Phylogeny and codivergence in the fig-fig wasp mutualism: sycoecine and agaondid fig wasps (Chalcidoidea, Hymenoptera) associated with *Ficus* section *Galoglychia* (Moraceae)

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PLAGIARISM DECLARATION

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Jenny Underhill, August 2008

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THESIS ABSTRACT

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The interaction between figs and figs wasps is idealised as a classic example of coevolution through codivergence and cospeciation. Traditionally, the mutualism has been distinguished by a one-to-one ratio of host-specificity, whereby each species of fig tree (*Ficus*, Moraceae) is pollinated by a unique species of fig wasp (Agaonidae, Chalcidoidea, Hymenoptera). Recent studies conclude, however, that extreme host-specificity is no longer as ubiquitous as previously considered. Nevertheless, there are many factors that are thought to constrain host-switching events and maintain, to some degree, the host specificity of both pollinating and non-pollinating fig wasps within the fig wasp community.

This dissertation investigates the extent of codivergence between the host fig trees of *Ficus* section *Galoglychia*, associated agaonid pollinators and sycoecine non-pollinating fig wasps (Pteromalidae, Chalcidoidea, Hymenoptera) through cophylogenetic analysis. All phylogenies used in the analyses were constructed de novo using parsimony and Bayesian methods; new DNA sequence data were generated and combined with sequences retrieved from GenBank. In addition, the evolution of head shape in agaonid and sycoecine fig wasps was explored through ancestral character state reconstruction.

A robust hypothesis of Sycoecine phylogeny, well supported by bootstrap values and Bayesian posterior probabilities, was elucidated through phylogenetic analyses of a combined dataset of mitochondrial and nuclear gene regions. Although the monophyly of the genera *Crossogaster*, *Seres*, *Sycoecus* and *Diaziella* was supported, the sycoecine genus *Philocaenus* appeared paraphyletic. A taxonomic revision of the sycoecinae is

necessary; more taxa will need to be included in the analyses before new generic delimitations may be determined.

Molecular phylogenetic analysis of *Ficus* section *Galoglychia,* constructed using ETS and ITS gene regions, revealed a distinct lack of resolution among a number of species within subsection *Chlamydodorae*. Divergence times within the phylogeny of *Ficus* section *Galoglychia* were dated using Bayesian methods. The origin of this complex of species may be linked to the emergence of the savannah biome since the Miocene, around 8 MYA, in response to changes in African climate or shifts in climate variability. Similar studies appear to be an interesting avenue for future research; coupling paleoenvironmental changes with divergence events within the *Ficus* mutualism may yield fascinating insights into the coevolution of figs and fig wasp lineages, and the factors that drive the speciation of each partner.

The tree-based and distance-based methods of cophylogenetic analysis revealed both significant and non-significant levels of codivergence between the three lineages. Phylogenetic reconstruction of ancestral head shapes of agaonid and sycoecine fig wasps suggested that this character is generally evolutionarily conserved within these two independent lineages. However, the presence of distinct reversals of head shape within the reconstruction indicates that host-specific ostiolar morphology may not prevail to constrain host-shifting events. These results hint at complex history of codivergence between figs and fig wasps, in corroboration with similar, recently published studies.

PREFACE

The chapters of this thesis have been written following the conventions used for the submission of papers to journals: the text is followed by the tables and figures. The only departure from the standard format is that where more than one successive table or figure can fit on a page, this has been done. Each chapter has its own set of references. This results in the replication of some material in each chapter, but I have endeavoured to keep this to a minimum.

I am the primary author of all chapters. I was responsible for the laboratory work, data analysis and writing of papers, including incorporating comments on drafts by the thesis supervisors, Dr Krystal Tolley, Dr Simon van Noort and Prof. Terry Hedderson. Simon van Noort provided samples and fundamental ideas for the thesis. Krystal Tolley provided ideas and supervised laboratory work and data analysis. Publications resulting from this dissertation shall be co-authored by Jenny Underhill, Krystal Tolley and Simon van Noort.

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The University of Cape Town Postgraduate Funding Office (PGFO) and the South African Biosystematics Initiative (SABI) provided financial assistance for my studies. In addition, the PGFO approved a Scholarship for International Travel that allowed me to perform a significant portion of my laboratory work in France. Dr Jean-Yves Rasplus, Dr Roula Zahab and Dr Gwenaelle Genson welcomed me into the molecular laboratories at the Centre de Biologie et de Gestion des Populations, Campus International de Baillarguet, Montferrier-sur-Lez. Roula's efficient assistance meant that together we managed to accomplish more DNA sequencing than I had ever imagined possible.

It has been a pleasure to get to know fellow students and colleagues at SANBI, particularly those working in the Leslie Hill Molecular Laboratory. I am indebted to Kholiwe Balele and Laché Rossouw who trained me in molecular laboratory techniques. Dr Ruan Veldtman commented on early manuscripts, and Dr Michael McLeish permitted me to use a small number of his unpublished sequences in my analyses.

And lastly, to my husband, Jorn Das, my parents, Jane and Les Underhill, my sister, Carynn, family and friends old and new, and especially my 2004 UCT zoology honours classmates: the influence of your love and kindness is immeasurable.

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CHAPTER 1: General Introduction

1.1 Intraspecific mutualisms

Intraspecific mutualisms, where both partners benefit from the interaction, represent a broad spectrum of species associations. Mutualist partners are found in all ecosystems and in all organismal kingdoms (Boucher 1985). Hummingbirds and the flowers they pollinate, gut symbionts in the digestive tracts of animals, sea anemones that protect hermit crabs from predation, and mychorizal fungi that exchange carbohydrates with plants, are a few examples of the diversity of mutualistic interactions (Hoeksema & Bruna 2000). While some mutualistic associations are facultative rather than obligate, other mutualisms are characterized by partners that depend exclusively on each other for reproduction and survival, and complex morphological, physiological or behavioural adaptations to the species interaction may be recognized.

The abundance and diversity of mutualisms has ensured that these interactions remain the focus of many empirical studies (Bronstein 1994, Hoeksema & Bruna 2000). Their potential for determining community structure (van der Heijden *et al.* 1998, Hay *et al.* 2004), and for promoting coevolution (Thompson 1994), has resulted in a multitude of investigations pertaining to the evolution of mutualistic behaviour, the maintenance and stability of mutualisms, and the role of mutualisms in organizing communities (Hoeksema & Bruna 2000, Hay *et al.* 2004).

The fig-fig wasp mutualism (Addicott *et al.* 1990, Herre & West 1997, Weiblen & Bush 2002), examined in this thesis, is one of a few obligate mutualisms recorded between plants and pollinating insects. Other such specialized mutualisms are found in the interactions between yucca plants (Agavaceae) and the yucca moth (Lepidoptera; Riley 1892, Pellmyr *et al.* 1996, Pellmyr 2003), globe flowers (Ranunculaceae) and globeflower flies (Anthomyiidae; Pellmyr 1992) and the association between *Glochidion* trees (Phyllantacea) and Epicephala moths (Gracillariidae) (Kato *et al.* 2003, Kawakita *et al.* 2004).

1.2 The fig-fig wasp mutualism

Figs (*Ficus* spp., Moraceae) and their pollinating fig wasps (Agaonidae, Chalcidoidea, Hymenoptera) present a specialized case of an obligate pollination mutualism (Hill 1967, Galil & Eiskovitch 1971, Janzen 1979, Cook & Rasplus 2003). With few exceptions, neither organism can complete its life-cycle without the other. Each *Ficus* species is reliant on fig wasps for pollination and pollen dispersal and, in return, the pollinating fig wasp depends on the fig for reproduction and larval development (Ramírez 1970, Wiebes 1979, Berg & Wiebes 1992, Weiblen 2002).

There are in excess of 750 species of *Ficus* worldwide, comprising one of the largest angiosperm genera (Berg 1986, Frodin 2004). All fig trees produce the distinctive fig fruit or syconium, essentially an enclosed inflorescence containing numerous tiny male and uniovulate female florets (Verkerke 1989, Berg 1990). Figs have a pan-tropical distribution and are described as keystone species in tropical and subtropical ecosystems (Leighton & Leighton 1983, Terborgh 1986, Lambert & Marshall 1991). They fruit aseasonally, providing an essential and continual supply of food to frugivorous birds and mammals (but see Gautier-Hion & Michaloud 1989). While synchrony exists in intra-tree fruit production, inter-tree asynchrony promotes genetic out-crossing and is essential for the cycling of the mutualism; it is crucial that, when one tree is producing fig wasps, another tree is flowering (Ramírez 1970, Janzen 1979, Bronstein 1987, 1992, Bronstein *et al.* 1990, Compton *et al.* 1994, Anstett *et al.* 1997).

Both monoecious and functionally dioecious reproductive strategies are employed within the genus *Ficus*. Monoecious figs predominate in Africa and Madagascar, while dioecy is prevalent in the New World, the East and in Indo-Australasia (Ramírez 1974, Weiblen 2000). Monoecious fig trees produce inflorescences that perform both male and female functions: pollen production and pollen dispersal and seed production and seed dispersal, respectively. These functions are split in gynodioecious (functionally dioecious) *Ficus*.

When the syconia on a fig tree become receptive to pollination, a suite of host-specific volatiles is released from the fig that attracts female pollinating fig wasps (Barker 1985, van Noort *et al.* 1989, Ware *et al.* 1993, Hossaert-McKey *et al.* 1994, Grison-Pige *et al.* 2001). Pollen-laden females enter the fig cavity via the ostiole, a narrow bract-lined opening located at the apex of the fig. It is a tight squeeze and the females often lose their wings and antennae in the process (Ramírez 1974, Galil & Eiskovitch 1969, Nefdt & Compton 1996). Inside, the female wasps pollinate the stigmas of the florets that line the inside of the fig and oviposit in a proportion of the ovules. Once pollination has occurred, the ostiole closes, sealing the fig cavity (Wieblen 2000).

The pollinating fig wasp larvae develop within the ovules, feeding on galled endosperm tissue. Wasp larval development and host fig development are correlated and within three to twenty weeks the larvae will mature (van Noort & Rasplus 2008). Adult males are the first to emerge into the fig cavity and fights ensue as the wasps compete for access to emerging females (Bronstein & Mckey 1989, Berg & Wiebes 1992). After mating occurs, male wasps will chew an exit hole through the wall of the fig, allowing females to escape (Bronstein & McKey 1989, Berg & Wiebes 1992, Cook *et al.* 1997). Female wasps actively or passively gather pollen from their natal fig and emerge in search of receptive syconia to complete their reproductive cycle, often flying long distances to locate trees with figs at the right stage of development (van Noort 2003). Once the female wasps have exited the fig, the fruit ripens and becomes attractive to frugivores that disperse the seed.

1.3 The fig wasp community

Any chalcid wasp (Chalcidoidea, Hymenoptera) dependent on fig inflorescences for reproduction and larval development is referred to as a "fig wasp" (van Noort & Rasplus 2008); exceptions are the chalcid wasps that parasitize, for example, the lepidopteran and dipteran larvae that facultatively exploit the fig infloresencence. The fig wasp community comprises a diverse assemblage of both pollinating and non-pollinating fig wasps (West *et al.* 1996). Up to 30 fig wasp species may be associated with a single *Ficus* host

(Compton *et al.* 1994). Non-pollinating fig wasps are in direct competition with the pollinators for the resources of the fig. While their biology is generally poorly known, they do appear to be as host-specific and dependent on the fig for their reproduction and development as the pollinators (Ulenberg 1985, van Noort & Compton 1996; Jousselin *et al.* 2008). In essence, they are parasites of the pollination mutualism (Compton *et al.* 1994, West & Herre 1994, West *et al.* 1996, Cook & Rasplus 2003), and appear to provide no benefit to the host tree, although a recent study suggests that parasitic wasps may assist in maintaining the stability of the mutualism (Dunn *et al.* 2008). In addition, there is evidence that a minority of parasitic wasps may play a role in the pollination of their *Ficus* hosts (Jousselin *et al.* 2001).

Previously, the majority of the fig wasps were placed under the Agaonidae, a family comprised of six subfamilies: Agaoninae, Epichrysomallinae, Otitesellinae, Sycoryctinae, Sycoecinae and Sycophaginae (Bouček 1988). Under this taxonomy, however, the Agaonidae were paraphyletic (Machado *et al.* 1996, Kerdelhué *et al.* 1997, Rasplus *et al.* 1998). Subsequent morphological and molecular studies revealed that the different groups of fig wasps are not closely related (Cook & Rasplus 2003). This implies that, through evolutionary time, the various groups of wasps have colonised the fig niche on a number of separate occasions. Current taxonomy maintains that only the pollinating fig wasps remain in the Agaonidae; the Sycoecinae, Otitesellinae and Sycoryctinae have been assigned to the family Pteromalidae (Rasplus *et al.* 1998, Campbell *et al.* 2000). Consequently, five families of chalcid wasp contain wasps that are associated with figs. These are the families Agaonidae, Pteromalidae, Ormyridae, Eurytomidae and Torymidae, although only a small proportion of each family's total species are fig wasps (van Noort & Rasplus 2008). There are two other chalcid groups, the Sycophaginae and Epichrysomallinae, which contain fig wasps, but family affiliations remain undecided. In general, the taxonomic relationships of all these groups are unresolved and are currently under investigation (van Noort & Rasplus 2008).

In contrast to the pollinators, most non-pollinating fig wasps do not enter the fig cavity, and thus disperse no pollen. Instead, oviposition occurs externally through the wall of the

fig (Kerdelhué *et al.* 2000). All the Sycoecinae, however, as well as a few scattered non-pollinating genera (*Sycophaga* Westwood from the Sycophaginae, *Grasseiana* Abdurahiman & Joseph, *Lipothymus* Grandi and *Eujacobsonia* Grandi from the Otitesellinae) are internal ovipositors, mimicking the pollinating fig wasps and entering the fig to lay their eggs in the florets (Compton & van Noort 1992, Cook & Rasplus 2003). Because all internal ovipositors must enter the fig while the ostiole is open, oviposition by internally ovipositing non-pollinating fig wasps and the pollinating fig wasps will coincide (Galil *et al.* 1970, van Noort 2003). External ovipositors may oviposit at any time during fig development, but all fig wasp larval development must coincide with fig maturation (Kerdelhué *et al.* 2000).

Fig wasp larvae may be phytophagous or parasitic; developing larvae feed on endosperm tissue within the galled ovules, or on developing phytophage larvae. Larvae may also be inquiline; initially feeding on the developing larvae and later on galled endosperm tissue (Bronstein 1991, West *et al.* 1996, Kerdelhué 2000). Non-pollinating wasps either mate within the fig or both inside and outside the fig in the case of winged males (Jousselin *et al.* 2004). Emerging females are generally reliant on the males of pollinating fig wasps to chew the exit hole to enable their escape (S. van Noort, pers. comm.).

1.4 Host specificity and codivergence of figs and fig wasps

Traditionally, each species of fig tree was thought to be pollinated by its own species of fig wasp, a relationship that was regarded as the one-to-one ratio of host-specificity between figs and pollinating fig wasps (Ramírez 1970, 1974, Janzen 1979, Wiebes 1979, Herre *et al.* 1997, Cook & Rasplus 2003). The interaction has been idealised as a classic example of coevolution through strict codivergence and cospeciation (Ramírez 1970, Wiebes 1979, Bronstein & McKey 1989, Herre *et al.* 1996, Anstett *et al.* 1997, Jousselin *et al.* 2003). Early taxonomic investigations emphasized that related figs were pollinated by related wasps and found that, on a broad scale, *Ficus* sections or subsections are usually pollinated by a single fig wasp genus (Berg 1989, Berg & Wiebes 1992). More

recently, independent estimates for 10 pairs of fig and pollinator lineages suggest highly significant temporal congruence and a coevolutionary history of between 60 and 100 million years (Rønsted *et al.* 2005).

Taxonomical and molecular studies are revealing more and more exceptions to the one-to-one ratio of host-specificity between fig and pollinating fig wasps (Compton & van Noort 1992, Rasplus 1996, Kerdelhué *et al.* 1999, Lopez-Vaamonde *et al.* 2001, Cook & Rasplus 2003, Molbo *et al.* 2003; Jackson 2004, Machado *et al.* 2005, Haine *et al.* 2006, Erasmus *et al.* 2007, Marussich & Machado 2007, Jousselin *et al.* 2008). Separate studies conclude that host specificity, though consistent, is no longer as ubiquitous as previously considered. For instance, the association of more than one pollinating fig wasp per *Ficus* host, often from more than one genus, as well as one pollinating fig wasp associated with more than one *Ficus* species, are extensively documented both in Old World and Neotropical taxa (Ramirez 1970, Michaloud *et al.* 1985, Compton 1990, Compton *et al.* 1991, Ware & Compton 1992, Rasplus 1996, Keldelhue *et al.* 1999, Molbo *et al.* 2003, Erasmus *et al.* 2007). Multiple pollinators associated with multiple hosts suggests that hybridization and introgression are occurring within *Ficus* lineages, for which there is some evidence on two different continents (Parrish *et al.* 2003, Machado *et al.* 2005). Early published exceptions tended to be viewed as "special cases" (Michaloud 1996, Cook & Lopez-Vaamonde 2001) and were often thought to be a product of erroneous taxonomy (Berg 1989, Wiebes 1987, Rasplus 1996, Weiblen 2002). However, the breakdown of host specificity now appears to be a reflection of an intricate evolutionary history between the fig and fig wasp lineages. Such complex associations imply that events such as host-switches, losses (lineage extinctions, omissions, or lineage sortings) and duplications are common occurrences in the evolutionary history of these independent lineages (Molbo *et al.* 2003, Jackson 2004, Marussich & Machado 2007, Jousselin *et al.* 2008).

Although deviations from host-specificity are now well-documented, both pollinators and non-pollinators are still expected to display some degree of host fidelity with their *Ficus* hosts because of the various morphological, ecological and chemical constraints that act

to constrain rampant host switching (Cook & Rasplus 2003, Marussich & Machado 2007). These constraints are most likely to increase proportionately with taxonomic distance. Preservation of pollinator host-specificity is thought to ensure the stability of the mutualism and the genetic integrity of each species.

The unique signature of volatiles released by the figs when they are receptive for pollination is believed to be the most significant constraint to host-switching events in both pollinating and non-pollinating fig wasps. Female pollinating wasps locate receptive figs by homing in on these host-specific chemical cues (van Noort *et al.* 1989, Ware *et al.* 1993, Hossaert-McKey *et al.* 1994), and appear to be able to identify their particular host species by its volatile profile (Grison-Pigé *et al.* 2002). Non-pollinating fig wasps are documented to respond to the same volatile signals emitted by the figs to attract their pollinators. The volatile profile varies in its composition at different stages of fig development, thus making it possible for wasps to locate figs in the correct phase of development for oviposition (Proffit *et al.* 2007).

While generally considered to be atypical behaviour, female pollinating fig wasps have been recorded to enter fig trees that are not their usual hosts, most often in unusual circumstances such as when trees are planted outside of their natural distribution (Ramírez 1970, Compton 1990, Ware & Compton 1992). If a pollinator host-switch should occur, low pollination success may cause *Ficus* host trees to abort their present crop of figs. Several non-pollinating wasps, however, are able to thwart the abortion of figs (Bronstein 1991, West *et al.* 1996, Marussich & Machado 2007). It has also been suggested that some non-pollinating male fig wasps may be capable of chewing an exit hole through the fig wall allowing females to escape (Marussich & Machado 2007); a behaviour that was previously exclusively assigned to male pollinating fig wasps. Alternatively, if successful pollination of hosts does transpire, pollinator reproduction may fail due to differences in physiological conditions. Suitable temperature conditions and temporal congruence of larval and host fig development are required. In addition, the presence of other wasp competitors and parasitoids already associated with a given

species, particulary those with similar ecology, reduce the likelihood of host switches through niche exclusion (Marussich & Machado 2007).

In internally ovipositing wasps, style length may be an important factor constraining host-switching events. Ovipositor length is a character that is correlated with the mean style length of the associated fig host species (Nefdt 1989). Egg-laying success in external ovipositors is also limited by ovipositor length and strength; certain fig wasps will not be able to exploit figs that have walls that are too hard or too thick for successful oviposition (Marussich & Machado 2007).

Host shifts are thought to be more likely in lineages of externally ovipositing non-pollinating figs than in internally ovipositing pollinating fig wasps (Machado *et al.* 2001, Weiblen & Bush 2002, Cook & Rasplus 2003, Jackson 2004). The rationale is that female non-pollinating fig wasps do not have to conform to the morphological adaptations that are imposed on female pollinating fig wasps for entry into the fig cavity through a host-specific ostiole (Cook & Rasplus 2003). Ostiolar morphology may prevent general entry into the fig cavity for wasps that are not specifically adapted to the ostiolar morphology of that particular *Ficus* species (Janzen 1979, Verkerke 1989, van Noort & Compton 1996). Most non-pollinators have the option to oviposit into the ovules of multiple syconia of multiple fig trees with which they are anatomically compatible (Jackson 2004). These ideas are still contentious, however, and a recent study has shown that externally ovipositing non-pollinating fig wasps may be no less host-specific than the associated pollinating fig wasps (Jousselin *et al.* 2008).

1.5 Cophylogenetic analyses: terminology, methodology and previous studies

Coevolution, codivergence and cospeciation are terms that are often used interchangeably. However, when clearly defined, they describe different evolutionary processes (Charleston & Perkins 2006). The concept of coevolution was first introduced by Erlich & Raven (1964) to describe an insect-plant relationship. No clear definition was

given, however, and usage in the biological literature became broad. Brooks (1979) defined coevolution as a combination of both microevolutionary and macroevolutionary processes described separately as coaccommodation and cospeciation. Coaccommodation was recognized as reciprocal change or mutual modification through time. The term coaccomodation was used with no inference of cospeciation, while the term cospeciation was described as the contemporaneous cladogenesis of the associated lineages (Brooks 1979). In contrast, Thompson (1989) defined coevolution simply as the process of reciprocal evolutionary change in two species or populations of interacting lineages; in short, both species must evolve in response to the interaction.

In the context of this study, codivergence will be considered to be "a process of parallel cladogenesis; the speciation of one biological entity resulting in the speciation of those entities that are associated with it" (Charleston & Perkins 2006). Cospeciation and codivergence may be thought of as special types, or possible consequences, of coevolution. Cophylogenetic analyses evaluate the congruence between two or more phylogenies of distinct lineages at any taxonomic level in order to reveal codivergence (Charleston & Perkins 2006). A pattern of contemporaneous cladogenesis in the host and parasite lineages is termed "cophylogeny". However, congruent cladograms are necessary, but may not be sufficient, to indicate cophylogeny (Thompson 1989, Jackson 2004). Cophylogeny may also arise as in closely associated lineages without a history of coevolution. For example, phylogenies may display parallel topologies due to resource tracking or the invasion of a habitat sequentially and not simultaneously.

In short, cophylogeny is a pattern while coevolution, codivergence and cospeciation are processes (Light 2005). A pattern of cophylogeny reflecting codivergence or cospeciation will emerge only if the taxa have displayed consistent host specificity over time. However, it should be remembered that host specificity does not necessarily imply cospeciation; host specificity may merely describe the current associations of taxa whilst the ancestors did not exhibited host-specific behaviour. When cophylogenetic analyses do not reveal significant congruency between two phylogenies, these results may be explained by the processes of host-switching, independent speciation of figs or fig wasps, extinction events, inaccurate phylogeny estimation and taxonomic error in identifying

species which may be cryptic (Charleston & Perkins 2006). In addition, evidence suggests that evolutionary histories that have been shaped through codivergence may be obscured over evolutionary time (van Noort 1992, Jackson 2004, Jousselin *et al.* 2008).

Thompson (1989) does not regard parallel cladogenesis within a host-parasite interaction as coevolution. Thus, although the interaction between fig trees and pollinating fig wasps may lead to codivergence and, perhaps, cospeciation in the context of coevolution, in many cases, the relationship between non-pollinating fig wasps and host fig trees may not be described as coevolution under Thompson's definition (van Noort 1992). However, the term coevolution is often used loosely, and is regularly defined merely as the evolution of adaptations in two or more species caused by the selective pressures each imposes on the other (Thain & Hickman 2000). Although it is unlikely that non-pollinating fig wasps have large effects on host fig speciation, non-pollinating wasps do pose certain selective pressures to which figs must adapt; the reverse is also true. Nevertheless, in this study, the general term codivergence will be used when coevolution *sensu stricto* cannot be assumed.

In recent years, a number of statistical methods of cophylogeny analysis have been developed and debated (Page 1994, Ronquist 1995, Paterson & Banks 2001, Charleston & Page 2002, Legendre *et al.* 2002, Brooks *et al.* 2004, Siddall 2004, Stevens 2004, Siddall 2005). These methods are most frequently used to explore relationships between hosts and their parasites, but can also be used to explore the relationships between any associated phylogenetic trees or DNA sequence data (Marussich & Machado 2007). The three most commonly used methods of cophylogenetic analysis, widely applied in host-parasite contexts (e.g. Hafner *et al.* 2002, Johnson & Clayton 2003, Hughs *et al.* 2007) as well as within the framework of the fig-fig wasp mutualism (Weiblen & Bush 2002, Cook & Lopez-Vaamonde 2001, Jousselin *et al.* 2006, Jackson 2004), are TreeMap 1.0 (Page 1995), TreeMap 2.02β (Charleston & Page 2002) and ParaFit (Legendre *et al.* 2002). All methods test the null hypothesis that the "host" and "parasite" phylogenies have evolved independently.

There are several tree-based methods for identifying cospeciation; both TreeMap 1.0 (Page 1995) and TreeMap 2.02β (Charleston & Page 2002) utilize a method that is known as reconciliation analysis. Only the topologies of independently derived host and parasite phylogenies are compared. The parasite phylogeny is mapped onto the phylogeny of the host in order to determine optimal reconstructions of the evolutionary history of the two lineages. TreeMap 1.0, the first version of the program, uses parsimony to determine the optimal reconciliations of parasite and host phylogenies that maximize cospeciation and minimize host-switching events. A large number of reconstructions are feasible for the comparison of any two phylogenies, and the search relies on heuristic methods for which it has been criticized (Page and Charleston 1997). In particular, the heuristic search method was blamed for presenting inconsistent internal reconstructions, some of which were deemed to be sub-optimal (Ronquist 1995).

In contrast, TreeMap 2.02β implements the Jungle event-cost algorithm (Page & Charleston 1998, Charleston 1998), rather than parsimony, to determine all the potentially optimal reconstructions of one tree mapped onto another in a way that accommodates specified "costs" and "bounds" in a more computationally efficient way (Charleston 1998). It is more sophisticated than TreeMap 1.0 in that additional sorting events that allow complex host switches, including those that involve successive sorting events to make source and destination contemporary, can be performed (these are termed "weakly incompatible switches"; Charleston 1998, Jackson 2004, Stevens 2004).

While TreeMap 1.0 may yield numerous solutions with an identical number of cospeciation events, TreeMap 2.02β uses the Jungle algorithm to search for all plausible reconstructions within specified bounds. It is possible to set the costs of four parameters: cospeciation, duplication, loss (lineage sorting, omission or extinction) and host switching, individually. This enables the total cost of each potentially optimal past association to be calculated. In addition, maximum and/ or minimum bounds for each parameter may be preset such that sub-optimal reconstructions may be discarded; the lowest cost being the most optimal. Finally, the significance of the observed codivergence is then determined using randomization tests. Reconciliation analysis is

computationally intensive, however, and the program is currently limited in terms of the size and complexity of datasets that can be inputted; the number of potentially optimal solutions increases exponentially with the number of associations in the dataset (Jackson 2004). Thus it is often necessary to start the analysis with strict bounds, and gradually make them more relaxed (Jackson 2004).

ParaFit (Lengendre *et al.* 2002) is, as yet, the only distance-based method of cophylogenetic analysis. This program uses distance matrices, rather than tree topologies, to test for congruence. It is also able to assess phylogenetic congruence globally (across both phylogenies), as well as identify specific host-parasite pairs that are significantly associated. In the analysis, the "Host" and "parasite" patristic distance matrices, taken from the phylogenies, are transformed into principle coordinates. The two matrices of principle coordinates and a third matrix of host associations are combined. A test statistic is then computed via a fourth-corner approach (Legendre *et al.* 2001) and compared to a randomized null distribution of host-parasite associations via a permutation procedure. Unlike TreeMap, ParaFit is able to accommodate uncertainty within tree topologies.

A number of cophylogenetic analyses have explored fig-fig wasp codivergence. Early studies compared fig phylogenies with pollinating fig wasp phylogenies (Herre *et al.* 1996, Weiblen 2000, 2001) and non-pollinating fig wasps (Machado *et al.* 1996). In the absence of species-level fig phylogenies, early studies focused on assessing codivergence between pollinating and non-pollinating fig wasps (Machado *et al.* 1996, Lopez-Vaamonde *et al.* 2001). Conclusions of quantitative studies exploring the host-specificity of figs and their non-pollinating fig wasps have generally indicated significant, but incomplete, codivergence between lineages of pollinators, non-pollinators and their host figs (Machado *et al.* 1996, Lopez-Vaamonde *et al.* 2001, Weiblen & Bush 2002, Jackson 2004, Jousselin *et al.* 2005, Machado *et al.* 2005, Marussich & Machado 2007). Certainly, there is support for codivergence among figs wasps from different ecological guilds (Kerdelhué *et al.* 2000, Marussich & Machado 2007, Jousselin *et al.* 2008). However, the lack of pervasive codivergence challenges the classical notion of strict-sense coevolution of figs and their associated pollinating and non-pollinating wasps; the

history of codivergence between figs and fig wasps is complex. Host-shifting and "diffuse coevolution" is now recognized as playing a significant role in the evolution of associations between figs and fig wasp (Jackson 2004, Machado *et al.* 2005, Marussich & Machado 2007, Jousselin *et al.* 2008).

There has been a strong perception (Machado *et al.* 2001, Weiblen & Bush 2002, Jackson 2004, Cook & Rasplus 2003) that externally ovipositing non-pollinating fig wasps are more likely to experience host shifts than the internally ovipositing pollinating fig wasps. A recent analysis performed on the phylogenies of three monophyletic groups of fig wasps, the Agaonidae, Otitesellinae and two *Phylotrypesis* clades (Sycoryctinae), and the phylogeny of the associated *Ficus* section *Galoglychia,* showed significant congruence between the phylogenies of non-pollinating fig wasps and the host fig phylogeny (Jousselin *et al.* 2008). Non-pollinating fig wasps may be at least as constrained to a host as their associated pollinating fig wasps.

1.6 Study taxa

1.6.1 *Ficus* section *Galoglychia*

The genus *Ficus*, comprised of ca. 750 species, is structured into four subgenera, 18 sections and numerous subsections. Berg (1992) recognised 105 species of *Ficus* in the Afrotropical region. This number has increased to 112 species with the publication of a new treatise of south-central and southern African *Ficus* (Burrows & Burrows 2003). In this work a number of species previously synonymised under *F. thonningii* have been resurrected, and *F. modesta* and *F. salicifolia* have been recognised as good species (van Noort & Rasplus 2008). Section *Galoglychia* belongs to the monoecious subgenus *Urostigma* and contains approximately 78 species (Figure 1.1; Berg & Wiebes 1992; Burrows & Burrows 2003; van Noort & Rasplus 2008). All occur within the Afrotropical region, a biogeographic area that includes Africa south of the Sahara, the southern Arabian Peninsula, Madagascar and the Mascarene Islands (Berg & Wiebes 1992).

Subgenus *Urostigma* displays a worldwide distribution and, besides the section *Galoglychia*, it contains the sections *Urostigma*, *Americana* and *Stilpnophyllum*. The section *Galoglychia* has been closely associated with the Neotropical subsection *Americana*. Recent molecular phylogenies of *Ficus* based on ETS, ITS and G3pdh nuclear sequence data suggest that section *Galoglychia* is paraphyletic with respect to section *Americana* (Rønsted *et al.* 2005, 2007, 2008). However, this placement is not supported by boostrap values or Bayesian posterior probabilities, and the closest relatives of section *Americana* remain uncertain (Rønsted *et al.* 2007).

The recent molecular analysis has shown that the six subsections of section *Galoglychia* are largely monophyletic and fall into two major clades (Rønsted *et al.* 2007). The first clade comprises subsections *Platyphyllae* and *Chlamydodorae*. Distributions of species within this clade are concentrated in eastern Africa extending to Madagascar and the Mascarene Islands. The subsections *Caulocarpae*, *Cyathistipulae*, *Galoglychia* and *Crassicostae* form the second major clade, and species distributions centre in west and central Africa. Rønsted (2007) proposed that the distribution of the species within each separate clade coincides with two of six important centers of endemism located within sub-Saharan Africa, as delimited by Linder (2001).

Rønsted *et al.* (2005) used a calibrated phylogenetic tree of *Ficus* using both non-parametric rate smoothing and penalized likelihood methods to account for deviations from a molecular clock. The published ultrametric tree contained 146 taxa of *Ficus*; to date, it is the largest phylogeny of the genus *Ficus* (Rønsted *et al.* 2005). According to dates published in Rønsted *et al.* (2007), but determined from Rønsted *et al.* (2005), section *Galoglychia* originated 40 million years ago (MYA), followed by the divergence of two main clades around 38 MYA. The clade containing the subsections *Platyphyllae* and *Chlamydodorae* diverged approximately 31 MYA ago, while 33 MYA is the estimate for the divergence of the clade comprising *Caulocarpae*, *Cyathistipulae* and *Galoglychia*.

Within section *Galoglychia*, a group of species belonging to subsection *Chlamydodorae* have, over various taxonomic revisions, either been lumped into complexes or split into separate entities. Section *Chlamydodorae* consists of 13 savannah woodland and rainforest fig species, and species distributions centre in eastern Africa, although many are widespread species (Berg & Wiebes 1992). The *Ficus thonningii* complex was instated by Berg (Berg & Wiebes 1992, van Greuning 1990) to override a number of entities with confusing variation. Berg (1989) asserted that the group was either a species complex or a complex currently undergoing speciation.

Burrows & Burrows (2003) opposed this grouping and maintained that variation in geography, pollination and ecology is likely to reveal distinct entities within the complex. Although they recognized many of the forms as separate species, they admitted that the complex is most probably a recently evolved lineage and suggest that the various entities may not yet be reproductively isolated species. Where distribution ranges of fig species overlap, hybrid taxa may be common (Burrows & Burrows 2003, Rønsted *et al.* 2007).

