacontane	0.13	±	0.05	0.17	±	0.17	0.25	±	0.04	1.79	±	0.09	1.41	±	0.10
47	11-decyldocosane	0.55	±	0.24	6.08	±	0.25	4.51	±	0.53	5.13	±	0.53	3.95	±	0.64
48	unknown[4]		–		0.02	±	0.02	0.03	±	0.02	0.10	±	0.04	1.08	±	0.19
49	9-octyltetracosane	9.94	±	1.10	0.03	±	0.03		–		3.21	±	0.28	2.91	±	0.54
50	unknown[5]	0.57	±	0.25	4.68	±	0.16	16.71	±	2.73	4.62	±	0.88	1.97	±	0.27
51	unknown[6]	0.02	±	0.02		–		12.92	±	2.13	3.44	±	0.74	0.02	±	0.02

Peak	Compound	*E. stuckenbergi*			*E. glumosae*			*C. capensis*			Uluzi species-group			Sesqui species-group		
		Mean	±	SE	Mean	±	SE	Mean	±	SE	Mean	±	SE	Mean	±	SE
52	3-methylhentriacontane	0.13	±	0.08	1.21	±	0.09	9.30	±	1.80	0.15	±	0.14		–	
53	3,11-dimethylnonacosane	1.39	±	0.11	0.10	±	0.10	1.24	±	0.52	3.75	±	0.59	2.55	±	0.34
54	unknown[7]	0.07	±	0.03	1.19	±	0.11	0.37	±	0.04	2.36	±	0.38	1.45	±	0.27
55	dotriacontane	0.44	±	0.05	0.15	±	0.08	0.47	±	0.05	0.24	±	0.04	0.30	±	0.07
56	branched alkane[3]	0.02	±	0.01	0.96	±	0.13	0.45	±	0.05	5.30	±	0.82	1.33	±	0.25
57	1-hentriacontanol	0.64	±	0.15	0.97	±	0.11	0.76	±	0.11	0.06	±	0.03		–	
58	n-methyldotriacontane	1.95	±	0.18	0.06	±	0.04	0.77	±	0.07	0.98	±	0.13	1.03	±	0.15
59	methyl branched ester of carboxylic acid	0.62	±	0.14	1.17	±	0.23		–		2.05	±	0.28	1.99	±	0.58
60	10-methyldotriacontane	1.31	±	0.15	0.52	±	0.13	2.10	±	0.12	0.45	±	0.11	1.41	±	0.49
61	dodecyl decanedioate	0.41	±	0.07	1.68	±	0.08	0.23	±	0.03	4.74	±	0.35	1.58	±	0.21
62	n-tritriacontene	0.04	±	0.02	1.51	±	0.05	0.22	±	0.02	2.72	±	0.20	1.73	±	0.16
63	α-amyrin	0.16	±	0.04	1.01	±	0.08	0.04	±	0.01	0.57	±	0.13	0.31	±	0.06
64	tritriacontane	0.03	±	0.01	0.04	±	0.04	0.03	±	0.01	0.74	±	0.14	1.09	±	0.13
65	15-methyltritriacontane	0.14	±	0.06	2.49	±	0.11	0.82	±	0.08	8.07	±	0.45	16.37	±	1.66
66	15,19-dimethyltritriacontane	0.41	±	0.18	1.73	±	0.20	3.04	±	0.45	4.07	±	0.93	5.16	±	1.04
67	11-decyltetracosane	0.06	±	0.03	0.85	±	0.03	0.35	±	0.03	3.77	±	0.35	5.76	±	0.59
68	β-amyrin acetate	0.06	±	0.03	0.45	±	0.06	0.17	±	0.02	1.96	±	0.10	2.82	±	0.32
69	branched alkane[4]	0.41	±	0.13	0.32	±	0.05	0.16	±	0.01	0.58	±	0.07	0.83	±	0.11
70	urs-12-en-24-oic acid, 3-oxo, methyl ester	0.36	±	0.09	0.20	±	0.09	0.01	±	0.01	0.14	±	0.05	0.49	±	0.07
71	1,30-triacontanediol	0.31	±	0.07	1.27	±	0.12	0.37	±	0.04	0.22	±	0.05	0.71	±	0.23
72	unknown[8]	0.04	±	0.04	0.19	±	0.03		–		0.68	±	0.13	0.08	±	0.03
73	octadecyl octadec-9-enoate	0.03	±	0.01	0.58	±	0.04	0.17	±	0.04	0.80	±	0.08	2.15	±	0.26
74	13-undecylpentacosane	0.07	±	0.03	0.87	±	0.09	1.13	±	0.02	2.18	±	0.23	7.27	±	0.60
75	unknown[9]	0.01	±	0.01	0.20	±	0.03	0.06	±	0.01	0.52	±	0.07	1.23	±	0.11
76	icosyl octadec-9-enoate		–		0.08	±	0.04	0.01	±	0.00	0.09	±	0.04	0.07	±	0.02
77	11,15-dimethylpentatriacontane	0.04	±	0.02	0.16	±	0.06	0.38	±	0.02	0.17	±	0.05	0.19	±	0.03
78	branched alkane[5]		–		0.00	±	0.00	0.47	±	0.05		–			–	

The PCA identified 38 principal components (PCs) with Eigenvalues above one that explained 83.75% of the total variance in the dataset. Canonical discriminant analysis of these principal components (Figure 2.3; Wilks' $\lambda < 0.001$, $x^2 = 2155.744$, $df = 152$, $p < 0.001$) revealed a significant separation between species and species-groups. Discriminant function 1 explained 49.1% of the variance and was responsible for the separation between *E. stuckenbergi* and the rest of the groups, as well as separating *E. glumosae* and *C. capensis* from *E. stuckenbergi* and the *Otitesella* species-groups. Discriminant function 2 explained 33.9% of the variance between groups, and was largely responsible for separating *C. capensis* from the other groups. The slight divergence that occurred between the two *Otitesella* species-groups was mostly a result of discriminant function 2. A 100% of *E. stuckenbergi*, *E. glumosae*, Sesqui species-group and Uluzi species-group samples were correctly assigned to their groups, and only one sample of *C. capensis* (1.4%) was misclassified as coming from the Uluzi species-group. This low incidence of misclassification coupled with low Wilks' λ and an extremely significant p value indicates that fig wasp species as well as species-groups can be clearly discriminated based on their CHC profiles.

Table 2.2: Results from pairwise comparisons (ANOSIM) for significant differences between the GC profiles of different fig wasp species/species-groups (Global R = 0.54, p = 0.001). P-values that were significant after sequential Bonferroni correction was applied are in bold.

Fig wasp species and species-group analyses Pairwise comparisons		R	p
E. stuckenbergi	*C. capensis*	0.584	**0.001**
E. stuckenbergi	*E. glumosae*	0.789	**0.001**
E. stuckenbergi	Uluzi species-group	0.842	**0.001**
E. stuckenbergi	Sesqui species-group	0.853	**0.001**
C. capensis	*E. glumosae*	0.128	**0.008**
C. capensis	Uluzi species-group	0.394	**0.001**
C. capensis	Sesqui species-group	0.282	**0.002**
E. glumosae	Uluzi species-group	0.111	**0.035**
E. glumosae	Sesqui species-group	0.836	**0.001**
Uluzi species-group	Sesqui species-group	0.149	**0.011**

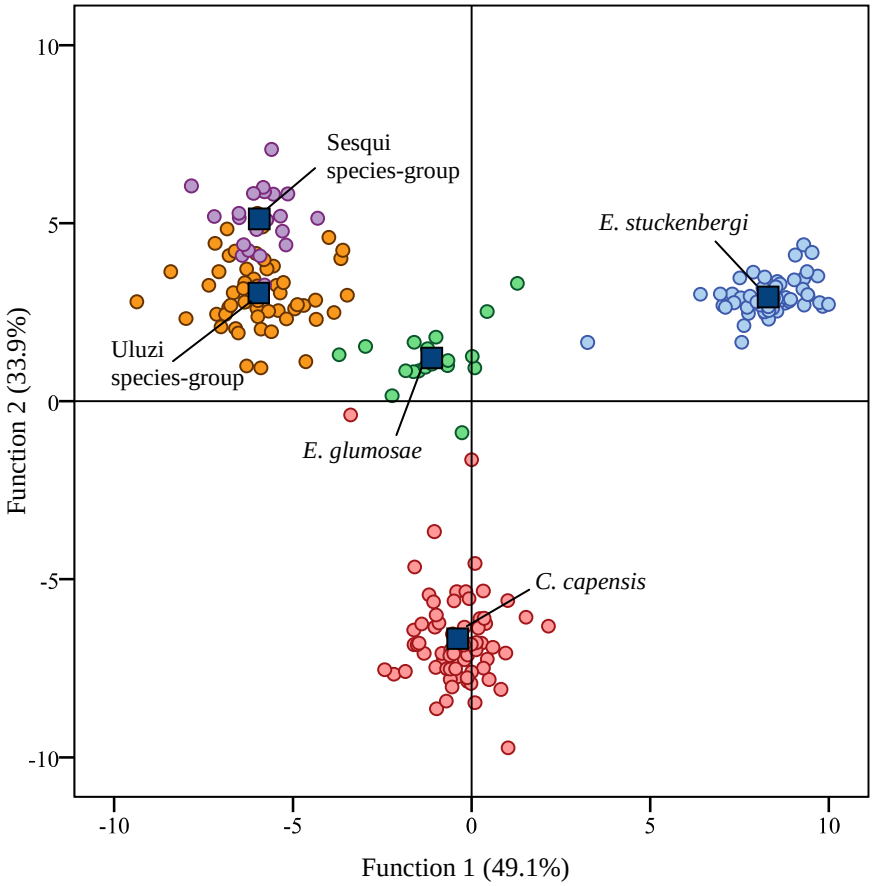


Figure 2.3: Canonical Discriminant Analysis of fig wasp CHCs based on 38 principal components, selected by PCA, of the compounds found in the CHC profiles of the Sesqui species-group (, n = 38), Uluzi species-group (, n = 40), *E. glumosae* (, n = 20), *E. stuckenbergi* (, n = 62) and *C. capensis* (, n = 73). All five species/species-groups can be clearly defined into groups (Group centroid =).

The CHC profiles of all species and species-groups were significantly different from each other (Table 2.2; ANOSIM: Global R = 0.54, p = 0.001, 999 permutations from a random sample of total possible permutations), even though some overlap existed between the two *Otitesella* species-groups as well as *E. glumosae* (Figure 2.3). There was a large divergence between CHC profiles between *E. stuckenbergi* and the *Otitesella* species-groups. The pairwise comparisons also confirm a large degree of dissimilarity between *E. stuckenbergi* and *E. glumosae* (R = 0.789, p = 0.001; as observed in Figure 2.3), despite their close taxonomic relationship. The CHC profiles of the two *Otitesella* species-groups are very similar (R = 0.149, p = 0.011), as was originally predicted.

Table 2.3: The average percentage contribution of the 10 major CHC compounds that contributed most (average,*) and most consistently (ratio,•) to the similarity (SIMPER) of the CHC profiles of each of the 5 fig wasp species/species-groups.

Compound	*E. stuckenbergi*		*E. glumosae*		*C. capensis*		Uluzi species-group		Sesqui species-group	
	Average	Ratio	Average	Ratio	Average	Ratio	Average	Ratio	Average	Ratio
Z-9-nonacosene	9.08*	3.20•	5.29	2.00	5.47	0.46	2.17	1.11	1.43	0.94
nonacosane	0	0	3.91	11.33	0.29	0.76	1.27	2.29	1.51	7.88•
n-dimethylnonacosane	0.56	1.48	1.04	13.56	0.70	7.40•	1.05	1.99	0.51	0.96
unknown[12]	8.49*	0.76	0	0	2.47	0.42	0	0	0	0
branched alkane[2]	6.21	4.74•	8.38*	12.57	4.74	4.53	7.06*	1.34	1.51	1.54
11-decyldocosane	0	0	5.08	15.00•	4.06	0.62	4.47	1.47	1.83	7.57
unknown[5]	0	0	3.92	11.54	11.99*	0.37	3.48	0.54	1.77	7.94•
unknown[6]	0	0	0	0	10.86*	0.42	0	0	0	0
15-methyltritriacontane	0	0	2.08	13.18	0.73	0.76	6.98*	3.60•	13.50*	2.43
unknown[16]	0	0	1.15	1.29	3.16	0.74	6.62*	0.71	15.30*	0.78
Average similarity (%)	60.46		83.25		52.66		58.10		69.66	

The compounds contributing the most (average) and most consistently (ratio) to the similarity within the GC profiles of the fig wasp species included a combination of methyl-branched alkanes, as well as nonacosane, Z-9-nonacosene and four compounds that could not be identified (Table 2.3). The compounds that contributed the most consistently to the dissimilarity between groups were methyl-branched alkanes and one alkene (n-tritriacontene), with 3-methylhentriacontane largely distinguishing between *E. glumosa* and all the other species except *C. capensis* (Table 2.4).

Table 2.4: The compounds responsible for dissimilarity (SIMPER) between CHC profiles of species, specifically peaks that contributed the most consistently (ratio) to differences between CHC profiles.

Species/species-groups	*E. stuckenbergi*	*E. glumosae*	*C. capensis*	Uluzi species-group
E. glumosae	(1) branched hexacosane (2) 3-methylhentriacontane (3) n-tritriacontene			
C. capensis	(1) unknown[11] (2) unknown[10]	(1) unknown[13]		
Uluzi species-group	(1) n-tritriacontene (2) 15-methyltritriacontane	(1) 3-methylhentriacontane	(1) unknown[11] (2) 9-octyltetracosane	
Sesqui species-group	(1) unknown[4] (2) unknown[9]	(1) 3-methylhentriacontane	(1) unknown[4] (2) unknown[5]	(1) unknown[26]

2.4 Discussion

Fig pollinators as well as non-pollinating galler fig wasp species-groups have species- and species-group-specific CHC profiles, respectively. This study supports the use of CHCs to differentiate between different fig wasp species in future taxonomic studies, and is in accordance with a number of published studies showing the usefulness of CHC profiles in chemotaxonomy (Carlson and Service, 1980; Sutton *et al.*, 1996; Haverty *et al.*, 1997; Bagnères and Wicker-Thomas, 2010). As predicted, the two *Otitesella* species-groups were more similar to each other than to the pollinators as a whole. The lower level of variation between the *Otitesella* species-groups as compared to other fig wasp groups support studies that have found that CHC profiles of insects are taxonomically significant and reflect phylogenetic relationships (Lockey, 1988; Sutton *et al.*, 1996; Nation, 2002; Copren *et al.*, 2005). The two pollinators from the genus *Elisabethiella* however did not have similar CHC patterns as predicted, and did not reflect their close taxonomic relationship.

The fact that the two *Otitesella* species-groups are more similar to each other than to any other species is a reflection of their close taxonomic relationship. It must also be said that despite the large probability that the Uluzi and Sesqui species-groups each represent multiple species, the CHC profiles of these potential species still have enough in common to yield CHC profiles specific enough that the two species-groups are significantly different from each other. Consequently if there are multiple species present within each species-group, their CHC profiles may only separate out when analysed at a finer scale. This has been found in other insects, where variation not only occurs on a species level, but at finer population scales as well (Singer *et al.*, 1998; Dapporto *et al.*, 2004a; Dapporto *et al.*, 2004b; Dapporto *et al.*, 2007). This will be addressed in Chapter 3, where the CHC profiles of the two *Otitesella* species-groups are investigated further.

Considering the fact that the *Otitesella* species-groups have so little variation between them, the large separation between the two pollinators from the *Elisabethiella* genus is unexpected (Figure 2.3). Not only do the two species belong to the same genus, but the host trees also belong to the same section within the genus *Ficus* (Berg and Wiebes, 1992). Possible drivers behind the large difference in CHC profile may be habitat-related, as the two host trees, *F. glumosa* (host of *E. glumosae*) and *F. burkei*

(host of *E. stuckenbergi*) tend to occur in different habitats, with *F. glumosa* found in savanna woodland and always associated with rocky outcrops, and *F. burkei* found in wooded grassland and wet or dry forest (Berg and Wiebes, 1992). An additional environmental factor affecting the large difference in CHC profiles between these two *Elisabethiella* species is that they occur in different host species, and that there is thus a difference in diet between *E. stuckenbergi* and *E. glumosae*. Studies have indicated that different diets strongly affects the CHC profiles of members of the same species (Stennett and Etges, 1997; Richard *et al.*, 2004; Buczkowski *et al.*, 2005; Etges *et al.*, 2009), so different diets may be enhancing the difference in CHC profiles of these two already genetically different species.

One of the ecological forces driving the species-level differentiation of CHCs in fig wasps may be that fig wasps are using CHCs to locate mates in the darkness of the fig cavity (i.e. mate recognition, Lorenzi *et al*., 1996; Blomquist, 2010; Millar, 2010). A large part of the stability of the fig – fig wasp mutualism depends on the maintenance of important species-specific morphological and behavioural characteristics of the pollinator. These include behavioural characteristics such as active pollination behaviour (Kjellberg *et al.*, 2001; Cook and Rasplus, 2003) and morphological characteristics such as head shape (van Noort and Compton, 1996). Active pollination behaviour involves female fig wasps opening anthers, collecting the pollen and placing it in pollen pockets (Galil and Meiri, 1981; Kjellberg *et al.*, 2001), as well as placing the pollen directly onto stigmas in receptive figs (Galil and Meiri, 1981; Compton and van Noort, 1992; Weiblen, 2002). It is also essential that certain morphological characteristics such as head shape be conserved in pollinating wasps, because a pollinator's head shape is specifically adapted to make their way through the ostiole of a specific fig species (van Noort and Compton, 1996), and the shape of the ostiole differs between fig species (Janzen, 1979). Species-specific CHC profiles would allow fig wasp males to locate the appropriate females and avoid interbreeding with other species that lack the necessary behavioural and morphological characteristics for successful pollination, as well as wasting time and energy attempting copulations that cannot yield viable offspring. Because of these reasons, the ability to recognise mates though species-specific CHCs may be essential in maintaining the mutualism. In a study on CHCs of the social wasp *Polistes dominulus*, Dani and colleagues (2001) found that alkenes and branched hydrocarbons

played a role as recognition cues between these wasps, which suggest that there is a possibility that they may be performing a similar function in fig wasps. As CHCs play an important role as semiochemicals in many other insects (Lockey, 1988; Howard and Liang, 1993; Lorenzi *et al*., 1996; Howard and Blomquist, 2005; Blomquist, 2010; Millar, 2010), it is highly likely that CHCs in fig wasps also act as mediators of behaviour.