1.6.2 Pollinators of *Ficus* section *Galoglychia*

Of all the fig wasps, taxonomy and host relationships are best known within the Agaonidae (Chalcidoidea, Hymenoptera), the monophyletic family of pollinating fig wasps (Rasplus *et al.* 1998). While widespread exceptions to the one-to-one ratio of host-specificity between pollinating fig wasps and host fig trees continue to emerge, the pollinators of section *Galoglychia* present particularly well-documented exceptions to the one-to-one ratio of host-specificity (Rasplus 1996, Erasmus *et al.* 2007).

Seven fig wasp genera pollinate the figs in section *Galoglychia*: *Alfonsiella*, *Elisabethiella*, *Nigeriella*, *Courtella*, *Agaon*, *Allotriozoon*, and *Paragaon* (Figure 1.2). The first major *Galoglychia* clade (Rønsted *et al.* 2007) contains four subsections, each of which is associated with one fig wasp genus: *Caulocarpae*, associated with *Courtella*; *Cyathistipulae*, associated with *Agaon; Galoglychia* associated with *Allotriozoon* and

Crassicostae, associated with *Paragaon*. Within the second of the *Galoglychia* clades (Rønsted *et al.* 2007) subsection *Chlamydodorae* is associated with the pollinator genera *Elisabethiella* and *Alfonsiella,* and section *Platyphyllae* with *Elisabethiella*, *Alfonsiella* and *Nigeriella*. This is unusual because most *Ficus* sections or subsections are pollinated by a single genus of fig wasp.

A recent molecular analysis supports the monophyly of these morphologically delimited genera, although conflict still surrounds their placement within the phylogeny (Wiebes 1982, Erasmus *et al.* 2007). The DNA sequence data indicate that the pollinators of *Ficus* section *Galoglychia* appear to be less constrained to a specific host than other pollinating fig wasp genera, suggesting frequent host-switching, duplication and lineage extinction events (Compton & van Noort 1992, Ware & Compton 1992, Erasmus *et al.* 2007, Jousselin *et al.* 2008). Estimates of African taxa suggest that more than one pollinator per host and one pollinator for two or more hosts occur in 17% and 15% of cases respectively (Rasplus 1996).

The lack of congruence between the classification of *Galogychia* pollinators and their fig phylogeny necessitates testing the validity of the current taxonomic delimitations of the taxa. In addition, species that are widespread and associated with numerous hosts may either be opportunistic parasites or may represent a suite of cryptic species. For example, a new species of *Alfonsiella* was recently delimited based on molecular evidence and subsequent morphological reappraisal (Erasmus *et al.* 2007).

1.6.3 The Sycoecinae

The Sycoecinae (Pteromalidae, Chalcidoidea, Hymenoptera) are a predominantly Old World group of non-pollinating fig wasps. Four of the six genera, *Crossogaster*, *Philocaenus*, *Sycoecus* and *Seres*, are restricted to the Afrotropical region and two genera, *Diaziella* and *Robertsia* occur in Indo-Australasia (Figure 1.3). Van Noort & Rasplus (2008) estimated from host associations that only 35% of the world's sycoecine

fig wasp species are described. Current taxonomy is based on morphological delimitation, and, as yet, no molecular analysis has been attempted.

In contrast to the other fig-associated pteromalid subfamilies, all sycoecines are internal ovipositors that enter the fig cavity via the ostiole to lay their eggs at the same time as the pollinators (van Noort 1992), emerging from the figs at the same time as the agaonids. It is thought that they are attracted to the same host-specific volatiles released by receptive figs to attract pollinating fig wasps (S. van Noort pers. comm.); there is evidence from studies of New World non-pollinators that suggests that non-pollinating fig wasps do use the same chemical cues as the pollinators (Marussich & Machado 2007, Proffit *et al.* 2007).

The Sycoecinae lack specialized pollen-carrying adaptations, and are generally thought to play no active role in pollination. However, Newton & Lomo (1979) reported a case of accidental pollination by a sycoecine and Jousselin *et al.* (2001) reported efficient pollination of passively pollinated Indo-Australasian *Ficus* species by the sycoecine genus *Diaziella*. The biology of these wasps is very similar to that of the agaonid wasps and it is not apparent why internal ovipositors do not establish a mutualistic relationship with their host fig (Herre 1999), but see Jousselin (2001).

Sycoecine larvae are phytophagous, feeding on galled endosperm tissue. There is no evidence that the Sycoecinae are typical seed predators that require pollinated ovules in which to oviposit. However, larval success of the Agaonidae is increased if they develop in pollinated ovules and the same may be true of the Sycoecinae (Galil & Eiskovitch 1971, Verkerke 1986). In addition, *Sycoecus thaumastocnema*, has been observed to enter the fig cavity to oviposit before the associated pollinator *Agaon fasciatum* (van Noort 1992), and sycoecines are capable of successfully reproducing and emerging from the fig in the absence of pollinators (S. van Noort pers. comm.), suggesting that sycoecine development may not be wholly dependent on successful pollination of the fig fruit.

Convergent evolution is believed to account for the morphological similarity between the sycoecine and pollinating fig wasps (van Noort & Compton 1996); both lineages have evolved similar adaptations under identical selection pressures due the constraints of internal oviposition. These morphological adaptations, such as elongated and dorso-ventrally flattened heads and thoraces, and the presence of tibial and mandibular modifications, enable both pollinators and sycoecines to crawl through dense bracts surrounding the ostiole and enter the fig cavity. Van Noort & Compton (1996) revealed a correlation between fresh fig diameter of fig trees in *Ficus* section *Galoglychia* and head shape (calculated as the ratio of head width to head length) of female agaonid and sycoecine fig wasps associated with those fig tree species.

It is supposed that there is increased potential for some extent of cophylogeny between the Sycoecinae and their *Ficus* hosts (Jackson 2004). This may be explained by the selective pressures imposed by ostiolar morphology that may mitigate against host-switching events (Janzen 1979, Verkerke 1989, van Noort 1992, van Noort & Compton 1996). Externally ovipositing non-pollinating fig wasps do not have to conform to the physical adaptations required for entry into the fig cavity through a host-specific ostiole. The majority of non-pollinating fig wasps oviposits externally through the fig wall, and is thus more likely to experience host shifting events than internal ovipositors (Cook & Rasplus 2003).

1.7 Study objectives

This dissertation has five key objectives. Firstly, to investigate the phylogenetic relationships of the sycoecine non-pollinating fig wasps using mitochondrial and nuclear DNA sequences and, in addition, to address the taxonomical, evolutionary and biogeographical implications of the phylogenetic inference. Secondly, to incorporate novel DNA sequence data into preceding molecular datasets and construct the phylogenies of *Ficus* section *Galoglychia,* and of the pollinators of *Ficus* section *Galoglychia.*

Thirdly, to investigate the extent of cophylogeny between the Sycoecinae, the pollinators of *Ficus* section *Galoglychia*, and *Ficus* section *Galoglychia*, using the three most commonly used methods of cophylogenetic analysis. Fourthly, to explore the evolution of head shape in agaonid and sycoecine fig wasps through ancestral character reconstruction. Fig wasp head shape may maintain host-specificity within the fig-fig wasp mutualism through the constraints imposed by ostiolar morphology, and, within monophyletic clades, conservatism of head shape should be observed.

The final objective was to obtain date estimates for divergence events within the molecular phylogeny of *Ficus* section *Galoglychia*. In order to provide insight into the mechanisms that instigate *Ficus* diversification, divergence times were linked to paleoenvironmental changes.

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Figure 1.1: The diversity of *Ficus*, subgenus *Urostigma*, section *Galoglychia*: (a–c) fig trees displaying the three broad categories of growth forms: (a) *Ficus tettensis*, rock-splitter, (b) *Ficus usambarensis*, strangler, (c) *Ficus lutea*, free-standing tree; (d–i) diversity of leaves and syconia: (d) *Ficus trichopoda*, (e) *Ficus natalensis graniticola*, (f) *Ficus stuhlmannii*, (g) *Ficus ottonifolia lucanda*, dissected galled fig, (h) *Ficus lingua depauperata*, (i) *Ficus tremula tremula* (van Noort & Rasplus 2008).

Figure 1.2: Scanning electron micrograph (SEM) and photographs of agaonid pollinators associated with *Ficus* section *Galoglychia*: (a) SEM of *Nigeriella* sp., female; (b) *Elisabethiella socotrensis*, female; (c) *Elisabethiella socotrensis*, female, ventral head view; (d) *Elisabethiella baijnathi*, female, lateral head view; (e) *Agaon kiellandi*, female; (f) *Agaon kiellandi*, female, antennal club; (g) *Agaon kiellandi*, male (van Noort & Rasplus 2008).

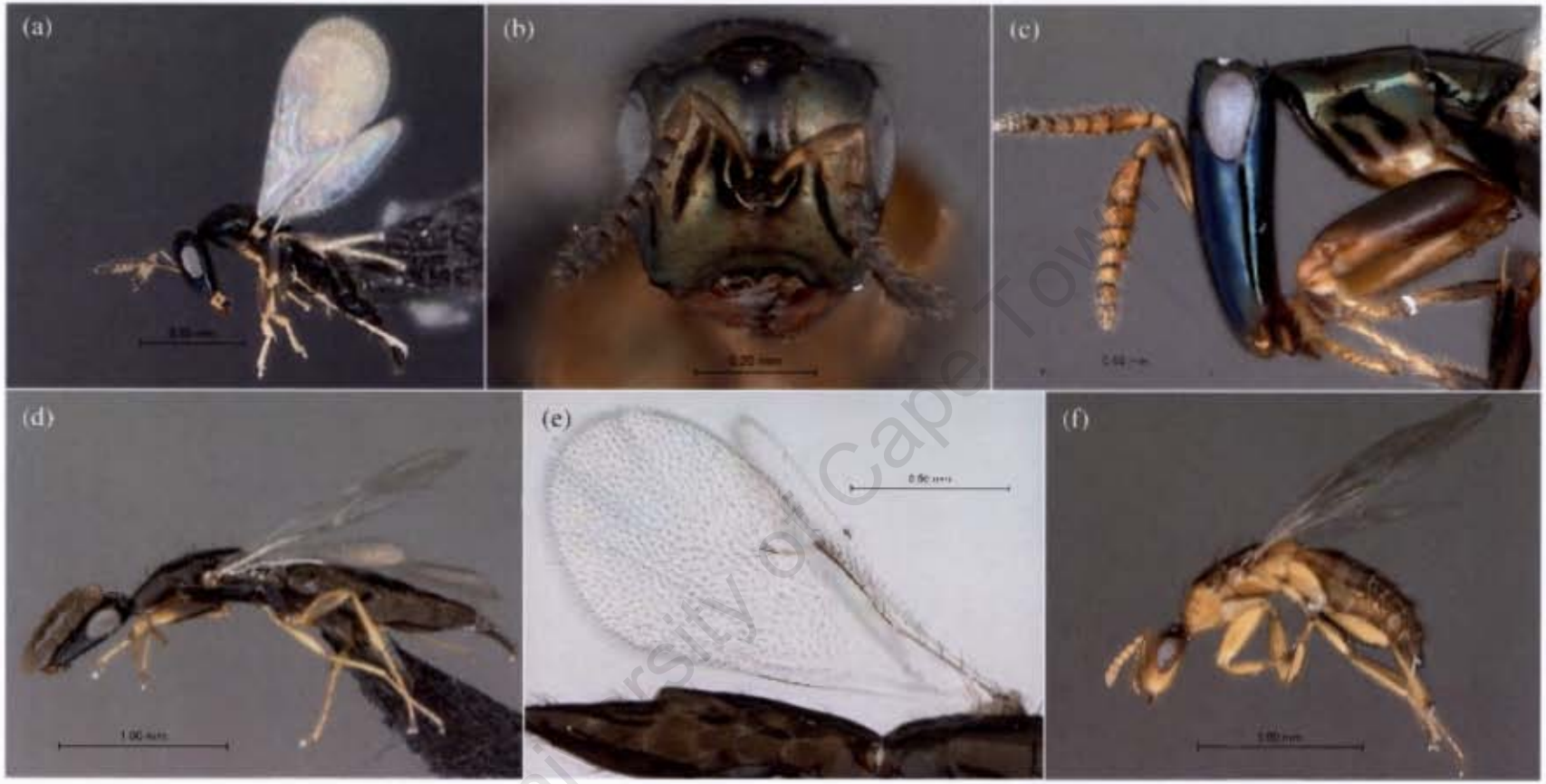

Figure 1.3: Photographs of sycoecine fig wasps. (a) *Philocaenus tiodontus*, female; (b) *Diaziella yangi*, female, frontal head view; (c) *Crossogaster insitata*, female, lateral head view; (d) *Sycoecus taylori*, female; (e) *Sycoecus taylori*, female, fore wing; (f) *Sycoecus taylori*, male (van Noort & Rasplus 2008).

CHAPTER 2: Molecular phylogeny of the Sycoecinae (Pteromalidae, Chalcidodea, Hymenoptera)

2.1 Abstract

The subfamily Sycoecinae (Pteromalidae, Chalcidoidea, Hymenoptera) is a predominantly Old World group of non-pollinating fig wasps comprising six genera. This study was the first attempt to elucidate the taxonomic relationships within the Sycoecinae through phylogenetic analysis using both mitochondrial and nuclear gene regions: COI, COII, Cytb and ITS2. The topologies of the parsimony and Bayesian analyses of the combined dataset were well supported by bootstrap values and Bayesian posterior probabilities, in contrast to the parsimony and Bayesian analyses of each individual gene region. Phylogenetic analyses of the combined dataset revealed three major clades. The monophyly of the genus *Crossogaster* was supported (97% BS, 100% PP), the genera *Philocaenus*, *Seres* and *Sycoecus* clustered together in one strongly supported clade (90% BS, 100% PP). The monophyly of the genera *Sycoecus* (98% BS, 100% PP) and *Seres* (99% BS, 100% PP) were well supported, however, the genus *Philocaenus* appeared paraphyletic, challenging the morphological delimitation of the sycoecine genera. The placement of the clade containing the Indo-Australiasian genus *Diaziella* is not supported, and differs between the Bayesian and parsimony phylogenies. A taxonomic revision of the Sycoecinae is necessary; further taxon sampling and molecular analysis will be necessary before new generic delimitations can be determined. Fne-scale phylogenetic studies of the Sycoecinae may also verify the occurrence of cryptic species, of which this phylogeny offers only insubstantial evidence.

2.2 Introduction

Every species of fig tree (*Ficus*, Moraceae) hosts a unique assemblage of fig wasps that reproduce exclusively within the fig fruit, or syconium, which is an enclosed inflorescence (Ramirez 1970, Galil & Eiskovitch 1971, Berg & Wiebes 1992). Pollinating fig wasps, in turn, pollinate the fig tree; neither partner of the fig-fig wasp mutualism can complete its lifecycle without the other (Hill 1967, Janzen 1979, Cook & Rasplus 2003). Female pollinating fig wasps enter receptive figs through the ostiole, a narrow, bract-lined opening located at the apex of the fig, in order to oviposit in the ovules of the florets that line the inside cavity of the syconium (Ramirez 1969, Berg & Wiebes 1992). This plant-insect relationship is, in many instances, highly host-specific, with a "one-to-one ratio" between figs and pollinating fig wasps purported to occur (Ramirez 1970, Janzen 1979, Wiebes & Compton 1990, Herre et al. 1997). However, a growing body of evidence shows that exceptions to the one-to-one ratio of host-specificity are ubiquitous (Rasplus 1996, Kerdelhué *et al.* 1999, Cook & Rasplus 2003 Jackson 2004, Machado *et al.* 2005, Erasmus *et al.* 2007), suggesting that processes other than strict cospeciation have shaped the evolutionary history of the fig and fig wasp lineages.

While all fig wasps belong to the hymenopteran superfamily Chalcidoidea (Bouček 1993), only those belonging to the family Agaonidae are pollen vectors (but see Jousselin *et al.* 2007). Besides the Agaonidae, four families of non-pollinating chalcid fig wasps have colonized the fig independently of the pollinating fig wasps (Rasplus *et al.* 1998). These are the families Pteromalidae (Sycoecinae, Sycoryctinae, Otitesellinae), Ormyridae, Eurytomidae and Torymidae plus two groups (Sycophaginae and Epichrysomallinae) whose family affiliations are undecided, although only a small proportion of each family's total species are associated with figs (van Noort & Rasplus 2008). Non-pollinating fig wasps are parasites of the fig-fig wasp mutualism and provide no obvious benefit to the host fig tree (Compton *et al.* 1994, West & Herre 1994, Cook & Rasplus 2003), although a recent study suggests that externally ovipositing parasitic wasps may play a role in maintaining the stability of the mutualism (Dunn *et al.* 2008).

The subfamily Sycoecinae (Pteromalidae, Chalcidoidea, Hymenoptera) is a predominantly Old World group of non-pollinators restricted to the *Ficus* subgenus *Urostigma*. Four of the six genera, *Crossogaster*, *Philocaenus*, *Seres* and *Sycoecus*, are restricted to the Afrotropical region and are associated with *Ficus* section *Galoglychia*. Two genera occur in Indo-Australasia: the genus *Diaziella* is restricted to south-east Asia and is associated with *Ficus* section *Urostigma*, subsection *Conosycea*, and the Australasian genus *Robertsia* is associated with *Ficus* section *Stilpnophyllum*, subsection *Malvanthera*. The delimitation of these genera is based on morphological taxonomy (van Noort 1992, 1993a, 1993b, 1994a, 1994b, 1994c) and their monophyly requires evaluation using molecular phylogenetic methods.

Although current taxonomy of the Sycoecinae supports a degree of host specificity between the wasps and their *Ficus* hosts, strict cospeciation cannot be supposed (van Noort 1992). For instance, the association of more than one sycoecine per *Ficus* host, often from more than one genus, and one sycoecine associated with more than one *Ficus* species, occurs frequently. Such complex associations imply that events such as host-switches, losses (lineage extinctions, omissions, or lineage sortings) and duplications are common incidents in the evolutionary history of these independent lineages. In addition, sycoecine species that are associated with many hosts may either be opportunistic parasites or may represent a suite of cryptic species. The genera *Crossogaster* and *Philocaenus* are morphologically very similar and are believed to be sister taxa (van Noort 1992). *Crossogaster* and *Philocaenus* species are often found on the same host, particularly within the subsections *Platyphyllae* and *Chlamydodorae* of *Ficus* section *Galoglychia*.

The Sycoecinae are internal ovipositors that enter the fig through the ostiole to oviposit within the syconium at the same time as the pollinating fig wasps and show remarkable convergent morphological adapations with the pollinators (van Noort & Compton 1996). In contrast, most non-pollinating fig wasps oviposit externally through the fig wall and are thus more likely to experience host shifts than the internal ovipositors because they do

not have to conform to the physical adaptations necessary for passage through a host-specific ostiole (Cook & Rasplus 2003). The potential for some extent of cophylogeny between the Sycoecinae and their *Ficus* hosts may be higher (Jackson 2004) because the selective pressures imposed by ostiolar morphology may mitigate against host-switching events (Janzen 1979, Verkerke 1989, van Noort 1994, van Noort & Compton 1996).

Cophylogenetic analyses require independent phylogenies for both parasites and their hosts. The objective of this study was to attempt to elucidate the taxonomic relationships within the Sycoecinae through the phylogenetic analysis of three mitochondrial genes and one nuclear gene region. I hypothesized that the evolutionary relationships would correspond to the morphological taxonomy. The resulting phylogeny will be used to facilitate investigations of cophylogeny between the sycoecine fig wasps, and their associated pollinating fig wasps and *Ficus* hosts. The taxonomical, evolutionary and biogeographical implications of the phylogenetic inference were addressed.

2.3 Methods

2.3.1 Sampling protocols and species representation

Sycoecine fig wasp specimens were obtained from fig trees in southern and central Africa, with a small amount of additional material obtained from collections made in Indo-Australasia (Table 2.1). Wasps were collected by sampling fig fruit containing developing wasp larvae. The fruit, containing wasps no more than a few days short of their emergence, were placed in handmade wasp-rearing chambers. Once emerged, adult wasps were killed and preserved in 96% ethanol. Fieldwork protocols followed those of van Noort & Compton (1999).

All sycoecine fig wasps associated with a single *Ficus* species were collected at one tree. When a single collection did not represent all known sycoecine fig wasps associated with that *Ficus* species, two or three collections from different trees were included. Species were also replicated whenever possible, particularly when they were associated with several host species.

2.3.2 DNA extraction, PCR amplification and sequencing

Phylogenetic relationships within the Sycoecinae were determined using partial sequences of four gene regions; cytochrome b (Cytb), cytochrome oxidase subunit I and subunit II (COI and COII) and the second internal transcribed spacer (ITS2) of the nuclear-encoded 18S-26S cistron. COI and COII, and, more recently, ITS2, have been used extensively in determining fig wasp phylogenetic relationships (Weiblen 2001, Machado *et al.* 2001, Jousselin *et al.* 2006, Erasmus *et al.* 2007). Seven unpublished ITS2 sequences of *C. odorans* (Coetzee 2004) were also included in the dataset (Table 2.1).

Genomic fig wasp DNA was extracted using the Puregene and Qiagen Tissue Kits. Each extraction was performed on a single wasp to avoid sequence contamination. In a minority of cases, where specimens were older than five years and the DNA was degraded, up to five wasps, all obtained from a single collection, were used in a single extraction.

All wasp PCRs were performed as 25 µl volume reactions with a quantity of 2 µl of 25 ng/ul DNA template per reaction. All fragments were amplified in reactions containing 0.7 µM of each primer, 2.5 mM $MgCl_2$, 0.05 mM dNTPs, and 0.025 U/µl Taq polymerase. An additional reagent of 5 × Solution Q (Qiagen) was added to the Cytb amplifications. Solution Q is a PCR additive that changes the melting behaviour of DNA to encourage the amplification of difficult templates and enhance amplification yields.

Fragments of COI and COII were amplified using primers TL-2-N 3014 and C1-J-2183 and CO2SCAF and CO2BSCAR (Table 2.2). Primers ITS2F and ITS2R were used to amplify the ITS2 nuclear gene region and the Cytb gene fragment was amplified using primers CP1 and CB2 (Table 2.2). PCR conditions varied with the gene region amplified (Table 2.3).

PCR product of the chalcid outgroup taxa (see below) was sent to the Macrogen commercial sequencing facility, Korea, for purification and single strand sequencing with forward primers. Both strands of sycoecine PCR product, with few exceptions, were sequenced after purification at Genoscope, France.

2.3.3 Sequence alignment

Sequence Navigator v1.01 (Perkin-Elmer) was used to edit all single strand sequences, while Sequencher v3.1 was used to create and edit contiguous sequences where both strands were sequenced. Sequence alignments were performed using the default settings on ClustalX (Thompson *et al.* 1997). Alignments were checked by eye for misalignments, and gaps were manually inserted or deleted. Protein coding sequences were verified by translation to amino acid sequences in MacClade v4.0 (Maddison & Maddison 2000) to ensure that pseudogenes had not been amplified.

2.3.4 Phylogenetic analysis

While the relationships between the pteromalid subfamilies remain controversial, three genera, *Philotrypesis, Sycoscapter* and *Watshamiella*, of the subfamily Sycoryctinae were chosen to be the outgroup taxa for the phylogenetic analysis of the Sycoecinae. Based on morphological analysis (van Noort 1992), the tribe Sycoryctini (Sycoryctinae, Chalcidoidea, Hymenoptera) is considered to be the most closely related sister group to the Sycoecinae.

Prior to combining the sequence data into a single matrix, Incongruence Length Difference (ILD) tests (Farris *et al.* 1994) were performed in PAUP* v4.0 (Swofford 2000) using the partition homogeneity test. Tests were run with 1000 replicates, 50 random additions of taxa, and tree-bisection-reconnection (TBR) branch swapping. Each possible pair of data partitions was evaluated for congruence.

Unweighted parsimony analyses of both separate and combined datasets were performed using heuristic searches with 1000 random sequence additions and the TBR branch swapping option. Internal branch support (Felsenstein 1985) was assessed using 1000 bootstrap replicates with random sequence addition and TBR branch swapping.

Modeltest v3.06 (Posada & Crandall 1998) was used to determine the nucleotide substitution models that best described the data. Out of the 56 models of substitution, one model providing the most complex approximation to each data partition was chosen through hierarchical likelihood ratio tests (hLRTs) and the Akaike Information Criterion (AIC; Table 2.4; Huelsenbeck & Rannala 1997, Posada & Crandall 1998, Posada and Buckley 2004).

Bayesian analyses were performed in MrBayes v3.1.1 (Huelsenbeck & Ronquist 2001) using the Markov Chain Monte Carlo (MCMC) algorithm. Two independent Bayesian analyses were preformed to ensure that the search strategy was not limited by local optima. Each analysis consisted of two parallel runs, each comprising one cold and three heated chains. The data were analyzed using flat priors and four or six rate categories according to the model selected for each partition. Starting trees were randomly chosen. The first, second and third codon positions of the protein coding sequence data (COI, COII, Cytb) were allowed to run with separate values for the model parameters in MrBayes. The MCMC was run for 10 million generations with trees sampled every 1000 generations.

Log likelihood scores were compared for stationarity (Huelsenbeck & Bollback 2001, Leaché & Reeder 2002). The standard deviations of split frequencies indicated the

generation at which the topologies of the parallel Bayesian runs converged, and the number of trees to be discarded as burn-in. A 50% majority rule consensus tree was generated from the remaining trees in each run. The percentage of times each node was recovered indicated the Bayesian posterior probabilities of that node (Huelsenbeck & Ronquist 2001). Nodes that obtained Bayesian posterior probabilities ≥95% were considered supported, and are indicated on the relevant phylogram.

2.4 Results

Five of the six sycoecine genera, *Crossogaster*, *Philocaenus*, *Seres*, *Sycoecus* and *Diaziella* were represented in this study. *Robertsia* specimens consistently failed to amplify, most likely due to degraded DNA. *Robertsia* is restricted to Papua New Guinea and it was not possible to procure fresh material. Sequence data were recovered for a total of 61 sycoecine specimens representing 25 species, although a small proportion (8%) of the four partial gene regions, Cytb, COI, COII and ITS2, were not successfully amplified. The phylogeny contains approximately 34% of the total of described sycoecine species, but only 13% of the estimated world total of extant species (van Noort & Rasplus 2008).

The ILD tests gave significant results for three out of the six tests (Table 2.5); each possible pair of the four gene regions was assessed for combinability. The phylogenetic signals present in the partitions Cytb and ITS2 (P = 0.46), COI and ITS2 (P = 0.1) and COII and ITS2 (P = 0.14) were not in conflict. In certain situations, ILD tests have been demonstrated to fail to accurately assess the combinability of data partitions (De Queiroz *et al.* 1995, Dowton & Austin 2002, Darlu & Lecointre 2002). An ILD test may indicate that the data partitions represent different gene histories, but this assessment of the homogeneity of the phylogenetic signal may be incorrect, particularly when the substitution rate of evolution in the data partitions is not homogenous, if few characters are present, or partitions differ markedly in size (De Queiroz *et al.* 1995, Dowton & Austin 2002, Darlu & Lecointre 2002). Due to these considerations, maximum parsimony

and Bayesian analyses were performed in the combined dataset, as well as on each separate partition.

Similar patterns of relationship were identified in the analyses of the different gene regions; however, topological conflict did occur. Nevertheless, inconsistent nodes were generally poorly supported by bootstrap and Bayesian posterior probabilities (Appendices 2.1–2.4).

The aligned Cytb data partition consisted of 745 base pairs, 240 of which were parsimony informative (32%). The parsimony analysis yielded 50 equally parsimonious trees (Appendix 2.1; Length = 1044, Consistency Index (CI) = 0.40, Retension Index (RI) = 0.64, Rescaled Consistency Index (RC) = 0.25). Support for internal nodes in both the Bayesian and Parsimony trees was poor. The parsimony analysis of the Cytb analyses differed from the analyses of the remaining three gene regions in the placement of the *Sycoecus* clade sister to the *Crossogaster* clade.

Of the 779 base pairs in the aligned data matrix of COI, 261 (33%) were parsimony informative. A total of 102 most parsimonious trees was found (Appendix 2.2; Length = 1062, CI = 0.46, RI = 0.64, RC = 0.30). The placement of *Crossogaster inusitata* together with *Diaziella* in the Bayesian and Parsimony analyses of COI was an anomaly since the analyses of the remaining gene regions all suggest the placement of *C. inusitata* at the base of the *Crossogaster* clade (Appendix 2.2).

Due to ambiguous alignment, 49 base pairs of the tRNA region located at the start of the COII sequences were excluded from analysis; the remaining 581 base pairs yielded 346 (59%) parsimony informative sites. Parsimony analysis of the COII gene produced eight equally parsimonious trees (Appendix 2.3; Length = 1051, CI = 0.53, RI = 0.72, RC = 0.38). Nodes of both Bayesian and Parsimony phylograms were well supported.

A total of 102 ambiguously aligned base pairs of the alignment and two full sequences were excluded from the ITS2 analyses; the ITS2 sequences of *Diaziella bizarrea* and

Diaziella yangi sequences proved difficult to align with the remainder of the sycoecine and outgroup sequences. The ITS2 analyses were thus performed with a total of 489 aligned base pairs, 231 of which were parsimony informative (47%). A total of 117 trees was found to be equally parsimonious. Both parsimony and Bayesian analyses revealed a deep divergence between the clade comprised of the genus *Crossogaster* and the clade containing the genera *Philocaenus, Seres,* and *Sycoecus*. The 10 additional sequences of *Crossogaster odorans* from Coetzee (2004) did not reveal any clear-cut structuring within the clade based on host associations. Branch lengths separating the taxa were markedly short. However, *C. odorans* specimens associated with *Ficus burkei* and *F. natalensis* did cluster into a separate clade (99% BS) (Appendix 2.4).

A total of eight outgroup and 45 ingroup taxa was included in the analysis of the combined (Cytb, COI, COII and ITS2) dataset. Sequences from all four markers were not obtained for every individual; seven taxa for which only three of the four gene regions were obtained were included. The analysis also incorporated three taxa that were represented by only one or two gene regions because they were associated with under-represented *Ficus* subsections; two of the four gene regions amplified successfully in *Philocaenus* sp. (ex. *F. usambarensis*) and *Crossogaster* sp. (ex. *F. chirindensis*), whereas only one gene region could be amplified in *Sycoecus* sp. (ex *F. scassellatii*).

The combined dataset consisted of 2594 base pairs, 1089 of which were parsimony informative (41%). The maximum parsimony analysis resulted in eight equally parsimonious trees (Length = 3953, CI = 0.50, RI = 0.72, RC = 0.36). The Bayesian phylogeny was constructed using 4000 trees; the first six million generations of the two runs were discarded as burn-in. In contrast to the individually analysed data partitions, the topologies of the parsimony and Bayesian analyses of the combined dataset were well supported by bootstrap values and Bayesian posterior probabilities (Figures 2.1 & 2.2). Maximum parsimony and Bayesian analyses were in general agreement, with only two major differences: the placement of *Philocaenus warei*, and the clade containing the two *Diaziella* species. However, neither placement was supported by Bayesian posterior probabilities ≥95% (Figures 2.2), or the bootstrap (53% BS; Figure 2.1).

The Sycoecinae clustered into three major clades: Clades A, B and C (Figures 2.1 & 2.2). The phylogenies of the combined datasets supported the monophyly of the genera *Crossogaster* corresponding to Clade A (97% BS, 100% PP), *Sycoecus* (98% BS, 100% PP) and *Seres* (99% BS, 100% PP). The genera *Philocaenus*, *Seres* and *Sycoecus* clustered together in one strongly supported clade (Clade B, 90% BS, 100% PP; Figures 2.1 & 2.2), however, the genus *Philocaenus* appeared paraphyletic within this clade. The two Diaziella species formed the unsupported Clade C (Figures 2.1 & 2.2).

Philocaenus barbarus, *P. quatuordentatus*, *P. liodontus*, *P. rotundus, P. medius* and *P. warei* formed a strongly supported clade (86% BS, 100% PP), although branch lengths between these taxa were relatively short. *P. barbarus*, *P. liodontus* and *P. medius* specimens were collected from a number of different *Ficus* hosts. Although host-specific clustering within the clades was not obvious, the placement of these species may loosely reflect a pattern that was observed within the well-supported clade containing *Crossogaster odorans* (100% BS, 100% PP). *Crossogaster odorans* specimens associated with *F. burkei* and *F. natalensis,* and those associated with *F. stuhlmannii*, *F. petersii and F. louisii,* clustered together.

The placement of four *Philocaenus* species challenged the morphological delimitation of the sycoecine genera. *Philocaneus silvestrii* and *Philocaneus* sp. (ex. *F. usambarensis*) were revealed to be more closely related to the *Sycoecus* clade than the remainder of the *Philocaenus* species. *Philocaenus hippopotamus* was placed sister to the clade containing both *Philocaenus* and *Seres* specimens, while *Philocaenus levis* was placed sister to the *Seres* clade.

2.5 Discussion

The combined analysis of the partial sequences of Cytb, COI, COII and ITS2 enables a robust phylogenetic hypothesis of relationships among the Sycoecinae. Although the genera *Crossogaster*, *Seres* and *Sycoecus* appeared monophyletic, the identification of the

genus *Philocaenus* as paraphyletic challenges the morphological delimitation of the sycoecine genera. A revision of the Sycoecinae clearly needs to be undertaken. However, more taxa will need to be included in the molecular analysis before new generic delimitations can be determined; sampling of *Seres* and *Sycoecus* was limited. In future revisions, the inclusion of further taxa presumed closely related to *Philocaenus hippopotamus*, *P. levis*, *P. silvestrii* and *Philocaenus* sp. (ex. *F. usambarensis*) will be essential, these individual taxa may represent clades conceivably warranting generic status. Within the phylogeny, *P. silvestrii* represents the only sycoecine taxon of a total of three known species associated with subsection *Galoglychia*. *Philocaneus* sp. (ex. *F. usambarensis*) is the only species associated with the subsection *Crassicostae,* a subsection with a species distribution centered in the poorly sampled Congo basin. There are a total of eight species in subsection *Crassicostae*, which may potentially host as many as eight *Crossogaster* species.

Further investigations should focus attention on species that are associated with numerous fig hosts, the majority of which fall within *Ficus* section *Galoglychia* subsection *Chlamydodorae*. Contrary to the conclusions based on the morphological analysis of the Sycoecinae (van Noort 1992), the molecular phylogenies showed that *Philocaenus* and *Crossogaster*, although morphologically similar, were not each other's closest relative. This is corroborated by studies that propose that different lineages of fig wasps display convergent morphology, evolved in response to the constraints of host fig morphology. This has been demonstrated to have occurred between the sycoecines and their associated pollinating fig wasps (van Noort & Compton 1996).

Morphologically, the genus *Philocaenus* comprises four distinct species-groups: the *P. silvestrii* group; *P. barbatus* group; *P. levi* group and the *P. liodontus* group (van Noort 1994b; 1994c). *Philocaenus silvestrii* Grandi was originally described as a species of *Crossogaster*, but was transferred to *Philocaenus* by van Noort (1994b), a decision supported by the molecular analyses (Figures 2.1 & 2.2). The *Philocaenus silvestrii* species-group was considered to warrant a genus in its own right, but a conservative approach of retaining the group within *Philocaenus,* based on the absence of apomorphies

and the nesting of the clade within *Philocaenus* was adopted at the time (van Noort 1994b). Nevertheless, there now appears to be some support for defining the species-group at a higher level based on the molecular phylogeny; morphologically this is also the only species-group distinguishable in both sexes while the remaining *Philocaenus* species-groups cannot be separated from each other based on male morphology.

The *P. barbatus* species-group (including *P. barbarus, P. medius* and *P. hippopotamus*) and the *P. liodontus* species-group (including *P. liodontus, P. warei, P. rotundus* and *P. quatuordentatus*) did not form mutually exclusive clades in the molecular analyses. However, with the exception of *P. hippopotamus*, together the two groups form a well supported clade. The short branches separating these taxa in both the parsimony and Bayesian phylogenies suggested incomplete lineage sorting; the history of taxon splitting is not reflected in the gene histories due to the recent divergence of these taxa.

Philocaenus levi was originally described in *Seres*, but was transferred to *Philocaenus* when four new species were described and placed within the *P. levi* group (van Noort 1994c). Although only a single representative of this group was included in the phylogeny, the nesting of *Seres* within the *Philocaenus* clade may suggest that all *Seres* species belong within the clade of *Philocaenus*.

The phylogeny of the combined datasets offered only insubstantial evidence of potential cryptic species. Both the *Philocaenus barbarus* and *Crossogaster odorans* clades appeared to cluster into two subclades, one containing individuals associated with *F. burkei* and *F. natalensis natalensis* and the other containing individuals associated with *F. stuhlmanni* and *F. petersii*, which may reflect relationships with *Ficus* section *Chlamydodorae.* There is some support for these subclades but further data are required to confirm this result. Fine-scale phylogenetic studies of numerous specimens sampled throughout their geographic range may uncover divergences within these clades. Nevertheless, as previously stated, the short branches separating these taxa suggested incomplete lineage sorting, potentially confounding interpretations of the phylogenetic relationships between these species. This may be indicative of a recent speciation event.

In order to resolve the deeper nodes within the Sycoecinae, in particular the relationship between the Indo-Australasian and African sycoecine fauna, further studies with extensive sampling and slowly evolving markers are required. The combined parsimony and Bayesian analyses of Cytb, COI, COII and ITS2 were in conflict with regard to the placement of the *Diaziella* clade, although nodal support is lacking for either of the possible placements. *Diaziella* species are associated with *Ficus* subgenus *Urostigma*, section *Conosycea.* The molecular phylogeny of *Ficus* (Rønsted *et al.* 2005) placed the sections *Conosycea* and *Malvanthera* (host section of *Robertsia* species) as sister clades, sister to the sections *Galoglychia* and *Americana*, which also share a sister relationship. It is plausible to hypothesize a common ancestor relationship between the Indo-Australasian taxa, *Robertsia* and *Diaziella*, a view that is supported by morphological analysis (van Noort 1992), and a basal split between Indo-Australasian and Afrotropical Sycoecinae. However, without phylogenetic evidence, these hypotheses are purely speculative.

The clades established through the molecular phylogenetic analysis of the Sycoecinae (Figures 2.1 & 2.2) are consistent with the morphological findings (van Noort 1992) that processes other than strict cospeciation have shaped the evolutionary history of the Sycoecinae, because *Ficus* subsections do not correspond precisely with the sycoecine clades (Table 2.1). It appears that the constraints of internal oviposition may not be enough to prevent host-switching events. The extent of cospeciation between the Sycoecinae and their *Ficus* hosts will be assessed quantitatively through cophylogenetic analysis methods (Chapter 3). Generally, this study reveals incongruence between the morphological phylogeny of the sycoecinae (van Noort 1992) and the phylogeny constructed from molecular data, thus refuting the hypothesis that the molecular data would reflect the morphological hypothesis of taxonomic relationships.

2.6 References

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Table 2.1: Host associations and collection details of the sycoecine fig wasps included in this study

Ficus Host	Sycoecine	Collection number	Collection locality & date	Cytb	COI	COII	ITS2
Section *Galoglychia*							
Subsection *Platyphyllae*							
F. glumosa	*Philocaenus warei*	SA05-F19	RSA, Limpopo, Makhado, 11-2005	1	1	1	
	Crossogaster stigma	SA06-F97	RSA, KwaZulu-Natal, Port Edward, 05-2006	1	1	1	1
	Crossogaster quadrata	SA06-F97	RSA, KwaZulu-Natal, Port Edward, 05-2006	1	1	1	1
	Philocaenus warei	SA06-F97	RSA, KwaZulu-Natal, Port Edward, 05-2006	1	1	1	1
	Crossogaster stigma	FMK13	Tanzania, Mkomazi Game Reserve, 11-1995				1
*F. stuhlmannii**	*Philocaenus barbarus*	SA05-F55B	RSA, KwaZulu-Natal, False Bay Park, 11-2005	1	1	1	1
	Crossogaster odorans	SA05-F56	RSA, KwaZulu-Natal, Hluhluwe region, 11-2005	1		1	1
	Philocaenus liodontus	MW06-F60	Mozambique, Niassa Province, Mandimba, 05-2006	1	1	1	1
	Philocaenus medius	MW06-F60	Mozambique, Niassa Province, Mandimba, 05-2006	1	1	1	1
	Crossogaster odorans	IP3A[a]	RSA, Limpopo, Nelspruit, 02-2002				1
	Crossogaster odorans	IP8A[a]	RSA, Limpopo, Nelspruit, 2001				1
	Crossogaster odorans	FMK21[a]	Tanzania, Mkomazi Game Reserve , 04-1996				1
F. abutilifolia	*Philocaenus rotundus*	SA05-F23	RSA, Limpopo, Soutpansberg, R524, 11-2005	1	1	1	1
F. trichopoda	*Crossogaster robertsoni*	SA05-F67	RSA, KwaZulu-Natal, Umlalazi Nature Reserve, 11-2005		1	1	1
	Philocaenus hippopotamus	SA05-F67	RSA, KwaZulu-Natal, Umlalazi Nature Reserve, 11-2005	1	1	1	1
Subsection *Chlamydodorae*							
F. craterostoma	*Philocaenus quatuordentatus*	SA05-F59	RSA, KwaZulu-Natal, Ngome Forest, 11-2005	1	1	1	1
	Crossogaster odorans	SPB29a[a]	RSA, Limpopo, Soutpansberg, 03-2002				1
	Crossogaster odorans	SPB47a[a]	RSA, Limpopo, Soutpansberg, 03-2002				1
	Crossogaster odorans	SPB11a[a]	RSA, Limpopo, Soutpansberg, 03-2002				1
F. natalensis natalensis	*Philocaenus barbarus*	SA05-F75	RSA, KwaZulu-Natal, Mtunzini, 11-2005	1	1	1	1
	Philocaenus medius	ZA06-F14	Zambia, Central Province, T2 between Kapiri Mposhi and Mkushi, 05-2006	1	1	1	1
	Philocaenus liodontus	ZA06-F14	Zambia, Central Province, T2 between Kapiri Mposhi and Mkushi, 05-2006	1	1	1	1
	Crossogaster odorans	ZA06-F14	Zambia, Central Province, T2 between Kapiri Mposhi and Mkushi, 05-2006	1	1	1	1
	Philocaenus medius	MW06-F89	Mozambique, 06-2006	1	1	1	1
F. natalensis graniticola	*Philocaenus barbarus*	SA05-F08	RSA, Limpopo, Soutpansberg, 11-2005	1	1	1	1
	Crossogaster odorans	SPB59a[a]	RSA, Limpopo, Soutpansberg, 03-2002				1
F. burtt-davyi	*Philocaenus liodontus*	SA05-F82	RSA, Eastern Cape, Woody Cape Nature Reserve, 11-2005	1	1	1	1
F. petersii	*Crossogaster odorans*	SA05-F45	RSA, Mpumalanga, Louws Creek, 11-2005	1	1	1	1
	Philocaenus liodontus	SA05-F45	RSA, Mpumalanga, Louws Creek, 11-2005	1	1	1	1
	Philocaenus liodontus	ZA06-F46	Zambia, Northern Province, 70 km southeast of Isoka, 05-2006	1	1	1	1
	Philocaenus barbarus	ZA06-F46	Zambia, Northern Province, 70 km southeast of Isoka, 05-2006	1	1	1	1
	Crossogaster odorans	ZA06-F46	Zambia, Northern Province, 70 km southeast of Isoka, 05-2006	1	1	1	1

Table 2.1: *Continued*

Ficus Host	Sycoecine	Collection number	Collection locality & date	Cytb	COI	COII	ITS2
F. burkei	*Philocaenus barbarus*	SA05-F28	RSA, Limpopo, Abel Erasmus Pass, 11-2005	1	1	1	1
	Crossogaster odorans	SA05-F28	RSA, Limpopo, Abel Erasmus Pass, 11-2005	1	1	1	1
	Philocaenus barbarus	SA06-F98	RSA, KwaZulu-Natal, Port Edward, 06-2006	1	1	1	1
Subsection *Crassicostae*							
F. louisii	*Crossogaster* sp.	GA00-F03	Gabon, Réserve des Monts Doudou, 03-2000	1			1
F. usambarensis	*Philocaenus* sp.	ZA06-F32	Zambia, Northern Province, 70 km southwest of Mporokoso, 05-2006	1			1
Subsection *Galoglychia*							
F. lutea	*Philocaenus silvestrii*	SA05-F18	RSA, Limpopo, Makhado, 11-2005	1		1	1
	Philocaenus silvestrii	SA05-F61	RSA, KwaZulu-Natal, Ongoye Forest, 11-2005	1		1	1
Subsection *Cyathistipulae*							
F. cyathistipula cyathistipula	*Sycoecus* sp.	MW06-F67	Mozambique, Mount Namuli, 05-2006	1	1		1
F. cyathistipula pringsheimiana	*Sycoecus taylori*	UG05-F02	Uganda, Kibale National Park, 08-2005	1	1		1
F. scassellatii	*Sycoecus* sp.	FMK29	Tanzania, Lake Chala, 11-1996				1
Subsection *Caulocarpae*							
F. bizanae	*Crossogaster* sp.	SA05-F69	RSA, KwaZulu-Natal, Ongoye Forest, 11-2005	1	1	1	1
	Crossogaster sp.	SA05-F81	RSA, Port St Johns, 11-2005	1	1	1	1
F. chirindensis	*Crossogaster* sp.	UG05-F03	Uganda, Kibale National Park, 08-2005	1	1		1
F. sansibarica sansibarica	*Seres solweziensis*	SA05-F27	RSA, Limpopo, Legalameetse Nature Reserve, 11-2005	1	1	1	1
	Seres solweziensis	SA05-F40	RSA, Mpumalanga, Krododilpoort, 11-2005	1	1	1	1
F. sansibarica macrosperma	*Seres* sp.	ZA06-F18	Zambia, Luapula Province, 20 km west of Kawambwa, 05-2006	1	1	1	1
	Crossogaster inusitata	ZA06-F18	Zambia, Luapula Province, 20 km west of Kawambwa, 05-2006	1	1	1	1
F. ottoniifolia lucanda	*Philocaenus levis*	UG05-F01	Uganda, Kibale National Park, 08-2005	1	1	1	1
F. ovata	*Seres solweziensis*	ZA06-F19A	Zambia, Luapula Province, Kawambwa, 05-2006	1	1	1	1
Section *Urostigma*							
Subsection *Conosycea*							
F. glaberrima	*Diaziella bizarrea*		China, Xishuangbanna, Kunming	1	1	1	1[b]
F. curtipes	*Diaziella yangi*		China, Xishuangbanna, Cheng Zi village	1	1	1	1[b]
Outgroup taxa							
	Philotrypesis tridentata	GenBank		DQ270084			DQ270076
	Philotrypesis longicaudata	GenBank		DQ270090			DQ270067

Table 2.1: *Continued*

Ficus Host	Sycoecine	Collection number	Collection locality & date	Cytb	COI	COII	ITS2
	Philotrypesis sp.			1 [c]	1 [c]		
	Sycoscapter sp. *1*	GenBank		DQ270079			DQ270056
	Sycoscapter sp. *2*			1 [c]	1 [c]		
	Sycoscapter sp. *3*			1 [c]	1 [c]	1	
	Sycoscapter sp. *4*					1	
	Watshamiella sp.			1 [c]	1 [c]	1	

The classification of *Ficus* presented in this table is from Berg (1986), Berg & Wiebes (1992) and Burrows & Burrows (2003)

*A recent molecular phylogeny (Rønsted et al. 2005) suggests that *F. stuhlmanni* should be placed within subsection Chlamydodorae rather than within the subsection Platyphyllae.

[a] Unpublished sequences used with permission (Vinet 2005).

[b] Excluded from analyses. For explanation, see text.

[c] Unpublished sequences used with permission from M. Mcleish.

Table 2.2: Sequences of Primers used in the amplification of sycoecine DNA.

Primer Name	Sequence	Region	Reference
TL-2-N 3014	TCCATTGCACTTATTCTGCCATATTA	COI	Simon *et al.* 1994
C1-J-2183	CAACATTTATTTTGATTTTTTGG	COI	Simon *et al.* 1994
CO2SCAF	GCAGATTAGTGCAATGAATTTAA	COII	Villalba *et al.* 2002
CO2BSCAR	GCTCCACAAATTTCTGAGCATTG	COII	Villalba *et al.* 2002
ITSF	ATTCCGCACCACGCCTGGCTGA	ITS2	Campbell *et al.* 1993, Lopez-Vaamonde *et al.* 2001
ITSR	CGCCTGATCTGAGGTCGTGA	ITS2	Campbell *et al.* 1993, Lopez-Vaamonde *et al.* 2001
CP1	GATGATGAAATTTTGGATC	Cyt B	Harry *et al.* 1998
CB2	ATTACACCTCCTAATTTATTAGGAAT	Cyt B	Jermin & Crozier 1994

Table 2.3: PCR conditions for the amplification of Cytb, COI, COII, and ITS2 gene regions.

	Cytb		COI & COII		ITS2	
Initial Denaturation	94 °C	3 min	94 °C	3 min	94 °C	3 min
Denaturation phase	92 °C	30 s	92 °C	30 s	92 °C	1 min
Annealing phase	48 °C	1 min 30 s	48 °C	1 min 30 s	50 °C	1 min
Extension phase	72 °C	1 min 30 s	72 °C	2 min 30 s	72 °C	1 min
No of cycles	40		35		35	
Final Extension	72 °C	10 min	72 °C	5 min	72 °C	10 min

Table 2.4: Nucleotide substitution models chosen for each sycoecine data partition; models were selected using Modeltest 3.06 (Posada & Crandall 1998).

Gene Region	Model
Cytb	GTR + I + G
COII	GTR + I + G
COII	K81uf + G
ITS2	TVMef + G

Table 2.5: Results of Incongruence Length Difference (ILD) Tests. The sycoecine Cytb, COI, COII, and ITS2 data partitions were assessed for combinability. Probabilities ≤0.05 reveal significant incongruence between the pairs of data partitions, and are indicated in bold.

	Cytb	COI	COII
COI	0.001		
COII	0.03	0.04	
ITS2	**0.46**	**0.1**	**0.14**

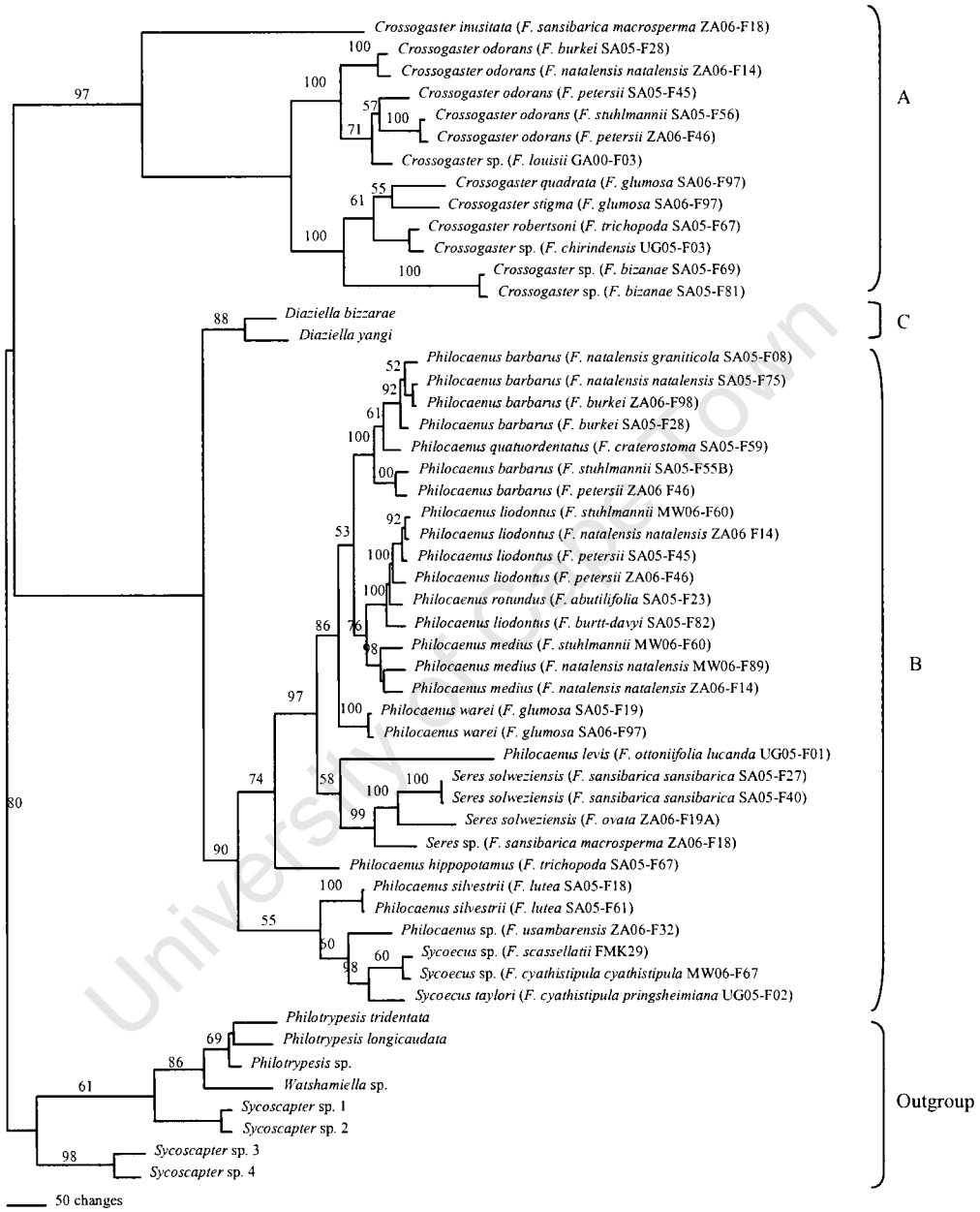

Figure 2.1: One of eight most parsimonious trees from the combined analysis of three mitochondrial genes Cytochrome b, COI, COII and the ITS2 nuclear gene region of the Sycoecinae. Boostrap values (≥50) are indicated above each node. Clades mentioned in the text are denoted by letters. Species from three genera of the subfamily Sycoryctinae (Pteromalidae, Chalcidoidea) form the outgroup.

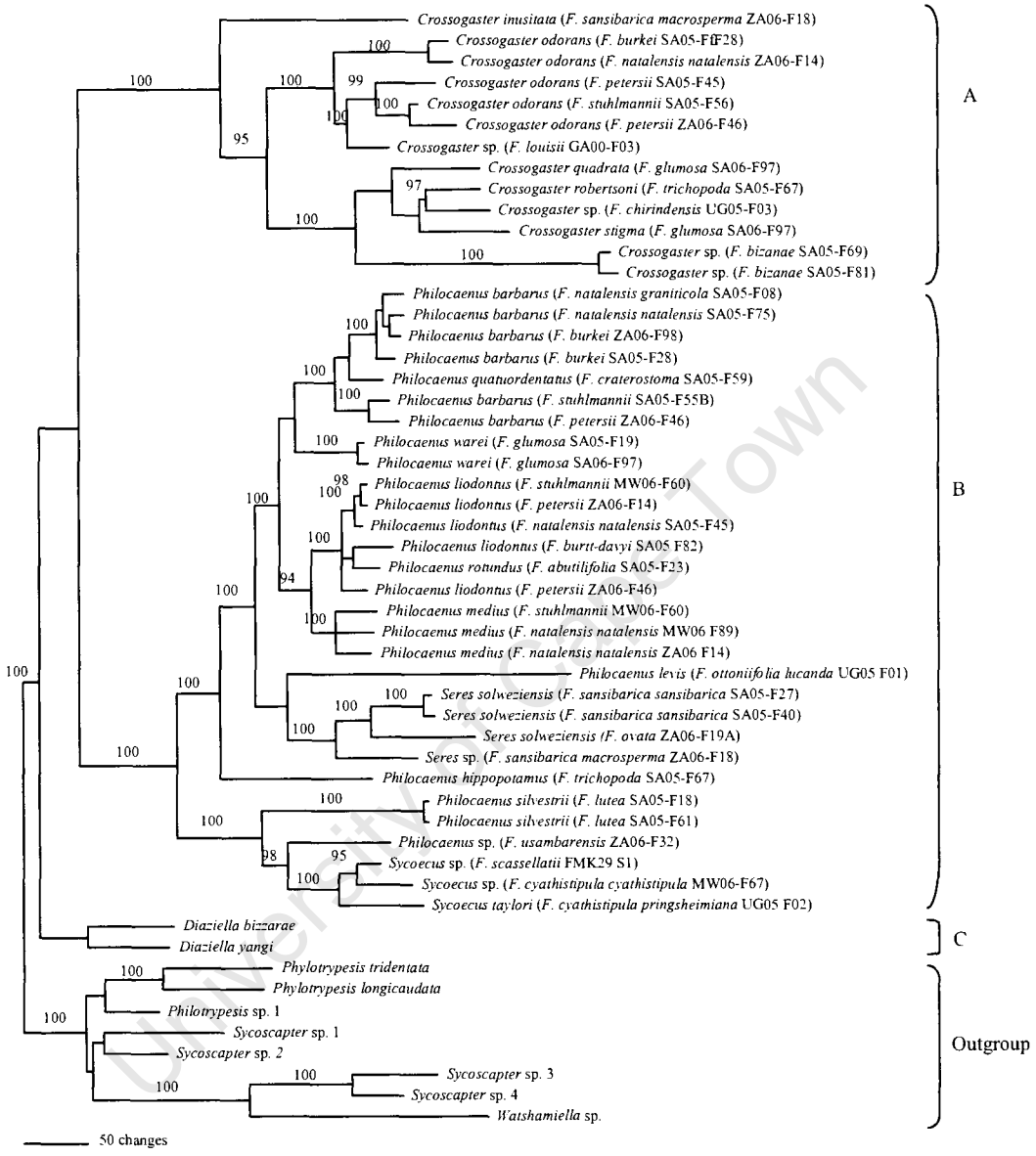

Figure 2.2: Bayesian consensus tree from the combined analysis of three mitochondrial genes Cytochrome b, COI, COII and the ITS2 nuclear gene region of the Sycoecinae. Bayesian posterior probabilities (≥95%) are indicated above each node. Clades mentioned in the text are denoted by letters. Species from three genera of the subfamily Sycoryctinae (Pteromalidae, Chalcidoidea) form the outgroup.

CHAPTER 3: Untangling the trees: cophylogenetic analyses of agaonid and sycoecine fig wasps (Chalcidoidea, Hymenoptera) associated with *Ficus* section *Galoglychia* (Moraceae)

3.1 Abstract

The fig-fig wasp mutualism was traditionally distinguished by a one-to-one ratio of host to pollinating fig wasp. However, recent studies conclude that extreme host specificity, although frequent, is no longer as ubiquitous as previously considered, thus challenging strict coevolutionary hypotheses. This study investigated the extent of codivergence between the host fig trees of *Ficus* section *Galoglychia*, their associated agaonid pollinators, and the associated non-pollinating Sycoecinae fig wasps (Pteromalidae, Chalcidoidea, Hymeoptera) through cophylogenetic analysis. The *Ficus* and agaonid phylogenies were constructed de novo; new sequence data were generated and combined with sequences retrieved from GenBank. The sycoecine phylogeny was obtained form Chapter 2. The tree-based and distance-based methods of cophylogenetic analyses revealed both significant and negligible evidence of codivergence between the three lineages. These results hint at a complex history of codivergence between figs and fig wasps, in corroboration with similar, recently published studies. Sycoecines enter the fig to oviposit, imitating the pollinators, in contrast to the majority of externally ovipositing non-pollinating fig wasps. Internal oviposition requires the sycoecines to adapt to the same selective pressures as the pollinators and potentially promotes the maintenance of host-specificity; both lineages display convergent morphology. Phylogenetic reconstruction of ancestral character states of agaonid and sycoecine head shapes suggested that the character is generally evolutionarily conserved within these two independent wasp lineages. However, the presence of distinct reversals of head shape within the reconstruction indicates that host-specific ostiolar morphology may not prevail to constrain host-shifting events.

3.2 Introduction

Neither pollinating fig wasps (Agaonidae, Chalcidoidea, Hymenoptera) nor fig trees (*Ficus*, Moraceae) can complete their life cycles without each other (Hill 1967, Galil 1977, Janzen 1979, Cook & Rasplus 2003). Figs are entirely reliant on fig wasps for pollination and pollen dispersal while fig wasps reproduce exclusively within the fig fruit, or syconia. Female pollinating fig wasps enter the syconium through the ostiole, a tiny opening located at the apex of the fig, thereby pollinating the stigmas. Once inside, the female oviposits in the ovules of a proportion of the tiny florets that line the fig cavity (Ramírez 1969, Berg & Wiebes 1992, Cook & Power 1996). The fig wasp larvae feed on galled endosperm tissue, and, once mature, emerge into the fig cavity. Wingless male wasps mate with females before chewing an exit hole through the fig wall, allowing the pollen-laden female wasps to escape. Dispersing females are attracted to conspecific fig trees through distinctive chemical cues released by the fig fruit. Males generally do not disperse, (but see Greeff *et al.* 2003) and most die within their natal fig (Galil & Eiskovitch 1971, Wiebes 1979, Bronstein & McKey 1989, Berg & Wiebes 1992, Cook *et al.* 1997).

Besides the pollinating fig wasps, a diverse community of non-pollinating fig wasps (Chalcidoidea, Hymenoptera) also utilizes the fig syconia for reproduction. Four groups of chalcid wasp, in addition to the family Agaonidae, contain wasps that are associated with figs. They are parasites of the fig-fig wasp mutualism and are thought to provide no benefit to the host fig tree (Compton *et al.* 1994, West & Herre 1994, West *et al.* 1996, Cook & Rasplus 2003) although Dunn *et al.* (2008) suggests that externally ovipositing parasitic wasps may play a role in maintaining the stability of the mutualism. In contrast to the pollinators, most non-pollinating fig wasps do not enter the fig cavity; oviposition occurs externally through the fig wall and thus no pollen is dispersed. The fig wasp subfamily Sycoecinae (Pteromalidae, Chalcidoidea, Hymenoptera) is one exception. These non-pollinating fig wasps enter the fig via the syconium to oviposit in the same way as the pollinating fig wasps (van Noort & Compton 1996). The Sycoecinae are a

predominantly Afrotropical fig wasp group, with four of the six genera associated with *Ficus* section *Galoglychia*.

Traditionally, fig wasps were thought to display extreme host fidelity, with a unique species of fig wasp pollinating each *Ficus* species (Ramírez 1970, 1974, Janzen 1979, Wiebes & Compton 1990, Herre *et al.* 1996, Cook & Rasplus 2003). The fig-fig wasp mutualism was upheld as classic example of strict coevolution. On a broad scale, *Ficus* sections or subsections are usually pollinated by a single fig wasp genus. These findings provided evidence for the hypothesis of a long history of codivergence and cospeciation between pollinator and host (Wiebes 1979, 1987, Berg & Wiebes 1992, Herre *et al.* 1996, Kerdelhué *et al.* 1999, Weiblen 2000, 2001, 2004, Cook & Lopez-Vaamonde 2001, Jousselin *et al.* 2003, Rønsted *et al.* 2005). However, recent taxonomical and molecular studies are revealing more and more exceptions to the one-to-one ratio of host-specificity between fig and pollinating fig wasps (Rasplus 1996, Kerdelhué *et al.* 1999, Lopez-Vaamonde *et al.* 2001, Cook & Rasplus 2003, Molbo *et al.* 2003, Jackson 2004, Machado *et al.* 2005, Haine *et al.* 2006, Erasmus *et al.* 2007, Marussich & Machado 2007, Jousselin *et al.* 2008).

Within *Ficus,* subgenus *Urostigma,* section *Galoglychia* (a predominantly African section of the genus *Ficus*), breakdowns in host specificity have been documented in 15–17% of fig species (Rasplus 1996). For instance, the association of more than one pollinating fig wasp per *Ficus* host, often from more than one genus, as well as one pollinating fig wasp associated with more than one *Ficus* species, has been extensively documented both in Old World and Neotropical taxa (Rasplus 1996, Keldelhue *et al.* 1999, Molbo *et al.* 2003, Erasmus *et al.* 2007, Marussich & Machado 2007). Multiple pollinators associated with multiple hosts suggest that hybridization and introgression are occurring within *Ficus* lineages (Parrish *et al.* 2003, Machado *et al.* 2005). Once thought to be a product of erroneous taxonomy (Wiebes 1987, Rasplus 1996) the breakdown of host specificity now appears to be a reflection of an intricate evolutionary history between the fig and fig wasp lineages. Such complex associations imply that events such as host-switches, losses (lineage extinctions, omissions, or lineage sortings) and

duplications are common occurrences in the evolutionary history of these independent lineages.

There is some evidence (Machado *et al.* 2001, Weiblen & Bush 2002, Marussich & Machado 2007) and an implicit perception (Cook & Rasplus 2003) that externally ovipositing non-pollinating fig wasps are more likely to experience host shifts than the internally ovipositing pollinating fig wasps. The rationale for this hypothesis is that female non-pollinating fig wasps do not have to conform to the morphological adaptations that are imposed on female pollinating fig wasps that are required to enter the fig cavity through a host-specific ostiole (Cook & Rasplus, 2003). Ostiolar morphology may prevent general entry into the fig cavity for wasps that are not specifically adapted to the ostiolar morphology of that particular *Ficus* species (Janzen 1979, Verkerke 1989, van Noort & Compton 1996). Most non-pollinators have the option to oviposit into the ovules of multiple syconia of multiple fig trees with which they are anatomically compatible (Jackson 2004). However, these ideas are still contentious; a recent study has shown that externally ovipositing non-pollinating fig wasps may be at least as host specific as their associated pollinating fig wasps (Jousselin *et al.* 2008).

Nevertheless, there are many factors that are thought to constrain host-switching events and maintain, to some extent, the host-specificity of both pollinating and non-pollinating fig wasps within the mutualism. If a pollinator host-switch should occur, low pollination success may cause *Ficus* host trees to abort their present crop of figs. Alternatively, if successful pollination of hosts does transpire, pollinator reproduction may fail due to differences in physiological conditions within the fig (such as temperature or developmental time) required by the developing wasp larvae. Other constraining factors may be chemical, ecological or morphological in nature; both non-pollinating and pollinating fig wasps locate receptive figs by recognizing and homing in on host-specific volatile cues (Ware *et al.* 1993, Grison-Pigé *et al.* 2002, Proffit *et al.* 2007). Competition from other fig wasps already associated with the host may deter host-switching, and fig size, style length and ostiolar diameter may effectively thwart successful oviposition of

fig wasps in figs to which they are not specifically adapted (Herre 1989, van Noort & Compton 1996, Jackson 2004, Marussich & Machado 2007).

The ostiole is an effective physical barrier for internally ovipositing fig wasps, because the selective pressures imposed by the ostiole size and shape may mitigate against host-switching events (Janzen 1979, Verkerke 1989, van Noort 1992, van Noort & Compton 1996). Sycoecine wasps are internally ovipositing parasitic fig wasps. Therefore, the potential for codivergence between the Sycoecinae and their *Ficus* hosts may be higher than between externally ovipositing fig wasps and their hosts (Jackson 2004). Futhermore, van Noort & Compton (1996) reveal a correlation between fresh fig diameter of fig trees in *Ficus* section *Galoglychia* and head shape (calculated as the ratio of head width to head length) of female agaonid and sycoecine fig wasps associated with those fig tree species. They ascribe the significant morphological similarity of agaonid and sycoecine fig wasps to convergent evolution as a result of identical selection pressures that enforced parallel adaptations to host fig morphology. These similarities include elongation and dorso-ventral flattening of the head and thorax, and tibial and mandibular modifications that assist the movement of the wasp through the bracts surrounding the ostiole (van Noort & Compton 1996).

The first objective of this study was to investigate the extent of cophylogeny between the phylogenies of the Agaonidae, Sycoecinae and associated host fig trees of *Ficus* section *Galoglychia* through cophylogenetic analysis. Both tree-based (TreeMap 1.0 and TreeMap 2.02β) and distance-based (ParaFit) methods were used; these were originally developed for research into host-parasite coevolution. I hypothesized a history of codivergence between figs of *Ficus* section *Galogychia* and their associated agaonid and sycoecine fig wasps. Due to similar ecological, morphological and chemical constraints, it was predicted that both of the internally ovipositing fig wasp lineages should display phylogenetic congruence with their hosts. The implications of the results are addressed.

Secondly, head shapes of the agaonid and sycoecine fig wasps were plotted onto their respective phylogenies. Ancestral head shape characters of the internal nodes were

reconstructed to explore the evolution of head shape in a phylogenetic context, determining whether head shape is an evolutionarily conserved or labile character within these two wasp lineages. I hypothesized that the constraints of ostiolar morphology help to maintain host-specificity in the fig- fig wasp mutualism and that, within monophyletic clades, conservatism of head shape should be observed. In contrast, evolutionary lability of head shape characters would indicate that host-specific ostiolar morphology may not constrain host-shifting events.