This study provides novel information regarding the cuticular composition of fig wasps. The suite of chemical classes that were identified on the cuticles of fig wasps are similar to compounds that have been identified from other insects (Lockey, 1988; Lockey, 1991; Nation, 2002; Leonhardt *et al.*, 2009, reviewed in Juárez and Fernández, 2007; Gołebiowski *et al.*, 2010), with straight-chain alkanes, branched alkanes, alkenes and esters playing a prominent role in the CHC profile, and alcohols, aldehydes and triterpenes featuring on a smaller scale (Table 2.1). The number of different compounds occurring on these fig wasps' cuticles may seem large, but it is not entirely surprising considering that insect cuticular profiles have the potential to contain highly complex mixtures comprising of well over a hundred different compounds (Blomquist and Bagnères, 2010).

Studies on insect CHCs have identified the presence of various compounds that are not synthesised by the insect, but instead originate from their environment (Nowbahari *et al.*, 1990; Liang and Silverman, 2000; Leonhardt *et al.*, 2009; Millar, 2010). Likewise, some of the compounds that were identified on fig wasp cuticles have the potential to be environmental in origin. Examples include urs-12-en-24-oic acid-3-oxo-methyl ester, a volatile compound that has been found in the shrub *C. roseus* (Wu *et al.*, 2009), and two triterpenes (α-amyrin and β-amyrin acetate), which also have the potential to come from the insect's environment (e.g. tree resins, as suggested by Leonhardt *et al.*, 2009). The presence of these known environmental compounds suggests that some of the other chemicals found on fig wasps may also have originated from the environment (Millar, 2010), and that part of the CHC signal may therefore be environmental in nature, with evidence that CHC profiles do vary with changes in environment (Lockey, 1988; Nielsen *et al.*, 1999; this issue will be discussed further in Chapters 3 and 4). It is thus necessary to investigate the potential environmental influences on CHC profiles in fig wasps.

It is in the nature of CHCs to vary on all levels of organisation (Lockey, 1988; Gibbs and Pomonis, 1995), with differences occurring between families (Jacob, 1979; Lockey, 1988), between species (Carlson and Service, 1980; Lavine and Carlson, 1991; Howard and Liang, 1993; Lorenzi *et al*., 1996; Everaerts *et al.*, 1997; Singer *et al*., 1998; Howard and Blomquist, 2005; Dapporto, 2007; Juárez and Fernández, 2007; Antonialli Jr. *et al.*, 2008; Bagnères and Wicker-Thomas, 2010), between colonies or populations within a species (Howard and Liang, 1993; Chapman *et al.*, 1995; Lorenzi *et al*., 1996; Singer *et al*., 1998; Etges and Ahrens, 2001; Dapporto *et al.*, 2004b; Dapporto, 2007; Tannure-Nascimento *et al.*, 2007; Leonhardt *et al.*, 2009; Bagnères and Wicker-Thomas, 2010; Baracchi *et al.*, 2010), and finally on a very fine scale even between individuals in the same colony or population (Gibbs and Pomonis, 1995). We have observed variation between different species-groups (*Otitesella*) and between species (*E. stuckenbergi, E. glumosae* and *C. capensis*), which at this broad level make CHCs useful in chemotaxonomy, and could contribute to solving some of the remaining uncertainties in fig wasp phylogenies. However, we predict that intra-species variation in CHC profiles will become apparent at finer scale analyses. This potential fine-scale variation will be addressed in the following two chapters.

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Chapter 3. **Intra-species-group variation in cuticular hydrocarbon profiles of *Otitesella* fig wasps.**

3.1 Introduction

Previous studies on insect hydrocarbons have indicated that while a large part of an insect's CHC profile is determined by genes, the environment can also influence CHC profiles to a varying degree (Lorenzi et al., 1996; Liang and Silverman, 2000; Dapporto *et al.*, 2009). Two ways that the environment can cause changes in insect CHC profiles are either through the adsorption or assimilation of hydrocarbons from the surroundings (Nowbahari *et al.*, 1990; Liang and Silverman, 2000; Millar, 2010), or by causing an indirect change in the phenotypic expression of CHCs (i.e. phenotypic plasticity). The latter has been shown in ants (Richard *et al.*, 2004) and moths (Piskorski *et al.*, 2010). However, identifying the role of genes and/or the environment in influencing CHC profiles is a contentious issue, and studies have yielded results along a continuum with some studies having shown no detected environmental influence on the CHC profile (Howard and Liang, 1993; Floreani *et al.*, 2006) or marginal environmental influences (Lavine and Carlson, 1991; Nielsen *et al.*, 1999; Etges and Ahrens, 2001; Baracchi *et al.*, 2010; Piskorski *et al.*, 2010), while other studies have shown CHC profiles to be largely influenced by environmental factors (Bagnères and Wicker-Thomas, 2010; Ferreira-Caliman *et al.*, 2010). These incongruent findings could potentially be the result of the diverse life histories found in insects, and as such each species has the potential to be affected by a unique combination of environmental factors.

Fig wasps from the *Otitesella* species-groups (the Uluzi species-group and Sesqui species-group, as defined in Chapter 2) occur in multiple fig tree host species and regions across South Africa, from Stellenbosch in the Western Cape to Mabibi in Kwa-Zulu Natal (Chapter 2). This allows us to investigate potential environmental and genetic influences on the CHC profile of this group. In fig wasps, one of the possible environmental causes of variation could be changes in diet, as has been shown in ants (Liang and Silverman, 2000; Etges and Ahrens, 2001; Richard *et al.*, 2004; Buczkowski *et al.*, 2005) and *Drosophila* (Stennett and Etges, 1997). Fig wasp larvae feed on the endosperm of galled fig flowers (Verkerke, 1989), and different species of fig may produce galls that have differences in chemistry and nutritional

content, which could then possibly affect the CHC profile of the fig wasps eating those galls. Regional differences may also influence the CHC profile of *Otitesella* fig wasps, for three reasons. Firstly, the fig wasp community in the syconium of a specific fig species may differ between regions. This is because moving between regions will also in some cases mean moving between habitats, and the fig wasp communities of a fig species is affected by differences in habitat across even very short distances (Compton et al., 1994). Additionally, fig wasp communities can be influenced by chance colonisation events (Hawkins and Compton, 1992), which refers to the presence or absence of a fig wasp species in a fig, not because of a specific ecological reason, but simply as a result of whether the fig tree was discovered by that species of fig wasp during the timeframe when the fig wasp could oviposit in the fig. This introduces a random element to fig wasp community composition which is difficult to account for. The fact that the fig wasp community is highly likely to change between regions means that fig wasps will be exposed to different combinations of CHCs based on which other species of fig wasp are present in a syconium, which will present opportunities to exchange CHCs with other species by adsorption of CHCs onto the cuticle. This has been shown to occur when different species of termite or cockroach live in close proximity, with CHCs being exchanged between species (Everaerts *et al.*, 1997; Vauchot *et al.*, 1998; Liang and Silverman, 2000; Millar, 2010). Secondly, habitat differences (Lockey, 1988) between regions may directly affect the CHC profiles of fig wasps. A previous study has found that differences in vegetation between regions influenced the CHC profiles of two grasshopper species (Buckley *et al.*, 2003), indicating that vegetation may have an indirect effect on the CHC profiles of fig wasps occurring in different regions. Thirdly, some biogeographical studies investigating regional variation in the CHC profiles of social paper wasps have found that geographic distance between populations causes genetic variation between populations, and thus potentially affect the CHC profile as the result of genetic differences (e.g. Dapporto *et al.*, 2004).

In a study on *Otitesella* in Africa, Jousselin *et al.* (2006) indicated that there is a high probability that these fig wasps are more host-specific than might be expected from a parasitic lineage, and that they may consequently occur as separate genetic lineages on different fig species. Fig wasps from the two *Otitesella* species-groups can occur in more than one fig species over different regions (Jousselin *et al.*, 2006), which may

result in divergent lineages from both species-groups occurring in the same fig. This situation offers us the opportunity to investigate both host species and environmental differences as possible influences on CHC profiles. Cuticular hydrocarbons have been found to be important in distinguishing between closely related species in the past (Bagnères and Wicker-Thomas, 2010), thus there is a possibility that CHC profiles may be used to distinguish between different groups within *Otitesella*. This will be done by exploring the genetic relationships within and between the two species-groups, as well as regional- and host species-induced variation in CHC profiles.

3.2 Materials and Methods

3.2.1 Fig wasp collection and study sites

Fig wasps of the Uluzi and Sesqui species-groups were collected using the fig wasp collection methods described in Chapter 2. The data used for analyses in this chapter were from three collection sites: Baviaanskloof Nature Reserve in the Eastern Cape Province, Ithala Game Reserve and Mabibi Nature Reserve in Kwa-Zulu Natal Province (see Figure 2.1; voucher specimens lodged at the Iziko Museum Cape Town). Collection data for all sites are given in Table 6.1b (Appendix). Due to the aseasonal nature of fig production in the *Ficus* species used for this study (reviewed in Weiblen, 2002; Cook and Rasplus, 2003), finding fig trees carrying figs at the right stage of development posed a challenge, and resulted in small sample sizes in some cases.

3.2.2 Analyses of host species-associated influences on CHC profiles

The GC-MS protocol (see Chapter 2 for details) was used to distinguish CHC profiles of fig wasps from different host species. Data were analysed using multivariate statistics in both SPSS (v. 18) and Primer (v. 5.2.9). For multivariate analyses in SPSS, the CHC peaks were standardised as the percentage contribution to the total hydrocarbon blend for the sample in question and then transformed using Aitchison's equation as described in Chapter 2 (Aitchison, 1986). Principal components analysis and stepwise discriminant analysis were performed using the same methods as those described in Chapter 2. For analyses in Primer, the data were standardised as the percentage contribution as before, and then transformed using double square root transformations as described in Chapter 2. Analysis of similarity (ANOSIM, 999

permutations from a random sample of total possible permutations) was performed as described in Chapter 2, followed by adjustments of significant α values using sequential Bonferroni correction for pairwise comparisons.

To explore the effect that host species (*F. glumosa* and *F. burtt-davyi*, in this case) has on the CHC profiles of fig wasps in the Sesqui species-group collected from Ithala, multidimensional scaling (MDS, Primer) was used (as only two groups were compared, an MDS was more appropriate than discriminant analysis). Multidimensional scaling displays categories based on how similar or dissimilar they are from each other (Quinn and Keough, 2002) and represents the relationships between groups in multidimensional space, with the spatial manner in which the groups are displayed indicating their underlying differences (Quinn and Keough, 2002). The closer two points are to each other the more similar their CHC profiles are. Stress values are used to indicate dissimilarity and level of match between groups, and are an indication of the goodness of fit, with stress values below 0.15 indicating good fit (Clarke and Gorley, 2001). Analysis of similarity (ANOSIM, 999 permutations from a random sample of total possible permutations) was performed to test for significant pairwise differences between fig wasps from different host species, with the application of sequential Bonferroni corrections to determine significant α values.

To test for possible host species-associated effects on the CHC profiles of fig wasps within the Uluzi species-group, stepwise discriminant analysis based on principal components identified by PCA was used. Fig wasps were collected from the host species *F. glumosa*, *F. burkei*, *F. polita*, *F. burtt-davyi* and *F. lutea* in Ithala and Mabibi. Analysis of similarity (ANOSIM, 999 permutations from a random sample of total possible permutations) was used to test for significant pairwise differences between fig wasps from different host species. Sequential Bonferroni corrections were used to determine significant α values.

3.2.3 Regional differences within the Uluzi species-group

To investigate possible regional influences on the CHC profiles of fig wasps from the Uluzi species-group, PCA followed by stepwise discriminant analysis was performed for fig wasps from this species-group that were collected in Mabibi, Baviaanskloof and Ithala. Samples from Ithala included fig wasps collected from *F. glumosa*, *F.*

burtt-davyi and *F. burkei*, samples from Mabibi included fig wasps collected from *F. polita* and *F. lutea*, and samples from Baviaanskloof were collected from *F. burtt-davyi*. Once again ANOSIM (999 permutations from a random sample of total possible permutations) was used to determine significant differences for individual pairwise comparisons between groups, with sequential Bonferroni correction used to determine significant α values. For the purpose of this study, a region was defined as an area that is broadly homogenous in habitat and covers a maximum area of a size that fig wasps can conceivably easily disperse across. For example, all collections from Ithala Game Reserve, which covers 290 km^2, are considered to have come from the same region, since calculations based on dispersal ability have estimated that potential breeding population sizes of fig wasps can cover areas of this size (Nason *et al.*, 1996). For this reason a particular species of fig wasp collected within a region was also considered to be part of the same population. Due to the fact that fig wasps from the Sesqui species-group were only collected in one region (Ithala), this analysis could not be repeated for the Sesqui species-group.

To investigate the relative importance of different factors responsible for the underlying variation in CHC profiles, permutational multivariate analysis of variance (PERMANOVA v. 1.0.3 in PRIMER v. 6.1.13) was performed to determine the importance of species-group membership, host species-associated effects and region. The results from this test indicates whether the factors under investigation have a significant influence on the variation in the dataset, as well as yielding an estimate of how much variation each factor is responsible for (Anderson *et al*., 2008).

3.2.4 Interaction between host species and genetic relationships in Otitesella species-groups

Multi-dimensional scaling (MDS, Primer) was used to explore the variation in the CHC profiles of *Otitesella* as a function of host species and species-group. Samples from both the Uluzi and Sesqui species-groups collected from *F. glumosa* and *F. burtt-davyi* in Ithala were used. The MDS was followed by ANOSIM (999 permutations from a random sample of total possible permutations) to test for significant pairwise differences between groups. Sequential Bonferroni correction was used to determine significant α values for the multiple pairwise comparisons.

3.2.5 Genetic analyses

Sequences from two mitochondrial gene regions [cytochrome oxidase I (COI ~ 630 bp) and cytochrome b (Cytb ~380 bp)] as well as a nuclear gene region [elongation factor – one alpha F2 copy (EF-1α ~510 bp)] was used to infer genetic divergences within and between lineages collected from different fig species. Genetic divergences were inferred using a Bayesian phylogenetic approach. This gave an indication of the relationships between the two species-groups and among samples collected from different regions and host species. Samples from both the Uluzi and Sesqui species-groups were sequenced in all of the fig tree collections where they were present, which consisted of 18 Uluzi samples from five different host species across eight different collections, and 6 Sesqui samples from two different host species across three collections (Appendix, Table 6.2). To more stringently assess genetic variation among the samples collected for the GC analysis, we included sequence data (McLeish, M.J., unpublished) for 35 additional *Otitesella* specimens in the phylogenetic inference, which in total included 7 outgroup taxa belonging to the fig wasp genus *Philocaenus* and 59 ingroup taxa.

The DNA extractions used for the sequencing data were from tissue preserved in > 96% ethanol. DNA was extracted from single whole fig wasps using a QIAGEN® QIAamp DNA Micro Kit. The PCR reactions included SuperTherm® DNA Polymerase (100U @ Enzyme Concentration: 5u/ml) and 10X Buffer (1ml @ pH 8.5). Amplifications of mitochondrial DNA were performed using the following protocol: 94°C, 3 minute polymerase incubation period for the first cycle only; 92°C, 30 seconds denaturation; 48°C, 1.5 minute annealing; 72°C, 1.5 minute extension for 35 cycles; with a final cycle of 72°C, 7 minute extension. The PCR mixture was a 25 µl reaction including: 2.5 µl 10X buffer, 0.2 µl of 5 U/ml of polymerase, 2.5 µl of $MgCl_2$ (25mM), 2.5 µl (10 mg/ml) of dNTPs, 1.0 µl (0.2 pmol/µl) of each primer, and 2.0 µl of unknown concentrations of template DNA. Amplifications of nuclear DNA were performed using the following protocol: 94°C, 3 minutes polymerase incubation period for the first cycle only; 92°C, 45 second denaturation; 56°C, 1.5 minute annealing; 72°C, 1.5 minute extension for 45 cycles; with a final cycle of 72°C, 7 minute extension. The PCR mixture was a 25 µl reaction including: 2.5 µl 10X buffer, 0.2 µl of 5 U/ml of polymerase, 0.75 µl of $MgCl_2$ (25mM), 1.5 µl (10 mg/ml) of

dNTPs, 0.75 µl (0.2 pmol/µl) of each primer, and 2.0 µl of unknown concentrations of template DNA. All primers were specifically designed for use in fig wasps (McLeish *et al.*, 2010; McLeish unpublished) and are given in Table 3.1.

Sequence editing was performed using SeqEd version 1.0.3 (Applied Biosystems, 1992). Sequence alignment was carried out by hand and matched to an existing dataset. No insertions or deletions were present. All sequence data has been submitted to GenBank (accession numbers as well as collection information are given in the Appendix, Table 6.2).

We used a Bayesian approach implemented in MrBayes v.3.1.1 (Huelsenbeck and Ronquist, 2001) to infer a consensus phylogeny. The DNA sequence was partitioned into gene fragments and each of these into codon positions (3 x coding gene fragments x 3 codon positions each = 9 partitions total). A general time reversible DNA substitution model (GTR) was used with gamma distributed (+G) rates, a default rate category prior of 4, and with a proportion of invariant sites (+I). This substitution model incorporates specific models that potentially emerge from the Markov-Chain Monte Carlo (MCMC) parameter search space. Posterior probabilities and mean branch lengths were derived from 30000 trees sampled every 1000 trees from generations 10 to 40 million. The trees were derived from post-burnin generations of Markov chains that had reached apparent stationarity. The MCMC Tracer Analysis Tool v.1.4.1 (Drummond and Rambaut, 2007; available from http://beast.bio.ed.ac.uk/) was used to assess the point in the MCMC chain where stable likelihood values were reached. The mean of standard deviations of the post-burnin split frequencies were used to assess the consistency between runs. All Bayesian reconstructions were run four times to verify consistency of the inferences. A phylogram consensus phylogeny was used to visualise branch length differences between individual fig wasp samples.

Table 3.1: Primers used in PCR reactions

Primer	Reference	Primer sequence (5'-3')
COI-070368	McLeish *et al.*, 2010	F: TTATCTTTACCAGTATTAGC
COI-070029	McLeish *et al.*, 2010	R: AATGTTGAGGGAAAAATGT(CT)
Cytb-070330	McLeish *et al.*, 2010	F: CTACCATGAGGACAAATATC
Cytb-070326	McLeish *et al.*, 2010	R: (AG)GAAT(TA)GATCG(TA)A(AG)AAT(TA)GC
EF-1α-080588	McLeish unpublished	F: GGTCTTGGACAAACTGAAGG
EF-1α-073534	McLeish unpublished	R: TTGTC(AG)GT(TG)GG(CT)CTGCT(TG)GG

3.3 Results

3.3.1 Analyses of host species-associated influences on CHC profiles

Sesqui species-group:

There was a distinct significant difference between CHCs of fig wasps from the Sesqui species-group collected from *F. glumosa* and those collected from *F. burtt-davyi*. Samples from different host species separate clearly in the MDS (Figure 3.1), and the low stress value (0.05) and significant results from the ANOSIM (Global R = 0.941, p = 0.001) indicates a large difference in CHC profiles between the two groups (94.1% dissimilarity of CHC profiles between groups).