3.3 Methods

DNA sequence data of *Ficus* section *Galoglychia* were generated and combined with data obtained from Rønsted *et al.* (2007; Table 3.1). Similarly, sequence data of the pollinators of *Ficus* section *Galoglychia* were generated and combined with sequences retrieved from GenBank, largely those published by Erasmus *et al.* (2007; Table 3.2). All phylogenies were reconstructed *de novo*. The Bayesian consensus tree of the combined dataset (Figure 2.2) from the molecular phylogenetic analysis of the Sycoecinae (Chapter 2) was used in the tests of phylogenetic congruence.

3.3.1 Sampling protocols

Fig leaf and wasp material were obtained from fig trees in southern and central Africa (Tables 3.1 & 3.2). Fig leaves were dried in silica to absorb moisture and preserve the integrity of the DNA. For all new data, associated sycoecine and agaonid fig wasps were collected at a single tree wherever possible. When a single collection did not represent all known agaonid and sycoecine fig wasps associated with that *Ficus* species, two or three collections from different trees were included. It was attempted, with limited success, to make the specimens of the two lineages used in the following cophylogenetic analysis true associates.

Fig wasps were collected by sampling fig fruit containing developing wasp larvae. The fruit, containing wasps no more than a few days short of their emergence, were placed in handmade wasp-rearing chambers. Once emerged, adult wasps were killed and preserved in 96% ethanol. Fieldwork protocols followed those of van Noort & Compton (1999).

3.3.2 DNA extraction, PCR amplification and sequencing

3.3.2.1 *Ficus* section *Galoglychia*

The internal transcribed spacer (ITS) and external transcribed spacer (ETS) of the eukaryotic ribosomal DNA (rDNA) 18S-26S transcriptional unit were targeted for sequencing to construct the phylogeny of *Ficus* Section *Galoglychia*. Genomic DNA was extracted using the CTAB procedure (Doyle & Doyle 1987). An average of 0.3 g of *Ficus* leaf material was ground in 100 µl of 2 x CTAB extraction buffer. Standard protocols of the Qiagen Purification Kit were used to purify the DNA extract.

Primers ITSF and ITSR (Sun *et al.* 1994) and Hel-1 and 18S-ETS (Baldwin & Markos 1998) were used to amplify ITS and ETS respectively (Table 3.3). PCR amplifications were performed in 50 µl reaction volumes with 4 µl of 25 ng/µl DNA template. Each reaction amplifying the ITS or ETS region contained 0.3 µM of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.024 U/µl Taq polymerase. Adjuvants BSA and DMSO were added to all PCR reactions, in final concentrations of 0.004% and 5% respectively. ITS and ETS PCR conditions (Table 3.4) followed those described by Rønsted *et al.* (2007). *Ficus* PCR product was sent to the Macrogen commercial sequencing facility, Korea, for purification and single strand sequencing with forward primers ITSF and Hel-1.

3.3.2.2 Pollinators of *Ficus* section *Galoglychia*

Phylogenetic relationships within the pollinators of *Ficus* section *Galoglychia* were investigated using partial sequences of five gene regions; cytochrome oxidase subunit I and subunit II (COI and COII), 18S, 28S (sequences retrieved entirely from GenBank) and the second internal transcribed spacer (ITS2) of the nuclear-encoded 18S-26S cistron were used to construct the phylogeny of the *Galoglychia* pollinators. COI and COII, and, more recently, ITS2, have been used extensively in determining chalcid phylogenetic relationships (Machado *et al.* 2001, Weiblen 2001, Jousselin *et al.* 2006, Erasmus *et al.* 2007).

Genomic fig wasp DNA was extracted using the Puregene and Qiagen Tissue Kits. Each extraction was performed on a single wasp to avoid sequence contamination. In a minority of cases, where specimens were older than five years and the DNA was degraded, up to five wasps, all obtained from a single collection, were used in an extraction. All wasp PCRs were performed as 25 µl volume reactions with a quantity of 2 µl of 25ng/ul DNA template per reaction. All fragments were amplified in reactions containing 0.7 µM of each primer, 2.5 mM $MgCl_2$, 0.05 mM dNTPs, and 0.025 U/µl Taq polymerase.

Fragments of COI and COII were amplified using primers TL-2-N 3014 and C1-J-2183 and CO2SCAF and CO2BSCAR (Table 3.3). Primers ITS2F and ITS2R were used to amplify the ITS2 nuclear gene region, and the fragment of 18S was amplified using primers 18SH-17F and 18SH-35R (Table 3.3). PCR conditions varied with gene region amplified (Table 3.5).

Both strands of wasp PCR product, with few exceptions, were sequenced after purification at Genoscope, France. Chalcid outgroup PCR product was sent to the Macrogen commercial sequencing facility, Korea, for purification and single strand sequencing.

3.3.3 Sequence alignment

Sequence Navigator v1.01 (Perkin-Elmer) was used to edit all single strand sequences, while Sequencher v3.1 was used to create and edit contiguous sequences where both strands were sequenced. Sequence alignments were performed using the default settings on Clustal X (Thompson *et al.* 1997). Alignments were checked by eye for misalignments, and gaps were manually inserted or deleted. Protein coding sequences were verified by translation to amino acid in MacClade v4.0 (Maddison & Maddison 2000) to ensure that pseudogenes were not present.

3.3.4 Phylogenetic analysis

3.3.4.1 Phylogeny of *Ficus* section *Galoglychia*

Following the recent phylogeny of *Ficus* section *Galoglychia* (Rønsted *et al.* 2007), eight species of *Ficus* from section *Americana*, suggested to be paraphyletic with respect to section *Galoglychia,* were incorporated into the *Ficus* dataset. Three taxa from the remaining sections of the subgenus *Urostigma* were designated as the outgroup: *F. drupacea* (*Conosycea*), *F. rubignosa* (*Malvanthera*) and *F. superba* (*Urostigma).*

Recent studies exploring the phylogeny of *Ficus* (Jousselin *et al.* 2003, Rønsted *et al.* 2005, 2007) combined ETS and ITS sequence data into a supermatrix on the basis that they are part of a tandem repeat within the nuclear ribosomal DNA. Nevertheless, an Incongruence Length Difference (ILD) test (Farris *et al.* 1994) was performed in PAUP* 4.0 (Swofford 2002) to evaluate the topological congruence of the two data partitions. The ILD test was run with 1000 replicates, 50 random additions of taxa, and tree-bisection-reconnection (TBR) branch swapping.

Two separate analyses were run. For some individuals, amplification of ITS or ETS failed and both sequences could not be obtained for each specimen. Thus, the first

analysis comprised a subset of taxa for which both ITS and ETS sequences data was available. The second analysis was performed by incorporating all sequences of all taxa into the ITS and ETS data matrix, regardless of missing data, in order to determine the subsectional placement of species for which only one of the two gene regions could be obtained. Parsimony, bootstrap and Bayesian analyses followed identical protocols in both analyses.

An unweighted parsimony analysis was performed on the combined dataset in PAUP* v4.0 (Swofford 2002). Trees were obtained using a heuristic search with 1000 random addition sequences and the TBR branch swapping option, and 100 trees saved per replicate. Internal branch support was assessed (Felsenstein 1985) using 1000 bootstrap replicates with a random sequence addition, TBR branch swapping and the option "max trees" set to 1000. Nodes that obtained bootstrap support values ≥70% were considered supported.

Bayesian analyses were performed in MrBayes v3.1.1 (Huelsenbeck & Ronquist 2001) using the Markov Chain Monte Carlo (MCMC) algorithm. Modeltest v3.06 (Posada & Crandall 1998) was used to determine the nucleotide substitution models that best described the ITS and ETS data. The TrN + G model was selected for both the partitions, specifying six rate categories with uniform priors and gamma distributed rate variation. Each analysis consisted of two parallel runs, each comprising one cold and three heated chains. Starting trees were randomly chosen and ITS and ETS data partitions were allowed to run with separate values for the model parameters. The MCMC was run for 10 million generations with trees sampled every 1000 generations. Two independent Bayesian analyses were performed to ensure that local optima were not limiting the search strategy.

Log likelihood scores of trees were compared for stationarity (Huelsenbeck & Bollback 2001, Leaché & Reeder 2002) and the standard deviations of split frequencies indicated the generation at which the topologies of the two parallel Bayesian runs converged, and the number of trees to be discarded as burn-in. A 50% majority rule consensus tree was

generated from the remaining trees in each run. The percentage of times each node was recovered indicated the Bayesian posterior probabilities of that node (Huelsenbeck & Ronquist 2001). Nodes that obtained Bayesian posterior probabilities ≥95% were considered supported, and are indicated on the phylogram.

3.3.4.2 Phylogeny of the pollinators of *Ficus* section *Galoglychia*

Five genes were combined for the dataset, including 28S sequence data from Erasmus *et al.* (2007) retrieved from GenBank, along with the neotropical agaonid taxa that were chosen as the outgroup of the pollinators of *Ficus* section *Galoglychia*. Difficulty was experienced in combining agaonid sequence data produced in this present study with those from Erasmus *et al.* (2007), because sequences were not obtained for every targeted gene region for each individual sample included in this present study. The molecular supermatrix dataset therefore contains 40% missing data. Consequently, two separate analyses were run with combined analyses of 18S, COI and COII, and thereafter of ITS2 and 28S. In addition, the COI sequence divergence (p-distances) within and between the main clades or lineages of the genus *Elisabethiella* were separately assessed in MEGA4 (Tamura *et al.* 2007)

Prior to combining the sequence data, Incongruence Length Difference (ILD) tests (Farris *et al.* 1994) were performed in PAUP* v4.0 (Swofford 2002) using the partition homogeneity test. Tests were run with 1000 replicates, 50 random additions of taxa, and tree bisection reconnection (TBR) branch swapping. Each possible pair of data partitions was evaluated for congruence. However, in certain situations, ILD tests have been demonstrated to fail to assess the combinability of data partitions accurately (De Queiroz *et al.* 1995, Dowton & Austin 2002, Darlu & Lecointre 2002). An ILD test may indicate that the data partitions represent different gene histories, but this assessment of the homogeneity of the phylogenetic signal may be incorrect, particularly when the substitution rate of evolution in the data partitions is not homogenous, if few characters are present, or partitions differ markedly in size (De Queiroz *et al.* 1995, Dowton &

Austin 2002, Darlu & Lecointre 2002). In view of these findings, separately analysed data partitions were compared by eye for strongly supported inconsistencies.. When none were determined, the partitions were combined.

Unweighted parsimony analyses of both separate and combined datasets were performed using a heuristic search with 1000 random addition sequences with the TBR branch swapping option. Internal branch support was assessed (Felsenstein 1985) using 1000 bootstrap replicates with random sequence addition and TBR branch swapping.

The protocols of the agaonid Bayesian analyses, and for the selection of substitution models in Modeltest v3.06 (Posada & Crandall 1998; Table 3.6), followed those used to determine the *Ficus* phylogeny. However, in contrast to the *Ficus* analyses, the first, second and third codon positions of the protein coding sequence data (COI & COII) were allowed to run with separate values for the model parameters in MrBayes v3.1.1 (Huelsenbeck & Ronquist 2001). The number of generations to be discarded as burn-in was assessed individually in the combined and separate analyses.

3.3.5 Cophylogenetic analyses

The extent of codivergence between the three lineages was assessed using three commonly applied methods of cophylogenetic analysis. TreeMap 1.0 (Page 1995), TreeMap 2.02β (Charleston & Page 2002) and ParaFit (Legendre *et al.* 2002) have been widely applied in host-parasite contexts (e.g. Hughs *et al.* 2007, Hafner & Page 1995, Johnson *et al.* 2002) as well as within the framework of the fig-fig wasp mutualism (Cook & Lopez-Vaamonde 2001, Jousselin *et al.* 2003, Jackson 2004, Weiblen 2004, Jousselin *et al.* 2008). Both methods test the null hypothesis that the "host" and "parasite" phylogenies have evolved independently. Both TreeMap 1.0 and 2.02β were implemented due to the computational constraints encountered in the latter version, and to ensure results are comparable with previous cophylogenetic studies.

TreeMap 1.0 and 2.02β stipulate strictly bifurcating trees and cannot interpret polytomies. In addition, each parasite must be associated with at least one host. Thus it was necessary to prune the phylogenies of extraneous taxa using MacClade v4.0 (Maddison & Maddison 2000) to remove or resolve any polytomies. Although ParaFit does not stipulate the same requirements, for consistent comparison, identically pruned phylogenies were used across all tests. Generally, a pruned tree, as opposed to a tree rebuilt from a subset of taxa, has been recognized to be a better estimate of the true phylogeny (Rannala *et al.* 1998, Zwickl & Hillis 2002).

Phylogenies were pruned independently for each analysis, thus a different subset of the taxa are represented in each test of codivergence. The *Ficus* Bayesian consensus tree (Figure 3.1), the sycoecine Bayesian consensus tree constructed with the combined COI, COII, Cytb & ITS2 sequence data (Figure 2.2, Chapter 2), and the parsimony phylogram of the pollinators of *Ficus* section *Galoglychia* constructed with 28S and ITS2 sequence data (Figure 3.7) were selected as the phylogenies of choice for the three analyses.

The tests of cophylogeny considered the pruned wasp phylogenies to be the "parasite" trees and were individually compared to the pruned *Ficus* "host" phylogeny. Thereafter, the agaonid and sycoecine phylogenies were compared to determine whether they displayed congruent phylogenies. This analysis was performed twice in TreeMap 1.0, TreeMap 2.02β and ParaFit; the host and parasite roles of the agaonids and sycoecines were swapped because the results of the TreeMap tests differ depending on which tree is assigned to be the "host".

3.3.5.1 Tree-based method

TreeMap 1.0 (Page 1995) and TreeMap 2.02β (Charleston & Page 2002) utilize a method that is known as reconciliation analysis; only the topologies of the host and parasite phylogenies are compared. The parasite phylogeny is mapped onto the phylogeny of the host in order to determine optimal reconstructions of the evolutionary history of the two

lineages. TreeMap 1.0, the first version of the program, uses parsimony to determine the optimal reconciliations of parasite and host phylogenies that maximize cospeciation and minimize host-switching events.

In contrast, TreeMap 2.02β implements the Jungle event-cost algorithm (Charleston 1998), rather than parsimony, to determine all the potentially optimal reconstructions of one tree mapped onto another. It is more sophisticated than TreeMap 1.0 in that additional sorting events that allow complex host switches can be performed (Chapter 1; Charleston 1998, Jackson 2004). The default settings of TreeMap 2.02β were used in the analyses; a cost of zero for codivergence events, and a cost of one for host switches, losses, and duplications were implemented. However, reconciliation analysis is computationally intensive and the program is currently limited in terms of the size and complexity of datasets that can be inputted (the number of potentially optimal solutions increases exponentially with the number of associations in the dataset; Jackson 2004). Computational limits were reached on the analyses that were performed in TreeMap 2.02β. Thus, the analysis was repeated several times for each cophylogeny comparison, each time increasing the maximum number of host switching events by one, until a solution with the highest number of cospeciation events and the lowest cost was found.

To determine whether the number of cospeciation events recovered in TreeMap 1.0 was significant, the parasite trees were randomized 10 000 times using a Markovian model. The observed number of cospeciation events was then compared to the null distribution of cospeciation events derived from this randomization procedure. The null hypothesis was tested by determining whether more cospeciation events are observed than expected by chance alone. An identical protocol was implemented in TreeMap 2.02β, but only the reconciliations with the lowest cost and highest number of cospeciation events were selected to be randomized, and parasite trees were randomized only 1000 times due to computational constraints.

3.3.5.2 Distance-based method

ParaFit (Lengendre *et al.* 2002) is able to assess phylogenetic congruence globally (across both phylogenies), as well as identify specific host-parasite pairs that are significantly associated. "Host" and "parasite" patristic distance matrices, taken from the phylogenies, are transformed into principle coordinates. The two matrices of principle coordinates and a third matrix of host associations are combined. A test statistic is then computed via a fourth-corner approach (Legendre *et al.* 1997) and compared to a randomized null distribution of host-parasite associations via a permutation procedure.

Each Parafit cospeciation analysis was implemented using two approaches following McLeish *et al.* (2007). The first method used true patristic distance matrices calculated directly from the branch lengths of the respective phylogenies, the second used a matrix determined from the phylogenies with all branch lengths equalized. The two different approaches enabled the analysis to be performed including tree topology and branch length variation, and with topology alone.

Matrices of the patristic distances with branch lengths equalized were transformed into principle coordinates, using DistPCoA (Legendre & Anderson 1989). None of the true patristic distance matrices could be transformed into principle coordinates due to the incidence of negative eigenvalues that standard correction protocols could not amend. As an alternative, patristic distance matrices were transformed into matrices of multidimensional scaling (MDS) coordinates in Genstat (2003). Tests of random associations were performed with 9999 permutations globally across both phylogenies. Both the ParaFitGlobal test statistic and the tests on the individual host-parasite links were assessed; test statistics of $P \leq 0.05$ were considered to be significant (Legendre *et al.* 1997). ParaFitLink1, and not ParaFitLink2, was used because it is a more conservative test statistic and has been observed to reduce type I error (Legendre *et al.* 2002).

3.3.6 Head shape evolution

Head lengths and widths of sycoecine and *Galoglychia* associated agaonid fig wasps were measured by S. van Noort, or gathered from the literature (Compton & van Noort 1994). These measurements were used to calculate head shape, measured as a ratio of head length to head width. Multiple measurements of the same wasp species collected from different *Ficus* hosts were included where available.

The sycoecine combined Bayesian tree of Cytb, COI, COII and ITS2 data and the agaonid Bayesian tree of ITS2 and 28S data were pruned of taxa for which no measurements were available. Head shapes were plotted onto the phylogeny and a parsimony reconstruction of ancestral character states was performed with the software Mesquite v2.01 (Maddison & Maddison 2007). Ancestral character state reconstruction with maximum likelihood criteria on a continuous character dataset cannot be performed in Mesquite v2.0 so only the parsimony reconstruction was implemented.

3.4 Results

3.4.1 Phylogeny of *Ficus* section *Galoglychia*

A total of 12 new sequences were generated for ITS and 29 for ETS; however, in only six cases could both sequences be amplified because the ITS gene region proved persistently difficult to amplify (Table 3.1). Both ITS and ETS were amplified for *F. burkei*, *F. craterostoma*, *F. natalensis*, *F. petersii*, *F. lingua* (subsection *Chlamydodorae*) and *F. abutilifolia* (subsection *Platyphyllae*). These six sequences were added to the *Ficus* sequence data generated by Weiblen (2000), Jousselin *et al.* (2003) and Rønsted *et al.* (2005, 2007) (Table 3.1). The ILD test indicated significant conflict between the ITS and ETS data partitions ($P = 0.002$). Nevertheless, following previous analyses (e.g. Rønsted *et al.* 2007), both data partitions were combined.

The aligned ITS and ETS data matrix consisted of 1343 base pairs, 131 (10%) of which were parsimony informative. The parsimony analysis of the combined ITS and ETS dataset resulted in 24825 equally parsimonious trees (Figure 3.1; Length = 487, Consistency Index (CI) = 0.74, Retention Index (RI) = 0.83, Rescaled Consistency Index (RC) = 0.61). The first 6 million generations of each run of the two parallel Bayesian runs of the combined ETS and ITS analysis were discarded as burn-in, thus the Bayesian consensus tree was constructed from 4000 trees. The topology of the parsimony phylogeny for the *Ficus* taxa was near-identical to the recent phylogeny of *Ficus* section *Galoglychia* (Rønsted *et al.* 2007) although bootstrap values were not as high and, similarly, the posterior probabilities from the Bayesian analysis indicated that support for clades was not as extensive (Figure 3.1).

Ficus section *Galoglychia* was paraphyletic with respect to the neotropical section *Americana* in both parsimony and Bayesian analyses, although placement of the *Americana* clade varied between the two (Figure 3.1). However, this paraphyly was not supported by bootstrap (BS) or posterior probabilities (PP) in these analyses nor in the analyses of Rønsted *et al.* (2007).

The two major clades identified in the phylogenies of *Ficus* section *Galoglychia* were termed Clades A and B (Figure 3.1). Clade A contained the *Ficus* subsections *Caulocarpae*, *Crassicostae*, *Cyathistipulae*, and *Galoglychia*. Subsections *Caulocarpae* (70% BS, 99% PP), *Cyathistipulae* (88% BS; 100% PP) and *Galoglychia*, although represented by only two species, (97% BS, 100% PP) were monophyletic and well supported by both bootstrap and Bayesian posterior probabilities. *Ficus platyphylla*, taxonomically assigned to subsection *Platyphyllae*, was placed in the clade containig subsection *Crassicostae* (100% PP). The subsections *Chlamydodorae* (97% BS, 100% PP) and *Platyphyllae,* excluding *F. platyphylla,* (100% PP) were identified as sister clades that together comprised Clade B (Figure 3.1).

While the phylogenies constructed through both parsimony and Bayesian methods were generally consistent with the preceding study (Rønsted *et al.* 2007), the inclusion of the

the six taxa, five of which were placed in *Ficus* subsection *Chlamydodorae*, revealed a distinct lack of resolution among species within the subsection *Chlamydodorae* (Figure 3.1). The clade that contained the polytomy was well supported (79% BS, 95% PP), but ITS and ETS sequence data did not recover relationships at the species level within the clade. *Ficus lingua*, *F. petersii*, *F. natalensis natalensis*, *F. craterostoma*, *F. buxifolia, F. calyptrata*, *F. burkei* and *F. thonningii* fall within an unresolved polytomy. In addition, duplicate taxa did not group as sister taxa (Figure 3.1). For example, the two *F. burkei* and two *F. craterostoma* samples embedded within the polytomy were not each other's closest relative.

The second set of analyses incorporated the indivual specimens for which only one of the two gene regions were obtained into the combined supermatrix. A total of 21 ITS (six new sequences and 15 retrieved from GenBank) and 23 new ETS sequences were incorporated into the supermatrix to test the subsectional placement of species. The parsimony and Bayesian analyses of this dataset were in general agreement with traditional taxonomy (Appendix 3.1). The substantial number of missing sequences (18%) may account for the lack of resolution and poor support, and for the multiple occasions where duplicate taxa did not appear as sister taxa (e.g. *F. polita*).

The placement of *F. stuhlmannii* differed from traditional taxonomy where it is placed in section *Platyphyllae*, and in these analyses grouped within the polytomy of subsection *Chlamydodorae* (Appendix 3.1), in corroboration with Rønsted *et al.* (2005). The positions of *F. modesta* within subsection *Caulocarpae*, and *F.* sp. "samfya fig" and *F. rokko* within subsection *Chlamydodorae*, for which sequences were not included in analyses performed by Rønsted *et al.* (2007), were expected, but not supported. As anticipated, *Ficus barteri* was placed within subsection *Cyathistipulae* (99% PP). Three placements differed from traditional taxonomy: *Ficus bussei* (subsection *Platyphyllae*) joins *F. platyphyllae (*subsection *Platyphyllae;* Rønsted *et al.* 2007) in subsection *Crassicostae* (99% PP), and *F. nigropunctata (*subsection *Platyphyllae*; Rønsted *et al.* 2007) was placed within the subsection *Chlamydodorae* (Appendix 3.1).

3.4.2 Phylogeny of the pollinators of *Ficus* section *Galoglychia*

All of the seven agaonid genera that pollinate species within *Ficus* section *Galoglychia*: *Nigeriella*, *Paragaon* (single species), *Agaon, Allotriozoon*, *Elisabethiella*, *Alfonsiella* and *Courtella* were represented in this study. DNA sequences from 43 fig wasp specimens were added to the sequence data analysed in the recent molecular phylogeny of the pollinators of *Ficus* section *Galoglychia* (Erasmus *et al.* 2007), although not every targeted gene regions could be amplified for every new specimen. Additional data included sequences from two supplementary gene regions, 18S and COII, and from seven species not included in the analysis by Erasmus *et al.* (2007). As previously stated, combining the agaonid sequence data produced in this study with sequence data from Erasmus *et al.* (2007) produced a molecular supermatrix with approximately 40% missing data.

The aligned COI data partition consisted of 819 base pairs, 298 (36%) of which were parsimony informative. The parsimony analysis, including a total of 47 ingroup taxa, yielded 49 equally parsimonious trees (Figure 3.2; Length = 1117, CI = 0.43, RI = 0.69, RC = 0.30);. Measures of support for internal nodes in both the Bayesian and Parsimony trees were poor (Figures 3.2 & 3.3). In contrast, support for terminal clades was present and the placement of taxa within the genus *Elisabethiella* was well supported and consistent between parsimony and Bayesian analyses.

The COI phylogenies (Figures 3.2 & 3.3) did not show *Elisabethiella stuckenbergi* specimens clustering based on host association. However, specimens appeared to cluster geographically; *E. stuckenbergi* individuals sampled in South Africa (100% BS, 100% PP) and those collected in Zambia, Tanzania and Mozambique grouped together (54% BS, 100% PP; Figure 3.2, Table 3.2). Similarly, *Alfonsiella binghami* and *A. longiscapa* individuals also did not group together into a single clade, nor did *E. socotrensis* individuals.

The mean percentage sequence divergence (p-distance; ± standard deviation) between clades or lineages of *Elisabethiella* (marked A-K; Figure 3.2) was 6% ±1.5% (Table 3.7). The smallest sequence divergence was between *E. allotrizoon* (Clade A) and the clade of *E. socotrensis* individuals (Clade B). The demarcated clades (C, F & G; Figure 3.2) containing *E. stuckenbergi* individuals showed comparable levels of sequence divergence to the remaining clades that generally each comprised a different species. The mean percentage sequence divergence (± standard deviation) within species clades or lineages of the *Elisabethiella* genus was 1% ±1.5%, but ranged from 0% to 4%; the 4% within clade divergence was obtained from Clade G containing *E. stuckenbergi* individuals (Figure 3.2).

Of the 595 base pairs in the aligned data matrix of COII, 308 (51%) were parsimony informative. One most parsimonious tree was found (Appendix 3.1; Length = 852, CI = 0.56, RI = 0.71, RC = 0.40). The 18S data partition consisted of 782 base pairs, of which 12 were parsimony informative (2%). With so few informative base pairs, the parsimony and Bayesian phylogenies (not shown) constructed with 18S displayed little resolution. Nevertheless, this partition was included in a combined analysis with the COI and COII data partitions.

The ILD tests gave inconsistent results for the three tests assessing the combinability of the COI, COII and 18S data partitions; each possible pair of the three gene regions was assessed for combinability. No conflict in phylogenetic signal was detected between the COI and 18S ($P = 1.00$) and COII and 18S ($P = 0.99$) data partitions. In contrast, the ILD test indicated that the phylogenetic signals present in the partitions COI and COII ($P = 0.01$) were in conflict. Nevertheless, parsimony and Bayesian analyses were performed on the combined dataset.

The combined analysis of the COI, COII and 18S gene regions included 33 ingroup taxa and a total of 2206 characters, 556 of which were parsimony informative (25%). The parsimony analysis yielded eight most parsimonious trees (Figure 3.4; Length = 1836, CI = 0.53, RI = 0.64, RC = 0.34). The Bayesian phylogeny was constructed using 5000

trees; the first five million generations of the two runs were discarded as burn-in (Figure 3.5). The clades containing *Alfonsiella* (74% BS, 94% PP), *Elisabethiella* (69% BS, 99% PP) were monophyletic and supported. This phylogeny represented a smaller subset of the taxa than the COI analysis, with similar placement of taxa maintained.

A total of 101 ambiguously aligned base pairs of the alignment were excluded from the parsimony and Bayesian analyses of the ITS2 data partition. The final ITS2 partition consisted of 278 base pairs, 109 of which were parsimony informative (39%). The parsimony analysis retrieved 183 most parsimonious trees (Appendix 3.2; Length = 449, CI = 0.58, RI = 0.73, RC = 0.42).

The ILD test indicated significant conflict between the ITS2 and 28S data partitions ($P = 0.002$). Nevertheless, following Erasmus *et al.* (2007), both data partitions were combined. The combined ITS2 and 28S analyses were thus performed on 44 ingroup taxa, with a molecular matrix containing a total of 1227 aligned base pairs, 221 of which were parsimony informative (18 %) Under parsimony, 4034 equally optimal trees were retained (Figure 3.5; Length = 848, CI = 0.63, RI = 0.70, RC = 0.44). Both parsimony and Bayesian analyses revealed high resolution of internal nodes, in contrast to the analyses that included sequence data from the mitochondrial genes COI and COII.

The clades containing *Alfonsiella* (86% BS, 100% PP), *Elisabethiella* (87% BS, 100% PP) and *Nigeriella* (75% BS, 95% PP) are monophyletic and well supported (Figures 3.6 & 3.7). The placement of these three clades differs between the parsimony and Bayesian analyses; the parsimony analysis places *Alfonsiella* and *Elisabethiella* as sister genera, while the Bayesian consensus tree shows *Nigeriella* sister to *Alfonsiella*, a clade which formed a polytomy with *Elisabethiella* and the weakly supported clade containing *Paragaon* and *Agaon* (66% BS) specimens. *Courtella* specimens grouped into a poorly supported clade (60% BS only), while *Allotriozoon* did not form a monophyletic group in the parsimony analysis (Figure 3.6), but was well supported (100% PP) in the Bayesian analysis.

None of the phylogenies retrieved the three species of *Alfonisiella* as a monophyletic group, nor were the groupings based on host-association or geography (Figures 3.2–3.7, Appendices 3.2 & 3.3). Equally unexpected, *Courtella* sp. (ex. *F. modesta*) did not group within the clade containing the remaining *Courtella* specimens in the analyses containing mitochondrial COI sequence data (Figures 3.2–3.5); *Courtella* sp. (ex. *F. modesta*) was placed sister to all the pollinators of section Galoglychia. However, in the ITS2 (Appendix 3.2) and 28S and ITS2 (Figures 3.6 & 3.7) analyses, *Courtella* sp. (ex. *F. modesta*) grouped within the *Courtella* clade.

3.4.3 Cophylogenetic analyses

The *Ficus* Bayesian consensus tree was pruned for use in the cophylogenetic analyses. In order to remove the polytomy within the subsection *Chlamydodorae,* four pertinent species (*F. natalensis natalensis*, *F. petersii*, *F. craterostoma* and *F. burkei*) in the complex were treated as a single terminal taxon, and, in all subsequent analyses, the fig wasps associated with these taxa were linked to this complex (termed the *F. natalensis* sp- complex). There is large amount of overlap in the wasp fauna associated with the four fig tree species. For instance, in our limited and incomplete collections, the sycoecine fig wasp *Crossogaster odorans* is associated with all four of these *Ficus* species. *Philocaenus barbarus* is associated with *F. natalensis*, *F. petersii* and *F. burkei,* and *Philocaenus liodontus* is associated with *F. natalensis natalensis* and *F. petersii* (Chapter 2; Table 2.1). Similarly, the agaonid fig wasps *Elisabethiella stuckenbergi* and *E. socotrensis* pollinate many of the fig tree species in *Ficus* section *Chlamydodorae* (Table 3.2). In general, host-specificity of sycoecine and agaonid wasps within section *Chalmydodorae* appears to be less strict than in the remaining *Ficus* subsections.

The Treemap 1.0 cospeciation test of the *Ficus* section *Galoglychia* and sycoecine phylogenies identified a total of eleven cospeciation events (Figure 3.8) and rejected the null hypothesis of independent evolution (P = 0.007, Table 3.8). TreeMap 2.02β suggested a larger number of cospeciation events and produced a significant result

($P = 0.01$), although it was necessary to limit the number of host-switching events to three due to computational constraints. The ParaFitGlobal test statistic, calculated from the phylogenies with branch lengths equalized, was in agreement ($P = 0.03$). Five of the 20 ParaFitlinks were significantly associated (Figure 3.8). In contrast, the ParaFitGlobal test result based on matrices of true patristic distances was not significant ($P = 0.21$). The majority of the incongruence between the phylogenies lies within *Ficus* subsections *Chlamydodorae* and *Platyphyllae* (Clade B; Figures 3.1 & 3.8) and within subsection *Caulocarpae* (Clade A; Figure 3.1 & 3.8).

Treemap 1.0 did not detect significant cophylogeny between the *Ficus* phylogeny and the phylogeny of the pollinators of *Ficus* section *Galoglychia* ($P = 0.78$), and neither did TreeMap 2.02β (the number of host-switches was restricted to four events due to computational limits; $P = 0.38$; Table 3.8; Figure 3.9). However, both ParaFit tests, with equal branch lengths and with true patristic distances, rejected the hypothesis of independent evolution ($P = 0.001$ and $P = 0.002$ respectively). The ParaFit analysis of the phylogenies with branch lengths equalized revealed that 13 of the 20 host parasite links were significantly associated while the ParaFit test based on matrices of true patristic distances showed 16 significant links (Table 3.8).

The hypothesis of independent evolution was not rejected by any of the tests comparing the phylogenies of the pollinators of *Ficus* section *Galogylchia* and the Sycoecinae. (Table 3.8, Figure 3.10). Neither Treemap 1.0 nor both ParaFit analyses detected significant cophylogeny between the two wasp lineages regardless of which lineage was regarded as the host (Table 3.10). However, it is remarkable that, when mapping the Agaonid "parasite" tree onto the sycoecine "host" tree, only one cospeciation event was identified (a single cospeciation event is always enforced; Treemap postulates a cospeciation event at the ancestral node of the host phylogeny), while when the sycoecine "parasite" tree was mapped onto the agaonid "host" tree, a total of eight cospeciation events were identified (Table 3.8). Due to computational constraints, these analyses could not be performed in TreeMap 2.02β.

3.4.4 Head shape evolution

A total of 31 measurements of female sycoecine head shape and 23 measurements of female agaonid head shape were mapped onto the respective wasp phylogenies in order to perform ancestral character state reconstructions. Head shape, calculated as the ratio of head length to head width, ranged between 0.94–1.85 in the Sycoecinae, and between 0.8–1.52 in the pollinators of *Ficus* section *Galogychia* (Appendix 3.4).

The Mesquite v2.01 (Madison & Madison 2007) ancestral reconstruction using parsimony criteria data suggested that sycoecine and agaonid head shapes were generally evolutionarily conserved (Figure 3.11); generic level clades display broadly similar head shape dimensions. However, "square" and "elongate" head shapes have arisen independently on several occasions.