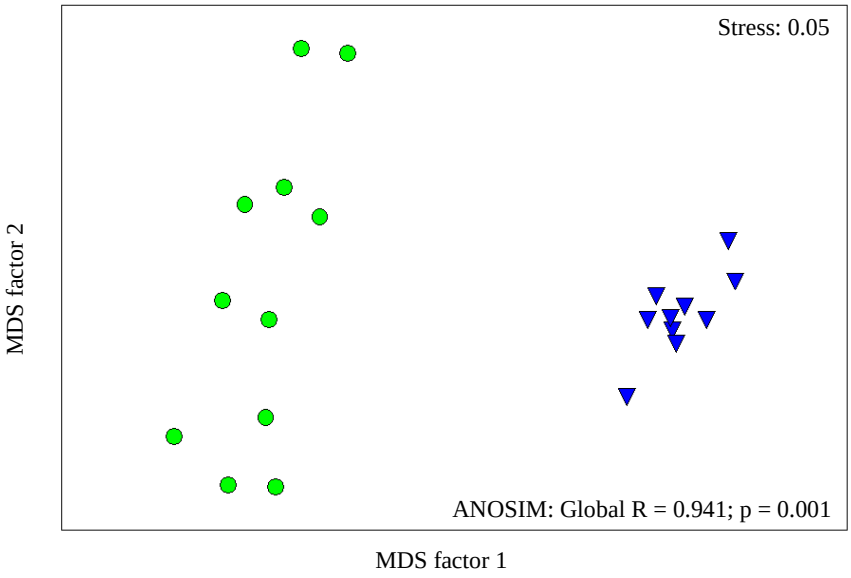

Figure 3.1: Multidimensional scaling indicating differences in CHC profiles between fig wasps from the Sesqui species-group collected from host trees *F. burtt-davyi* (▼ n = 10) and *F. glumosa* (●, n = 11).

Uluzi species-group:

There was a significant difference in CHC profiles between fig wasps from the Uluzi species-group that were collected from different host species. Principal component analysis identified 24 principle components (PCs) that were responsible for 92.89% of the variance within the dataset. Using these 24 principal components, stepwise discriminant analysis identified 12 PCs that were responsible for 85.2% of the separation of CHC profiles of fig wasps belonging to the Uluzi species-group by host species (Figure 3.2; Wilks' $\lambda < 0.001$, $x^2 = 301.457$, $df = 40$, $p < 0.001$). Discriminant

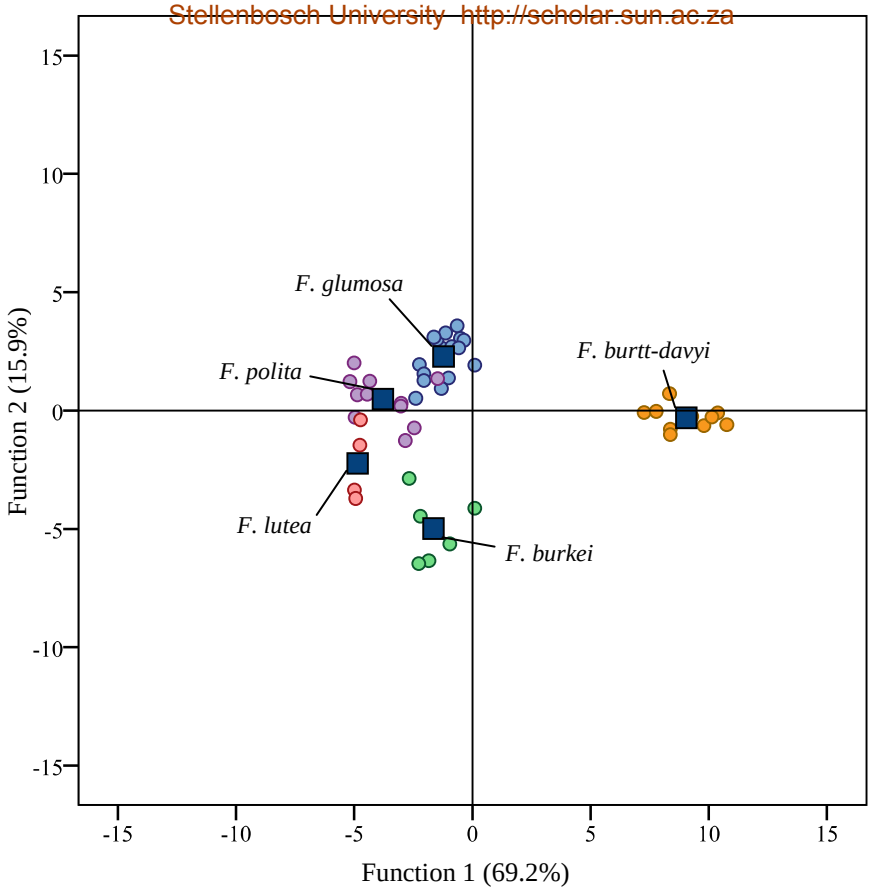

Figure 3.2: Stepwise Discriminate Analysis of regression factors identified by PCA contributing to the separation of CHC profiles of fig wasps from the Uluzi species-group collected on different host trees (*F. glumosa*, , n = 16; *F. burkei*, , n = 6 ; *F. lutea*, , n = 4; *F. polita*, , n = 11; and *F. burtt-davyi*, , n = 20). Wilks' $\lambda < 0.001$, $x^2 = 301.457$, $df = 40$, $p < 0.001$. (Group centroid =).

function 1 was responsible for 69.2% of the variation in the CHCs of fig wasps collected from different host species. Most of the separation between the CHCs of fig wasps collected from *F. burtt-davyi* and those collected from the remaining host species occurred on this function, as well as the separations between the CHCs of fig wasps collected from *F. glumosa* and *F. lutea* and between those collected from *F. burkei* and *F. lutea*. Discriminant function 2 accounted for 15.9% of the separation of CHCs by host species, and was responsible for most of the separation between the CHCs of fig wasps collected from *F. glumosa* and those collected from *F. burkei* and *F. lutea*. This discriminant function also highlighted the separation between the CHCs of fig wasps collected from *F. polita* and those collected from *F. burkei*. A 100% of samples were correctly classified by the host species they originated from. Results from Primer's ANOSIM indicated that all groups of fig wasps from the Uluzi species-group defined by different host species were significantly different from each other (Table 3.2a, Global R = 0.836, p = 0.001).

Table 3.2: ANOSIM results from pairwise comparisons of fig wasp CHCs from the Uluzi species-group by (a) host species and (b) region. Significant p values adjusted with the sequential Bonferroni correction are in bold.

a) Uluzi species-group host species analysis (Global R = 0.836, p = 0.001)			
Pairwise comparisons		R	p
F. glumosa	*F. burtt-davyi*	0.987	**0.001**
F. glumosa	*F. burkei*	0.958	**0.001**
F. glumosa	*F. polita*	0.841	**0.001**
F. glumosa	*F. lutea*	0.996	**0.001**
F. burtt-davyi	*F. burkei*	0.898	**0.001**
F. burtt-davyi	*F. polita*	0.729	**0.001**
F. burtt-davyi	*F. lutea*	0.996	**0.001**
F. burkei	*F. polita*	0.489	**0.001**
F. burkei	*F. lutea*	0.817	**0.005**
F. polita	*F. lutea*	0.499	**0.005**
b) Uluzi species-group regional analysis (Global R = 0.738, p = 0.001)			
Pairwise comparisons		R	p
Ithala	Baviaanskloof	0.737	**0.001**
Ithala	Mabibi	0.689	**0.001**
Baviaanskloof	Mabibi	0.890	**0.001**

3.3.2 Genetic variation between and within Otitesella species-groups

The Bayesian consensus phylogenetic inference revealed well-supported monophyletic clades of the Uluzi and Sesqui species-groups [Figure 3.3, posterior probability (PP): 99-100]. In addition to separating samples out by species-groups, samples within each group formed well-supported lineages that corresponded with the host species that the fig wasp were sampled from (PP: 99-100, a phylogeny with all posterior probabilities > 90% are given in the Appendix, Figure 6.1), indicating that within a species-group there are multiple lineages which specialise on different fig species. Fig wasps grouped together in both the Uluzi and Sesqui clades according to host species even if they were sampled from different individual trees found in different regions. Branch-lengths between the specimens collected from the same host species are generally very short, indicating a relatively low level of genetic divergence between them, which contrasted with the much longer branch-lengths separating the lineages associated with different fig species. These longer branch-lengths also appear to be consistent with species-level divergences. Interestingly, even though there are

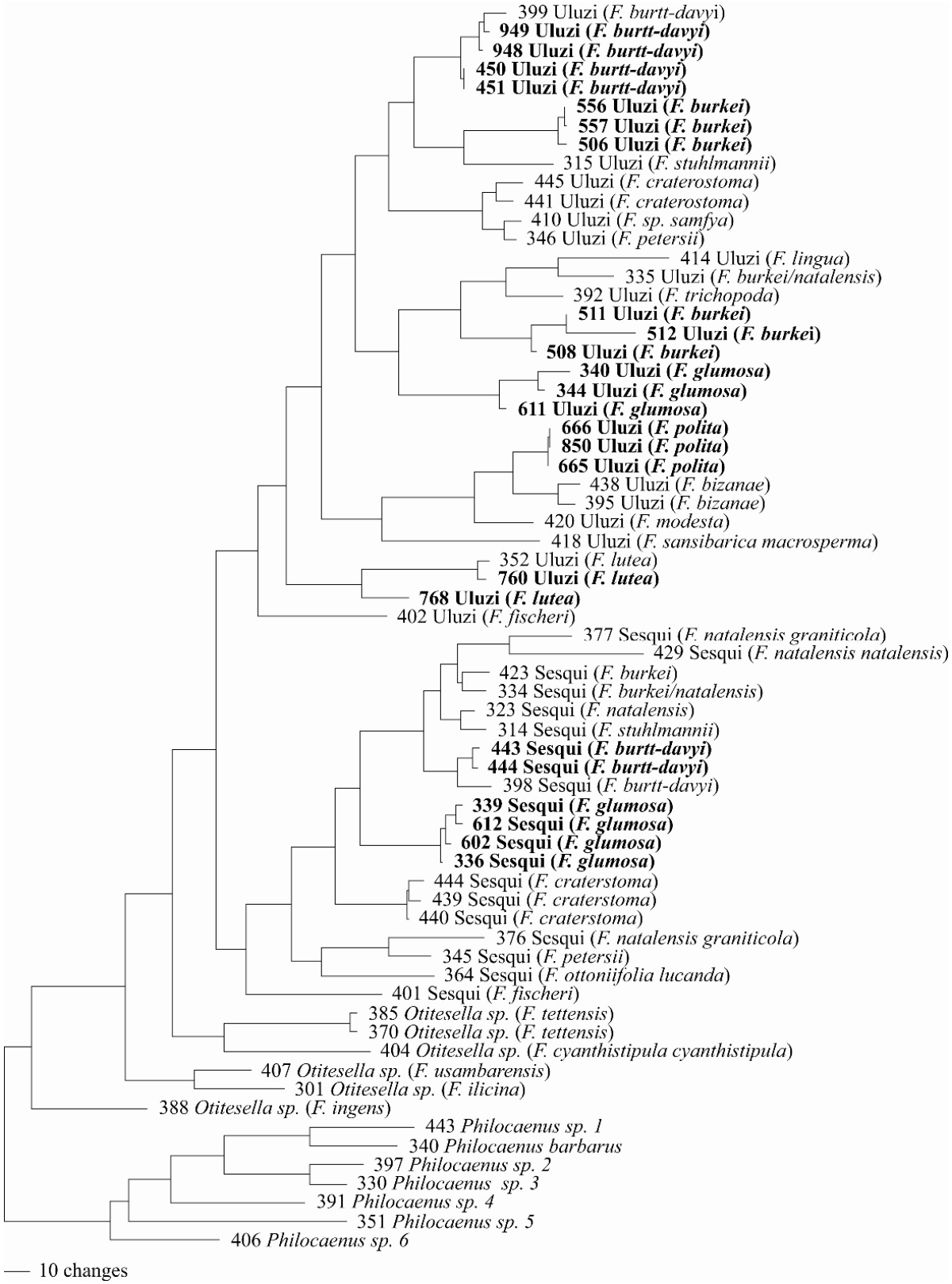

Figure 3.3: Consensus phylogram representing the phylogenetic relationships among fig wasps from the Uluzi and Sesqui species-groups. Samples in bold are those that formed part of the collections used in analyses of CHCs. Species names in brackets indicate which host species the fig wasp was sampled from. Fig wasp samples from the genus *Philocaenus* was used as outgroups.

longer branch-lengths between some fig wasps that occur on the same host species (e.g. Uluzi fig wasps on *F. lutea*, Figure 3.3), indicating a greater variation in genetic makeup within these lineages, the CHCs of all fig wasps still clearly separated out by host species (Figure 3.1 and 3.2), regardless of the degree of relatedness within each lineage.

3.3.3 Is host species or genetic lineage more important in determining CHC variation in Otitesella?

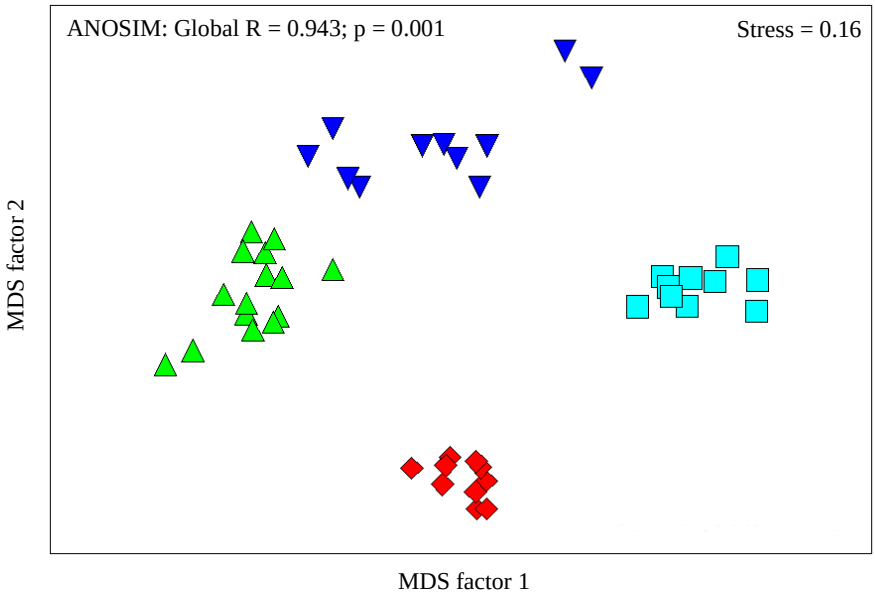

Figure 3.4: Multidimensional scaling indicating differences in CHC profile between fig wasps from both the Uluzi and Sesqui species-groups collected from host trees *F. burtt-davyi* (*O. uluzi*, ◆, n = 10; *O. sesquianellata*, ■, n = 10) and *F. glumosa* (Uluzi species-group, ▲, n = 16; Sesqui species-group, ▼, n = 11).

Thus far we have shown that CHC profiles are influenced by host species and that each host species supports a genetically distinct lineage from either one or both of the *Otitesella* species-groups. From these data, one can argue that the differences in CHC profiles of fig wasps sampled from different host species may be the result of genetic differences between fig wasps lineages on different host species, and not necessarily differences caused by the host species themselves. Figure 3.4 shows that all four groups investigated (fig wasps from the Uluzi species-group on *F. glumosa* and *F. burtt-davyi*, and fig wasps from the Sesqui species-group on *F. glumosa* and *F. burtt-davyi*) have CHC profiles that are significantly different from each other (ANOSIM: all pairwise comparisons significant at $p = 0.001$, all $R > 0.77$, sequential Bonferroni corrections applied, Table 3.3).

Table 3.3: ANOSIM results from pairwise comparisons of fig wasps from both the Sesqui and Uluzi species-groups by host species *F. glumosa* and *F. burtt-davyi*. Significant p values adjusted with the sequential Bonferroni correction are in bold.

CHC profiles of fig wasps from the Uluzi and Sesqui species-groups from *F. burtt-davyi* and *F. glumosa* (Global R = 0.943, p = 0.001)			
Pairwise comparisons		R	p
Uluzi sp. on *F. glumosa*	Sesqui sp. on *F. glumosa*	0.779	**0.001**
Uluzi sp. on *F. glumosa*	Uluzi sp. on *F. burtt-davyi*	0.99	**0.001**
Sesqui sp. on *F. glumosa*	Sesqui sp. on *F. burtt-davyi*	0.921	**0.001**
Sesqui sp. on *F. burtt-davyi*	Uluzi sp. on *F. burtt-davyi*	1	**0.001**

Hypothetically, one would expect that if CHCs were influenced by genetics alone, the two groups representing the CHC profiles of fig wasps from the Uluzi species-group would lie closer together, as would the groups representing the CHC profiles of fig wasps from the Sesqui species-group, with a greater distance between species-groups than between host species (Figure 3.5a). This pattern would have reflected the genetic relationships represented in Figure 3.3, where the fig wasps of the Sesqui species-group collected from *F. burtt-davyi* and *F. glumosa* are more closely related to each other than they are to the fig wasps from the Uluzi species-group from the same two host species. Instead, the pattern shows greater than expected convergence in CHC profiles dependant on host tree species, given the genetic relationships between the groups. Hypothetically, the influence of host species results in the convergence of CHC profiles of fig wasps sharing a common host species, e.g. Sesqui on *F. burtt-davyi* grouping with Uluzi on *F. burtt-davyi*, with a similar pattern for the two

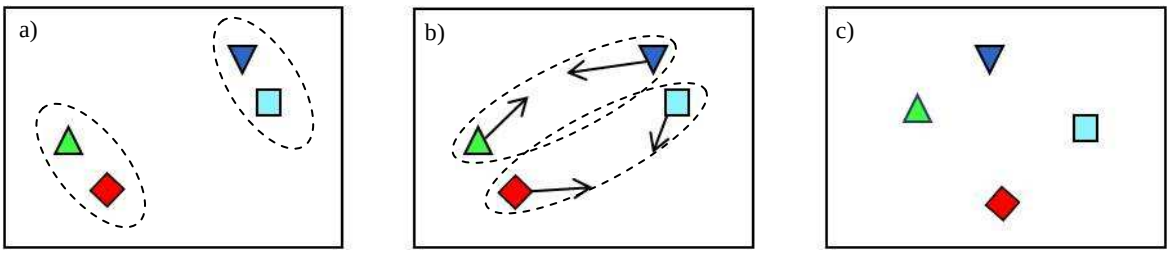

Figure 3.5: A graphic representation of a situation where a) the CHC profiles of fig wasps are more similar between fig wasps from the same species-group than between fig wasps from the same host species as a result of genetic relatedness (dashed circles represent samples from the same species-group), b) the hypothetical effect that host species-associated factors may have on increasing the similarity of CHC profiles between fig wasps from the same host species as a result of having a common host (arrows depict the influencing effect of host species on CHC profiles, dashed circles depict samples from the same host species), withc), depicting a simplified representation of Figure 3.4. The symbols represent the same groups as those in Figure 3.4.

species-groups found in *F. glumosa*. This effect of host species on fig wasp CHCs would be increased separation between the CHC profiles of fig wasps from the same species-group, with decreased separation between the CHCs of fig wasps from different species-groups collected from the same host species (Figure 3.5b). Consequently, the overall CHC pattern would be one of a more even distribution between groups (Figure 3.5c). The fact that the groups are not arranged in such a way that more closely related groups have more similar CHC profiles suggests that host species does, in fact, exert some influence on the CHC profile.