In the sycoecine analysis, the reconstructions showed the "square" head shape to be the pleisiomorphic state and the "elongate" shape to be derived. Elongate heads (head shape ratios ≤1.5) were reconstructed as evolving on three separate occasions on the pruned Baysesian consensus phylogeny of the Sycoecinae. Two reversals to more square head shapes (head shape ratios <1.5) were indicated (Figure 3.11)

In the agaonid head shape analysis, the ancestral reconstructions showed an intermediate form of headshape, of average 1.2, to be the pleisiomorphic state (Figure 3.11). Thus both elongate and square forms are derived. More elongate headshapes have arisen twice, first in the clade containing the genus *Courtella*, and secondly, in the branch containing *Nigeriella excavata*. Measurements for other *Nigeriella* species were not available.

3.5 Discussion

In corroboration with recent studies, these results present a complex evolutionary history of figs and fig wasps. Although the different cospeciation tests obtained mixed results, in essence, codivergence between fig and wasp lineages was revealed to some extent. However, it is far from ubiquitous. Furthermore, the phylogenetic results suggested that both fig and fig wasp taxonomy based on morphological characters conflicted, to various extents, with the evolutionary history determined by these molecular phylogenies. For example, there is evidence of crytic fig wasp pollinators and a number of *Ficus* species in subsection *Chlamydodorae* clade showed no resolution of species relationships.

3.5.1 Phylogeny of *Ficus* section *Galoglychia*

The molecular analysis showed that the six subsections of *Galoglychia* are largely monophyletic and fall into two major clades, clades A and B (Figure 3.1; Rønsted *et al.* 2007). Clade A comprises *Ficus* subsections *Caulocarpae*, *Crassicostae*, *Cyathistipulae*, and *Galoglychia*; with few exceptions, these four subsections contain fig species restricted to rainforest and forest habitats with species distributions that are concentrated in west and central Africa. Clade B is comprised of subsections *Chlamydodorae* and *Platyphyllae*; the majority of the species in these two subsections are associated with relatively dry savannah woodland, although a few species do inhabit rainforest. Distributions are concentrated in eastern Africa extending to Madagascar and the Mascarene Islands. Rønsted *et al.* (2007) proposed that the distribution of the species of the two clades coincides with two of Linder's (2001) six important centres of endemism located within sub-Saharan Africa.

The molecular analyses of ITS and ETS sequence data placed four fig taxa, *F. platyphyllae*, *F. nigropunctata, F. stuhlmannii* and *F. bussei,* all assigned to subsection *Platyphyllae*, in different subsections than predicted by traditional taxonomy. This suggested that the *Ficus* species residing within subsection *Platyphyllae* may require a

revision. The remaining subsections, delimited by Berg (1986) through morphological analysis, were upheld in the present molecular analyses.

The inclusion of the six taxa for which both ITS and ETS were successfully amplified revealed a polytomy within *Ficus* subsection *Chlamydodorae* (Clade B; Figure 3.1). This subsection contains 13 savannah woodland and rainforest fig species. Species distributions centre in eastern Africa, although many have widespread ranges (Berg & Wiebes 1992). Over various taxonomic revisions, these species have either been lumped into complexes or split into separate entities. Berg (1989) asserted that the group is either a species complex or a complex currently undergoing speciation. The polytomy within subsection *Chlamydodorae* may well be explained as a species complex. In agreement with Rønsted *et al.* (2005), *Ficus stuhlmannii* was placed within the subsection *Chlamydodorae*, rather than within subsection *Platyphyllae* in which it is currently classified (Berg 1986). This was unsurprising considering that *F. stuhlmannii* shares agaonid and sycoecine wasp fauna with many *Ficus* species from subsection *Chlamydodorae* (Table 2.1, Table 3.2).

Paleobotanical pollen records and carbon isotopes from west and east Africa date the earliest record of the savannah biome to the Middle Miocene, and reveal that grass-dominated habitats were widespread by the Late Miocene around eight million years ago (Jacobs 2004, Beerling & Osborne 2006). The expansion of the savannah biome has been linked with paleoclimatic changes as a result of global cooling events (deMenocal 2004, Beerling & Osborne 2006). In addition, many studies have shown congruence between climate change and radiation events (e.g. deMenocal 1995, Linder 2003). I hypothesized that the emergence of the savannah biome since the Miocene in response to change in African climate or shifts in climate variability instigated the recent radiation of the arid-adapted subsection *Chlamydodorae*. This radiation may be explained by special faunal adaptation and speciation due to novel selection pressures. Molecular dating of the phylogeny of *Ficus* section *Galoglychia* will enable these hypotheses to be explored further (Chapter 4).

3.5.2 Pollinators of *Ficus* section *Galoglychia*

The parsimony and Bayesian analyses of the five gene regions reached the same limitations and conclusions as both traditional taxonomy and the study performed by Erasmus *et al.* (2007). In general, it was found that the separate and combined analyses of the data partitions support the monophyly of the genera of the pollinators of section *Galogychia*.. The placement of the genera, however, remains unresolved, and the relative positions of congeneric taxa within the clades are remain in conflict (Erasmus *et al.* 2007); with each separate analysis suggesting a different hypothesis for the placement of the taxa. However, phylogenetic analyses incorporating new sequence data as reported here did yield various new insights.

In particular, the separately analysed COI phylogeny (Figures 3.2 & 3.3) reveals possible geographical divergence in *Elisabethiella stuckenbergi*, pollinator of several host figs within *Ficus* subsection *Chlamydodorae*. Sequence divergences between the clades comprising *E. stuckenbergi* indiduals were similar to clades of the remaining *Elisabethiella* species (Table 3.7). This suggests that the delimitation of this species is not valid and that a cryptic species complex is involved. A critical reassessment of morphological traits in combination with molecular data is required. Further studies will be necessary to delimit these taxa and investigate host specificity. For example, fine-scale phylogenetic studies of numerous specimens sampled throughout their geographic range will be needed to confirm divergences within these clades. Similarly, species validity of *E. socotrensis* and within the genus *Alfonisiella* requires further review.

The presence of cryptic species has implications for the combinability of datasets and the potential for incorporating additional sequence data from novel gene regions into existing DNA sequence data matrices. Combining different evolutionary signals from divergent lineages within a species should be carefully avoided, especially when attempting to reach fine-scale phylogenetic conclusions. Haine *et al.* (2006) discovered large divergences in mitochondrial Cytb sequences which correspond to four well-supported lineages within one Australian species of pollinating fig wasp. Therefore, combining

sequences from individuals putatively representing the same species collected either at different hosts, or from the same hosts in a different geographic location is not justifiable. Significantly, this lack of combinability limits supermatrix approaches to phylogeny reconstruction.

3.5.3 Cophylogenetic analyses

While it was attempted to make the wasp specimens used in the following cophylogenetic analysis true associates, limited success was experienced. Sampling was not exhaustive, thus representation of associated species across phylogenies was not uniform. Extensive pruning of each phylogeny was unavoidable and a different subset of taxa was represented in each test. Therefore, the cophylogenetic analyses omitted a large number of species present in the various phylogenetic reconstructions. In order to draw broad conclusions, and relate them to our relatively small-scale cophylogenetic analyses, the extent of host specificity between the pollinating agaonid fig wasps and non-pollinating sycoecine fig wasps and their associated host fig trees of Section *Galoglychia* will first be discussed qualitatively.

It is well established that species in the genera *Courtella*, *Agaon* and *Allotriozoon* are host-specific to, and the sole pollinators of, the subsections *Caulocarpae*, *Cyathistipulae,* and *Galoglychia* respectively. *Paragon* is restricted to subsection *Crassicostae*, but this subsection is not exclusively pollinated by this genus. The genera *Nigeriella*, *Alfonsiella* and *Elisabethiella* are not constrained to one specific subsection, rather, each is associated with two or three *Ficus* subsections. *Nigeriella* species pollinate the *Ficus* hosts within Cra*ssicostae* and *Platyphyllae* subsections, *Alfonsiellla* species pollinate *Chlamydodorae* and *Platyphyllae*, and *Elisabethiella* species pollinate *Crassicostae*, *Chlamydodorae* and *Platyphyllae* subsections. Similarly, *Sycoecus* is the only sycoecine genus associated with subsection *Cyathistipulae* and although there are other sycoecine genera associated with subsection *Caulocarpae,* the genus *Seres* is restricted to this subsection. The genera *Crossogaster* and *Philocaenus* are associated with three and four

subsections respectively; *Crossogaster* individuals have been recorded with *Ficus* subsections *Crassicostae*, *Platyphyllae* and *Chlamydodorae,* while *Philocaenus* individuals have been documented to be associated with *Ficus* subsections *Crassicostae*, *Platyphyllae*, *Chlamydodorae and Galoglychia*.

While the sycoecine genus *Philocaenus* may well require revision (*Seres* incorporated into *Philocaenus,* or *Philocaenus* split into a number of separate entities with generic status; see Chapter 2), the phylogenetic reconstructions of these independent analyses showed widespread support for the monophyly of the genera and subsections delimited by traditional taxonomy. If strict cospeciation was shaping the evolutionary trajectories of these phylogenies, clades of fig wasps would be strictly associated with clades of host figs. The general pattern of association between these pollinator and non-pollinator lineages and their *Ficus* hosts reveals break-down in host-specificity.

A cophylogenetic analysis of agaonid pollinators and their host figs has been attempted before (Jousselin *et al.* 2008), with mixed results dependent on which the phylogeny was used However, the source phylogenies used in the present study were slightly different; new ITS2 sequence data were included in the ITS2 & 28S phylogeny of the pollinators *Ficus* section *Galoglychia*. While Parsimony and Bayesian phylogenies using mitochondrial COI and COII sequence data indicated resolution of terminal clades, the parsimony phylogeny constructed with ITS2 and 28S incorporated more genera, and displayed greater support for deeper nodes. Thus, the the latter phylogeny was chosen to be used in the cospeciation analyses. The *Ficus* phylogeny was also different; in accordance with the polytomy revealed in the phylogeny of *Ficus* section *Galoglychia* (Figure 3.1), the fig species *F. natalensis natalensis*, *F. petersii*, *F. craterostoma* and *F. burkei* were treated as a single terminal taxon, namely the *F. natalensis* sp-complex.

This is the first cophylogenetic study including sycoecine fig wasps. Results from the cophylogentic analyses revealed different levels of codivergence dependent on the method of cospeciation analysis used. Only the tests comparing the Sycoecinae and *Ficus* showed significant codivergence in TreeMap 1.0 and TreeMap 2.02β. Both the sycoecine

and the pollinators when compared to their associated *Ficus* phylogenies obtained significant results for the ParaFit test with branch lengths equalized. However, only the *Ficus*-pollinator comparison showed a significant result in the ParaFit test including true patristic distances (Table 3.8). This may indicate that while distance matrices of the sycoecine and *Ficus* topologies alone display congruence, distance matrices including branch lengths between nodes do not correspond. Phylogenetic reconstructions based on diverse gene regions that evolve at different rates were used: Cytb, COI, COII and ITS2 (mitochondrial and nuclear gene regions) in the sycoecine phylogeny and ITS and ETS (nuclear gene regions only) in the *Ficus* phylogeny. Consequently, the results of the cophylogenetic analyses were not consistent between the tree-based and distance-based methods. Although these methods are commonly used in contemporary research contexts, they are not without their limitations. Both are reliant on the quality of the input phylogenies and the rejection of source phylogenies enforces rejection of the reconciled trees or the ParaFit result. Nodal support and confidence values are not incorporated into either version of TreeMap or ParaFit, and the phylogenies are assumed to be free of error.

Other studies have also experienced inconsistent results between ParaFit and TreeMap 1.0 and TreeMap 2.02β (e.g. Marussich & Machado 2007) and many authors prefer ParaFit as a more "biologically plausible" test of codivergence (e.g. Marussich & Machado 2007, Jousselin *et al.* 2008). Tree-based methods are often viewed as restrictive; fully resolved trees are mandatory, and results of these tests change dramatically depending on which lineage is considered as the "host" or "parasite"; this is most conspicuous when the roots of the phylogenies are different. Another disadvantage of TreeMap 1.0 is that it limits host-switching events. However, because TreeMap 2.02β is computationally intensive, the number of host switching events was limited and analyses comparing the agaonid and sycoecine phylogenies were unable to be performed.

Furthermore, all cophylogenetic analyses are sensitive, to differing degrees, to the subsets of taxa included. Jousselin *et al.* (2007) demonstrated that the results of the cophylogenetic analyses between the pollinators of *Ficus* section *Galoglychia* and their host fig trees differed when they used two different phylogenetic hypotheses; the

discrepancy resulted from the number of taxa included rather than from differing topologies. It is therefore likely that repeating the analyses reported here with larger or smaller subsets of taxa could reveal different results. The results of these tests appear to be especially sensitive to the taxonomic level of taxa that are incorporated into the analyses; a mix of distantly and closely related taxa introduces nodes of inconsistent age, potentially artificially inflating the level of codivergence determined (Jackson 2004). When cophylogenetic analyses consist of distantly related taxa, less host switching may be expected because these taxa are more likely to have evolved adaptations that result in morphological, ecological and chemical incompatibilities that prevent host-switching events. To be clear, if cophylogenetic analyses concentrated on coarse scale phylogenies indicate cospeciation, it does not automatically follow that fine scale phylogenies will indicate the same pattern (Machado *et al.* 2005).

The results presented here verify the recent recognition of "diffuse" codivergence within the fig-fig wasp community (Marussich & Machado 2007). Internal oviposition of non-pollinating fig wasps appears to impose no greater constrait to host-switching than external ovipostition. In short, these analyses confirmed that sorting events such as host-switches, losses (lineage extinctions, omissions, or lineage sortings) and duplications are regular occurrences in the fig-fig wasp community. The cophylogenetic analyses confirmed that fig divergence has influenced both pollinating and non-pollinating fig wasp divergence (Table 3.8). However, despite the ecological, physiological, chemical and morphological constraints on sycoecines and pollinating fig wasps to maintain host-specificity, codivergence was not pervasive. The analyses also suggested that, within our small sample, the associated agaonid and sycoecine fig wasps have not evolved through strict codivergence (Figure 3.10, Table 3.8). However, these results may well be an artefact of the subset of taxa involved in the analyses; 72% of the Agaonidae and 81% of the Sycoecinae were from the subsections *Chlamydodorae* and *Platyphyllae*. The *Ficus* hosts in these subsections are notorious for having shared fig wasps, hence the highly convoluted tanglegram (Figure 3.11). Nevertheless, the successful development of sycoecines within fig fruit is thought to be independent of pollinating fig wasps (van Noort 1992). While lack of successful pollination may cause figs to abort their fruit,

oviposition and galling of ovules by sycoecines appears to prevent this from occurring. Thus host-switching events of sycoecines may well occur independently of the pollinators.

Cospeciation and coevolution, processes that revealed by a pattern of codivergence (Chapter 1), may now be postulated between *Ficus* and their pollinators, although dated phylogenies are still necessary to confirm synchronized cocladogenesis. Similarly, without a dated molecular phylogeny of the Chalcidoidea, it is difficult to assess whether the sycoecine patterns of codivergence have occurred through contemporaneous codivergence or whether they represent a more recent colonization of the fig niche and subsequent speciation by sequential radiation. Nevertheless, given the significant results of the codivergence tests, it is not impossible that codivergence events of sycoecines and their *Ficus* hosts are contemporaneous.

Because the subset of taxa included in each cophylogenetic analysis has a large effect on the results of the tests, Jousselin *et al.* (2007) recommend that future studies sample "host" and "parasite" lineages exhaustively. Equally validly, Jackson (2004) postulated that, due to extinction events, there can never be exhaustive sampling and that cophylogeny tests will always be limited, and confounded, by this factor. Historical events, such as duplications, host-switches and extinctions may be obscured over time, thus a phylogeny of extant taxa will always be a poor indication of the evolutionary complexity to which a lineage is subjected. Thus, while these tree- and distance-based methods of cophylogenetic analysis may attempt to identify specific codivergence events and construct codiversification scenarios by assigning costs to different events, a conservative interpretation of these results as confirmation of the complex evolutionary history of the fig-fig wasp community is suggested.

3.5.4 Head shape evolution

Phylogenetic reconstruction of head shape suggests that it is an evolutionarily conserved character within these two independent wasp lineages. Head shapes within clades were similar, thus ostiolar morphology may well play an important role in limiting host-switching events. Both phylogenetic reconstructions showed intermediate to small headshapes as the ancestral character, with both larger and smaller headshapes evolving from this state. Nevertheless, the most parsimonious reconstructions of both sycoecine and agaonid phylogenies did show distinct reversals of head shape. Although exhaustive sampling was not obtained, these may have represented host-switching events, and an adaptation to the fig morphology of a new host. For example, *C. inusitata* (ex. *F. sansibarica sansibarica)*, the only large head-shape in the *Crossogaster* clade may have represented a host-switching event (Figure 3.11).

3.5.5 General conclusions

These results, and those of other studies (e.g. Jackson 2004, Jousselin *et al.* 2007, Marussich & Machado 2007), revealed a complex evolutionary history of codivergence within the fig-fig wasp community. Both tree-based and distance-based methods of cophylogenetic analyses are imperfect in their ability to determine duplication, host-switching and extinction events that may have become obscured over time (the matter is made worse when sampling is not exhaustive). Nevertheless, the general pattern of "diffuse" codivergence of both pollinating and non-pollinating fig wasps, revealed through cophylogenetic analyses, has prompted a robust modification of the widely-held hypotheses of strict, one-to-one codivergence. While figs and fig wasps do codiversify, codivergence is not pervasive.

More interesting are the questions that address the processes underlying these diversification patterns. There may be merit in investigating why certain subsections generally display high host-specificity (e.g. *Cyathistipulae* and *Caulocarpae*; Figure 3.1, Clade A) and why others generally display less host-specificity (e.g. *Chlamydodorae* and

Platyphyllae; Clade B, Figure 3.1). Identifying the processes (such as climate fluctuations and wasp ecology) that drive speciation of *Ficus*, and isolating the differences in these processes across temporal and spatial scales will be invaluable.

Machado *et al.* (2005) reiterated, and provided evidence for, the hypothesis of Baker (1961) that proposed that the large diversity of *Ficus* may be driven by hybridization and introgression; genetically well-defined pollinating wasp species are associated with multiple, but poorly-defined, groups of figs. Over time, some of these lineages disappear through extinction events. Therefore, ancient patterns that reveal codivergence and coevolution at broad scales may break down when lower taxonomic levels and contemporary processes are examined (Jackson 2004); effectively, tree comparison will never be able to provide a true account of evolutionary history. In the context of *Ficus* section *Galoglychia*, the complex of fig trees forming the polytomy within the *Chlamydodorae* may well be a result of contemporary hybridization. It is ideas such as these that will need to be explored in more depth in future studies.

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Table 3.1: Taxa included in the phylogenetic analyses of *Ficus* section *Galoglychia*. Sequences retrieved from GenBank were published in Weiblen (2000) Jousselin *et al.* (2003), Rønsted *et al.* (2005) and Rønsted *et al.* (2007).

***Ficus* species**	**Collection number**	**Collection locality & date**	**ITS**	**ETS**
Subsection *Caulocarpae*				
Ficus bizanae	GenBank		DQ455636	DQ455670
	SA05-F69	RSA, KwaZulu-Natal, Ongoye Forest, 11-2005		1
	SA05-F81	RSA, Eastern Cape, Port St. Johns, 11-2005		1
Ficus bubu	GenBank		DQ455637	DQ455671
	SA05-F54	RSA, KwaZulu-Natal, Lake Sibaya, 11-2005		1
Ficus dryepondtiana	GenBank		DQ455638	
	GenBank		DQ455639	
Ficus modesta	MW06-F73	Mozambique, Zambezia Province, Mount Mulanje, 05-2006		1
Ficus ottoniifolia	GenBank		AY730109	AY730198
Ficus ovata	GenBank		DQ455640	
Ficus ovata	GenBank			DQ455672
Ficus ovata	Genbank		DQ455641	
Ficus polita	GenBank		DQ455642	DQ455673
	GenBank		DQ455643	
	SA05-F51	RSA, KwaZulu-Natal, Mkuze - Ubombo Road	1	
Ficus sansibarica	GenBank		AY730110	AY730199
	ZA06-F18	Zambia, Luapula Province, 20 km west of Kawambwa, 05-2006		1
	SA05-F27	RSA, Limpopo, Legalameetse Nature Reserve, 11-2005		1
Ficus tremula	GenBank		AY730111	AY730200
Ficus umbellata	GenBank		DQ455644	DQ455674
Subsection *Chlamydodorae*				
Ficus faulkneriana	GenBank		DQ455645	
Ficus amadiensis	GenBank		DQ455646	
Ficus burkei	GenBank		AY730095	AY730184
	SA05-F28	RSA, Limpopo, Abel Erasmus Pass, 11-2005	1	1
	SA05-F36	RSA, Mpumalanga, Blyde River Canyon Nature Reserve, Belvedere, 11-2005	1	
Ficus burtt-davyi	GenBank		DQ455647	DQ455675
	SA05-F82	RSA, Eastern Cape, Woody Cape Nature Reserve, Alexandria Forest, 11-2005		1
Ficus calyptrata	GenBank		DQ455648	DQ455676
Ficus craterostoma	GenBank		AY730097	AY730186
	SA05-F76	RSA, Eastern Cape, Mkambati Nature Reserve, Gwe Gwe forest, 11-2005	1	1
Ficus fischeri	GenBank		DQ455649	
	GenBank		AY730098	AY730187
Ficus lingua	GenBank		AY730099	AY730188
Ficus lingua depauperata	MW06-F86	Mozambique, 06-2006		1
Ficus lingua depauperata	MW06-F88	Mozambique, 06-2006		1
Ficus lingua lingua	GenBank		AY730096	AY730185
Ficus lingua lingua	SA05-F53	RSA, KwaZulu-Natal, Lake Sibaya, 11-2005	1	1
Ficus natalensis graniticola	SA05-F09	RSA, Limpopo, Soutpansberg, Lajuma, 11-2005	1	

Table 3.1: *Continued.*

***Ficus* species**	**Collection Number**	**Collection locality & date**	**ITS**	**ETS**
Ficus natalensis leprieurii	GenBank		AY730100	AY730189
Ficus natalensis natalensis	SA05-F75	RSA, KwaZulu-Natal, Mtunzini, 11-2005	1	1
	ZA06-F14	Zambia, Central Province, T2 between Kapiri Mposhi and Mkushi, 05-2006		1
	SA05-F57	RSA, KwaZulu-Natal, False Bay Park, Listers Point, 11-2005	1	
Ficus petersii	GenBank		AY730101	AY730190
	SA05-F45	RSA, Mpumalanga, Crocriver Mountain Reserve, 11-2005	1	1
	SA05-F43	RSA, Mpumalanga, Louws Creek, 11-2005	1	
Ficus reflexa	GenBank		DQ455650	
Ficus rokko	MW06-F74	Mozambique, Zambezia Province, Mount Mulanje, 05-2006		1
Ficus thonningii	GenBank		AY730102	AY730191
F. sp. samfya	ZA06-F41	Zambia, Northern Province, Mbala, 05-2006		1
Subsection *Crassicostae*				
Ficus elasticoides	GenBank		AY730103	AY730192
Ficus oreodryadum	GenBank		DQ455651	
	GenBank		DQ455652	
Ficus usambarensis	GenBank		DQ455653	DQ455677
Subsection *Cyathistipulae*				
Ficus ardisoides	GenBank		DQ455654	
	GenBank		DQ455655	DQ455678
Ficus barteri	ZA06-F34	Zambia, Northern Province, road between Mporokosa and Mbala, 05-2006		1
Ficus conraui	GenBank		DQ455656	
Ficus cyathistipula	GenBank		DQ455657	DQ455679
Ficus cyathistipula cyathistipula	ZA06-F20	Zambia, Luapula Province, 5km northwest of Chimpembe, Lumangwe Falls, Kalungwishi river, 05-2006		1
Ficus cyathistipuloides	GenBank		DQ455658	
	GenBank			AY063524
Ficus densistipulata	GenBank		DQ455659	DQ455680
Ficus lyrata	GenBank		AY730104	AY730193
Ficus preussi	GenBank		AY730105	AY730194
	GenBank		DQ455660	
Ficus sagittifolia	GenBank		AY730106	AY730195
Ficus scassellatii	GenBank		AY730107	AY730196
Ficus scott-elliotii	GenBank		DQ455661	DQ455681
Ficus tesselata	GenBank		DQ455662	DQ455682
Ficus wildemaniana	GenBank		AY730108	AY730197
Subsection *Galoglychia*				
Ficus lutea	GenBank		AY063564	AY063525
	SA05-F18	RSA, Limpopo, Machado, 11-2005		1
	SA05-F61	RSA, KwaZulu-Natal, Ongoye Forest, 11-2005		1
Ficus saussureana	GenBank		AY730090	AY730179

Table 3.1: *Continued.*

Ficus species	Collection Number	Collection locality & date	ITS	ETS
Subsection *Platyphyllae*				
Ficus abutilifolia	GenBank		AY730091	AY730180
	SA05-F23	RSA, Limpopo, Soutpansberg, R524, 11-2005	1	1
Ficus bussei	MW06-F84	Mozambique, 45 km northeast of Inhaminga, 06-2006		1
	ZA06-F02	Zambia, Lusaka Province, Lusaka, 05-2006		1
Ficus glumosa	GenBank		AY063562	AY063523
	SA05-F02	RSA, Limpopo, Blouberg Nature Reserve, 10-2005		1
	SA05-F19	RSA, Limpopo, Machado, 11-2005		1
Ficus nigropunctata	GenBank		DQ455663	
Ficus platyphylla	GenBank		AY730092	AY730182
	GenBank		DQ455664	
Ficus populifolia	GenBank		AY730093	AY730182
Ficus stuhlmannii	SA05-F55B	RSA, KwaZulu-Natal, False Bay Park, 11-2005		1
	SA05-F56	RSA, KwaZulu-Natal, Hluhluwe region, 11-2005		1
Ficus tettensis	GenBank		DQ455665	DQ455683
	SA05-F29	RSA, Mpumalanga, Blyde River Canyon Nature Reserve, Swadini dam, 11-2005	1	
Ficus trichopoda	GenBank		DQ455666	DQ455684
	SA05-F67	RSA, KwaZulu-Natal, Umlalazi Nature Reserve, 11-2005		1
Outgroup				
Ficus albert-smithii	GenBank		AY730069	AY063157
Ficus americana	GenBank		AY730070	AY730158
Ficus cestrifolia	GenBank		AY730076	AY063164
Ficus citrifolia	GenBank		AY730077	AY063165
Ficus crocata	GenBank		DQ455667	DQ455686
Ficus drupacea	GenBank		AY730066	AY063154
Ficus rubignosa	GenBank		AY063569	AY063530
Ficus schumacherii	GenBank		AY063567	AY063528
Ficus subandina	GenBank		DQ455668	DQ455687
Ficus superba	GenBank		AF165410	AY063149
Ficus trigona	GenBank		DQ455669	DQ455688

The classification of *Ficus* presented in this table is from Berg (1986), Berg & Wiebes (1992) and Burrows & Burrows (2003).

Table 3.2: Host associations and collection details of the agaonid pollinating fig wasps included in this study.

Ficus host	Agaonid	Collection Number	Collection Locality & Date	COI	COII	18S	ITS2	28S
Subsection *Galoglychia*								
F. lutea	*Allotriozoon heterandromorphum*	GenBank					AJ972651	AJ971646
	Allotriozoon heterandromorphum	SA05-F18	RSA, Limpopo, Makhado, 11-2005				1	
	Allotriozoon heterandromorphum	SA05-F61	RSA, KwaZulu-Natal, Ongoye Forest, 11-2005	1		1	1	
F. chlamydocarpa	*Allotriozoon nigeriense*	GenBank					AJ972652	
Subsection *Platyphyllae*								
F. glumosa	*Elisabethiella glumosae*	GenBank		AJ971654			AJ972647	AJ971639
	Elisabethiella glumosae	GenBank		AY014976				
	Elisabethiella glumosae	SA05-F19	RSA, Limpopo, Makhado, 11-2005	1	1	1	1	
	Elisabethiella glumosae	SA06-F97	RSA, KwaZulu-Natal, Port Edward, 05-2006	1	1	1		
F. stuhlmannii	*Alfonsiella binghami*	GenBank		AJ971648				
	Alfonsiella binghami	GenBank					AJ972633	
	Alfonsiella binghami	GenBank						AY616526
	Alfonsiella binghami	SA05-F55B	RSA, KwaZulu-Natal, False Bay Park, 11-2005	1	1	1	1	
	Alfonsiella binghami	SA05-F56	RSA, KwaZulu-Natal, Hluhluwe region, 11-2005					
	Alfonsiella binghami	MW06-F60	Mozambique, Niassa Province, Mandimba, 05-2006	1	1	1		
F. tettensis	*Nigeriella excavata*	GenBank		AJ971655			AJ972654	AJ971638
	Nigeriella excavata	SA05-F04	RSA, Limpopo, Blouberg Nature Reserve, 11-2005	1				
	Nigeriella excavata	SA05-F31	RSA, Mpumalanga, Blyde River Canyon, 11-2005	1				
F. abutilifolia	*Elisabethiella comptoni*	GenBank		AJ971652			AJ972645	
	Nigeriella fusciceps	GenBank					AJ972653	AJ971637
	Elisabethiella comptoni	SA05-F23	RSA, Limpopo, Soutpansberg, R524, 11-2005	1	1	1		
F. vasta	*Elisabethiella socotrensis*	GenBank					AJ972648	AJ971641
F. trichopoda	*Elisabethiella bergi*	GenBank					AJ972643	AJ971642
	Elisabethiella bergi	SA05-F67	RSA, KwaZulu-Natal, Umlalazi, 11-2005				1	
Subsection *Chlamydodorae*								
F. fischeri	*Elisabethiella platyscapa*	ZA06-F13		1	1	1	1	
F. craterostoma	*Alfonsiella pipithiensis*	GenBank		AJ971649			AJ972626	
	Alfonsiella pipithiensis	GenBank					AJ972638	AJ971635
	Alfonsiella pipithiensis	SA05-F59	RSA, KwaZulu-Natal, Ngome Forest, 11-2005				1	
F. lingua depauperata	*Elisabethiella stuckenbergi*	MW06-F86	Mozambique, 06-2006		1	1		
F. lingua depauperata	*Elisabethiella stuckenbergi*	MW06-F88	Mozambique, 06-2006	1	1	1	1	
F. natalensis natalensis	*Elisabethiella socotrensis*	GenBank		AM260706			AJ972650	AJ971640
	Elisabethiella socotrensis	GenBank		AM260707				
	Elisabethiella stuckenbergi	GenBank		AJ971651			AJ972641	AJ971644
	Elisabethiella socotrensis	SA05-F75	RSA, KwaZulu-Natal, Mtunzini, 11-2005					
	Elisabethiella stuckenbergi	SA05-F75	RSA, KwaZulu-Natal, Mtunzini, 11-2005					

Table 3.2: *Continued.*

Ficus host	Agaonid	Collection Number	Collection Locality & Date	COI	COII	18S	ITS2	28S
F. natalensis natalensis	*Alfonsiella binghami*	ZA06-F14	Zambia, Central Province, T2 between Kapiri Mposhi and Mkushi, 05-2006	1				
	Elisabethiella stuckenbergi	ZA06-F14	Zambia, Central Province, T2 between Kapiri Mposhi and Mkushi, 05-2006	1	1	1		
	Alfonsiella longiscapa	GenBank		AY014974				
	Alfonsiella longiscapa	GenBank					AY616525	
	Alfonsiella longiscapa	MW06-F89	Mozambique, 06-2006	1		1	1	
F. natalensis graniticola	*Elisabethiella allotriozoon*	SA05-F08	RSA, Limpopo, Soutpansberg, 11-2005	1		1		
F. burtt-davyi	*Elisabethiella baijnathi*	GenBank		AJ971653			AJ972639	
	Elisabethiella baijnathi	GenBank		AY014975				
	Elisabethiella baijnathi	GenBank						AY616557
	Elisabethiella baijnathi	SA05-F82	RSA, Eastern Cape, Woody Cape Reserve, 11-2005		1	1		
F. ilicina	*Elisabethiella enriquesi*	GenBank					AJ972646	AJ971643
F. petersii	*Alfonsiella binghami*	GenBank		AJ971650			AJ972634	
	Alfonsiella binghami	SA05-F45	RSA, Mapumulanga, Louws Creek, 11-2005	1		1	1	
	Alfonsiella binghami	ZA06-F46	Zambia, Northern Province, 70 km southeast of Isoka, 05-2006	1	1	1		
	Elisabethiella stuckenbergi	ZA06-F46	Zambia, Northern Province, 70 km southeast of Isoka, 05-2006	1	1	1		
F. burkei	*Elisabethiella socotrensis*	GenBank		AM260705			AJ972649	
	Elisabethiella stuckenbergi	GenBank		AM260704			AJ972640	
	Elisabethiella stuckenbergi	SA05-F28	RSA, Limpopo, Abel Erasmus Pass, 11-2005		1			
	Elisabethiella stuckenbergi	SA06-F98	RSA, KwaZulu-Natal, Port Edward, 06-2006	1	1			
F. sp. samfya fig	*Elisabethiella socotrensis*	ZA06-F41	Zambia, Northern Province, Mbala, 05-2006	1	1		1	
	Elisabethiella stuckenbergi	ZA06-F41	Zambia, Northern Province, Mbala, 05-2006	1				
Subsection *Crassicostae*								
F. usambarensis	*Elisabethiella* sp.	GenBank					AJ972644	
	Elisabethiella sp.	ZA06-F32	Zambia, Northern Province, 70 km southwest of Mporokoso, 05-2006	1	1		1	
F. elasticoides	*Elisabethiella articulata*	GenBank					AJ972642	
F. louissi	*Paragaon josephi*	GenBank					AJ972658	
Subsection *Cyathistipulae*								
F. cyathistipula cyathistipula	*Agaon fasciatum*	MW06-F67	Mozambique, Zambezia Province, Mount Namuli, 05-2006		1	1		
F. cyathistipula cyathistipula	*Agaon* sp.	ZA06-F20	Zambia, Luapula Province, 5km northwest of Chimpembe, Lumangwe Falls, Kalungwishi river, 05-2006		1			
F. scott-elliotti	*Agaon* sp.	GenBank					AJ972659	AJ971647
F. cyathistipula pringsheimiana	*Agaon kiellandi*	UG05-F02	Uganda, Kibale National Park, 08-2005			1		
F. tesselata	*Agaon taiense*	GenBank						AY616524

Table 3.2: *Continued.*

Ficus host	Agaonid	Collection Number	Collection Locality & Date	COI	COII	18S	ITS2	28S
Subsection *Caulocarpae*								
F. ottoniifolia lucanda	*Courtella scobinifera*	UG05-F01	Uganda, Kibale National Park, 08-2005	1	1	1		
F. polita polita	*Courtella bekeliensis*	GenBank		AY014977				AY616550
	Courtella bekeliensis	SA06-F95	RSA, KwaZulu-Natal, Lake Sibaya, 11-2005	1	1	1		
F. bizanae	*Courtella* sp.	GenBank					AJ972657	AJ971636
	Courtella sp.	SA05-F69	RSA, KwaZulu-Natal, Ongoye Forest, 11-2005		1			
	Courtella sp.	SA05-F81	RSA, Port St Johns, 11-2005		1			
F. modesta	*Courtella* sp.	MW06-F69	Mozambique, Zambezia Province, Mount Namuli, 05-2006	1	1	1	1	
	Courtella sp.	MW06-F70	Mozambique, Zambezia Province, Mount Namuli, 05-2006	1	1	1		
F. chrindensis	*Courtella malawi*	UG05-F03	Uganda, Kibale National Park, 08-2005			1		
F. sansibarica sansibarica	*Courtella armata*	GenBank		AY014978				
	Courtella armata	GenBank					AJ972655	
	Courtella armata	GenBank						AY616549
	Courtella armata	SA05-F27	RSA, Limpopo, Legalameetse, 11-2005			1		
	Courtella armata	SA05-F40	RSA, Mpumalanga, Krododilpoort, 11-2005		1	1		
F. sansibarica macrosperma	*Courtella armata*	ZA06-F18	Zambia, Luapula Province, 20 km west of Kawambwa, 05-2006		1	1		
F. bubu	*Courtella michaloudi*	GenBank					AJ972656	AY616551
F. ovata	*Courtella hamifera modesta*	ZA06-F17	Zambia, Luapula Province, Road north of Mansa along Luapula River	1	1	1		
	Courtella hamifera modesta	ZA06-F19A	Zambia, Luapula Province, Kawambwa, 05-2006	1		1	1	
Outgroup taxa								
	Tetrapus americanus	GenBank		AY014971	AY968014			
	Tetrapus costaricanus	GenBank		AY014973	AY968016			
	Pleistodontes imperialis	GenBank		AJ298405				AJ298405
	Pleistodontes froggatti	GenBank		AJ275085				AJ275085

Table 3.3: Sequences of primers used in the amplification of *Ficus* & agaonid DNA.