3.3.4 Regional influence on CHC profile in the Uluzi species-group

The CHC profiles of fig wasps from the Uluzi species-group could be grouped according to the region they originated from, regardless of the host species they were collected from (Figure 3.6). Principal components analysis identified 26 PCs that explained 93.15% of the variation in the dataset. Stepwise discriminant analysis of these principal components indicated that significant region-dependant differences exist in the CHC profiles of fig wasps from this species-group (Figure 3.6; Wilks' λ = 0.005; x^2 =235.872; *df* = 36; $p < 0.001$). Discriminant function 1 explained 67.6% of the between-region variation and was responsible for most of the separation between the CHCs of fig wasps collected at Mabibi and those collected from Baviaanskloof and Ithala. Discriminant function 2 explained 32.4% of the between-region variance and was also responsible for the separation between the CHCs of fig wasps collected from Baviaanskloof and Ithala, and between Mabibi and Baviaanskloof. A 100% of samples were correctly assigned to their groups by the DA, and ANOSIM indicated that all pairwise comparisons were significant (Table 3.2b; Global R = 0.738, p = 0.001). Cuticular hydrocarbon profiles show strong regional effects which override the host species influences. This is evident by the fact that fig wasp CHCs collected from *F. burtt-davyi* (the shared host species) from both Baviaanskloof and Ithala do not converge by host species but rather by region. This indicates that regional differences in the CHC profiles of fig wasps are important, but does not necessary mean that genetic influences are absent since CHC profiles are effective in separation fig wasp CHC profiles (see Chapter 2). This emphasises the importance of the level of analyses when interpreting variation in CHC profiles.

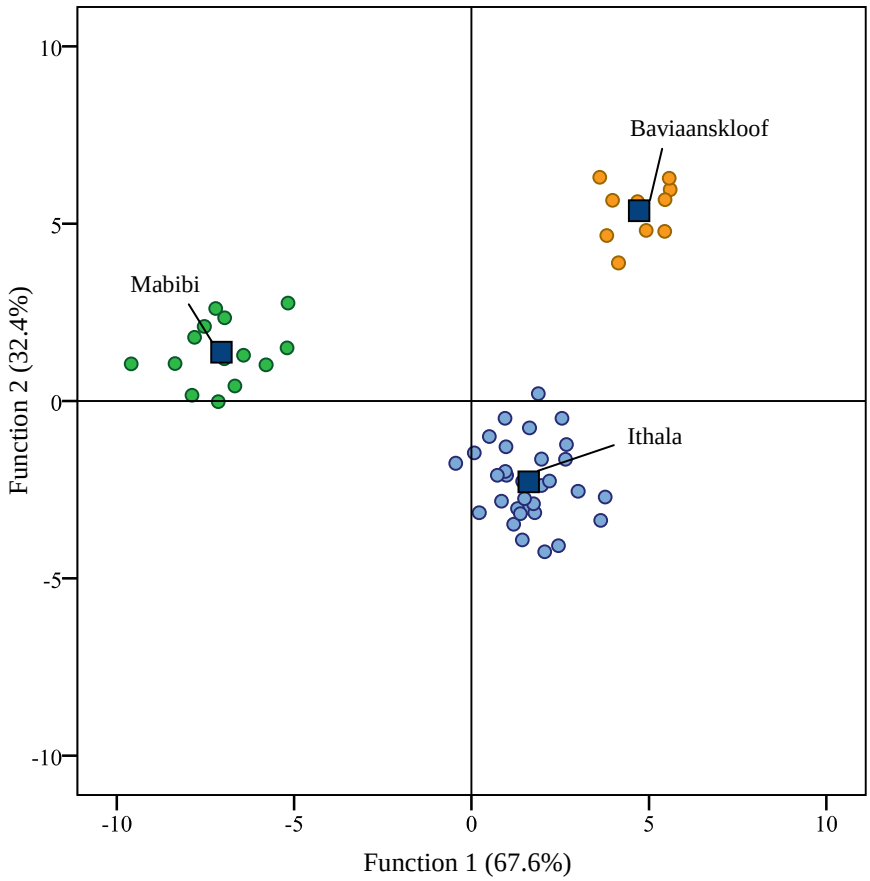

Figure 3.6: Stepwise Discriminate Analysis of regression factors identified by PCA contributing to separation of CHC profiles of Uluzi fig wasps collected in different regions (Baviaanskloof, , n = 10, *F. burtt-davyi*; Ithala, , n = 32, *F. burtt-davyi*, *F. burkei* and *F. glumosa*; and Mabibi, , n = 14, *F. polita* and *F. lutea*). Samples can clearly be separated based on their region of origin (Group centroid =).

Finally, permutational multivariate analysis of variance corroborated the results presented thus far by revealing that species-group membership, host species-associated effects as well as region were significantly responsible for explaining the underlying variation in fig wasp CHC profiles (Table 3.4). As has been inferred from the PCA results in this section, region explained the most of the variation in the dataset, followed by host species. It is clear that these three factors alone cannot explain all of the observed variation, indicating that there must be additional influences on fig wasp CHC profiles that remain to be investigated. As has been shown by the genetic results (section 3.3.2), separating host species-associated effects from the genetic effects of the different genetic lineages on different host species is challenging, and unfortunately further investigation of this is outside the scope of this study.

Table 3.4: PERMANOVA (PRIMER) results indicating the effects of species-group membership, host fig species and collection region on variation in the CHC profiles of fig wasps. Significant p-values are given in bold. The estimates of the relative importance of different components of variation are given as the percentage of the total variation that each component accounts for.

Source	df	SS	MS	Pseudo-F	P (perm)	%
Species-group	1	6957.3	6957.3	17.538	**0.001**	17
Host species	3	15511	5170.2	13.033	**0.001**	19.5
Region	1	8512.4	8512.4	21.458	**0.001**	24.3
Residuals	71	28165	396.69			19.9
Total	77	74114				

3.4 Discussion

We have shown that variation in CHCs of both the Uluzi and Sesqui species-groups belonging to the non-pollinating galling fig wasp genus *Otitesella* are determined in part by genetic lineage and that these differences are influenced and strengthened by host species influences. Moreover, in both species-groups the differences caused by host species and those caused by genetic lineage within a species-group are less important than region in determining the CHC profile of a fig wasp.

Previous studies investigating the relationship of regional variation and differences in CHC profile have also shown that geographic distances between groups were reflected in their CHC profiles. In paper wasps (*Polistes dominulus*, Dapporto *et al.*, 2004), fruit flies (*Drosophila mojavensis*, Etges and Ahrens, 2001), mosquitoes (Bagnères and Wicker-Thomas, 2010) and hover wasps (various species, Baracchi *et al.*, 2010) it was found that CHC variation was a function of the geographic distance between the populations investigated. The geographic variations in the CHC profiles of species are generally thought to be the result of a combination of variation in genetic population structure between sampling sites as well as environmental differences between sites, but the relative contribution of these two influences are hard to separate. What is clear is that the variation in CHC profiles between regions can often not be explained by regional genetic variation alone, indicating that exogenous influences must also play an important role in geographic CHC profile variation (Nielsen *et al.*, 1999; Dapporto *et al.*, 2009). Regarding our investigation, we must also add that the observed regional differences in CHC profiles were between fig wasps that belonged to closely-related species-groups, and that region may in this case only override genetic and host species effects in very closely related groups. This

effect that region has on CHC profile does not affect the species-group and species-level variation shown in Chapter 2, an indication that it is important to keep in mind at which level of organisation CHC profiles are being compared in order to correctly interpret any potential variation in CHC profiles.

Other studies support our finding that some of the differences between CHC profiles within a species can be caused by genetic variation between groups (distinct genetic lineages on different host species in the case of this study). Investigations on termites have indicated that genetic relationships between colonies reflected variation in CHC profiles (Dronnet *et al.*, 2006), and closely related triatomine species that form part of the same species-complex also have more similar CHC profiles (Juárez and Fernández, 2007). Regarding the genetic differences between fig wasps found on different host species, our results support the conclusions of Jousselin *et al.* (2006), who found that within *Otitesella* in both the Sesqui and Uluzi species-groups there were separate genetic lineages that appeared to be host-specific.

The host species influence on CHC profiles seen in both the Uluzi and Sesqui species-groups has also been shown for other species. Piskorski and colleagues (2010) showed that *Cydia pomonella* moths on different host species could be distinguished by the host tree species they occurred on by using their CHC profiles, and the same situation applies to some aphid species (Bagnères and Wicker-Thomas, 2010). When Argentine ants from different colonies that interacted aggressively were fed the same diet, their CHC profiles converged to such a degree that aggressive interactions were significantly reduced between colonies (Buczkowski *et al.*, 2005), and in a another study, being fed different diets affected the CHC profiles of ants so much that former nest mates acted aggressively toward each other (Liang and Silverman, 2000). In a study on the causes of differences in CHCs in *Drosophila mojavensis*, Stennett and Etges (1997) found the species of cactus used to rear larvae caused a significant difference in the CHCs of this species. This supports the possibility that the change in diet as a result of different host species use could affect the CHC profile of fig wasps to the degree observed here.

A possible reason why regional variation in CHC profile is overriding genetic and host species influences in determining CHC profile within a species-group could be the differences in vegetation (and therefore habitat) between the three regions

investigated. Ithala Game Reserve is located in the Savanna and Grassland biomes, Mabibi Nature Reserve is located in the Indian Ocean Coastal Belt as well as Forest biomes and Baviaanskloof Nature Reserve in the Albany Thicket biome (Mucina and Rutherford, 2006). Previous studies have found that changes in habitat can cause differences in CHC profiles of insects (Lockey, 1988; Bagnères and Wicker-Thomas, 2010; Ferreira-Caliman *et al.*, 2010, reviewed in Howard and Blomquist, 2005). The biomes in this study from where samples were collected are characterised by differences in vegetation, soils and amount of rainfall (Mucina and Rutherford, 2006), habitat differences which could conceivably be responsible for the observed differences in CHC profiles through a combination of habitat influences on both host trees and the fig wasp community that occurs in a syconium. In truth, such large differences in vegetation may not even be necessary to cause habitat-related changes, since habitat differences between closely situated areas can cause changes in fig wasp community composition in, for instance, *F. burtt-davyi* (Compton et al., 1994). While some fig species have the potential to occur in more than one type of habitat, this might not necessarily be true for all the fig wasp species that can potentially form a part of the fig wasp community in that fig tree. For example, in some fig species that are pollinated by more than one species of fig wasp, the species of pollinator associated with a particular fig tree population may depend on habitat (Michaloud *et al.*, 1996). This is an indication of how habitat differences can influence the distribution of a fig wasp species, and specifically how the fig wasp community composition of a fig tree may be influenced by a tree's habitat. This will determine to which other species a fig wasp is exposed, with the potential to exchange CHCs with these other species (Vauchot *et al.*, 1996; Everaerts *et al.*, 1997; Vauchot *et al.*, 1998). However, to truly investigate the effect that fig wasp community structure has on the CHC profiles of fig wasps one would need to determine the exact community composition of every syconium, an aspect not investigated in this study.

When the importance of CHC profiles in species- and mate-recognition is taken into account (Lorenzi et al., 1996; Nation, 2002; Millar, 2010), a possible consequence of our findings is that host species- and region-related changes in CHC composition may in the long term lead to pre-mating isolation (Bagnères and Wicker-Thomas, 2010). Pre-mating isolation could then result in populations of fig wasps which only occur on one type of host species, which may over time lead to speciation of non-pollinating

galling fig wasps inhabiting different host species through both adaptive changes to the new host as well as genetic drift. Cuticular hydrocarbons are important for mate recognition in *Drosophila mojavensis*, and it has been observed that populations from different regions exhibit pre-mating isolation. This has been attributed to the fact that *D. mojavensis* has switched hosts between regions, which caused changes in their CHC profiles, leading to pre-mating isolation (Etges and Ahrens, 2001). It had previously been shown that the CHC profiles of *D. mojavensis* are significantly affected by the species of host used to rear larvae (Stennett and Etges, 1997). This is highly relevant with regard to the potential changes in CHC profile caused by effects associated with different host species in the fig wasps in this study, as host switching in fig wasps may, hypothetically, in the long term also lead to genetic changes in the CHC profiles of individuals that can lead to pre-mating isolation. In other words, a fig wasp population diverged by host species or region over evolutionary time and the diverged populations were later reunited, this could be an explanation for the two separate genetic lineages encountered in fig wasps from the Uluzi species-group in *F. burkei*. This sets the stage for independent genetic lineages to develop within a group which may later lead to speciation on different host species. An example of sexual isolation caused by different host-use is apparent in the leaf beetle *Neochlamisus bebbianae* (Funk, 1998), where populations found on different host species were more reproductively isolated than those found on the same host species.

Common to many studies investigating the variation in CHCs is that although the authors identify a genetic or environmental influence on the CHC profile, they often follow this conclusion by emphasising that these identified influences only form part of a wider set of factors influencing CHC profiles, with both genes and environment responsible for the observed differences in CHC profiles (Lavine and Carlson, 1991; Nielsen *et al.*, 1999; Dapporto *et al.*, 2004; Dronnet *et al.*, 2006; Dapporto *et al.*, 2009). In the case of this study, we have identified three main factors which influence the CHC profiles of fig wasps belonging to two different species-groups: On a broad scale between and within species-groups the CHC profile is determined by genetic differences, within a species-group the CHC profile is further determined by regional differences, and on a finer within-region scale these differences are influenced by associated host species effects. As in the social paper wasp, *Polistes dominulus*, CHCs may be used in fig wasps as a “tool to emphasize biogeographical patterns of

similarity based both on gene flow and environmental characteristics" (Dapporto *et al.*, 2009).

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Chapter 4. **Intraspecies variation in the cuticular hydrocarbon profiles of two pollinating fig wasps, *Ceratosolen capensis* and *Elisabethiella stuckenbergi*.**

4.1. Introduction

There is no one aspect that governs how much variation commonly occurs in the cuticular hydrocarbon (CHC) profiles of insects, with variation occurring at the species, regional, colony, and individual level (Gibbs and Pomonis, 1995). The extent of variation in the CHC profiles of insects, in general, largely depends on the level of organisation being examined. The variation between individuals usually decreases as those individuals share more and more common environmental and genetic factors, with the largest variation occurring between species, less variation between populations and colonies, and members of the same population or colony having the most similar CHC profiles (Antonialli Jr. *et al.*, 2008, Chapter 2, Chapter 3). Populations that are geographically closer together will also in some cases have more similar CHC profiles than populations that are far apart, probably as a result of genetic similarity and shared environmental factors (as suggested by Dapporto *et al.*, 2004). However, this pattern does not always hold true as populations that are close together geographically do not always have similar CHC profiles (Dapporto *et al.*, 2009).

Unlike the non-pollinating galler fig wasp gallers investigated in Chapter 3, a pollinating fig wasp species generally only occurs in one host species (Janzen, 1979; Compton and van Noort, 1992; Cook and Rasplus, 2003). This is the result of the highly conserved relationship between pollinating fig wasps and their associated fig species which is essential for maintaining the mutualism (Weiblen, 2002). This conserved relationship includes factors like the life cycle of the pollinating wasp being highly synchronized with the development cycle of the fig (Compton and van Noort, 1992; Weiblen, 2002), as well as pollinating behaviour that involves actively gathering pollen and placing it on the stigmas of fig flowers in a different tree (Janzen, 1979; Compton and van Noort, 1992; Kjellberg *et al.*, 2001; Weiblen, 2002). The role of CHCs in this mutualism may be connected to mate recognition as has been shown in other species (Lorenzi et al., 1996; Howard and Blomquist, 2005; Millar, 2010), and to help pollinating wasps locate conspecifics in a fig that may host combinations of up to thirty different species of fig wasp (Compton and Hawkins, 1992). This reasoning leads to the expectation that pollinating fig wasps have species-

specific CHCs, which has been demonstrated in Chapter 2, but surprisingly these species-specific CHC profiles did not reflect the degree of taxonomic relatedness between species. It is likely that the large observed differences in CHC profiles between relatively closely related fig wasp species are the result of environmental factors such as host species, the habitat where the host tree generally occurs, the microclimate surrounding a specific tree (such as temperature, exposure to wind, relative humidity, soil type, aspect, etc.), as well as the fig wasp community that is characteristic of a specific host species or the region where the tree is situated (Chapter 3). The fig wasp community associated with a particular fig crop is highly differential in spatial recruitment, with species in a fig (or individuals within a species) potentially originating from an unpredictable geographical range, from within a couple of meters (if another conspecific tree is producing wasps within the immediate vicinity) to a recruitment area encompassing hundreds of kilometres. Fig wasps are capable of dispersing at least 160kms (Ahmed *et al.* 2009) and probably much further. The genetically determined component of the CHC profile that is influenced at a regional scale can therefore vary tremendously within a fig wasp community, even within a species where different individuals could have emanated from a variety of distances and source fig trees.

At this point it is unknown how much intraspecific CHC variation occurs in pollinating fig wasp species. As has been found in other insects, it is likely that in addition to potential environmental influences on CHC profiles (Lockey, 1988; Nielsen *et al.*, 1999; Liang and Silverman, 2000; Dapporto *et al.*, 2009; Ferreira-Caliman *et al.*, 2010), genetic variation between populations (Dronnet *et al.*, 2006) may also ultimately influence the CHC profile of pollinating fig wasps. It is possible that genetic population structure may be more important in determining CHC profiles than environmental differences. Previous studies on other wasp species have indicated that intra-specific genetic variation influences the CHC profile, and that it is often a combination of genetic and environmental differences between populations that are responsible for variation seen in the CHC profile (Lorenzi et al., 1996; Dapporto *et al.*, 2004; Dapporto *et al.*, 2009). In Chapter 3, factors responsible for variation in the CHC profiles of a non-pollinating galler fig wasp species-complex included host species-associated factors, genetic variation between fig wasps and regional environmental influences. Setting aside the possible host species-associated influence,

this suggests that potential factors that can influence the CHC profiles of pollinating fig wasps are intra-specific genetic variation and regional environmental influences.

The objectives of this chapter were to firstly investigate the potential intraspecies regional variation in the CHC profiles of two pollinating fig wasps, *Ceratosolen capensis* and *Elisabethiella stuckenbergi*, and secondly to investigate the genetic population structure in the same two species between the same regions. The purpose of these investigations was to determine to which degree intraspecies variation in CHC profiles could potentially be the result of population genetic structure between regions, and to what degree it could be influenced by environmental factors.

4.2. Materials and Methods

4.2.1. Fig wasp collection and study sites

Gas chromatography and genetic data from collections of *C. capensis* and *E. stuckenbergi* were used to investigate the intraspecies regional variation in these two pollinators. The GC data used in this chapter are a subset of the data used for analyses in Chapter 2 (see section 4.2.2) with 8 collection sites in Cape Town, Stellenbosch and Clanwilliam in the Western Cape Province, the Baviaanskloof Nature Reserve in the Eastern Cape Province and Ithala Game Reserve, Mtunzini and Mabibi Nature Reserve in Kwa-Zulu Natal Province (see Figure 2.1). Collection data for *C. capensis* and *E. stuckenbergi* for all sites are given in Table 6.1a (Appendix).

4.2.2. Gas Chromatography analysis

GC data were analysed using multivariate statistics in both SPSS (v. 18) and Primer (v. 5.2.9). For analyses in SPSS, peak area data were standardised as a percentage contribution to the total hydrocarbon blend for that sample, followed by transformation using Aitchison's equation as in Chapters 2 and 3 (Aitchison, 1986). For analyses in Primer, the data were standardised as the percentage contribution to the total hydrocarbon blend and then transformed using double square root transformations as in Chapters 2 and 3. Compounds that occurred in very low percentages were excluded from analyses, and *C. capensis* and *E. stuckenbergi* were analysed separately. The two pollinator species were analysed separately because of species-specific differences in CHC profiles between fig wasp species (see Chapter

2), and these differences may have overshadowed any other influences on variation if the two fig wasp species were analysed together.

C. capensis was collected from *F. sur* in Cape Town, Stellenbosch, Baviaanskloof, Mtunzini and Ithala, and *E. stuckenbergi* was collected from *F. burkei* in Stellenbosch, Clanwilliam and Ithala. Principal components analysis (PCA) and stepwise discriminant analysis (DA) were performed in SPSS, followed by analysis of similarity (ANOSIM, 999 permutations from a random sample of total possible permutations, PRIMER) as a measure of significant differences between regions. Sequential Bonferroni correction was used to determine significant α-values. PRIMER's SIMPER procedure was used to identify compounds responsible for dissimilarity between regions.

4.2.3. Genetic analyses

In order to assess variation in genetic structure in fig wasps between regions, at least five individual fig wasps, one each from five different samples, were genotyped for every collection. Fig wasps for both *C. capensis* and *E. stuckenbergi* were identified to species level. The same methods were used for DNA extraction, PCR, sequencing and alignment as in chapter 3. All sequence data have been submitted to GenBank (accession numbers as well as collection information are given in the Appendix, Table 6.3).

A phylogenetic approach was used to estimate the intraspecific genetic population structuring of *E. stuckenbergi* and *C. capensis*. Although phylogenetic inference violates intraspecific branching processes, the approach is useful for identifying clades that show negligible genetic divergences in relation to described species included in the inference (Posada and Crandall, 2001). Phylogenies were inferred using a Bayesian approach implemented in MrBayes v. 3.1.1. (Huelsenbeck and Ronquist, 2001). For *E. stuckenbergi*, species from *Ceratosolen* were chosen as outgroups based on their close relationship to the *Elisabethiella* genus (Cruaud *et al.*, 2010). For *C. capensis*, the outgroups were chosen based both on their close relationship as sister-species in the same genus (*Ceratosolen arabicus* and *Ceratosolen galili*, Jiang *et al.*, 2006), as well as more distantly related non-pollinating fig wasps (*Apocryptophagus sp.* and *Sycophaginae sp*, Rasplus *et al.*,

1998; Weiblen, 2002). Phylogenetic inferences for *C. capensis* incorporated six outgroup taxa (four samples from other species in the genus *Ceratosolen*, one sample from the genus *Apocryptophagus* and one sampled from the genus *Sycophaga*) and 37 ingroup taxa. Phylogenetic inferences for *E. stuckenbergi* incorporated six outgroup taxa (six samples from the genus *Ceratosolen*) and 32 ingroup taxa. The same analyses parameters were used as in Chapter 3. Haplotype networks were inferred using statistical parsimony (TCS v 1.18; Clement *et al.*, 2000) to estimate intraspecific genetic divergences and structuring of COI and Cytb loci to assess possible fig wasp population structuring among sampling sites.

4.3. Results

4.3.1. C. capensis

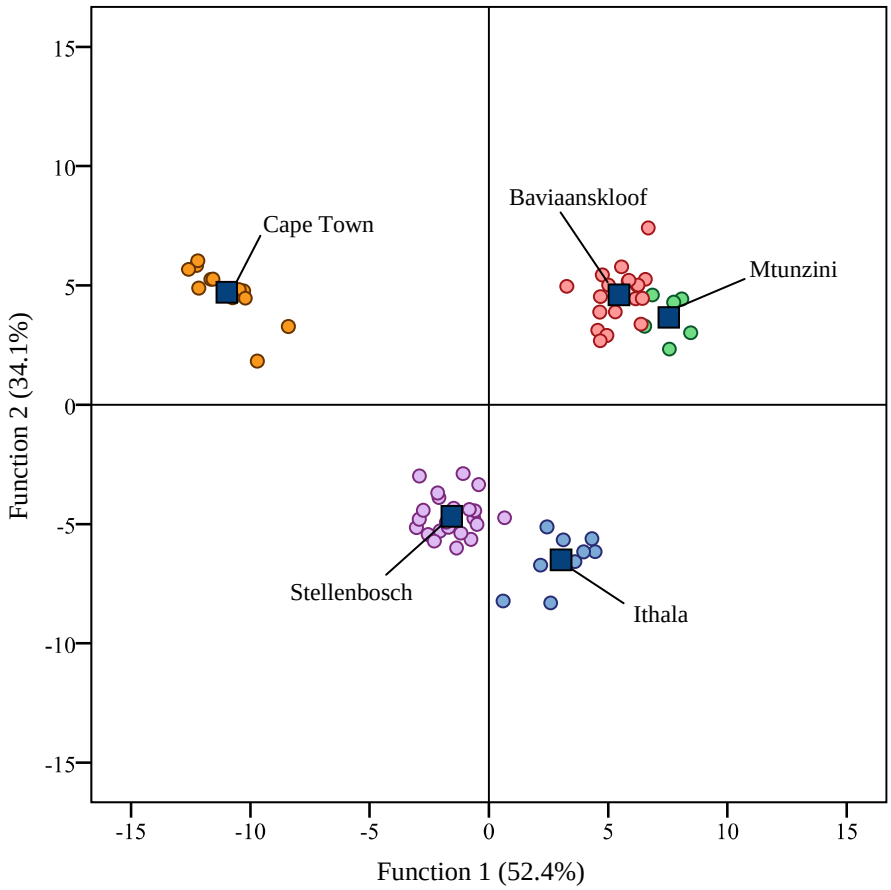

Figure 4.1: Stepwise discriminant analysis of regression factors identified by PCA contributing to separation of CHC profiles of *C. capensis* collected from the same fig species across different regions (Cape Town, , n = 13; Stellenbosch, , n = 25; Ithala, , n = 9; Baviaanskloof, , n = 20; and Mtunzini, , n = 6). All regions can be significantly separated based on their CHC profile (Table 4.1a). Group centroid =

The CHC profiles of *C. capensis* separated out significantly based on region. Principal component analysis of *C. capensis* data from Cape Town, Stellenbosch, Mtunzini, Baviaanskloof and Ithala revealed 21 principal components explaining 88.28% of the variation in the dataset. Stepwise discriminant analysis following the PCA identified four PCs as important in discriminating *C. capensis* between regions (Figure 4.1, Wilks' $\lambda < 0.001$, $x^2 = 626.379$, $df = 72$, $p < 0.001$) of which the first two functions accounted for 86.7% of the total variation between regions. Discriminant function 1 explained 52.4% of the variation and was responsible for the regional separation of the CHC profiles of fig wasps collected in Cape Town with the CHC profiles of wasps collected from all other sites, as well as the separation between the CHC profiles of fig wasps collected at Stellenbosch from the CHC profiles of fig wasps collected from all other sites. The CHC profiles of fig wasps collected from both Baviaanskloof and Ithala were also separated from those collected from Mtunzini on this function. Discriminant function 2 explained 34.1% of the variation and separated *C. capensis* collected from Cape Town, Baviaanskloof and Mtunzini from those collected from Stellenbosch and Ithala. Some overlap existed between the CHC profiles of fig wasps collected from Baviaanskloof and Mtunzini as well as between the CHC profiles of fig wasps collected from Stellenbosch and Ithala on this function. A 100% of the

a) *C. capensis* regional analysis (Global R = 0.585, p = 0.001)			
Pairwise comparisons		R	p
Stellenbosch	Cape Town	0.37	**0.001**
Stellenbosch	Ithala	0.266	**0.005**
Stellenbosch	Mtunzini	0.297	**0.001**
Stellenbosch	Baviaanskloof	0.159	**0.001**
Cape Town	Ithala	1	**0.001**
Cape Town	Mtunzini	1	**0.001**
Cape Town	Baviaanskloof	1	**0.001**
Ithala	Mtunzini	0.695	**0.001**
Ithala	Baviaanskloof	0.632	**0.001**
Mtunzini	Baviaanskloof	0.632	**0.002**
b) *E. stuckenbergi* regional analysis (Global R = 0.31, p = 0.03)			
Pairwise comparisons		R	p
Stellenbosch	Clanwilliam	-0.053	0.745
Stellenbosch	Ithala	0.978	**0.001**
Clanwilliam	Ithala	1	**0.001**

Table 4.1: ANOSIM results from pairwise comparisons of *C. capensis* and *E. stuckenbergi* CHC profiles collected from different regions. Significant p values adjusted with the sequential Bonferroni correction are in bold.

samples were correctly classified into their respective regions by the DA. Analysis of similarity indicated that all pairwise comparisons were significant (Table 4.1a). The CHC profiles of fig wasps collected from Cape Town were completely different to the profiles of fig wasps collected from Ithala, Mtunzini and Baviaanskloof (R = 1).

The compounds that were consistently responsible for differences in the CHC profiles of fig wasps collected between different regions (SIMPER) consisted of mostly branched alkanes, alkenes and esters (Table 4.2). Very few trends can be discerned in which compounds are responsible for regional differences, except for the role of dibutyl-1,2-benzene dicarboxylate and Z-14-nonacosene. Dibutyl-1,2-benzene dicarboxylate distinguishes the CHCs of fig wasps collected from Ithala with the CHCs of fig wasps collected in the Western Cape (Stellenbosch and Cape Town) and Eastern Cape (Baviaanskloof). Z-14-nonacosene plays an important role in separating the CHCs of fig wasps collected from Mtunzini and those collected from Ithala and Baviaanskloof, as well as separating the CHC profiles of fig wasps collected from Baviaanskloof and Cape Town.

Table 4.2: The compounds responsible for dissimilarity (SIMPER) between CHC profiles of regions within *C. capensis*, specifically compounds that contribute the most to differences between samples collected in different regions.

	Stellenbosch	Cape Town	Ithala	Mtunzini
Cape Town	(1) 11,15-dimethylpentatriacontane (2) unknown[21]			
Ithala	(1) dibutyl-1,2-benzene dicarboxylate	(1) n-tritriacontene (2) dibutyl-1,2-benzene dicarboxylate (3) 3,7-dimethylheptacosane (4) 7-hexyldocosane		
Mtunzini	(1) unknown[11] (2) n-methylnonacosane (3) 1,30-triacontanediol (4) Z-12-pentacosene	(1) squalene	(1) unknown[11] (2) Z-14-nonacosene (3) 9-hentriacontene (4) heneicosane	
Baviaanskloof	(1) n-dimethylnonacosane (2) tricosane (3) unknown[11] (4) unknown[22]	(1) n-methylnonacosane (2) Z-14-nonacosene (3) 1-nonacosene (4) dodecyl decanedioate	(1) dibutyl-1,2-benzene dicarboxylate	(1) n-hentriacontene (2) unknown[22] (3) 9-octyleicosane (4) Z-14-nonacosene

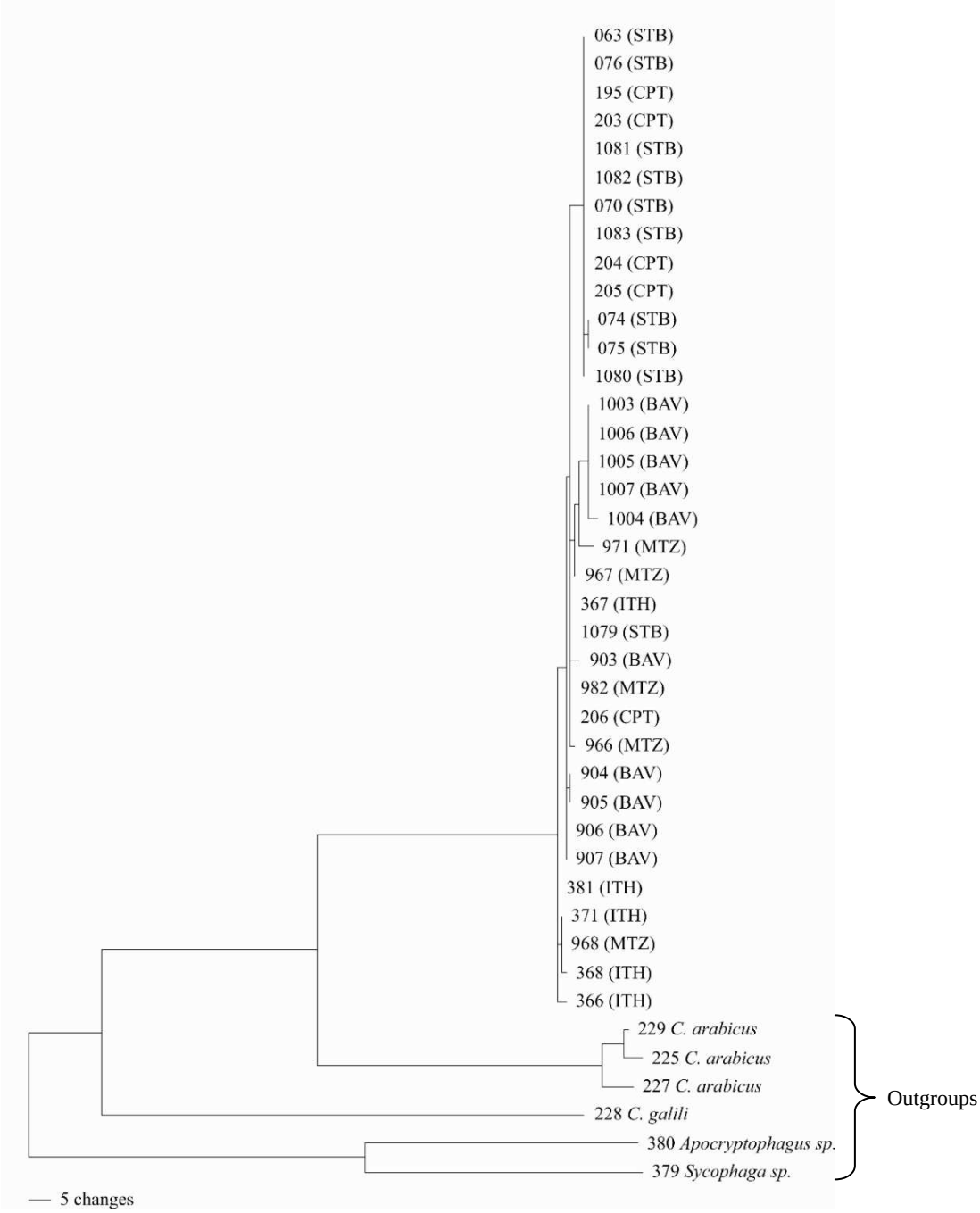

Figure 4.2: Consensus phylogram of *C. capensis* sampled from Cape Town (CPT), Stellenbosch (STB), Ithala (ITH), Baviaanskloof (BAV) and Mtunzini (MTZ).

Sampled individuals of *C. capensis* showed low intraspecific genetic variation between regions (phylogenetic inferences, Figure 4.2), as well as almost no genetic population structuring by region (haplotype network, Figure 4.3). The extremely short branch lengths of the consensus phylogram of samples from *C. capensis* derived from the Bayesian analysis (Figure 4.2) indicates that there is a high level of genetic relatedness between individual *C. capensis* fig wasps, considering that they were collected across South Africa from Cape Town in the Western Cape to Mtunzini and Ithala in north-western Kwa-Zulu Natal. Likewise, the haplotype network reveals that fig wasps sampled from different regions are genetically similar, and therefore within South Africa there seems to be a panmictic population (15 unique haplotypes identified, Figure 4.3). The observed lack of clear regional genetic structure indicates that the differences found in the CHC profiles of *C. capensis* between regions are unlikely to be the result of genetic variation of *C. capensis* between regions.

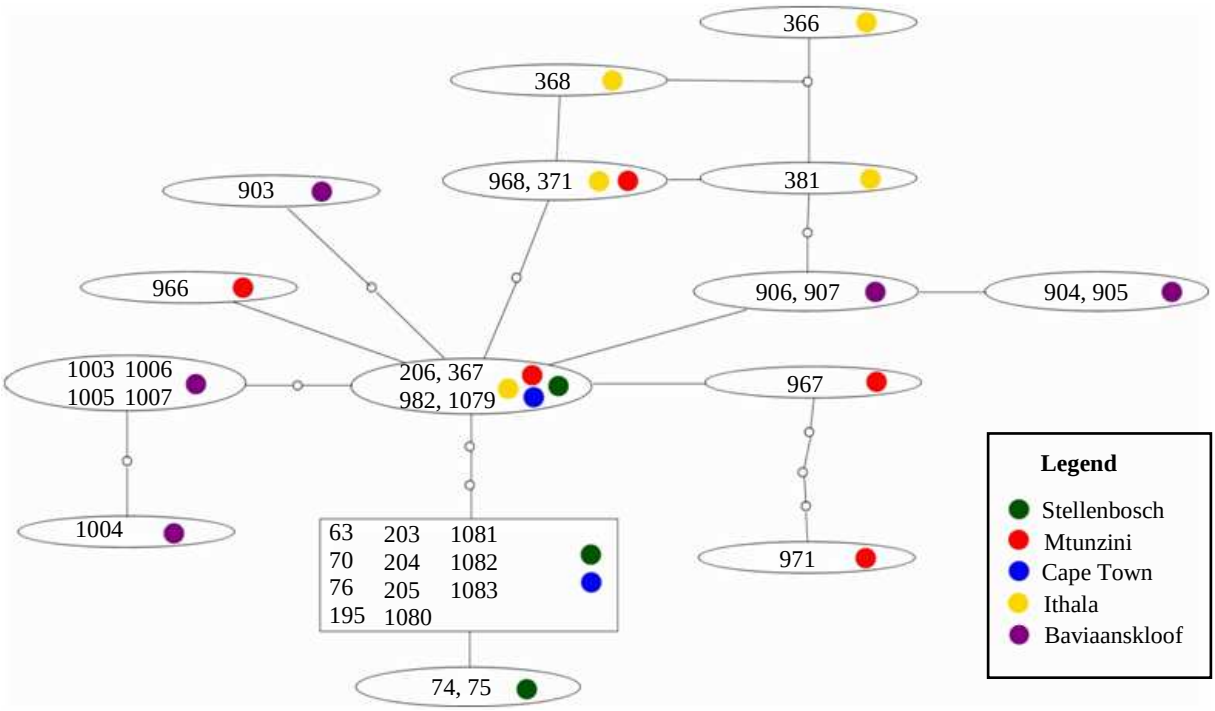

Figure 4.3: Haplotype network representing the distribution of *C. capensis* haplotypes across different regions in South Africa. The sample numbers of individual fig wasps matching each haplotype are given inside the ellipses and match the sample numbers of fig wasp individuals in Figure 4.2. Coloured circles indicate the different regions where a specific haplotype occurred. Small white circles indicate 1-step mutations.