Primer	Sequence	Region	Reference
ITS2F	ACGAATTCATGGTCCGGTGAAGTGTTCG	ITS	Sun *et al.* 1994
ITS2R	TAGAATTCCCCGGTTCGCTCGCCGTTAC	ITS	Sun *et al.* 1994
Hel-1	GCTCTTTTGCGCAACAACT	ETS	Baldwin & Markos 1998
18S-ETS	ACTTACACATGCATGGCTTAATCT	ETS	Baldwin & Markos 1998
TL-2-N 3014	TCCATTGCACTTATTCTGCCATATTA	COI	Simon *et al.* 1994
C1-J-2183	CAACATTTATTTTGATTTTTTGG	COI	Simon *et al.* 1994
CO2SCAF	GCAGATTAGTGCAATGAATTTAA	COII	Villalba *et al.* 2002
CO2BSCAR	GCTCCACAAATTTCTGAGCATTG	COII	Villalba *et al.* 2002
ITSF	ATTCCGCACCACGCCTGGCTGA	ITS2	Campbell *et al.* 1993, Lopez-Vaamonde *et al.* 2001
ITSR	CGCCTGATCTGAGGTCGTGA	ITS2	Campbell *et al.* 1993, Lopez-Vaamonde *et al.* 2001
18SH-17F	AAATTACCCACTCCCGGCA	18S	Heraty *et al.* 2004
18SH-35R	TGGTGAGGTTTCCCGTGTT	18S	Heraty *et al.* 2004

Table 3.4: *Ficus* PCR conditions for the amplification of ITS & ETS gene regions.

	ITS		ETS	
Initial Denaturation	94 °C	2 min	94 °C	2 min
Denaturation phase	94 °C	1 min	92 °C	1 min
Annealing phase	50 °C	1 min	50 °C	1 min
Extension phase	72 °C	1 min	72 °C	1 min
No of cycles	32		32	
Final Extension	72 °C	2 min	72 °C	2 min

Table 3.5: Agaonid PCR conditions for the amplification of COI, COII, ITS2 and 18S gene regions.

	COI & COII		ITS2		18S	
Initial Denaturation	94 °C	3 min	94 °C	3 min	94 °C	3 min
Denaturation phase	92 °C	30 s	92 °C	30 s	92 °C	30 s
Annealing phase	48 °C	1 min 30 s	48 °C	1 min 30 s	52 °C	45 s
Extension phase	72 °C	2 min 30 s	72 °C	2 min 30 s	72 °C	1 min
No of cycles	35		35		35	
Final Extension	72 °C	5 min	72 °C	5 min	72 °C	10 min

Table 3.6: Nucleotide substitution models chosen for each agaonid data partition; models were selected using Modeltest 3.06 (Posada & Crandall 1998).

Gene Region	Model
COII	GTR + I + G
COII	K81uf + G
ITS2	GTR + I + G
18S	TVMef + G

Table 3.7: COI sequence divergence (p-distances) within and between the main clades/lineages of the genus *Elisabethiella* (Agaonidae). No values were calculated (n/c) where only one individual represented a lineage.

	A	B	C	D	E	F	G	H	I	J	K
A	**n/c**										
B	0.013	**0.002**									
C	0.027	0.029	**0.002**								
D	0.054	0.062	0.057	**n/c**							
E	0.065	0.067	0.061	0.065	**0.017**						
F	0.041	0.054	0.052	0.067	0.070	**0.002**					
G	0.068	0.068	0.067	0.079	0.072	0.050	**0.041**				
H	0.060	0.062	0.060	0.071	0.073	0.073	0.077	**0.000**			
I	0.038	0.043	0.057	0.072	0.078	0.056	0.076	0.068	**0.005**		
J	0.076	0.078	0.058	0.087	0.078	0.078	0.088	0.082	0.076	**n/c**	
K	0.060	0.064	0.060	0.068	0.076	0.073	0.068	0.074	0.068	0.065	**n/c**

Table 3.8: Results of the cophylogentic analyses using Tree-based (TreeMap) and Distance-based (ParaFit) methods, performed on the three phylogenies to test the null hypothesis that they have evolved independently. Probabilities ≤0.05 were considered significant, and are indicated in bold.

"Host" (no. species)	**"Parasite" (no. species)**	**TreeMap 1.0**		**TreeMap 2.02β**			**ParaFit (branch lengths equal)**		**ParaFit (true patristic distances)**	
		No. cospeciation events	***P***	**No. opt. solutions**	**Codiversification scenario**	***P***	**No. significant links**	***P***	**No. significant links**	***P***
Ficus (13)	Sycoecine (20)	11	**0.007**	21	18≤C≤20,18≤D≤20, 17≤L≤1, S≤	**0.01**	5	**0.03**	2	0.21
Ficus (16)	Agaonid (20)	5	0.78	14	10≤C≤4,24≤D≤28, 18≤L≤1, S≤	0.38	13	**0.001**	16	**0.002**
Sycoecine (27)	Agaonid (18)	1	0.90	_	_		0	0.61	0	0.89
Agaonid (18)	Sycoecine (27)	8	0.21	_	_		0	0.56	0	0.93

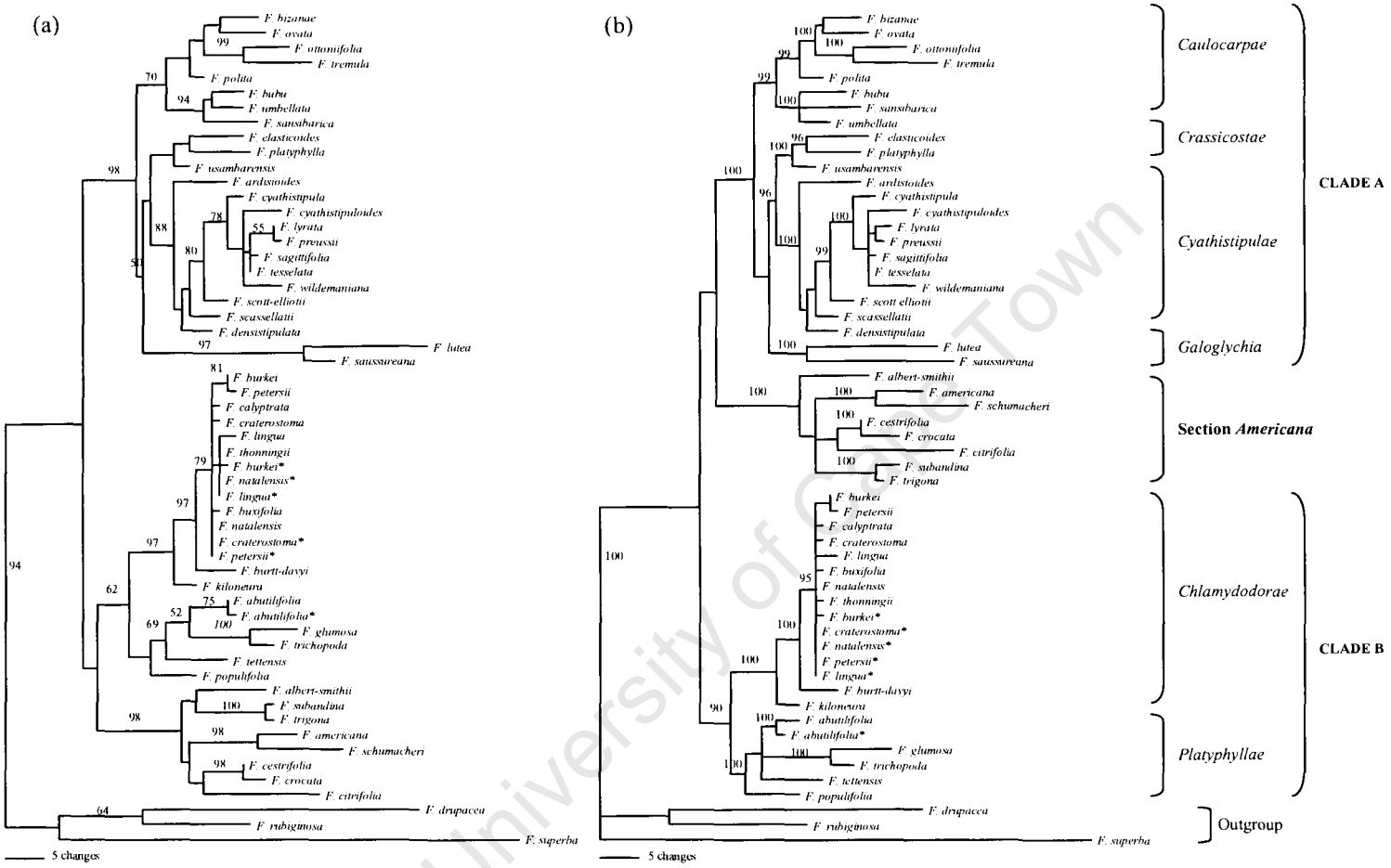

Figure 3.1: Maximum parsimony tree (a) and Bayesian consensus tree (b) from the combined analyses of the external and internal transcribed spacers (ITS and ETS) of *Ficus* section *Galoglychia* and *Americana*. The subsections of the *Galoglychia*, and the taxa belonging to *Ficus* section *Americana* are labelled. Taxa marked with an asterisk are new sequences that were added to the analyses performed by Rønsted *et al.* (2007). Bootstrap support values ≥50% and Bayesian posterior probabilities ≥95% are indicated on the parsimony phylogram and the Bayesian consensus tree respectively.

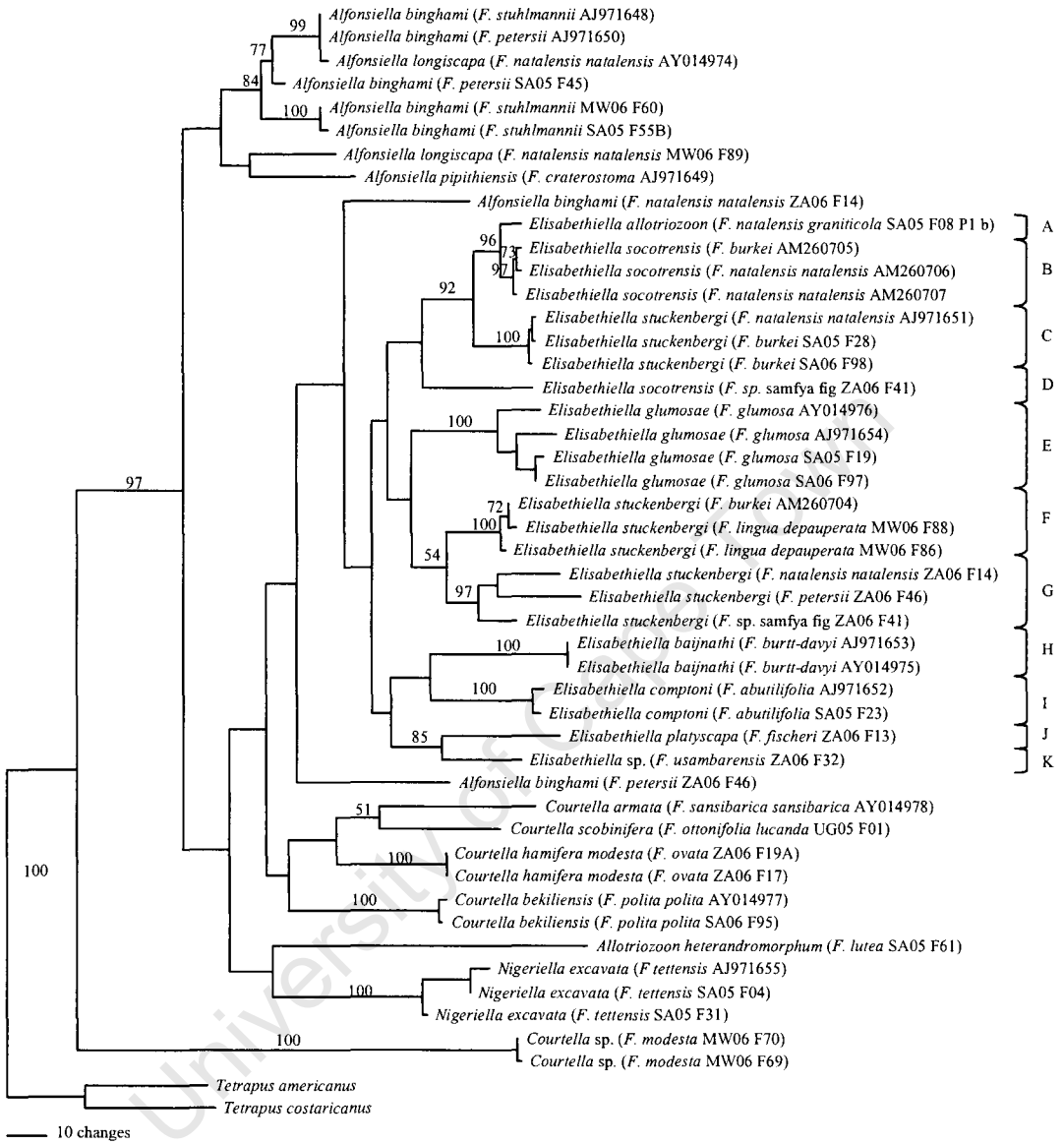

Figure 3.2: One of 49 most parsimonious cladograms resulting from parsimony analysis of the COI gene region of the agaonid pollinating fig wasps of *Ficus* section *Galoglychia*. Bootstrap support values (≥50%) are indicated above the nodes. Species names are followed by host association and collection number or GenBank accession (Table 3.2). The major clades/lineages of the genus *Elisabethiella* are labelled for the analysis of sequence divergence (see text).

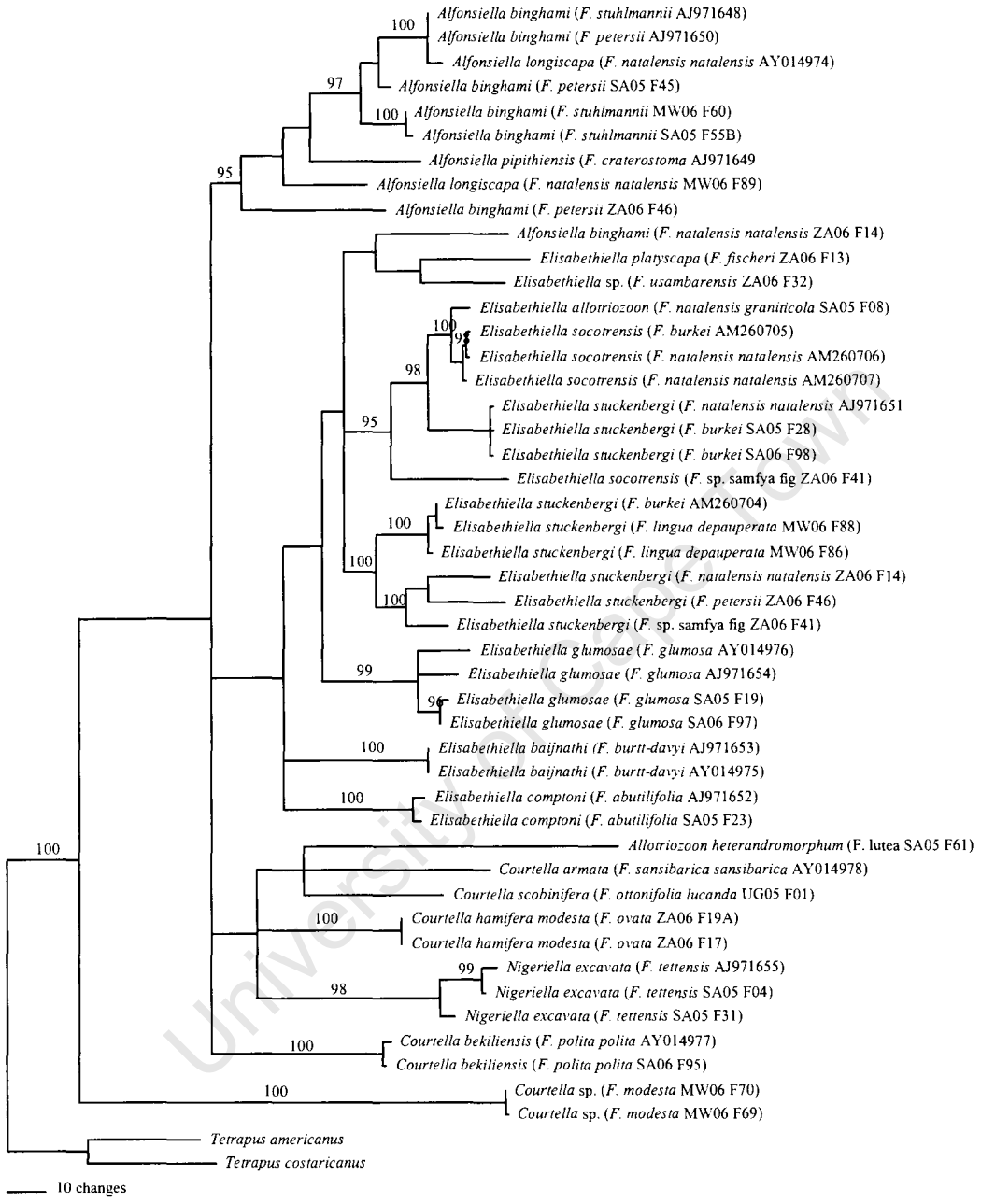

Figure 3.3: Bayesian consensus tree from analysis of the COI gene region of the agaonid pollinating fig wasps of *Ficus* section *Galoglychia*. Bayesian posterior probabilities (≥95%) are indicated above the nodes. Species names are followed by host association and collection number or GenBank accession (Table 3.2).

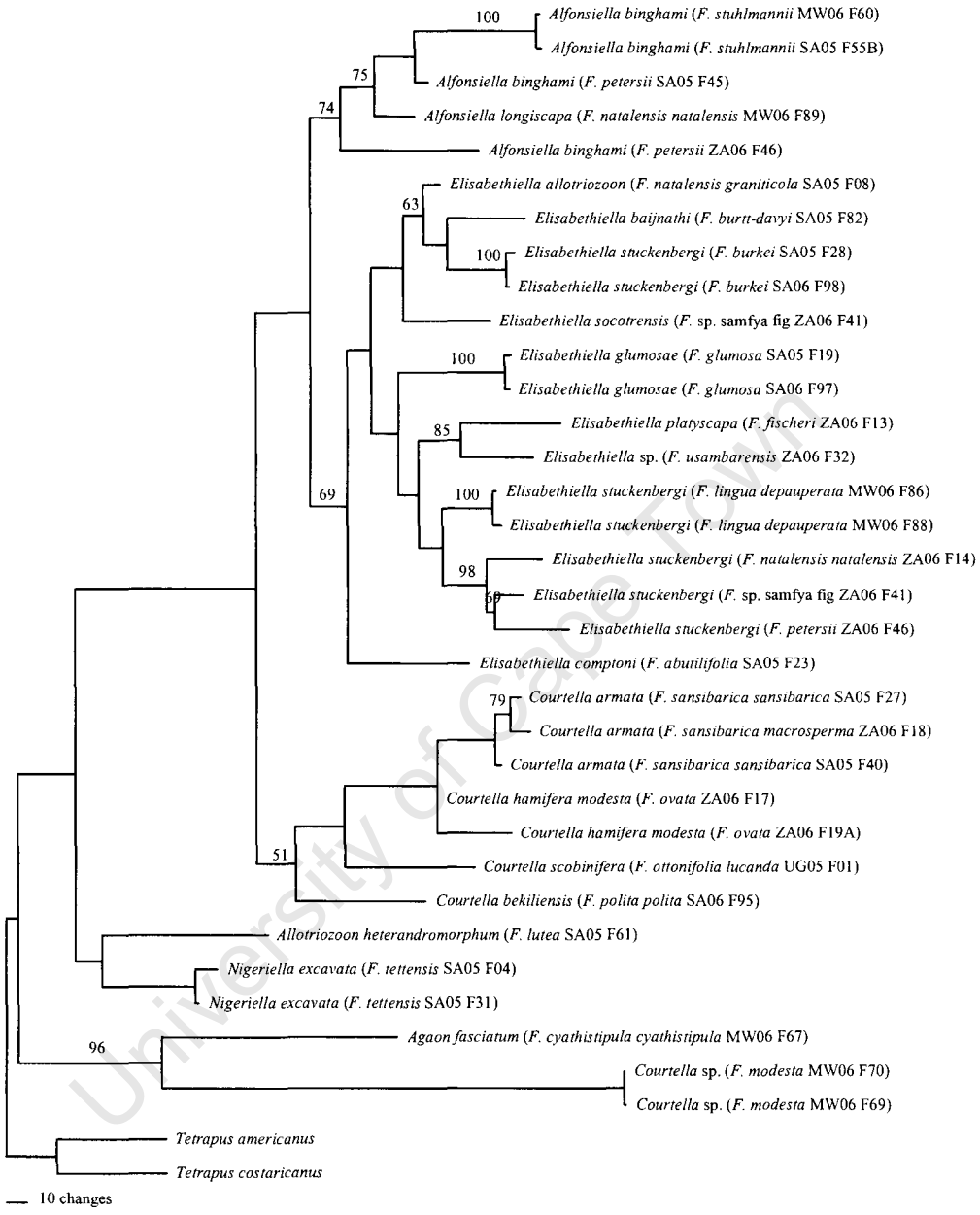

Figure 3.4: One of eight most parsimonious cladograms resulting from the combined parsimony analysis of the COI, COII and 18S gene regions of the agaonid pollinating fig wasps of *Ficus* section *Galoglychia*. Bootstrap support values (≥50%) are indicated above the nodes. Species names are followed by host association and collection number or GenBank accession (Table 3.2).

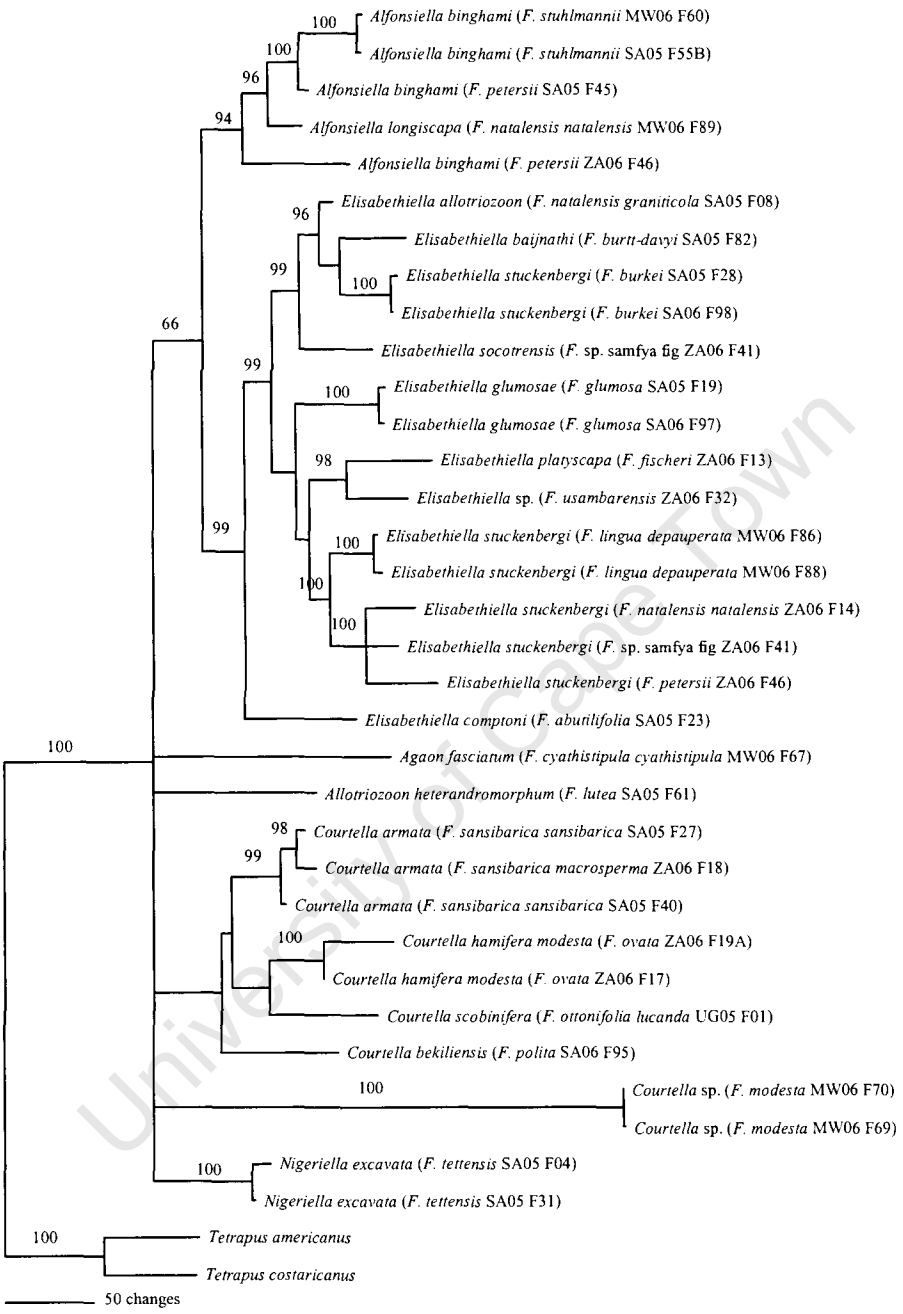

Figure 3.5: Bayesian consensus tree from the combined analysis of the COI, COII and 18S gene regions of the agaonid pollinating fig wasps of *Ficus* section *Galoglychia*. Bayesian posterior probabilities (≥95%) are indicated above the nodes. Species names are followed by host association and collection number or GenBank accession (Table 3.2).

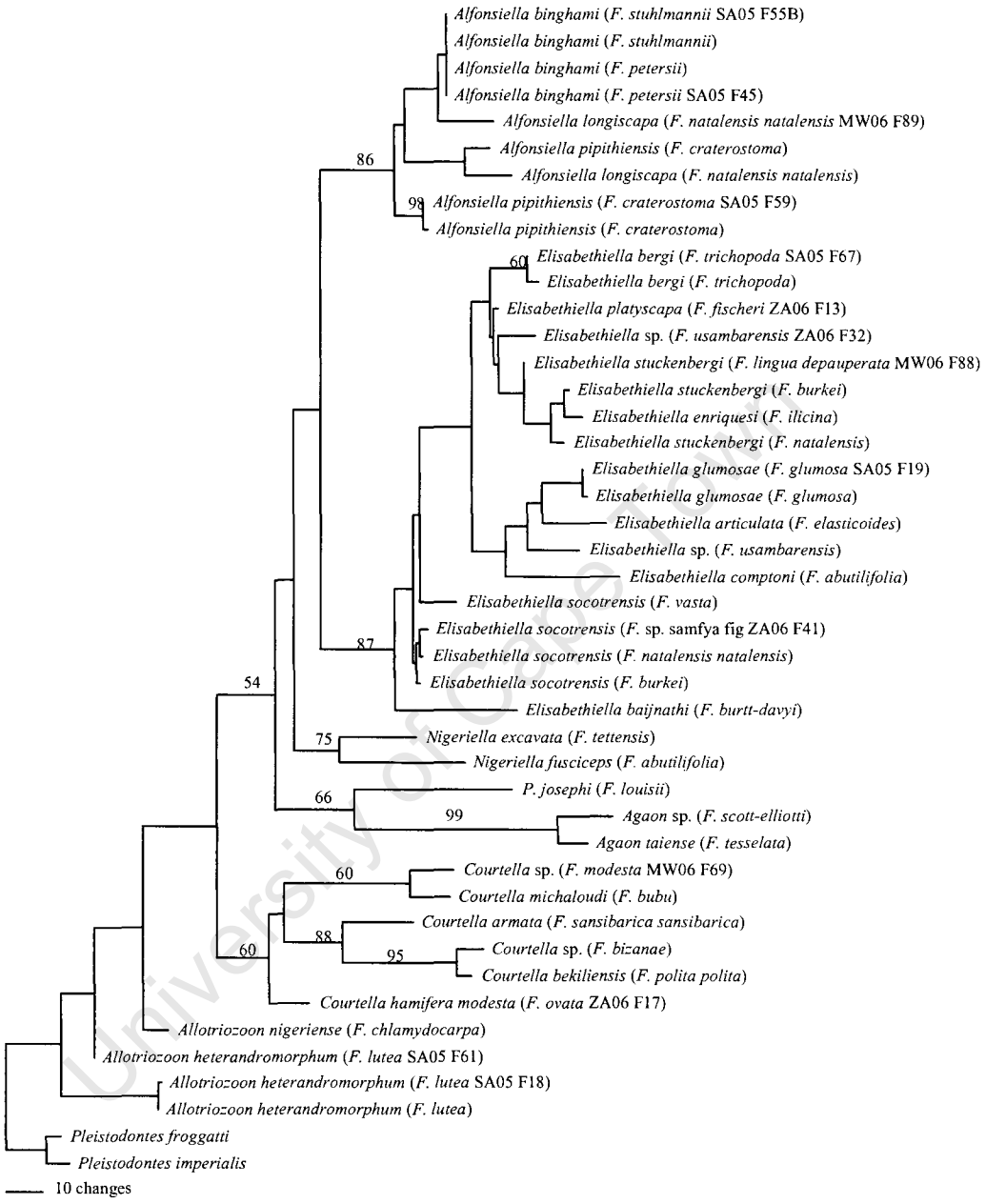

Figure 3.6: One of 4034 most parsimonious cladograms resulting from the combined parsimony analysis of the ITS2 and 28S gene regions of the agaonid pollinating fig wasps of *Ficus* section *Galoglychia*. Bootstrap support values (≥50%) are indicated above the nodes. Species names are followed by host association and collection number or GenBank accession (Table 3.2).

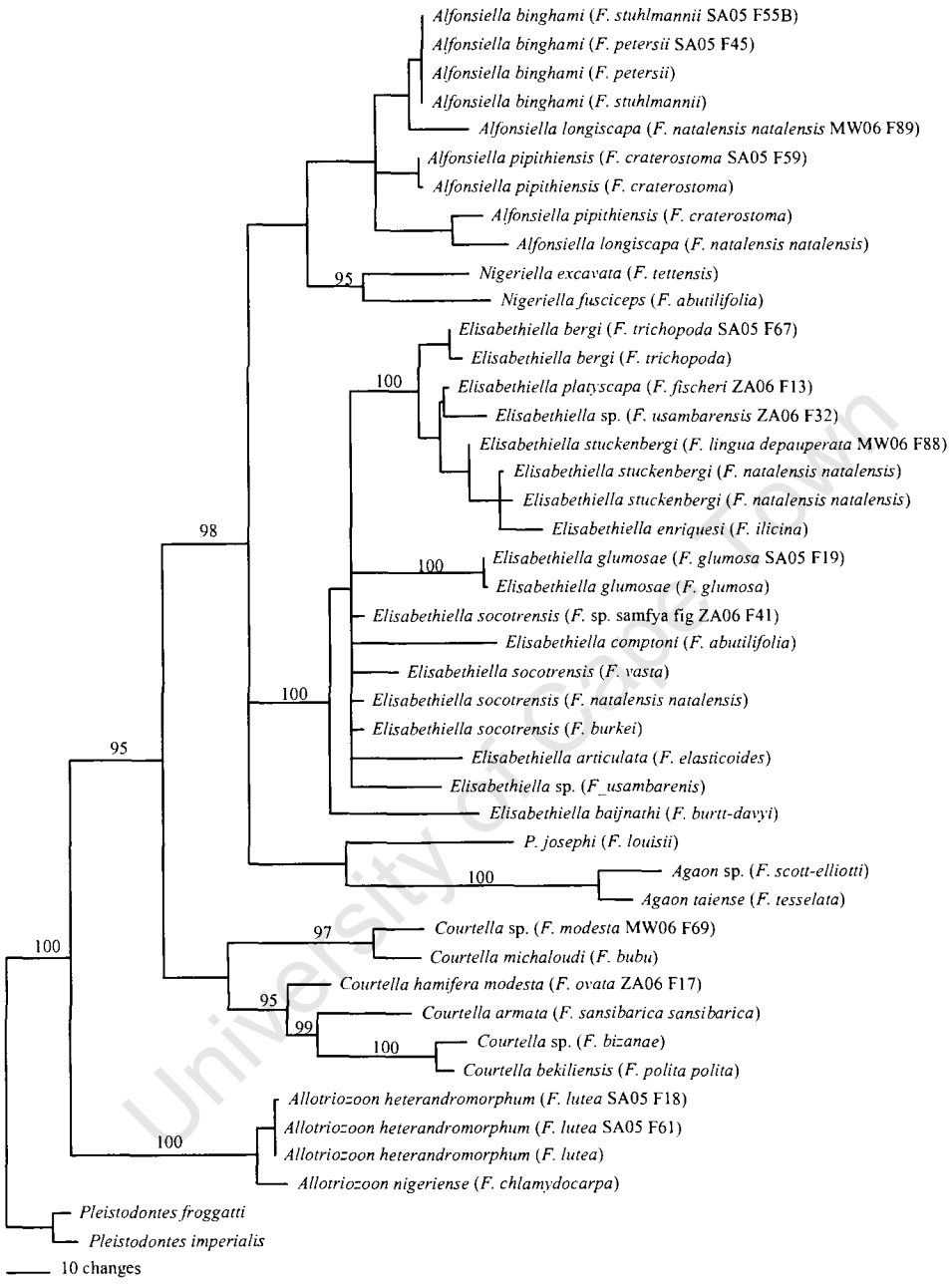

Figure 3.7: Bayesian consensus tree from the combined analysis of the ITS2 and 28S gene regions of the agaonid pollinating fig wasps of *Ficus* section *Galoglychia*. Bayesian posterior probabilities (≥95%) are indicated above the nodes. Species names are followed by host association and collection number or GenBank accession (Table 3.2).