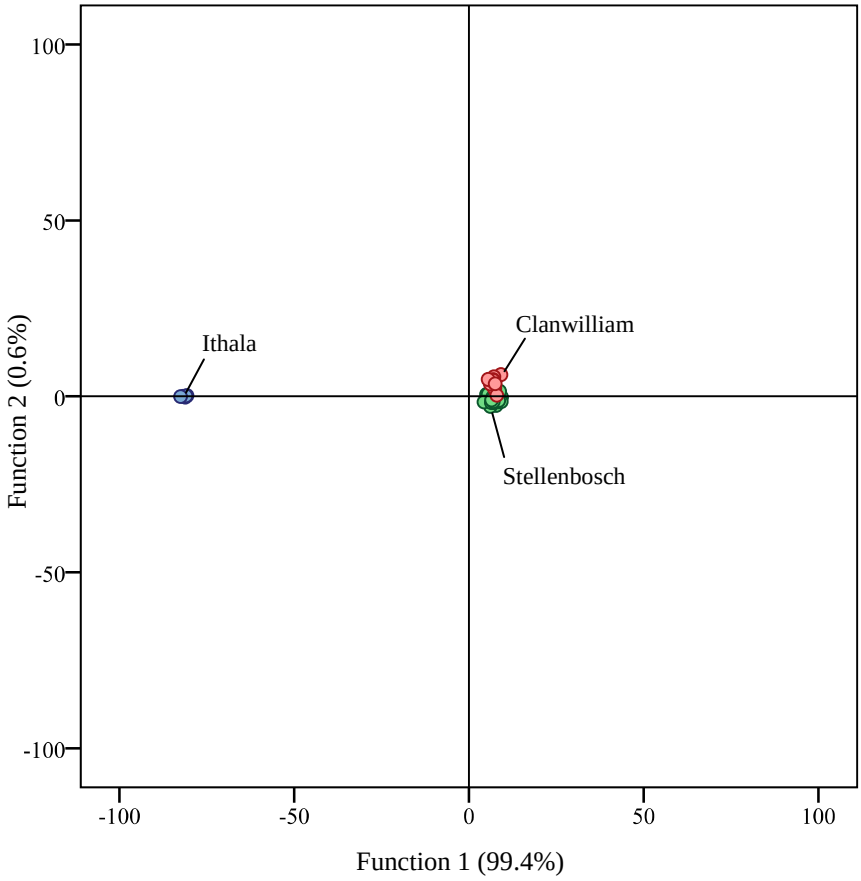

Figure 4.4: Stepwise discriminant analysis of regression factors identified by PCA contributing to separation of CHC profiles of *E. stuckenbergi* collected in different regions (Stellenbosch, Ithala and Clanwilliam). A significant difference exists between samples collected at Ithala (, n = 5) and samples collected from other sites, but no significance difference exists between Clanwilliam (, n = 12) and Stellenbosch (, n = 45).

4.3.2. E. stuckenbergi

Of the three regions where *E. stuckenbergi* were collected, the CHC profiles of fig wasps collected from Stellenbosch and Clanwilliam were not significantly different from each other, but the CHC profiles of fig wasps from both those regions were significantly different from the CHC profiles of fig wasps collected from Ithala (Table 4.1b, ANOSIM Global R = 0.31, p = 0.03). Principal components analysis of *E. stuckenbergi* data from Stellenbosch, Clanwilliam and Ithala revealed 16 principal components which explained 90.08% of the variation in the dataset. The stepwise discriminant analysis based on these components (Figure 4.4, Wilks' $\lambda < 0.001$, $x^2 = 419.411$, $df = 28$, $p < 0.001$) identified all 16 PC's as important in discriminating between regions for *E. stuckenbergi*, with discriminant function 1 explaining 99.4% of the variance, with *E. stuckenbergi* collected at Ithala clearly separating from those collected at Clanwilliam and Stellenbosch. Discriminant function 2 explained only

0.6% of the variance. All but one sample of the 12 samples collected in Clanwilliam were correctly classified as originating from Clanwilliam, and all but one sample collected in Stellenbosch were correctly classified as originating from Stellenbosch. A 100% of samples from Ithala were correctly classified. The compounds responsible for the dissimilarity in CHC profiles between sites (SIMPER) for *E. stuckenbergi* are not given since the majority of these compounds could not be identified by mass spectrometry.

There was no genetic structure in *E. stuckenbergi* between regions (Figure 4.5), with the bulk of *E. stuckenbergi* samples falling in a single undefined clade. Strangely, two *E. stuckenbergi* from Stellenbosch have been identified as extreme outliers from the rest of the samples. No explanation can be given for this difference, as three other samples from the same fig tree bears a genetic makeup that groups them with the majority of the other *E. stuckenbergi* from Stellenbosch, Ithala and Clanwilliam, and there was no morphological difference between these two samples and the rest of the collected *E. stuckenbergi*. The haplotype network indicated that there was even less variation in haplotypes in *E. stuckenbergi* than in *C. capensis*, with the majority of samples from all three regions having identical haplotypes (10 unique haplotypes identified, Figure 4.6). As in the phylogenetic inference, the only exception was two fig wasps from Stellenbosch that formed a network apart from the rest of the fig wasps collected in Stellenbosch, Clanwilliam and Ithala. The genetic separation between these two fig wasps from Stellenbosch are not reflected in the CHCs of these two individuals, as there are no outliers in the CHC profiles for Stellenbosch (Figure 4.4), indicating that in this case environment may be overriding potential genetic differences. All samples from Clanwilliam proved to possess identical haplotypes as to the majority of those from Stellenbosch which supports the extremely low R value in the pairwise analysis of CHC profiles between Clanwilliam and Stellenbosch (Table 4.1b). However, not only did three of the fig wasps sampled from Ithala have identical haplotypes to many samples collected in Stellenbosch and Clanwilliam, there was little genetic distance between the remaining two samples from Ithala and the bulk of the samples from Stellenbosch and Clanwilliam. Therefore, it is unlikely that the difference between CHC profiles of fig wasps from Ithala and those from Stellenbosch and Clanwilliam are the result of genetic differences. Similar to our results for *C. capensis*, the lack of regional genetic structure in *E. stuckenbergi*

indicates that it is unlikely that there is an underlying genetic cause for the clear differences seen between the CHC profiles of fig wasps collected in Ithala as opposed to those collected in Stellenbosch and Clanwilliam.

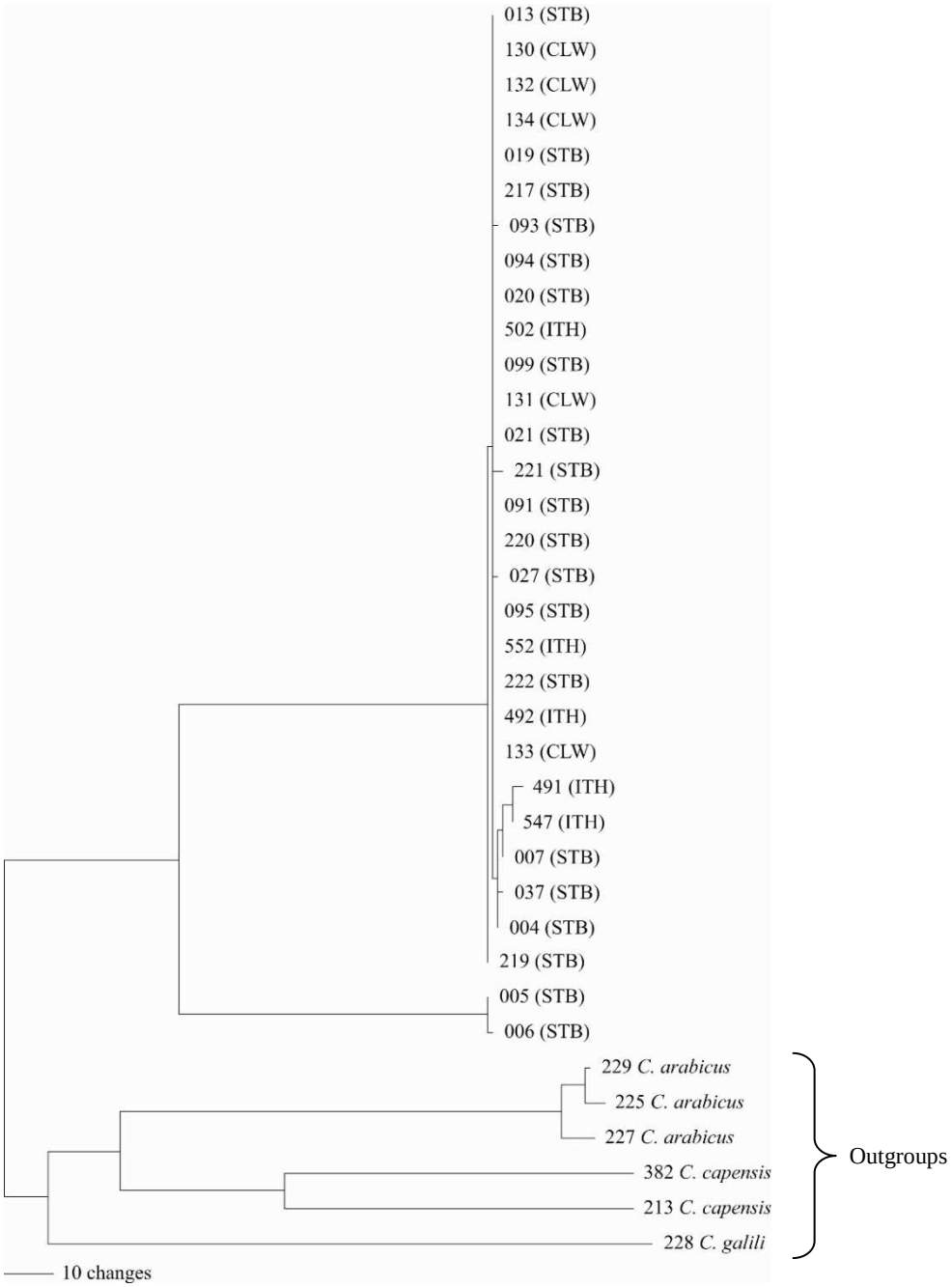

Figure 4.5: Consensus phylogram of samples of *E. stuckenbergi* collected from Clanwilliam (CLW), Stellenbosch (STB) and Ithala (ITH).

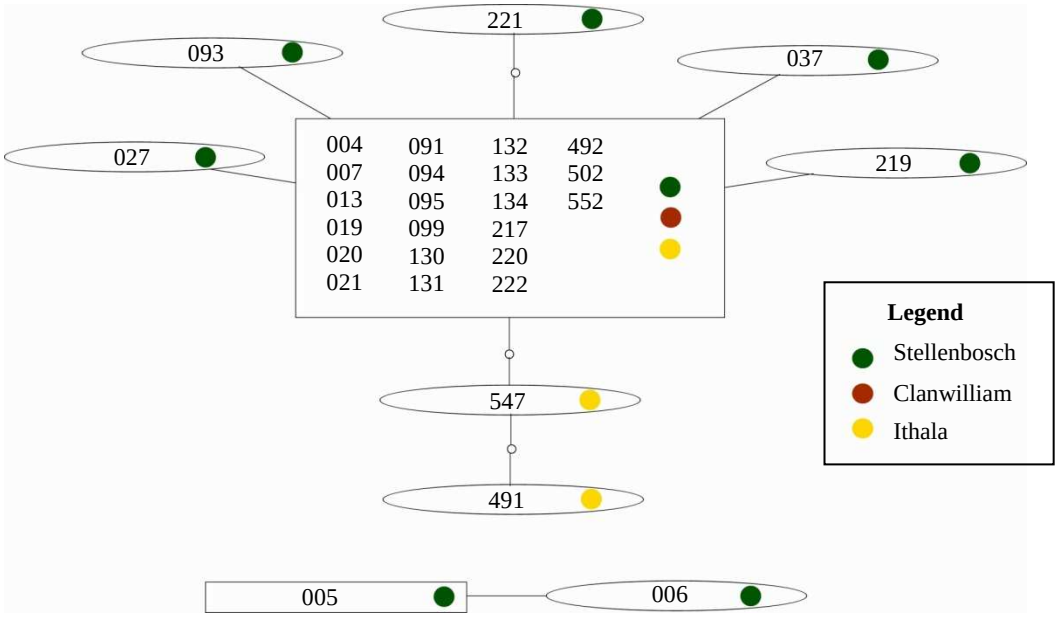

Figure 4.6: Haplotype network representing the distribution of *E. stuckenbergi* haplotypes across regions in South Africa. The sample numbers of individual fig wasps matching each haplotype are given inside the ellipses and match the sample numbers of fig wasp individuals in Figure 4.5. Coloured circles indicate the different regions where a specific haplotype occurred. Small white circles indicate 1-step mutations.

4.4. Discussion

This study found clear intraspecific regional differences in CHC profiles in two pollinating fig wasp species. The observed regional variation in CHC profiles was not supported by population genetic data in either *C. capensis* or *E. stuckenbergi*. Alternative causes of regional variation in CHC profiles of fig wasps must therefore be considered. One of these alternative explanations is that the regional differences in CHC profiles of the pollinating fig wasps investigated are the result of environmental factors.

Previous studies on the difference in CHC profiles in both social and non-social insects have found that CHC profiles varied between different populations (Chapman *et al.*, 1995; Dapporto, 2007; Bagnères and Wicker-Thomas, 2010; Baracchi *et al.*, 2010, reviewed in Howard and Liang, 1993) and regions (Lavine and Carlson, 1991; Lorenzi et al., 1996; Jenkins *et al.*, 2000; Etges and Ahrens, 2001; Dapporto *et al.*, 2004; Dapporto *et al.*, 2009; Bagnères and Wicker-Thomas, 2010). Interestingly, in one study on termites, CHC variation occurred in spite of a lack of genetic variation between the different regions (Jenkins *et al.*, 2000). These authors concluded that the

lack of genetic differentiation (in COII, in this case) indicated a high level of gene flow among populations. From the absence of genetic structuring among populations of the two fig wasp species under investigation here (Figures 4.2 and 4.5), combined with what is known regarding the exceptionally wide dispersal capability of fig wasps (Nason *et al.*, 1996; Ahmed *et al.*, 2009), it appears that a similar conclusion can be reached regarding our data – that the lack of genetic structure may indicate high levels of gene flow resulting in relatively random patterns of highly similar haplotypes across our sampling sites. However, this interpretation must be treated with caution, as a recent article has criticised the tendency of biogeographical studies to infer levels of gene flow from analyses based on assumptions of genetic models (e.g. Hardy-Weinberg equilibrium) that may not always apply (Marko and Hart, 2011). A previous study of fig wasps has already found that geographic distance is not a good indication of potential gene flow (Zavodna *et al.*, 2005), since an island population separated by 40 km from the mainland actually maintained better gene flow with the mainland than closely situated mainland populations did with each other. This suggests that factors other than geographic distance (or conventional barriers, in this case separation by the ocean) may influence gene flow in fig wasps. Similarly, the fact that fig wasps inhabiting regions that are geographically close to each other didn't always group closer together with respect to CHC profiles in this study indicates that in this instance the variation in CHC profiles are also not a simple case of geographic distance between populations, but may rather be heavily influenced by the spatial scale of recruitment of fig wasp communities.

Cuticular hydrocarbon expression has been shown to be a quantitative trait in *Drosophila* (Coyne, 1996; Etges *et al.*, 2009), and since the gene regions sequenced to determine regional genetic structuring in our study are not the specific gene regions responsible for CHC expression in fig wasps, we can only use the inferred results as an indirect indication of potential variation in quantitative trait loci responsible for CHC expression in fig wasps. Considering the absence of population genetic structuring as an explanation for the observed regional variation in fig wasp CHC profiles, we propose three possible environmental factors which may be responsible for the differences in CHC profiles between regions. These factors are possible genetic population structure in the host fig populations, differences in biomes and microhabitats between regions, or variation in fig wasp community structure between

regions. Studies on ants have found that differences in diet may lead to differences in CHC profile (Liang and Silverman, 2000; Buczkowski *et al.*, 2005), and since fig wasp pollinators eat galls produced by the host tree, they may be influenced by differences in the host tree that could be caused by changes in the genetic background of host trees, as well as changes in microhabitats or biomes between regions. So far there have been few studies investigating the genetic population structure of fig species (Dick *et al.*, 2008), but those studies that have examined genetic variation in fig species have reported that a level of genetic differentiation was present between populations (Dev *et al.*, 2011), even if those populations were close together (Wang *et al.*, 2009). Consequently there is a possibility that the differences in the CHC profiles of fig wasps between regions reported here may, to a certain extent, be a reflection of genetic differences between host trees found in different regions.

Another possible environmental influence that could explain these differences in CHC profiles between regions may be the variation in fig wasp community which has already been discussed in Chapter 3 – namely that regional changes as well as random colonisation events could also influence the fig wasp community composition of the figs in a tree, in turn influencing the CHC profile of the pollinators by inter-species transfer of CHCs. Hawkins and Compton (1992) have observed that in most cases, the fig wasp community in a fig will not be saturated, i.e. that not all the species of fig wasp that can potentially occur in a specific fig species will always be present in a syconium. It follows that the exact combination of fig wasp species in a fig can be highly variable, and that this would influence which CHCs the pollinators come into contact with before departing the fig. Before emerging from the fig, female fig wasps move around the inside of the syconium and may come into close contact with other fig wasp species that have also eclosed, which could lead to the transfer of CHCs between species as has been recorded in cockroaches (Everaerts *et al.*, 1997) and termites (Vauchot *et al.*, 1996; Vauchot *et al.*, 1998). Previous studies have found that the community composition of the fig wasps in a fig depends on chance colonisation events (Compton and Hawkins, 1992) and may also in some cases change along a latitudinal gradient (*F. sur*, Compton et al., 1994), so it is possible that the differences in CHC profiles in *C. capensis* and *E. stuckenbergi* may change by region as the fig wasp community changes.