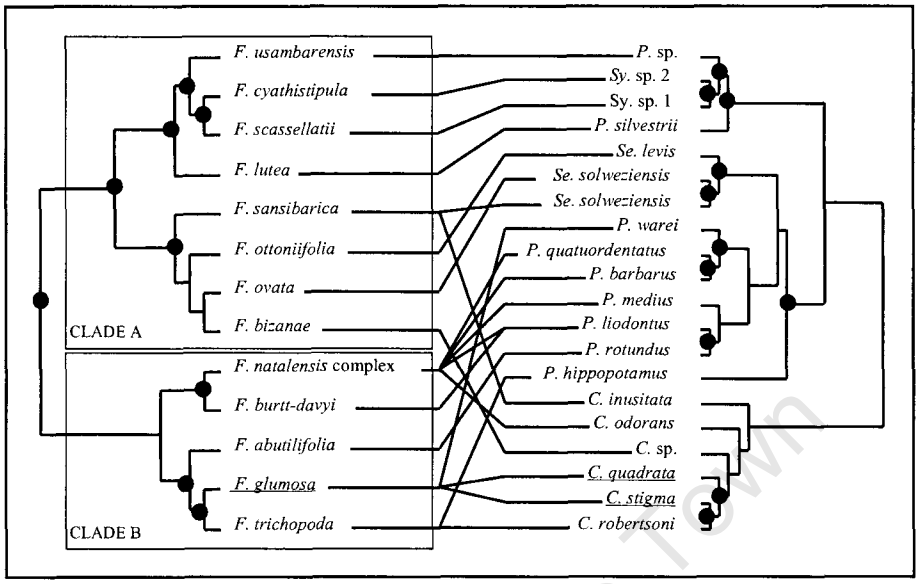

Figure 3.8: Tanglegram comparing the sycoecinae and their *Ficus* hosts. Lines connecting taxa indicate fig wasp-fig host associations. Black dots at nodes indicate instances of perfect cophylogeny identified in the TreeMap 1.0 reconciliation analysis. Underlined species represent significant links between taxa in ParaFit, determined with true patristic distances ($P \leq 0.05$). Clades A and B correspond to the two major *Ficus* clades identified in Figure 3.1. Abbreviations used in the figure: *F.* = *Ficus*, *P.* = *Philocaenus*, *Sy.* = *Sycoecus*, *Se.* = *Seres*, *C.* = *Crossogaster*.

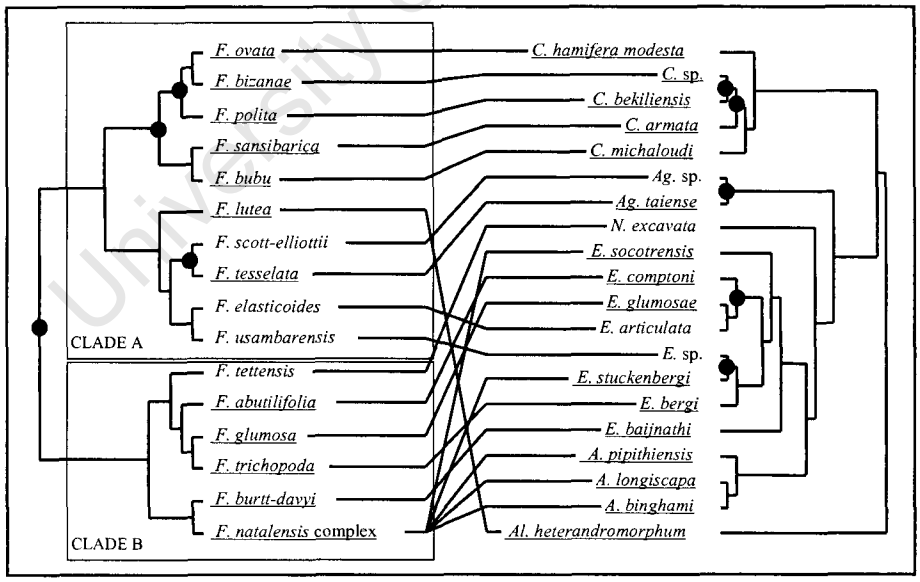

Figure 3.9: Tanglegram comparing the pollinators of *Ficus* section *Galoglychia* and their *Ficus* hosts. Lines connecting taxa indicate fig wasp-fig host associations. Black dots at nodes indicate instances of perfect cophylogeny determined in the TreeMap 1.0 reconciliation analysis. Underlined species represent significant links between taxa in ParaFit determined with ture patristic distances ($P \leq 0.05$). Clades A and B correspond to the two major *Ficus* clades identified in Figure 3.1. Abbreviations used in the figure: *F.* = *Ficus*, *C.* = *Courtella*, *Ag.* = *Agaon*, *N.* = *Nigeriella*, *E.* = *Elisabethiella*, *A.* = *Alfonsiella*, *Al.* = *Allotriozoon*.

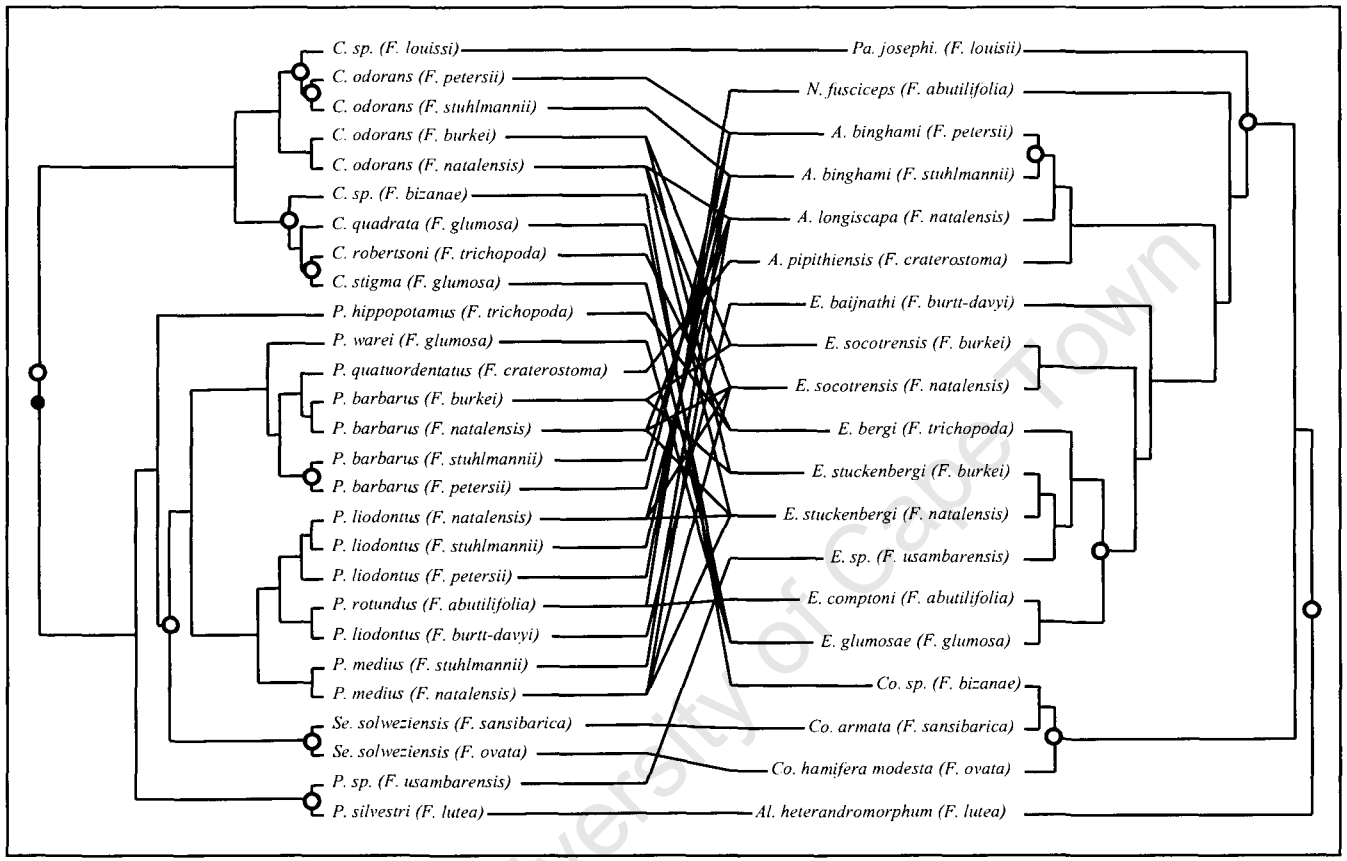

Figure 3.10: Tanglegram comparing the Sycoecinae and the pollinators of *Ficus* section *Galoglychia*. The lines connecting the taxa indicate shared hosts. Dots at nodes indicate instances of perfect cophylogeny identified in the TreeMap 1.0 analyses; black dots represent codivergence events in the analysis where the sycoecine phylogeny was considered as the "host" lineage, while black rings represent codivergence events when the agaonid phylogeny was considered as the "host" lineage. No significant links were found in the ParaFit analyses. Abbreviations used in the figure: *C.* = *Crossogaster*, *P.* = *Philocaenus*, *Se.* = *Seres*, *Pa.* = *Paragaon*, *N.* = *Nigeriella*, *A.* = *Alfonsiella*, *E.* = *Elisabethiella*, *Co.* = *Courtella*, *Al.* = *Allotriozoon*.

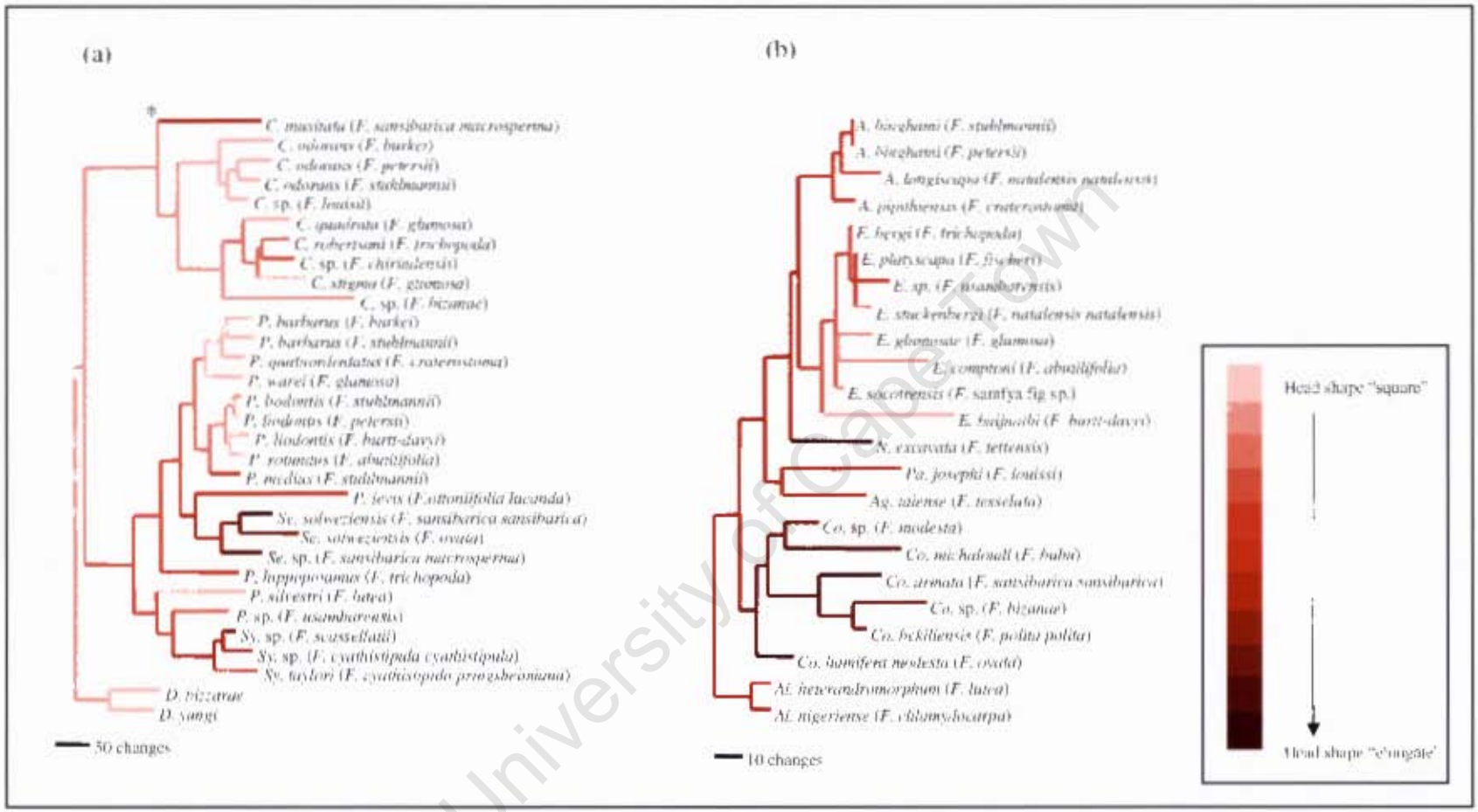

Figure 3.11: Ancestral character reconstructions of head shape using parsimony method implemented by MESQUITE v 2.01. Head shape ratios mapped onto the (a) sycoecine Bayesian consensus tree from the combined analysis of Cytb, COI, COII and ITS2 and the (b) parsimony tree from the combined analysis of the 28S and ITS2 gene regions of the agaonid pollinating fig wasps of *Ficus* section *Galoglychia*. Species names are followed by host association (Table 3.2). Abbreviations used in the figure: *C.* = *Crossogaster*, *P.* = *Philocaenus*, *Se.* = *Seres*, *Sy.* = *Sycoecus*, *D.* = *Diaziella*, *A.* = *Alfonsiella*, *E.* = *Elisabethiella*, *N.* = *Nigeriella*, *Pa.* = *Paragaon*, *Ag.* = *Agaon*, *Co.* = *Courtella*, *Al.* = *Allotriozoon*.

* The abrupt change from a square head shape to a more elongate head shape may indicate a potential host-switching event (see text for further details).

CHAPTER 4: Fig (subsect. *Chalmydodorae*, sect. *Galoglychia*, subg. *Urostigma*, *Ficus*, Moraceae) divergence linked to the emergence of African savannah

4.1 Abstract

The molecular phylogenetic analysis of *Ficus* section *Galoglychia* (Chapter 3) revealed a distinct lack of resolution among a number of species within subsection *Chlamydodorae*. The clade, namely the *F. natalensis* sp-complex, that contained the polytomy was well supported but analysis of the ITS and ETS sequence data did not recover relationships at the species level. Divergence times within the phylogeny of *Ficus* section *Galoglychia* (Chapter 3) were dated using Bayesian methods using three calibration points obtained from a dated phylogeny of *Ficus*. The node of the clade containing the *F. natalensis* sp-complex is estimated at around 6 million years ago (MYA; 95% highest posterior density (HPD): 2.96–9.76). Thus, the origin of this complex may be linked to the emergence of the savannah biome since the Miocene, around 8 MYA, in response to changes in African climate or shifts in climate variability. The presence of morphological diversity in the *F. natalensis* sp-complex, but lack of molecular variation, was accounted for and explained through speciation hypotheses pertaining to *Ficus*.

4.2 Introduction

The genus *Ficus* (Moraceae) is comprised of ca. 750 species and is one of the largest of the angiosperm genera (Frodin 2004). Figs and their pollinating fig wasps (Agaonidae, Chalcidoidea, Hymenoptera) present a specialized case of an obligate pollination mutualism (Hill 1967, Janzen 1979, Cook & Rasplus 2003). With few exceptions, neither organism can complete its life-cycle without the other. Each species of fig tree was traditionally thought to be pollinated by its own species of fig wasp, a relationship that was regarded as the one-to-one ratio of host-specificity between figs and pollinating fig wasps (Ramírez 1970, Cook & Rasplus 2003). On a broad scale, *Ficus* sections or subsections are usually pollinated by a single fig wasp genus (Wiebes 1979, Berg 1989,

Berg & Wiebes 1992); independent estimates for 10 pairs of fig and pollinator lineages suggest highly significant temporal congruence and a coevolutionary history of between 60 and 100 million years (Rønsted *et al.* 2005).

Nevertheless, recent taxonomical and molecular studies challenge the widely accepted hypothesis of strict-sense coevolution between figs and pollinating fig wasps (Chapter 3, Rasplus 1996, Kerdelhué *et al.* 1999, Lopez-Vaamonde *et al.* 2001, Cook & Rasplus 2003, Molbo *et al.* 2003; Jackson 2004, Machado *et al.* 2005, Haine *et al.* 2006, Erasmus *et al.* 2007, Marussich & Machado 2007, Jousselin *et al.* 2008). Separate studies, including cophylogenetic analyses, conclude that host specificity is no longer as ubiquitous as previously considered (Chapter 3, Compton & van Noort 1992, Erasmus *et al.* 2007, Marussich & Machado 2007, Jousselin *et al.* 2008). There are now a large number of documented exceptions to the one-to-one rule. For instance, the association of more than one pollinating fig wasp per *Ficus* host, often from more than one genus, as well as one pollinating fig wasp associated with more than one *Ficus* species, has been extensively documented both in Old World and Neotropical taxa (Compton *et al.* 1991, Rasplus 1996, Keldelhue *et al.* 1999, Molbo *et al.* 2003, Erasmus *et al.* 2007, Marussich & Machado 2007). In short, cophylogenetic analyses have revealed that while figs and fig wasps do codiversify, strict-sense codivergence is not pervasive (Chapter 3).

The Afrotropical *Ficus* section *Galoglychia* belongs to the monoecious *Ficus* subgenus *Urostigma* and comprises a total of 78 species (Berg & Wiebes 1992, Burrows & Burrows 2003, van Noort & Rasplus 2008). Molecular analysis has shown that the six morphologically delimited subsections of *Galoglychia* are largely monophyletic and fall into two major clades (Chapter 3, Rønsted *et al.* 2007). The subsections *Caulocarpae*, *Cyathistipulae*, *Galoglychia*, *Crassicostae* form the first major clade (Clade A) and species distributions centre in west and central Africa (Figure 3.1). Clade B is comprised of subsections *Platyphyllae* and *Chlamydodorae*. Distributions of species within this clade are concentrated in eastern Africa extending to Madagascar and the Mascarene Islands (Figure 3.1; Berg & Wiebes 1992).

Berg (1986) and Berg & Wiebes (1992) defined subsection *Chlamydodorae* to consist of 13 savannah bushveld and forest fig species, and species distributions centre in eastern Africa. Within subsection *Chlamydodorae* a group of species have, over various taxonomic revisions, either been lumped into complexes or split into separate entities. Berg recognized *F. natalensis* and *F. craterostoma* as separate species out of an initial species complex regarded as the *F. natalensis* complex (Berg 1989, 1990, Berg & Wiebes 1992, Burrows & Burrows 2003). The remaining species were grouped into the *F. thonningii*-complex by Berg (Berg & Wiebes 1992, van Greuning 1990) in order to override a number of "forms" that displayed confusing morphological variation. The complex included the species *F. burkei, F. petersii, F. rokko, F. psilopoga, F. persifolia* and numerous synonynomous entities (Berg & Wiebes 1992). Berg (1989) asserted that the group was either a species complex or a complex currently undergoing speciation. Burrows & Burrows (2003) opposed this grouping and argued that variations in distribution, pollination and ecology are likely to reveal distinct entities within the complex and resurrected a number of species previously synonymised under *F. thonningii*. For instance, Burrows and Burrows (2003) separate fig species *F. petersii* and *F. burkei* by their leaves; *F. petersii* has "relatively narrow, grey-green leaves borne on long, somewhat pendulous petioles", and propose that, although mostly occupying allopatric and sympatric distributions, where the species ranges overlap the distinction between the two species is obvious. While they recognize *F. petersii* and *F. burkei* as separate species, they nevertheless acknowledged that these two species, and others, are most probably recently evolved lineages and suggest that each entity may not yet be a reproductively isolated species. They concede that in overlapping distribution ranges, hybrid taxa may be common (Burrows & Burrows 2003, Rønsted *et al.* 2007).

The molecular phylogenetic analysis of the *Ficus* section *Galoglychia* (Chapter 3; Figure 3.1), determined *de novo* and constructed using the external and internal transcribed spacers (ITS & ETS) of the eukaryotic ribosomal DNA (rDNA) transcriptional unit, yielded tree topologies generally consistent with the preceding study (Rønsted *et al.* 2007). The inclusion of the six taxa, five of which were placed in *Ficus* subsection *Chlamydodorae*, revealed a distinct lack of resolution among a number of species within

the subsection *Chlamydodorae* (Figure 3.1). The clade that contained the polytomy was well supported (79% BS, 95% PP; Figure 3.1), but analysis of the ITS and ETS sequence data did not recover relationships at the species level within the clade. *Ficus lingua*, *F. natalensis natalensis*, *F. craterostoma*, *F. buxifolia*, *F. calyptrata*, *F. burkei, F. petersii* and *F. thonningii* fall within an unresolved polytomy. In addition, duplicate taxa did not group as sister taxa (Figure 3.1). For example, the two *F. burkei* and two *F. craterostoma* samples embedded within the polytomy were not each other's closest relative.

The *Ficus* species that are grouped within this clade, from now on referred to as the *F. natalensis* sp-complex, host numerous pollinating wasp species that are reported to be routinely and successfully shared (van Noort & Rasplus 2008). Based on subjective scrutiny of records of host associations, these fig-fig wasp associations appear to be less specific than host-pollinator relationships in the remainder of *Ficus* section *Galoglychia*. For example, within *Ficus* subsection *Chlamydodarae*, *Elisabethiella stuckenbergi* (Agaonidae, Chalcidoidea, Hymenoptera) has been documented to be associated with *F. burkei, F. natalenis natalenis*, *F. natalenis graniticola*, *F. lingua depauperata* and *F. psilopoga*. *Elisabethiella socotrensis* has been associated with *F. natalenis natalenis*, *F. natalenis graniticola, F. burkei* and *F. stuhlmannii*. In addition, *Alfonsiella binghami* has been associated with *F. petersii, F. burkei*, *F. natalensis natalensis* and *F. stuhlmanni.* While there is potential for these widespread fig wasp species to harbour cryptic species, evidence suggests that geographical genetic divergence, rather than divergence based on host association is to be expected (Chapter 3).

Many studies of African taxonomic groups have shown congruence between climate change and diversification events (e.g. deMenocal 1995, Linder 2003, Tolley *et al.* 2008), in particular, the expansion of the savannah biome has been linked with paleoclimatic changes as a result of global cooling events (deMenocal 2004, Beerling & Osborne 2006). Paleobotanical pollen records and carbon isotopes from west and east Africa date the earliest record of the savannah biome to the Middle Miocene, and reveal that grass-dominated habitats were widespread by the Late Miocene around 8 million years ago (Jacobs 2004, Beerling & Osborne 2006). Such changes could also have affected the

current biogeographic patterns of figs and could have influenced divergence events within the phylogeny.

The objective of this study was to obtain date estimates for divergence events within the molecular phylogeny of *Ficus* section *Galoglychia*. Divergence times, correlated to paleoenvironmental changes, may assist in providing insight into the mechanisms that support *Ficus* diversification. I hypothesized that the emergence of the savannah biome since the Miocene in response to change in African climate or shifts in climate variability is linked to the origin of the clade containing the *F. natalensis* sp-complex within subsection *Chlamydodorae*.

The nodes of the phylogeny of *Ficus* section *Galoglychia* (Chapter 3) were dated using Bayesian methods. Calibration points were obtained from Rønsted *et al.* (2005); a phylogenetic tree of *Ficus* dated using both non-parametric smoothing and penalized likelihood methods to account for deviations from a molecular clock. Rønsted *et al.* (2005) use fossilized *Ficus* achenes, determined to be 60 million years old, to constrain the minimum age of the earliest *Ficus*; the published ultrametric tree contains 146 taxa of *Ficus*; to date, it is the largest calibrated phylogeny of the genus *Ficus* (Rønsted *et al.* 2005).

4.3 Methods

Molecular sequence data of *Ficus* section *Galoglychia* were generated and combined with data obtained from Rønsted *et al.* (2007; Chapter 3, Table 3.1). Fig leaf material was collected from fig trees in southern and central Africa. Fig leaves were dried in silica to absorb moisture and to preserve the integrity of the DNA. The internal transcribed spacer (ITS) and external transcribed spacer (ETS) of the eukaryotic ribosomal DNA (rDNA) transcriptional unit were targeted for amplification (see Chapter 3 for DNA extraction, PCR amplification, sequencing and sequence alignment protocols).

Following the recent phylogeny of *Ficus* section *Galoglychia* (Rønsted *et al.* 2007), eight species of *Ficus* from section *Americana,* suggested to be paraphyletic with respect to section *Galoglychia,* were incorporated into the *Ficus* dataset. Two taxa from two of the three remaining sections of the subgenus *Urostigma* were designated as the outgroup: *F. drupacea* (*Ficus* section *Conosycea)* and *F. rubignosa* (*Ficus* section *Malvanthera*).

Time to most common recent ancestor (tMRCA) of various nodes were estimated using a relaxed phylogenetic approach implemented in BEAST v1.4.2 (Drummond & Rambaut 2008). This method of phylogenetic dating was developed by Drummond *et al.* (2006), and employs a relaxed molecular clock assumption in phylogenetic reconstruction, inferring rates and divergence times of nodes simultaneously with the topology estimation.

Three calibration dates were obtained from the dated phylogeny of *Ficus*, estimated by means of non-parametric rate smoothing (NPRS) and penalized-likelihood (PL) methods (Rønsted *et al.* 2005). These calibration points were applied as priors and were specified as normal distributions, with the standard errors of Rønsted *et al.* (2005) used to estimate the 95% confidence intervals. All three calibration points were employed simultaneously to constrain the age of nodes one to three (as numbered in Figure 4.1). Node one, the tMRCA of the clade containing *Galoglychia* and *Americana* was set to 39.47 million years ago (MYA) ± 2.68 million years (MY; age ± standard error), node two, the tMRCA of the *Americana* was set to 30.67 MYA ± 2.98 MY and, node three, the tMRCA for the outgroup was set to 44.08 MYA ± 2.39 MY (Rønsted *et al.* 2005). The outgroup was specified to be monophyletic.

The BEAST analyses of both the ETS and ITS data partitions were performed using six rate categories and gamma distributed rate variation. Six rate categories were choden because the substitution model (TrN + G) selected using Modeltest 3.06 (Posada & Crandall 1998) provided the most complex approximation to each data partition. The Yule process was specified as the tree prior and the UPGMA method was employed to construct a starting tree. The Auto Optimize function was enabled to maximize efficiency

of MCMC runs. Runs consisted of 10 million generations with parameter values sampled every 1000 generations. An uncorrelated relaxed lognormal clock model was assumed; this molecular clock model may be used to determine the presence of rate heterogeneity among lineages in order to establish whether the data conform or deviate from a strict molecular clock (Drummond *et al.* 2006). Although BEAST provides an assessment of rate heterogeneity within the dataset, a likelihood ratio test was performed in PAUP* 4.0 (Swofford 2002) to test the data for clocklike sequence evolution.

Results from the three independent BEAST runs for were combined to estimate the posterior distribution of the substitution model, tree model parameters and node ages. One million generations were discarded as burn-in for each run. Tracer v1.4 (Rambaut & Drummond 2008) was used to explore the analyses and to determine whether the effective sample size (ESS) estimates were adequate and that the independent runs show strong convergence. The posterior probability density was summarized using TreeAnnotator v.1.4.7 (Drummond & Rambaut 2008) to find the best supported tree. Figtree (Rambaut 2008) was used to construct the chronogram. This summary tree was annotated with the mean ages and the HPD ranges of nodes present in 50% of the trees.

4.4 Results

A total of 39 species (represented by 45 individuals) belonging to *Ficus* section *Galoglychia* comprised the taxon set incorporated into the BEAST analysis. This comprised approximately 50% of the total number of species attributed to *Ficus* section *Galoglychia*.

The use of the uncorrelated relaxed lognormal clock model in the analysis revealed that the data displayed rate heterogeneity: the mean coefficient of variation of the two independent runs was 0.497 (95% highest posterior density (HPD): 0.291–0.723). In addition, the likelihood ratio test rejected the ML phylogeny with an enforced molecular clock versus one without a molecular clock (-ln L with clock = 4717.46 and -ln L without

clock = 4666.97; $p < 0.001$) thereby providing additional evidence that the data departs from clock-like model of sequence evolution.

Analysis of parameters and tracer plots in Tracer v1.4 confirmed that the ESS estimates were acceptable (all ESS estimates were over 1000) and suggested that the three independent runs converged on the same results. Sampling the joint prior distribution by performing Markov chain Monte Carlo (MCMC) BEAST analyses without any sequence data suggested that the calibration priors did not have a strong influence on their estimated posterior distributions, and thus, the estimated divergence times of nodes.

The combined BEAST analyses estimated the tMRCA of the *F. natalensis* sp-complex as 6.12 MYA (95% highest posterior density (HPD): 2.96–9.76). The tMRCA of the two major *Galoglychia* clades, Clades A and B (Chapter 3; Figure 3.1) were estimated calculated as 29.47 MYA (95% HPD: 22.75–36.54) and 29.32 MYA (95% HPD: 18.97–40.75). Due to the large confidence intervals of the calibration points, the range between 95% HPD lower and upper intervals of the nodes was large (Figure 4.1).

4.5 Discussion

The estimated tMCRA of the clade containing the *F. natalensis* sp-complex within subsection *Chlamydodorae* was estimated at around 6 MYA given the relaxed dating method used and the calibration points available. The divergence time is generally compatible with the emergence of the savannah biome in Africa. Paleobotanical pollen records and carbon isotopes from west and east Africa date the earliest record of the savannah biome to the Middle Miocene, and reveal that grass-dominated habitats were widespread by the Late Miocene around 8 MYA (Jacobs 2004, Beerling & Osborne 2006), which falls within the 95% HPD lower and upper intervals of the node that ranged from 3.02–9.57 MYA.

A plausible hypothesis may be that the *F. natalensis* sp-complex may have arisen from a single ancestor associated with a forest habitat. This ancestral species radiated

morphologically when forest patches became isolated, due to special faunal adaptation to novel selection pressures. The emergence of arid niches in the savannah biome may have instigated these special adaptations. Pollinating wasps may well be shared across the complex, contributing to the lack of molecular variation shown in the phylogeny of *Ficus* section *Galoglychia.*

Various hypotheses of *Ficus* speciation have been proposed over the last half century, but no consensus has been reached on the mechanisms of speciation in figs and their pollinators. The emerging pattern of "diffuse" codivergence of fig wasps with their *Ficus* hosts, has prompted a robust modification of the widely-held hypotheses that advocate one-to-one codivergence. Baker (1961) drew attention to the incompatibility between the remarkable diversity of the genus *Ficus* and strict codiversification scenarios. In short, the chemical, physiological and morphological constraints, proposed as mechanisms that maintain the strict mutualistic relationship between fig and wasp, appear to be counter conducive to the enormous diversity displayed in the genus *Ficus* (Michaloud 1996).

Speciation of *Ficus* through allopatry was proposed by Ramírez (1970), Janzen (1979) and Michaloud *et al.* (1996). While evidence exists that specialist taxa are more prone to isolating events than generalist taxa (Eldredge & Cracraft 1980, Mitter *et al.* 1982), allopatric speciation seems unlikely given the exceptional dispersal abilities of pollinating fig wasps. In Neotropical figs, Nason *et al.* (1998) documented the largest distances of gene flow recorded from any tropical plant. Despite low densities and aseasonal fruiting, fig trees are visited by numerous individual fig wasps, and thus receive pollen from a large number of trees. Indeed, conservative estimates propose that pollen dispersal commonly occurs over a distance as great as 10 kilometers and that breeding populations of *Ficus* may cover areas of over 100 km^2 (Nason *et al.* 1996, 1998).

An alternative hypothesis is speciation of fig trees through allochrony, specifically temporal isolation of populations of trees through temporal asynchrony in flowering time (Kiester et al. 1984). This hypothesis too, has been refuted on the basis that populations of *Ficus* do not display evidence of population subdivision (Machado 2005).

A third hypothesis for speciation of figs can be traced to Baker (1961); he proposed that the large diversity of *Ficus* may be driven by hybridization and introgression. Machado *et al.* (2005) reiterated this hypothesis, and proposed that the large diversity of *Ficus* may be driven by "groups of genetically well-defined species of wasps [that] coevolve with groups of genetically less well-defined (frequently hybridizing) groups of Figs". The presence of multiple pollinators associated with multiple *Ficus* hosts provides support for this hypothesis, and there is documented evidence of hybridization and introgression in *Ficus* lineages on two different continents (Parrish *et al.* 2003, Machado *et al.* 2005).