Latitudinal changes in fig wasp community composition cannot, however, explain the little difference between the CHC profiles of *C. capensis* found in Stellenbosch and Ithala (Figure 4.1), which are separated the furthest in latitude. Moreover, *C. capensis* collected from Stellenbosch and Cape Town occurs on the same latitude, and yet these two regions show much less similarity to each other than would be expected, given their geographic proximity. As there is practically no genetic structuring in the fig wasps between Cape Town and Stellenbosch, the differences in CHCs between fig wasps in these two regions must be caused by some environmental influence. As the two regions are situated in the same biome (Mucina and Rutherford, 2006), it is unlikely that climatic or vegetative characteristics are responsible. Although both the collection areas were located in urban areas, Cape Town is a much more extensively urbanised area than Stellenbosch, with expected differences in temperatures caused by the heat island effect, as well as increased levels of air pollution associated with urban areas. The increased levels of pollution associated with the production of vehicle exhaust in cities influences the phenology of plants and increases the physiological stress experienced by the plant (Honour *et al.*, 2009), and increased temperatures can lead to increased emission of volatile organic compounds by trees in urban areas (Cardelino and Chameides, 1990, in Nowak et al., 1999). It could be possible that the fig wasps in Cape Town are also affected by these urban-related stresses, which then affected the expression of CHCs, or that changes in the trees brought on by urban stress affected their CHC profiles of the fig wasps in the syconia.

It is evident that there are multiple factors that could potentially contribute to the observed differences in CHC profiles of pollinating fig wasps between regions. These range from local environmental impacts such as diet, microhabitat variation and community assemblages inside the syconium to potential genetic population structure in the host species. What is clear is that the CHC signal appears to be a very complex attribute in fig wasps, with large scope for further studies in CHC variation between fig wasp pollinators.

4.5. References

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Chapter 5. **Discussion**

This study was the first to investigate the cuticular hydrocarbons (CHCs) of fig wasps. Our main purpose was to determine the composition of the CHC complement of both non-pollinating and pollinating wasps, as well as investigating the possible genetic and environmental influences that determine intraspecies and interspecies variation in the CHC profiles of these insects. Our overall conclusion is that the variation in CHCs in fig wasps is scale-dependant, and that the level of analysis (e.g. between different fig wasp species, between fig wasps collected from different host species, between fig wasps collected from different regions) reveals different patterns of variation. On the broadest scale – between species – there was clear variation between groups, but our findings indicated that on two finer scales – between fig wasps collected from different regions, as well as between fig wasps collected from different host species within a region – there was also clear variation. Large-scale regional differences between Ithala, Baviaanskloof and Mabibi overrode finer-scale differences between fig wasps collected from different host species within Ithala, and for this reason the most important influences on fig wasp CHC profiles are considered to be, from large to small scale: species level, regional level and finally on host species level. The regional differences in CHC profiles observed in this study have also been noted in other species (Lorenzi *et al.*, 1996; Jenkins *et al.*, 2000; Etges and Ahrens, 2001; Dapporto *et al.*, 2004b; Bagnères and Wicker-Thomas, 2010), as has the influence of host species-associated factors on CHC profiles (Stennett and Etges, 1997; Etges and Ahrens, 2001; Bagnères and Wicker-Thomas, 2010).

Our results imply that in conjunction with genetic contributions, other factors such as host fig species, habitat gradients, and other potential environmental sources such as substrate type and climate, as well as species interactions within a fig wasp community, strongly influences CHC variation among fig wasps. Previously CHCs have been used to examine species-level differences in insects for the purpose of determining taxonomy (Jacob, 1979; Lockey, 1988; Copren *et al.*, 2005; Bagnères and Wicker-Thomas, 2010; Blomquist and Bagnères, 2010; Gołebiowski *et al.*, 2010) and for examining intraspecies differences to investigate biogeography (Haverty *et al.*, 1997; Dapporto *et al.*, 2004a; Dapporto *et al.*, 2009), but our results indicate that in the case of fig wasps, CHCs also have the potential to aid in the investigation of insect

community-level interactions as well as fine-scale environmental interactions as an indication of an organism's ecology.

We found that fig wasps do possess species-specific CHC profiles as well as species-group-specific CHC profiles, suggesting that CHC profiles act as recognition cues. Thus CHCs clearly have the potential to be used for chemotaxonomy in fig wasps. However, when using CHCs for chemotaxonomic purposes, researchers will need to take into account the possible effect that genetic differentiation, factors associated with host species, and other regional environmental factors may have on the CHC profiles of fig wasps. Our results have indicated that on finer scale analyses variation in CHC profile by region is particularly important, so chemotaxonomists will have to account for possible regional variation in CHC profiles.

As discussed in Chapter 4, it is highly likely that the species-specificity of CHC profiles in pollinating fig wasps plays an important role in the maintenance of the genetic integrity of a pollinator species, and thus in maintaining host-specific morphological and behavioural features integral to the maintenance of the fig – fig wasp mutualism. Regarding the species-specificity of the groups of fig wasps investigated here, we have found that although multiple genetic lineages of *Otitesella* may occur on one species of tree, no lineage occurred on multiple species of fig. This suggests that the non-pollinating fig wasps used in this study show a high level of host conservatism. Additionally, the different CHC profiles found on fig wasps collected from different host species in the *Otitesella* species-groups may be an indication of speciation having occurred as the result of host tree influence on the CHC profile (which may lead to pre-mating isolation if individuals originating from different host species or populations come into contact again), coupled with changes in fig wasp preferences for different volatile organic compounds (VOCs) emitted by different host species, and fig wasps' preference for these VOCs (i.e. behavioural selection). From the differences in CHC profiles that can be attributed to the influence of factors associated with different host species, we can speculate that switching hosts may lead to altered CHCs as a result of changes in the environment associated with different host species. This, in turn, may lead to pre-mating isolation between other lineages of the same fig wasp species, which will then contribute to speciation by preventing fig wasps from different lineages from mating successfully. In other word, for a fig wasp

to remain specific to their host species probably helps in the maintenance of their CHC profile. This will help maintain the genetic integrity of the species, which will help maintain physiological and behavioural characteristics that helps maintain the mutualism. Other mutualistic arrangements also sometimes depend on maintaining the integrity of the CHC profile, such as in the case of chemical mimicry by parasites (Espelie and Hermann, 1988).

The CHC profiles of fig wasps also exhibited intraspecies and interspecies variation. This variation was observed between fig wasps from different genetic lineages, host species and regions in non-pollinating galling species-groups, as well between fig wasps collected from different regions in pollinating fig wasps. We proposed that the fig wasp CHC variation by host species is caused by a combination of differences in diet when fig wasps inhabit different species of tree, as well as exposure to host-specific fig wasp communities and subsequent exchange of CHCs between different fig wasp species in the syconium [as observed in termites and cockroaches (Everaerts *et al.*, 1997; Vauchot *et al.*, 1998)].

The clearest observed trend in these data is the role that region played in the intra-species and inter-species variation in CHC profiles of fig wasps. Studies on social insects have found that the CHC profile differs between colonies (Lorenzi *et al*., 1996; Dronnet *et al.*, 2006; Tannure-Nascimento *et al.*, 2007; Leonhardt *et al.*, 2009), and has been ascribed to either genetic differences between colonies (Dronnet *et al.*, 2006), environmental differences between colonies (Lorenzi *et al*., 1996), or a combination of both (Dapporto *et al.*, 2009). In fig wasps perhaps the closest analogy to the colony structure characteristic of social insects would be the fig wasp community within a syconium. In the light of this, our study supports studies on social insects that indicated that environmental differences were important in determining variation in CHC profiles between “colonies” (Lorenzi *et al*., 1996; Tannure-Nascimento *et al.*, 2007; Leonhardt *et al.*, 2009), as we found that pollinating fig wasps within a region which contained more than one collection showed less variation than between-region collections. As there was no clear mtDNA haplotype structuring between regions within pollinating fig wasp species, this indicated that environmental regional differences such as variation in the fig wasp community within the syconium, local habitat, and possibly genetic variation between host trees may also contribute to

the variation of CHC profiles. The surprising lack of haplotype structuring in pollinating fig wasps revealed here also invites further investigation into the population genetics of *C. capensis* and *E. stuckenbergi*. Another study which investigated the genetic population structure in two pairs of pollinating fig wasps of two different host species found that the populations investigated showed no geographic or temporal genetic population structure (Molbo *et al.*, 2004). However, the study by Molbo and colleagues (2004) was conducted in a very small geographic area, with most collections being separated by less than 20 kilometres, while our findings indicate that this lack of geographic genetic variation in pollinating fig wasps may not only apply to small geographic areas, but distances stretching thousands of kilometres. From the results of our study there appears to be high levels of gene flow between fig wasp populations across different regions, however, these results are only preliminary, and will need to be confirmed with more in-depth population genetic studies on *C. capensis*, *E. stuckenbergi* and the *Otitesella* fig wasps.

Many questions regarding the role of chemical ecology in the fig-fig wasp mutualism remains to be answered. Here we have presented the first exploration of the factors influencing fig wasp CHCs and the role they may play in fig wasp biology. There is a great range of possible future investigations in the chemical ecology of the fig-fig wasp mutualism in South Africa, including potential studies on the role fig tree VOCs play in pollinator and non-pollinating fig wasp host preference and CHC variation, experimental investigations into the effect of fig wasp community composition on fig wasp CHCs, as well as applying the use of CHCs to chemotaxonomic studies on the fig wasp phylogeny.

5.1. References

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Appendix

Table 6.1: List of the fig wasps used in chapters 2 to 4, as well as the host species they were collected from in each region.

Fig wasp species	Host species	Location	Number of samples
a) Pollinating fig wasps			
E. stuckenbergi	*F. burkei*	Stellenbosch	45
		Clanwilliam	12
		Ithala	5
E. glumosae	*F. glumosa*	Ithala	20
C. capensis	*F. sur*	Stellenbosch	25
		Cape Town	13
		Baviaanskloof	20
		Mtunzini	6
		Ithala	9
b) Non-pollinating fig wasps			
Uluzi species-group	*F. burkei*	Ithala	6
	F. glumosa	Ithala	16
	F. lutea	Mabibi	4
	F. polita	Mabibi	11
(*O. uluzi*)	*F. burtt-davyi*	Baviaanskloof	10
		Ithala	10
Sesqui species-group	*F. glumosa*	Ithala	11
(*O. sesquianellata*)	*F. burtt-davyi*	Ithala	10

Table 6.2: List of all fig wasps sequenced for genetic analyses, Chapters 3 and 4. All samples have been deposited with the Iziko museum, Cape Town.

Sample code	Sample code	Taxon	*Ficus* species	Ficus subsection	Voucher code	Location	Date	Acc. COI	Acc. Cytb	Acc. EF-1a
336_glum_sesq46	336	*Otitesella sp.* (sesqui)	*F. glumosa*	*Platyphyllae*	SA10-F46	Ithala	Oct 2010	JN617542	JN617455	JN704087
339_glum_sesq46	339	*Otitesella sp.* (sesqui)	*F. glumosa*	*Platyphyllae*	SA10-F46	Ithala	Oct 2010	JN617543	JN617456	JN704088
340_glum_uluzi46	340	*Otitesella sp.* (uluzi)	*F. glumosa*	*Platyphyllae*	SA10-F46	Ithala	Oct 2010	JN617559	JN617473	JN704107
344_glum_uluzi46	344	*Otitesella sp.* (uluzi)	*F. glumosa*	*Platyphyllae*	SA10-F46	Ithala	Oct 2010	JN617560	JN617474	JN704108
443_burtt_sesq222	443	*Otitesella sesquianellata*	*F. burtt-davyi*	*Chlamydodorae*	SA10-F222	Ithala	Oct 2010	JN704111	JN617457	JN704089
444_burtt_sesq222	444	*Otitesella sesquianellata*	*F. burtt-davyi*	*Chlamydodorae*	SA10-F222	Ithala	Oct 2010	JN617544	JN617458	JN704090
450_burtt_uluzi222	450	*Otitesella uluzi*	*F. burtt-davyi*	*Chlamydodorae*	SA10-F222	Ithala	Oct 2010	JN617553	JN617467	JN704100
451_burtt_uluzi222	451	*Otitesella uluzi*	*F. burtt-davyi*	*Chlamydodorae*	SA10-F222	Ithala	Oct 2010	JN617554	JN704083	JN704101
506_burk_uluzi205	506	*Otitesella sp.* (uluzi)	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN617555	JN617468	JN704102
508_burk_uluzi205	508	*Otitesella sp.* (uluzi)	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN704112	JN704084	JN704109
511_burk_uluzi205	511	*Otitesella sp.* (uluzi)	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN617545	JN617459	JN704091
512_burk_uluzi205	512	*Otitesella sp.* (uluzi)	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN617546	JN617460	JN704092
556_burk_uluzi205	556	*Otitesella sp.* (uluzi)	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN617556	JN617469	JN704103
557_burk_uluzi205	557	*Otitesella sp.* (uluzi)	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN704113	JN704085	JN704110
602_glum_sesq164	602	*Otitesella sp.* (sesqui)	*F. glumosa*	*Platyphyllae*	SA10-F164	Ithala	Oct 2010	JN617547	JN617461	JN704093
611_glum_uluzi164	611	*Otitesella sp.* (uluzi)	*F. glumosa*	*Platyphyllae*	SA10-F164	Ithala	Oct 2010	JN704114	JN617470	JN704104
612_glum_sesq164	612	*Otitesella sp.* (sesqui)	*F. glumosa*	*Platyphyllae*	SA10-F164	Ithala	Oct 2010	JN617548	JN617462	JN704094
665_poli_uluzi276	665	*Otitesella sp.* (uluzi)	*F. polita*	*Caulocarpae*	SA10-F276	Mabibi	Oct 2010	JN617549	JN617463	JN704095
666_poli_uluzi276	666	*Otitesella sp.* (uluzi)	*F. polita*	*Caulocarpae*	SA10-F276	Mabibi	Oct 2010	JN617550	JN617464	JN704096
760_lut_uluzi314	760	*Otitesella sp.* (uluzi)	*F. lutea*	*Galoglychia*	SA10-F314	Mabibi	Oct 2010	JN617551	JN617465	JN704097
768_lut_uluzi314	768	*Otitesella sp.* (uluzi)	*F. lutea*	*Galoglychia*	SA10-F314	Mabibi	Oct 2010	JN704115	JN617466	JN704098
850_poli_uluzi277	850	*Otitesella sp.* (uluzi)	*F. polita*	*Caulocarpae*	SA10-F277	Mabibi	Oct 2010	JN617552	JN704086	JN704099
948_burtt_uluzi433	948	*Otitesella uluzi*	*F. burtt-davyi*	*Chlamydodorae*	SA10-F433	Baviaanskloof	Oct 2010	JN617557	JN617471	JN704105
949_burtt_uluzi433	949	*Otitesella uluzi*	*F. burtt-davyi*	*Chlamydodorae*	SA10-F433	Baviaanskloof	Oct 2010	JN617558	JN617472	JN704106

Sample code	Sample code	Taxon	*Ficus* species	Ficus subsection	Voucher code	Location	Date	Acc. COI	Acc. Cytb	Acc. EF-1a
Additional *Otitesella* samples (McLeish, M.J., unpublished)										
301_ilc_Otitese	301	*Otitesella sp.*	*F. ilicina*	*Chlamydodorae*	Na07-Wk01	Namibia	Aug 2007	HM007865	HM007978	HM008091
314_stu_O_sesqu1	314	*Otitesella sp.* (sesqui)	*F. stuhlmannii*	*Platyphylla*	MW06-F60	Mozambique	Jun 2006	FJ886816	FJ886890	FJ886964
315_stu_O_uluzi1	315	*Otitesella sp.* (uluzi)	*F. stuhlmannii*	*Platyphyllae*	MW06-F60	Mozambique	Jun 2006	GQ898940	GQ899014	GQ899084
323_nat_O_sesqu1	323	*Otitesella sp.* (sesqui)	*F. natalensis*	*Chlamydodorae*	MW06-F89	Mozambique	Jun 2006	GQ898939	GQ899013	GQ899083
334_bur_O_sesqu1	334	*Otitesella sp.* (sesqui)	*F. burkei/natalensis*	*Chlamydodorae*	ZA06-F14	Zambia	Jun 2006	FJ886815	FJ886889	FJ886963
335_bur_O_uluzi1	335	*Otitesella sp.* (uluzi)	*F. burkei/natalensis*	*Chlamydodorae*	ZA06-F14	Zambia	Jun 2006	GQ898938	GQ899012	GQ899082
345_pet_O_sesqu1	345	*Otitesella sp.* (sesqui)	*F. petersii*	*Chlamydodorae*	ZA06-F46	Zambia	Jun 2006	FJ886814	FJ886888	FJ886962
346_pet_O_uluzi1	346	*Otitesella sp.* (uluzi)	*F. petersii*	*Chlamydodorae*	ZA06-F46	Zambia	Jun 2006	GQ898937	GQ899011	GQ899081
352_lut_O_uluzi1	352	*Otitesella sp.* (uluzi)	*F. lutea*	*Galoglychia*	SA05-F61	South Africa	Nov 2005	HM007853	HM007966	HM008079
364_ott_O_sesqu1	364	*Otitesella sp.* (sesqui)	*F. ottoniifolia lucanda*	*Caulocarpae*	UG05-F01	Uganda	Aug 2005	HM007864	HM007977	HM008090
370_tet_O_sesqu1	370	*Otitesella sp.* (sesqui)	*F. tettensis*	*Platyphyllae*	SA05-F04	South Africa	Nov 2005	HM007863	HM007976	HM008089
376_nat_O_sesqu1	376	*Otitesella sp.* (sesqui)	*F. natalensis graniticola*	*Chlamydodorae*	SA05-F08	South Africa	Nov 2005	HM007862	HM007975	HM008088
377_nat_O_sesqu1	377	*Otitesella sp.* (sesqui)	*F. natalensis graniticola*	*Chlamydodorae*	SA05-F08	South Africa	Nov 2005	HM007852	HM007965	HM008078
385_tet_O_sp2	385	*Otitesella sp.* (sesqui)	*F. tettensis*	*Platyphyllae*	SA05-F31	South Africa	Nov 2005	HM007861	HM007974	HM008087
388_ing_O_digi1	388	*Otitesella sp.*	*F. ingens*	*Urostigma*	SA05-F37	South Africa	Nov 2005	HM007851	HM007964	HM008077
392_tri_O_uluzi1	392	*Otitesella sp.* (uluzi)	*F. trichopoda*	*Platyphyllae*	SA05-F67	South Africa	Nov 2005	HM007850	HM007963	HM008076
395_biz_O_uluzi1	395	*Otitesella sp.* (uluzi)	*F. bizanae*	*Caulocarpae*	SA05-F71	South Africa	Nov 2005	HM007849	HM007962	HM008075
398_burd_O_sesq1	398	*Otitesella sp.* (sesqui)	*F. burtt-davyi*	*Chlamydodorae*	SA05-F82	South Africa	Nov 2005	HM007860	HM007973	HM008086
399_burd_O_uluz1	399	*Otitesella sp.* (uluzi)	*F. burtt-davyi*	*Chlamydodorae*	SA05-F82	South Africa	Nov 2005	HM007848	HM007961	HM008074
401_fis_O_sesqu1	401	*Otitesella sp.* (sesqui)	*F. fischeri*	*Chlamydodorae*	ZA06-F13	Zambia	Jun 2006	HM007859	HM007972	HM008085
402_fis_O_uluzi1	402	*Otitesella sp.* (uluzi)	*F. fischeri*	*Chlamydodorae*	ZA06-F13	Zambia	Jun 2006	HM007847	HM007960	HM008073
404_cya_O_uluzi1	404	*Otitesella sp.* (uluzi)	*F. cyathistipula cyathistipula*	*Cyathistipulae*	ZA06-F21	Zambia	Jun 2006	HM007846	HM007959	HM008072
407_usa_O_uluzi1	407	*Otitesella sp.* (uluzi)	*F. usambarensis*	*Crassicostae*	ZA06-F32	Zambia	Jun 2006	HM007845	HM007958	HM008071
410_sam_O_uluzi1	410	*Otitesella sp.* (uluzi)	*F. sp. samfya*	*NA*	ZA06-F41	Zambia	Jun 2006	HM007844	HM007957	HM008070
414_lin_O_uluzi1	414	*Otitesella sp.* (uluzi)	*F. lingua*	*Chlamydodorae*	MW06-F88	Mozambique	Jun 2006	HM007843	HM007956	HM008069
418_sanm_O_uluz1	418	*Otitesella sp.* (uluzi)	*F. sansibarica macrosperma*	*Caulocarpae*	ZA06-F18	Zambia	Jun 2006	HM007842	HM007955	HM008068
420_mod_O_uluzi1	420	*Otitesella sp.* (uluzi)	*F. modesta*	*Caulocarpae*	MW06-F69	Mozambique	Jun 2006	HM007841	HM007954	HM008067

Sample code	Sample code	Taxon	*Ficus* species	Ficus subsection	Voucher code	Location	Date	Acc. COI	Acc. Cytb	Acc. EF-1a
423_bur_O_sesqu1	423	*Otitesella sp.* (sesqui)	*F. burkei*	*Chlamydodorae*	SA05-F28	South Africa	Nov 2005	HM007858	HM007971	HM008084
429_nat_O_sesqu1	429	*Otitesella sp.* (sesqui)	*F. natalensis natalensis*	*Chlamydodorae*	SA05-F63	South Africa	Nov 2005	HM007857	HM007970	HM008083
438_biz_O_uluzi2	438	*Otitesella sp.* (uluzi)	*F. bizanae*	*Caulocarpae*	SA06-F100	South Africa	Jun 2006	HM007840	HM007953	HM008066
439_cra_O_sesqu1	439	*Otitesella sp.* (sesqui)	*F. craterostoma*	*Chlamydodorae*	SA05-F59	South Africa	Nov 2005	HM007856	HM007969	HM008082
440_cra_O_sesqu2	440	*Otitesella sp.* (sesqui)	*F. craterostoma*	*Chlamydodorae*	KN08-F52	South Africa	Jan 2008	HM007855	HM007968	HM008081
441_cra_O_uluzi1	441	*Otitesella sp.* (uluzi)	*F. craterostoma*	*Chlamydodorae*	KN08-F52	South Africa	Jan 2008	HM007839	HM007952	HM008065
444_cra_O_sesqu3	444	*Otitesella sp.* (sesqui)	*F. craterostoma*	*Chlamydodorae*	KN08-F15	South Africa	Jan 2008	HM007854	HM007967	HM008080
445_cra_O_uluzi2	445	*Otitesella sp.* (uluzi)	*F. craterostoma*	*Chlamydodorae*	KN08-F52	South Africa	Jan 2008	HM007838	HM007951	HM008064
Outgroups:										
443 cra P quatu1	443	*Philocaenus quatuordentatus*	*F. craterostoma*	*Chlamydodorae*	KN08-F15	South Africa	Jan 2008	HM007869	HM007982	HM008095
406 usa Philoc1	406	*Philocaenus sp.*	*F. usambarensis*	*Crassicostae*	ZA06-F32	Zambia	Jun 2006	HM007870	HM007983	HM008096
397 burd P liod1	397	*Philocaenus liodontus*	*F. burtt-davyi*	*Chlamydodorae*	SA05-F82	South Africa	Nov 2005	HM007872	HM007985	HM008098
391 tri P hippo1	391	*Philocaenus hippopotomus*	*F. trichopoda*	*Platyphyllae*	SA05-F67	South Africa	Nov 2005	HM007871	HM007984	HM008097
351 lut P silve1	351	*Philocaenis silvestrii*	*F. lutea*	*Galoglychia*	SA05-F61	South Africa	Nov 2005	HM007873	HM007986	HM008099
340 pet P barba1	340	*Philocaenus barbarus*	*F. petersii*	*Chlamydodorae*	ZA06-F46	Zambia	Jun 2006	HM007874	HM007987	HM008100
330 bur P mediu1	330	*Philocaenus medius*	*F. burkei*	*Chlamydodorae*	ZA06-F14	Zambia	Jun 2006	HM007876	HM007989	HM008102

Table 6.3: List of all *C. capensis* and *E. stuckenbergi* sequenced for genetic analyses, Chapter 4. All samples have been deposited with the Iziko museum, Cape Town.

Sample code	Sample code	Taxon	*Ficus* species	Ficus subsection	Voucher code	Location	Date	Acc. COI	Acc. Cytb
004_burk_stuck1001	4	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F01	Stellenbosch	Feb 2010	JN617596	JN617510
005_burk_stuck1001	5	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F01	Stellenbosch	Feb 2010	JN617597	JN617511
006_burk_stuck1001	6	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F01	Stellenbosch	Feb 2010	JN617598	JN617512
007_burk_stuck1001	7	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F01	Stellenbosch	Feb 2010	JN617599	JN617513
013_burk_stuck1001	13	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F01	Stellenbosch	Feb 2010	JN617600	JN617514
019_burk_stuck1003	19	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F03	Stellenbosch	Feb 2010	JN617601	JN617515
020_burk_stuck1003	20	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F03	Stellenbosch	Feb 2010	JN617602	JN617516
021_burk_stuck1003	21	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F03	Stellenbosch	Feb 2010	JN617603	JN617517
027_burk_stuck1003	27	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F03	Stellenbosch	Feb 2010	JN617604	JN617518
037_burk_stuck1003	37	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F03	Stellenbosch	Feb 2010	JN617605	JN617519
063_sur_cap04	63	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F04	Stellenbosch	March 2010	JN617561	JN617475
070_sur_cap04	70	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F04	Stellenbosch	March 2010	JN617562	JN617476
074_sur_cap04	74	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F04	Stellenbosch	March 2010	JN617563	JN617477
075_sur_cap04	75	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F04	Stellenbosch	March 2010	JN617564	JN617478
076_sur_cap04	76	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F04	Stellenbosch	March 2010	JN617565	JN617479
091_burk_stuck1005	91	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F05	Stellenbosch	March 2010	JN617606	JN617520
093_burk_stuck1005	93	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F05	Stellenbosch	March 2010	JN617607	JN617521
094_burk_stuck1005	94	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F05	Stellenbosch	March 2010	JN617608	JN617522
095_burk_stuck1005	95	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F05	Stellenbosch	March 2010	JN617609	JN617523
099_burk_stuck1005	99	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F05	Stellenbosch	March 2010	JN617610	JN617524
130_burk_stuck1006	130	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F06	Clanwilliam	March 2010	JN617611	JN617525
131_burk_stuck1006	131	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F06	Clanwilliam	March 2010	JN617612	JN617526
132_burk_stuck1006	132	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F06	Clanwilliam	March 2010	JN617613	JN617527
133_burk_stuck1006	133	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F06	Clanwilliam	March 2010	JN617614	JN617528
134_burk_stuck1006	134	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F06	Clanwilliam	March 2010	JN617615	JN617529
195_sur_cap08	195	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F08	Cape Town	April 2010	JN617566	JN617480
203_sur_cap08	203	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F08	Cape Town	April 2010	JN617567	JN617481

Sample code	Sample code	Taxon	*Ficus* species	Ficus subsection	Voucher code	Location	Date	Acc. COI	Acc. Cytb
204_sur_cap08	204	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F08	Cape Town	April 2010	JN617568	JN617482
205_sur_cap08	205	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F08	Cape Town	April 2010	JN617569	JN617483
206_sur_cap08	206	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F08	Cape Town	April 2010	JN617570	JN617484
217_burk_stuck1009	217	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F09	Stellenbosch	May 2010	JN617618	JN617532
219_burk_stuck1009	219	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F09	Stellenbosch	May 2010	JN617619	JN617533
220_burk_stuck1009	220	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F09	Stellenbosch	May 2010	JN617620	JN617534
221_burk_stuck1009	221	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F09	Stellenbosch	May 2010	JN617621	JN617535
222_burk_stuck1009	222	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SU10-F09	Stellenbosch	May 2010	JN617622	JN617536
366_sur_cap199	366	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F199	Ithala	Oct 2010	JN617571	JN617485
367_sur_cap199	367	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F199	Ithala	Oct 2010	JN617572	JN617486
368_sur_cap199	368	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F199	Ithala	Oct 2010	JN617573	JN617487
371_sur_cap199	371	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F199	Ithala	Oct 2010	JN617574	JN617488
381_sur_cap199	381	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F199	Ithala	Oct 2010	JN617575	JN617489
491_burk_stuck10205	491	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN617623	JN617537
492_burk_stuck10205	492	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN617624	JN617538
502_burk_stuck10205	502	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN617625	JN617539
547_burk_stuck10205	547	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN617626	JN617540
552_burk_stuck10205	552	*Elisabethiella stuckenbergi*	*F. burkei*	*Galoglychia*	SA10-F205	Ithala	Oct 2010	JN617627	JN617541
903_sur_cap386	903	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F386	Baviaanskloof	Oct 2010	JN617576	JN617490
904_sur_cap386	904	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F386	Baviaanskloof	Oct 2010	JN617577	JN617491
905_sur_cap386	905	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F386	Baviaanskloof	Oct 2010	JN617578	JN617492
906_sur_cap386	906	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F386	Baviaanskloof	Oct 2010	JN617579	JN617493
907_sur_cap386	907	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F386	Baviaanskloof	Oct 2010	JN617580	JN617494
966_sur_cap367	966	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F367	Mtunzini	Oct 2010	JN617581	JN617495
967_sur_cap367	967	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F367	Mtunzini	Oct 2010	JN617582	JN617496
968_sur_cap367	968	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F367	Mtunzini	Oct 2010	JN617583	JN617497
971_sur_cap367	971	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F367	Mtunzini	Oct 2010	JN617584	JN617498
982_sur_cap367	982	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F367	Mtunzini	Oct 2010	JN617585	JN617499

Sample code	Sample code	Taxon	*Ficus* species	Ficus subsection	Voucher code	Location	Date	Acc. COI	Acc. Cytb
1003_sur_cap429	1003	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F429	Baviaanskloof	Oct 2010	JN617586	JN617500
1004_sur_cap429	1004	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F429	Baviaanskloof	Oct 2010	JN617587	JN617501
1005_sur_cap429	1005	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F429	Baviaanskloof	Oct 2010	JN617588	JN617502
1006_sur_cap429	1006	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F429	Baviaanskloof	Oct 2010	JN617589	JN617503
1007_sur_cap429	1007	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA10-F429	Baviaanskloof	Oct 2010	JN617590	JN617504
1079_sur_cap10	1079	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F10	Stellenbosch	Dec 2010	JN617591	JN617505
1080_sur_cap10	1080	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F10	Stellenbosch	Dec 2010	JN617592	JN617506
1081_sur_cap10	1081	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F10	Stellenbosch	Dec 2010	JN617593	JN617507
1082_sur_cap10	1082	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F10	Stellenbosch	Dec 2010	JN617594	JN617508
1083_sur_cap10	1083	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SU10-F10	Stellenbosch	Dec 2010	JN617595	JN617509
Outgroups:									
213_sur_Cera_sp	213	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	KN08-F01	South Africa	Jan 2008	HM007909	HM008022
225_sym_Ce_arab1	225	*Ceratosolen arabicus*	*F. sycomorus*	*Sycomorus*	KN08-F56	South Africa	Jan 2008	HM007908	HM008021
227_sym_Ce_arab2	227	*Ceratosolen arabicus*	*F. sycomorus*	*Sycomorus*	KN08-F58	South Africa	Jan 2008	HM007907	HM008020
228_Ce_galili	228	*Ceratosolen galili*	*F. sycomorus*	*Sycomorus*	KN08-F58	South Africa	Jan 2008	HM007906	HM008019
229_sym_Ce_arab3	229	*Ceratosolen arabicus*	*F. sycomorus*	*Sycomorus*	KN08-F62	South Africa	Jan 2008	HM007905	HM008018
372_nat_E_stuck1	372	*Elisabethiella stuckenbergi*	*F. natalensis graniticola*	*Chlamydodorae*	SA05-F08	South Africa	Nov 2005	HM007916	HM008029
379_sur_Sycopha1	379	*Sycophaga sp.*	*F. sur*	*Sycomorus*	SA05-F25	South Africa	Nov 2005	HM007926	HM008039
380_sur_Apocgus1	380	*Apocryptophagus sp.*	*F. sur*	*Sycomorus*	SA05-F25	South Africa	Nov 2005	HM007925	HM008038
382_sur_Cer_capen1	382	*Ceratosolen capensis*	*F. sur*	*Sycomorus*	SA05-F27	South Africa	Nov 2005	HM007904	HM008017

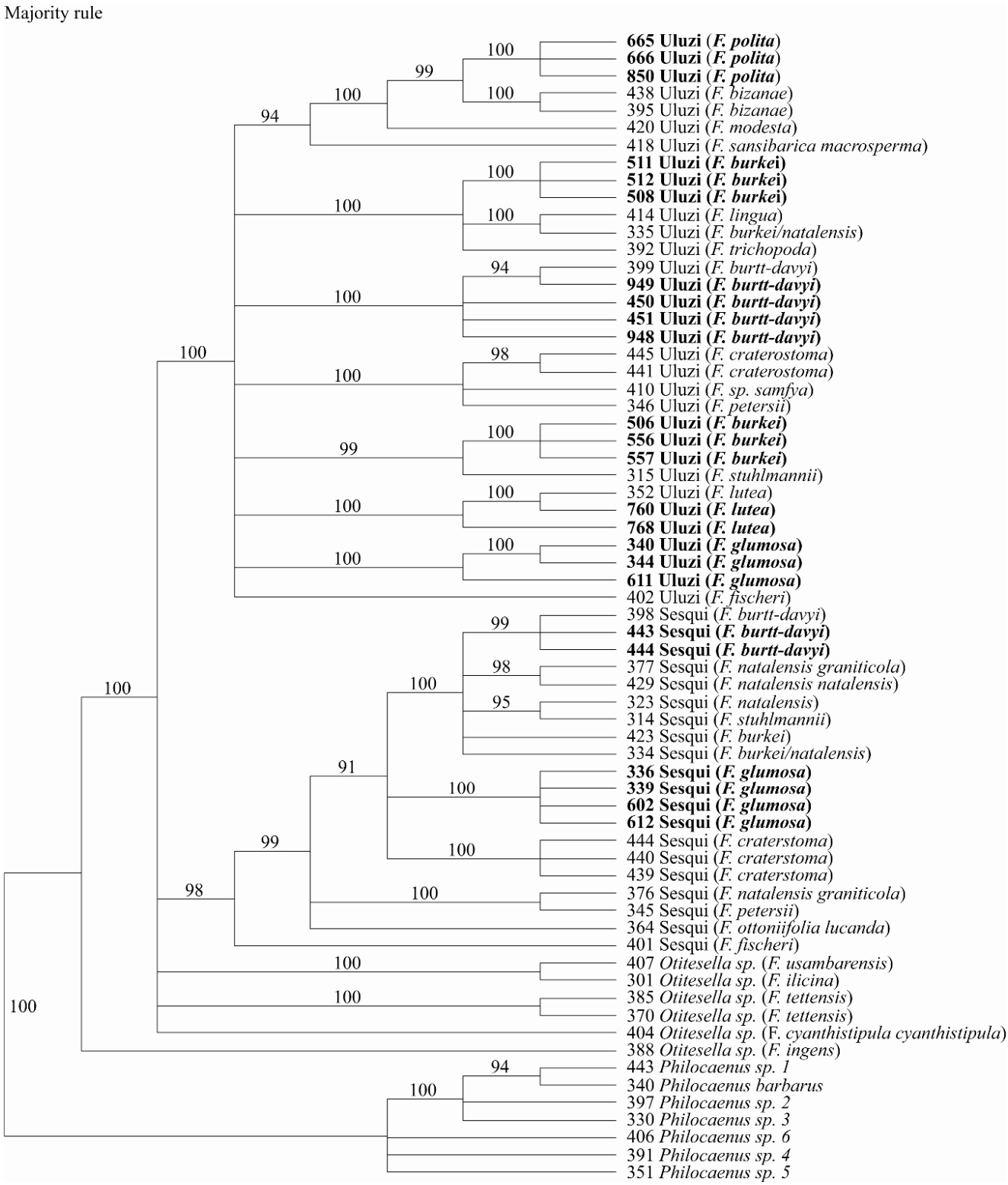

Figure 6.1: Phylogeny with posterior probabilities (>90%) based on the COI, Cytb and EF-1α gene regions of fig wasps from the Uluzi and Sesqui species-groups, as well as additional fig wasps from the genus *Otitesella*. Samples collected for this study and belonging to collections used for GC analyses are in bold. Species names in brackets indicate which host species the fig wasp was sampled from. Fig wasps from the Uluzi species-group were collected from Ithala (bold) as well as Mozambique, Zambia, Uganda and South Africa. Fig wasps from the Sesqui species-group were collected from Ithala, Mabibi and Baviaanskloof (bold), as well as Mozambique, Zambia and South Africa. Remaining *Otitesella* species were collected from Zambia, Namibia and South Africa. Fig wasp samples from the genus *Philocaenus* were used as outgroups.