Nevertheless, ancient patterns that reveal codivergence and coevolution on a broad scale in the fig-fig wasp mutualism must be reconciled with the latter model of fig speciation that appears to undermine the empirical basis of strict-sense codivergence. Jackson (2004) suggested that the influence of extinction on the fig-fig wasp interaction is substantial; "any extant pollinator clade can be expected to lose a proportion of lineages through extinction as it diversifies, historical traces will be lost and the picture presented by a phylogeny later will not mirror the complexity today". Over time, lineages will disappear through stochastic extinction events, the illusion of a simpler pattern of speciation, albeit comprising host-switching and duplication events (Jackson 2004).

While anlayses of ITS and ETS DNA sequence data did not resolve phylogentic relationahips within the *F. natalensis* sp-complex, future studies should focus on comprehensive fine-scale phylogenetic studies that may reveal the relationships between these taxa. These studies should be comprised of numerous specimens sampled throughout their geographic ranges, coupled with the use of more variable molecular markers (Rønsted et al. 2007). Should supplementary analyses fail to resolve the polytomy, further research should focus on the extent to which these species share pollinators, whether sharing of pollinators is based on geographic distribution, and the degree of reproductive isolation amongst the entities. The presence or absence of cryptic species of pollinating fig wasps should also be established.

Berg & Wiebes (1992), when describing the species synnomised under *F. thonningii*, state that "several of these 'forms' are widespread, others have a more restricted distribution or occur disjunctly. Differences in ecology can be recognized. In certain regions the morphological entities can be easily distinguished and intermediate forms appear absent, which suggests the occurrence of reproductive isolation. However, in other regions the differences between those entities fade away". This account seems to apply to many of the species in the *F. natalensis* sp-complex (Figure 3.1, Chapter 3). For example, *Ficus lingua* closely resembles *F. natalensis*, however, the former may be identified by smaller, oval-shaped figs and delicate leaves and is largely confined to the coastal belt of Mozambique. *Ficus craterostoma, F. natalensis, F. petersii* and *F. burkei* possess overlapping distribution ranges, and are easily confused (Burrows & Burrows 2003). Distinction between the species is based on such characters as fruit hairiness, the presence or absence of a fruit stalk, or variation in leaf shape or venation. *F. craterstoma* is associated with moist evergreen forest where it grows predominantly as a strangler (Burrows & Burrows 2003).

In many respects, this *F. natalensis* sp-complex appears to be a less well-defined, potentially hybridizing group of figs, coevolving in association with genetically well-defined species of wasps, reminiscent of the hypothesis of Baker (1961). One may argue that this complex represents one widespread species of *Ficus* displaying remarkable morphological variation, with shared pollinators preventing lineage sorting from being completed. However, there seems to be a continuum between the processes of incomplete lineage sorting and hybridization. Introgression may be thought of as a component of the lineage sorting process until lineage sorting has gone on to completion. Thus, I propose the idea that this complex may represent a model of contemporary *Ficus* speciation whereby a paleoenvironmental change has created new niches that have led to morphologically divergent entities. These are coevolving with shared pollinators, thereby contributing to the remarkable diversity of *Ficus*. In time, reproductive barriers would be established, lineage sorting completed, and certain lineages subsequently weeded out by stochastic extinction events.

If indeed this is a robust model of contemporary *Ficus* speciation, then ancient patterns are inconsistent with contemporary processes. This raises important questions regarding the accuracy of cophylogenetic studies. Pattern changes over time as lineages disappear would cause tree comparison to give an altered account of history. Indeed, the results of these tests appear to be especially sensitive to the taxonomic level of taxa that are incorporated into the analyses; a mix of distantly and closely related taxa introduces nodes of inconsistent age, potentially artificially inflating the level of codivergence determined (Jackson 2004).

This study attempted the calibration of various divergence events within the phylogeny of *Ficus* section *Galoglychia* using published date estimations obtained from a previous research paper (Rønsted *et al.* 2005) as calibration points. While the tenuous nature of this dating exercise should not be overlooked (see Graur & Martin 2004), the objective was a general attempt to link paleoenvironmental changes to diversification events in order to provide insight and to stimulate further discussion into the mechanisms that might be responsible for instigating *Ficus* diversification. However, the date estimations appear satisfactory; divergences of the two major clades of *Ficus* section *Galoglychia* were estimated by Rønsted *et al.* (2007) to be approximately 33 MYA for Clade A, containing the subsections *Caulocarpae*, *Cyathistipulae*, *Galoglychia and Crassicostae,* and 31 MYA for Clade B, comprising the subsections *Platyphyllae* and *Chlamydodoraae;* no confidence intervals were given. Although these ages are slightly older than the estimates proposed in this study, they are roughly of the same age as the mean age estimates proposed in this study, 29.47 and 29.32 MYA, and fall within the HPD ranges for Clades A and B respectively.

The extreme complexity of the fig-fig wasp mutualism is continually being revealed and the notion of strict-sense, one-to-one codivergence and cospeciation has largely been undermined. Future research linking paleoenvironmental changes with *Ficus* divergence events may yield fascinating insights into the coevolution of figs and fig wasp lineages, and the factors that drive the speciation of each.

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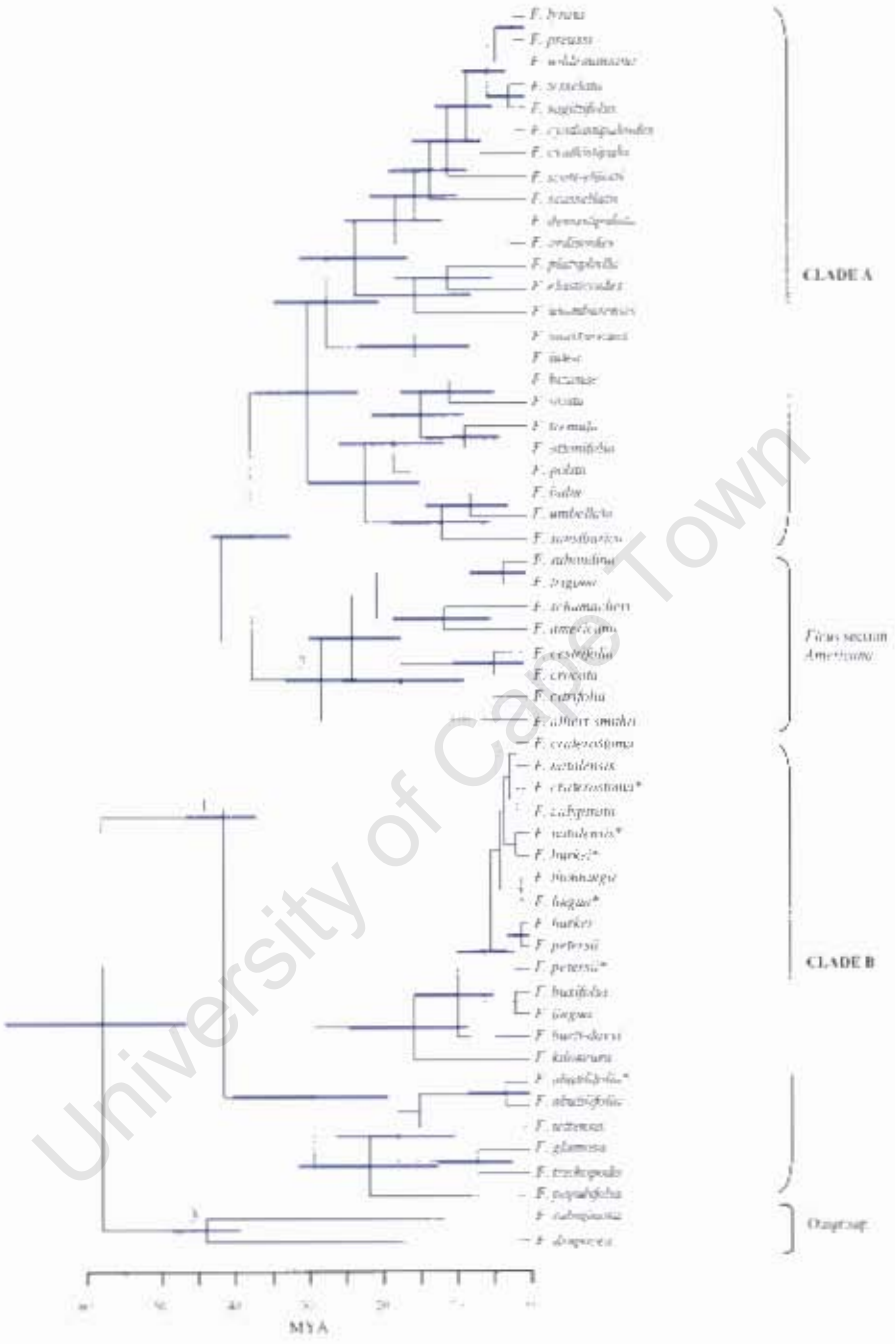

Figure 4.1: Dated phylogeny for *Ficus* section *Galoglychia* based on a Bayesian analysis, implemented in BEAST v1.4.7, of the combined data set of ITS and ETS. Taxa marked with an asterisk are new sequences that were added to the analyses performed by Rønsted *et al.* (2007). Uncertainty in the divergence times is indicated by bars on internal nodes; their length corresponds to the 95% highest posterior density (HPD) around mean estimates of divergence times, only nodes represented in 50% of trees are displayed. Numbered internal nodes (1-3) correspond to the constraints used to estimate divergence times.

CHAPTER 5: General Conclusions

Numerous studies have favoured the notion of strict-sense cospeciation between partners of the obligate mutualism between figs and their pollinating fig wasps (Ramírez 1970, 1974, Janzen 1979, Wiebes 1979, Herre *et al.* 1997). However, incongruencies between the phylogenies of wasp and *Ficus* hosts have been widely documented, thereby challenging the empirical basis of strict-sense, one-to-one, cospeciation (Compton & van Noort 1992, Rasplus 1996, Kerdelhué *et al.* 1999, Lopez-Vaamonde *et al.* 2001, Cook & Rasplus 2003, Molbo *et al.* 2003; Jackson 2004, Machado *et al.* 2005, Haine *et al.* 2006, Erasmus *et al.* 2007, Marussich & Machado 2007, Jousselin *et al.* 2008). This dissertation has followed previous endeavors to investigate the extent of cophylogeny between specific taxa of fig wasps and their hosts, specifically, the phylogenies of the fig-pollinating Agaonidae (Chalcidoidea, Hymenoptera), non-pollinating Sycoecinae (Pteromalidae, Chalcidoidea, Hymenoptera) and associated host fig trees of *Ficus* section *Galoglychia* (Moraceae).

Prior to this study, elucidation of the taxonomic relationships within the Sycoecinae through the molecular phylogenetic analysis had not been attempted. The combined analysis of mitochondrial and nuclear gene regions enabled a robust phylogenetic hypothesis of relationships among the four afrotropical genera, *Philocaenus, Crossogaster*, *Seres* and *Sycoecus*. While the genera *Crossogaster*, *Seres* and *Sycoecus* appeared monophyletic, the identification of the genus *Philocaenus* as paraphyletic has challenged the morphological delimitation of the genera. It is clear that a revision of the Sycoecinae needs to be undertaken, however, denser taxon sampling will be necessary before new generic delimitations can be determined. In order to resolve the deeper nodes within the Sycoecinae, in particular the relationship between the four afrotropical sycoecine genera and the two Indo-Australasian genera, further studies with extensive sampling and slowly evolving markers will be required.

The addition of a small amount of DNA sequence data to the phylogeny of *Ficus* section *Galoglychia* (Rønsted et al. 2007) revealed a polytomy within subsection *Chlamydodorae*. Bar four taxa that were placed in subsections other than their morphologically delimted subsections, the phylogenies were not in conflict with traditional taxonomy.

Similarly, new data were generated for the pollinators of *Ficus* section *Galoglychia* and combined with sequences retrieved from GenBank, largely those published by Erasmus *et al.* (2007; Table 3.2). The analyses reached the same limitations and conclusions as both morphological taxonomy and the study performed by Erasmus *et al.* (2007). Nevertheless, these data provide evidence for sub-clades of certain pollinator species; specimens of *Elisabethiella stuckenbergi* appeared to cluster based on geography and not on host association. The analyses also revealed the diificulty involved in adding new sequence data to existing phylogentic studies. The presence of cryptic species has implications for the combinability of datasets and the potential for incorporating additional sequence data from novel gene regions into existing DNA sequence data matrices. Combining different evolutionary signals from divergent lineages within a species should be carefully avoided, especially when attempting to reach fine-scale phylogenetic conclusions.

The three lineages studied in this dissertation offered a unique opportunity to investigate cophylogeny within the context of the fig-fig wasp mutualism. The sycoecine non-pollinating fig wasp lineages are internal ovipositors, like the pollinators. Testing for parallel phylogenies through cophylogentic analyses can been seen as an essential first step toward understanding cospeciation, coadaptation, and ecological relationships between fig wasps and their host fig trees. Wasp and fig phylogenies were assessed for similarity using tree-based and distance-based cophylogenetic methods. These methods attempt to explain the history between associated taxa, but they do have their limitations.

If strict cospeciation was shaping the evolutionary trajectories of these phylogenies, clades of fig wasps would be strictly associated with clades of host figs. From qualitative

observance of the phylogenies, the general pattern of association between these pollinator and non-pollinator lineages and their *Ficus* hosts reveals break-down in host-specificity. The results from the cophylogentic analyses revealed both significant results and results that showed no significant codivergence between lineages. Because these analyses are sensitive to the of subset taxa included in the analysis, the results were viewed in terms of their corroboration with recent, comparable studies, revealing that while figs and fig wasps do codiversify, codivergence is not pervasive. Results also reflect the findings of Jousselin *et al.* (2008) that non-pollinating figs wasps are at least as constrained to host-specificity as pollinating fig wasps.

Head shapes of the agaonid and sycoecine fig wasps were plotted onto their respective phylogenies. Internal nodes were reconstructed to explore the evolution of head shape in a phylogenetic context, determining whether head shape is an evolutionarily conserved or labile character within these two wasp lineages. Results suggested that head-shapes are generally evolutionarily conserved within these two independent wasp lineages. However, the presence of distinct reversals of head shape within the reconstruction indicates that host-specific ostiolar morphology may not prevail to constrain host-shifting events.

The last section of the thesis focused on the polytomy that was revealed in the *Ficus* phylogeny. The nodes of the phylogeny of *Ficus* section *Galoglychia* (Chapter 3) were dated using Bayesian methods using calibration points obtained from Rønsted *et al.* (2005). The origin of the clade containing the *F. natalensis* sp-complex within subsection *Chlamydodorae* was linked to the emergence of the savannah biome since the Miocene, around eight million years ago, in response to changes in African climate or shifts in climate variability (Jacobs 2004, Beerling & Osborne 2006). The presence of morphological diversity, but lack of molecular variation, was accounted for through speciation hypotheses. Similar studies appear to be an interesting avenue for future research; coupling paleoenvironmental changes with divergence events within the *Ficus* mutualism may yield fascinating insights into the coevolution of figs and fig wasp lineages, and the factors that drive the speciation of each partner.

This dissertation adds a small measure to the growing body of work investigating the obligate mutualism between figs and fig wasps. It is clear that this insect-plant interaction will continue to be upheld as a model system for exploring coevolutionary hypotheses for many years into the future.

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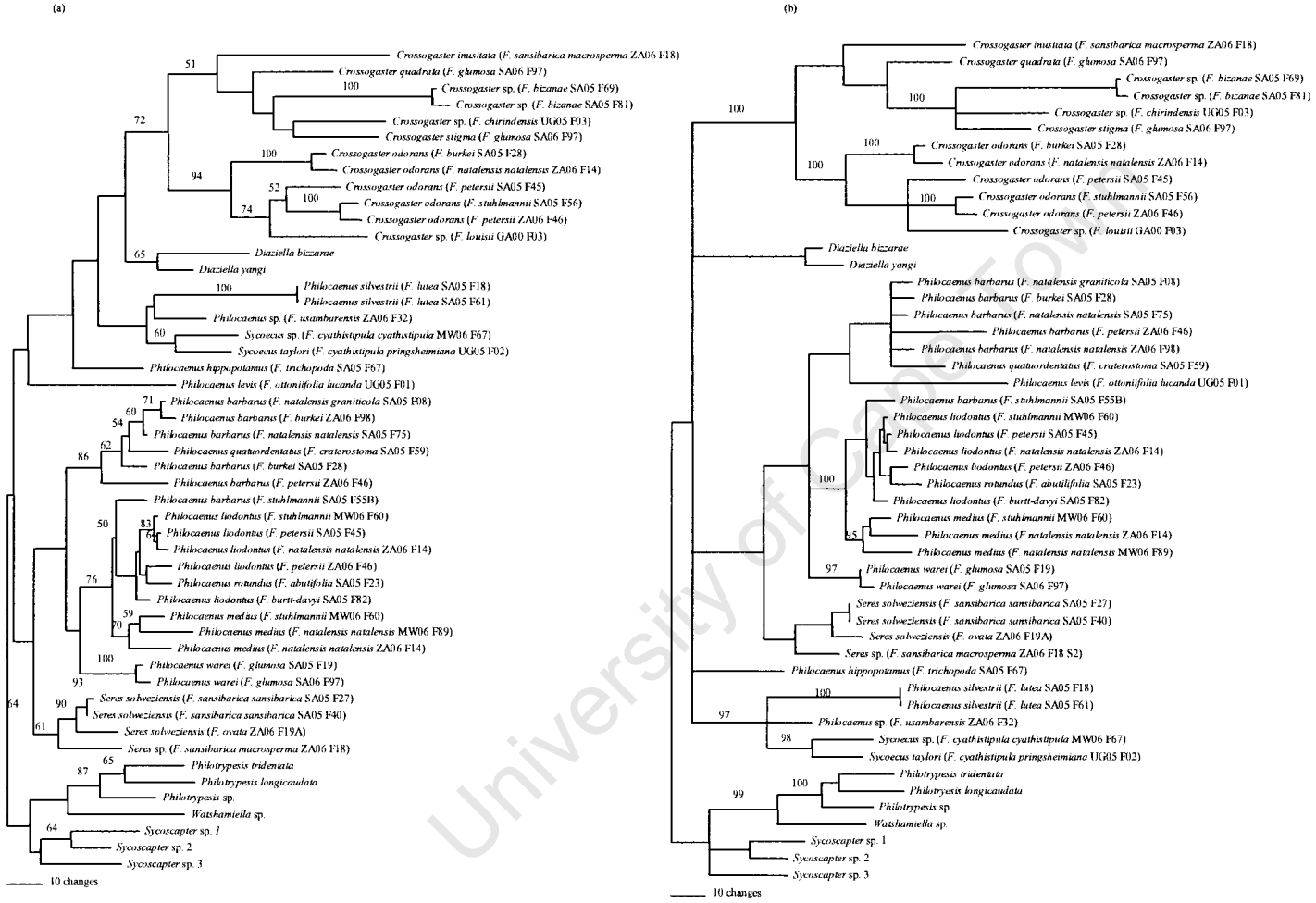
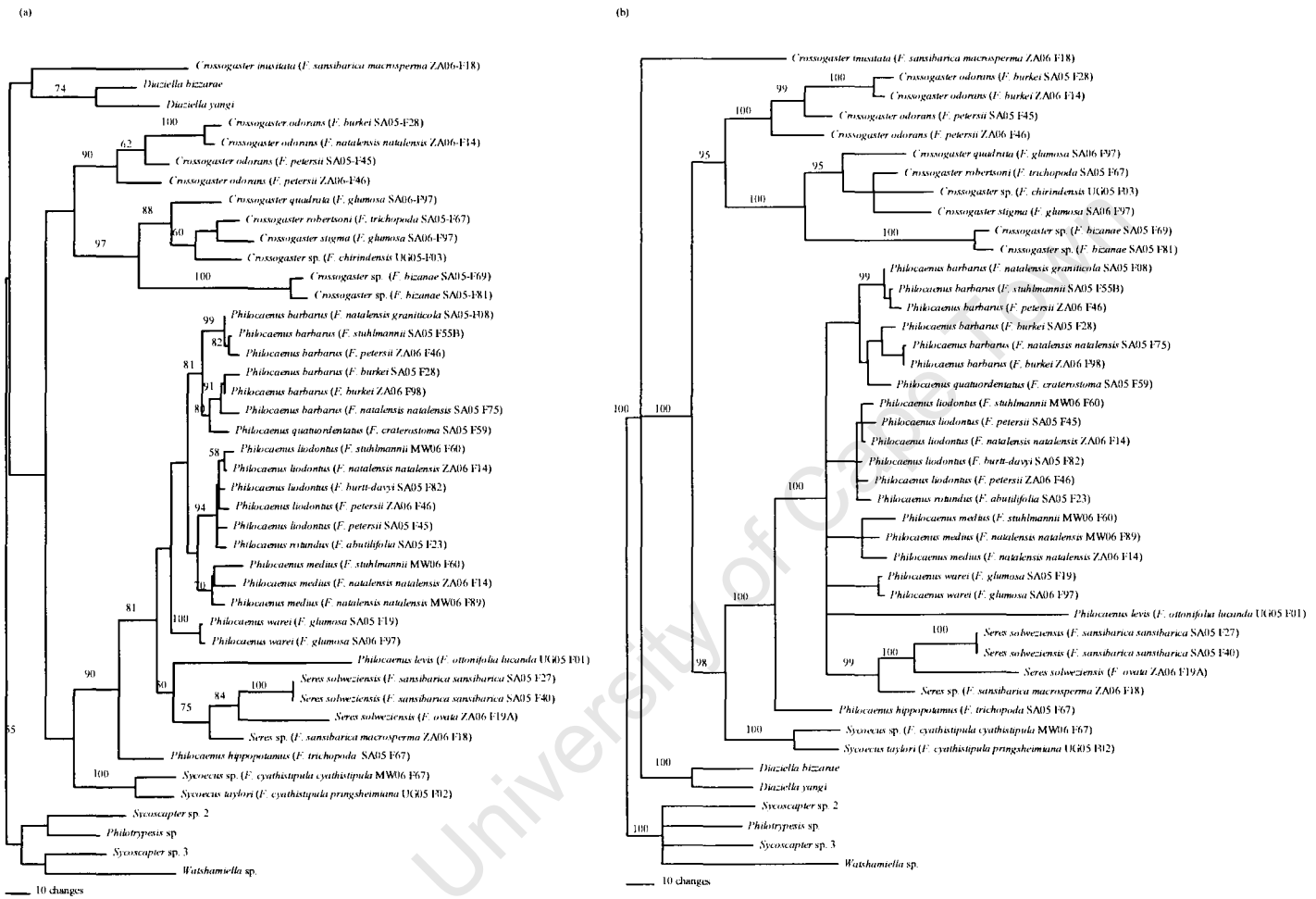
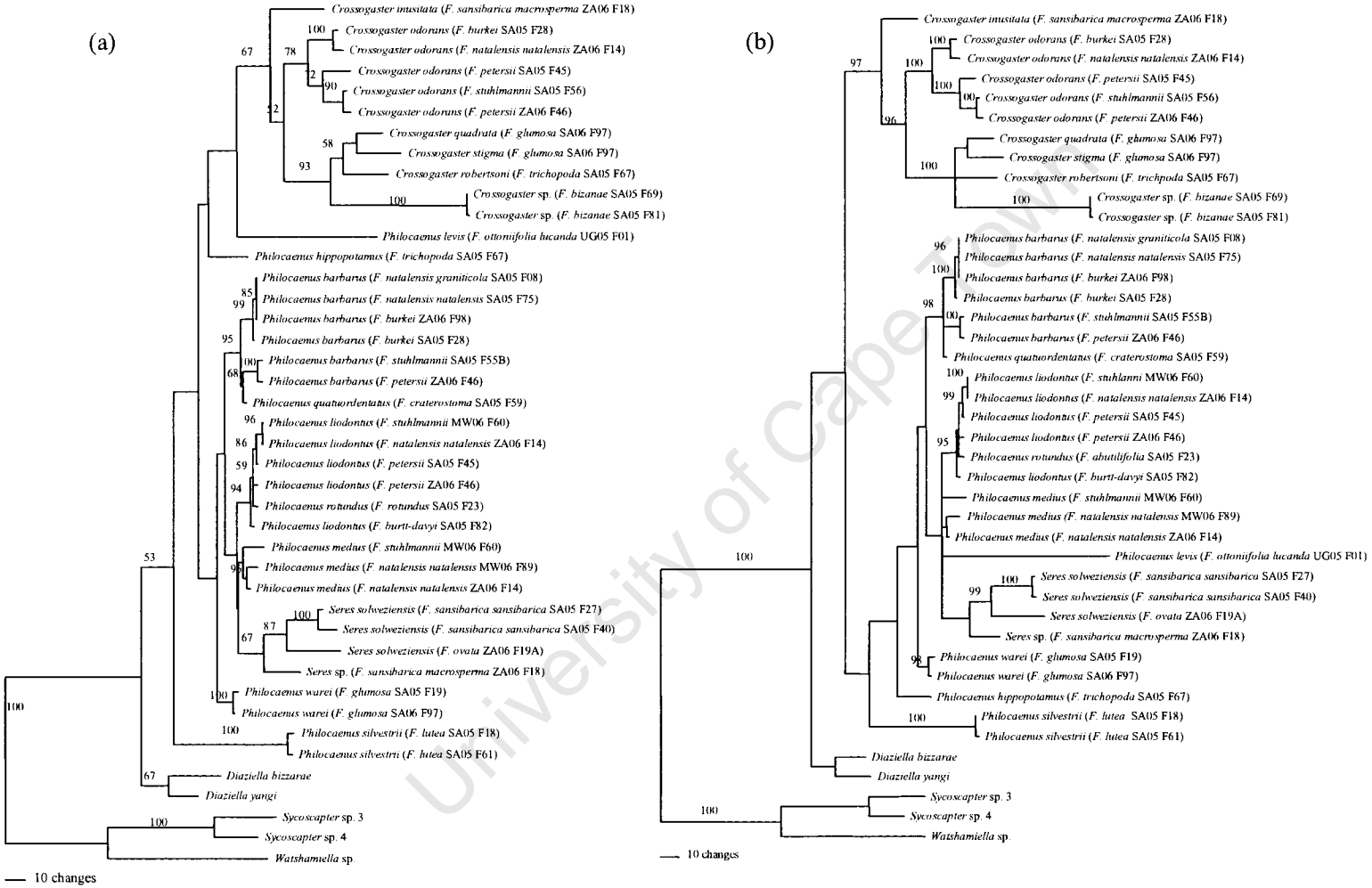
APPENDICES



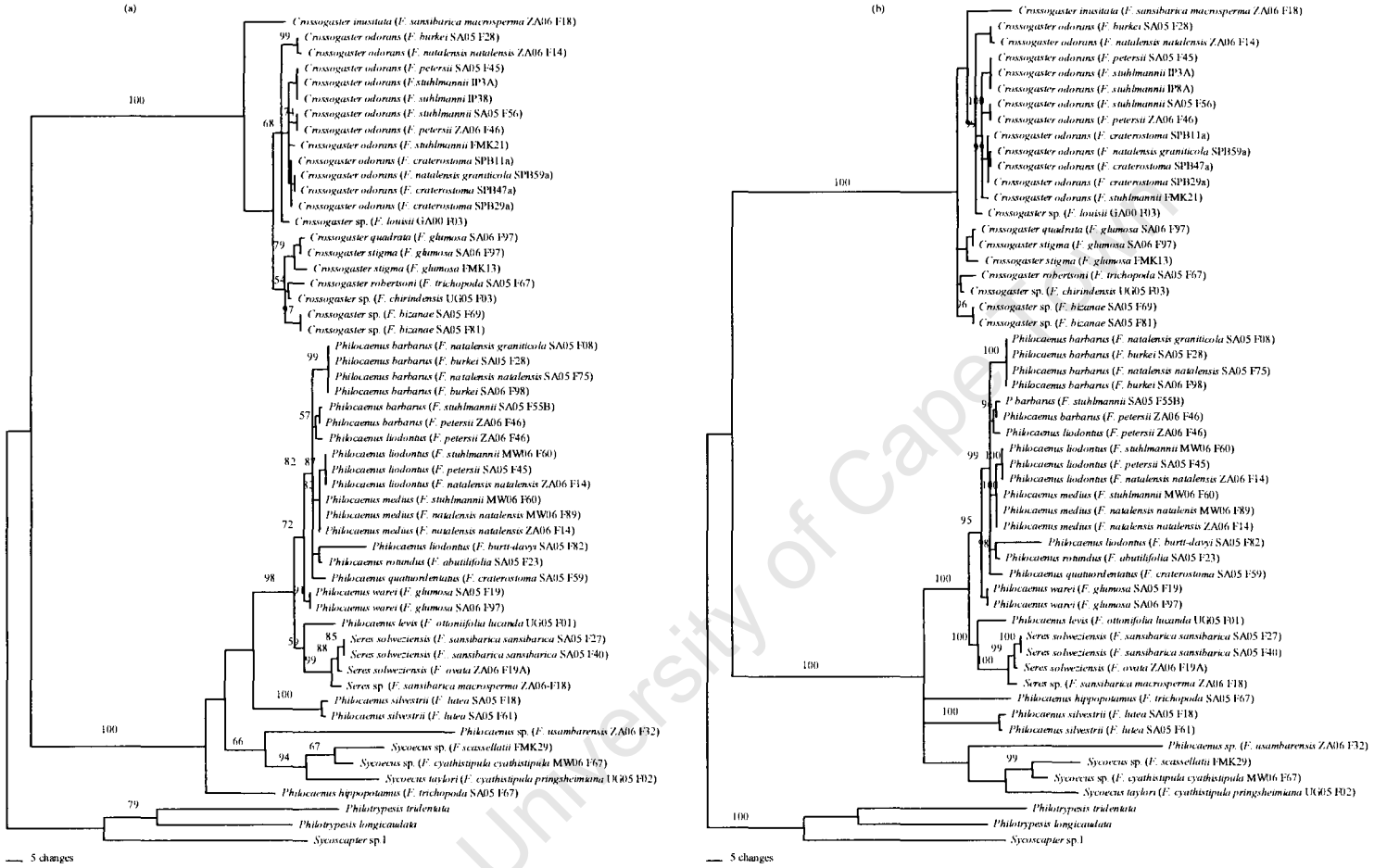
Appendix 2.1: Maximum parsimony phylogram (a) and Bayesian consensus tree (b) constructed from the sycoecine Cytb dataset. Host associations and collection numbers of the taxa are indicated in parentheses. Bootstrap support values ≥50% are indicated above the nodes. Posterior probabilities ≥95% are indicated on the Bayesian consensus tree; seven million generations of the Bayesian runs were discarded as burn-in.



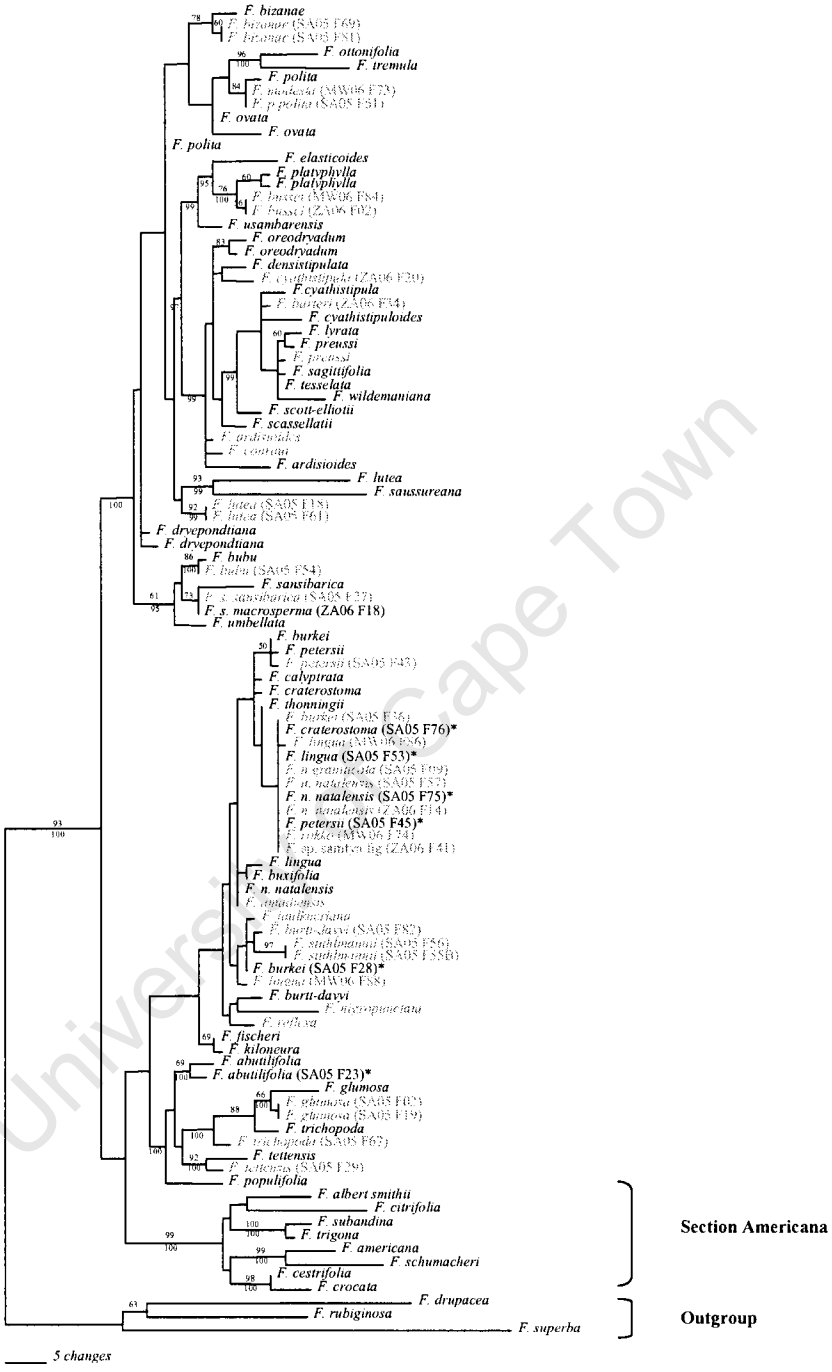
Appendix 2.2: Maximum parsimony phylogram (a) and Bayesian consensus tree (b) constructed from the sycoecine cytochrome oxidase subunit I (COI) dataset. Host associations and collection numbers of the taxa are indicated in parentheses. Bootstrap support values ≥50% are indicated above the nodes. Posterior probabilities ≥95% are indicated on the Bayesian consensus tree; six million generations of the Bayesian runs were discarded as burn-in.



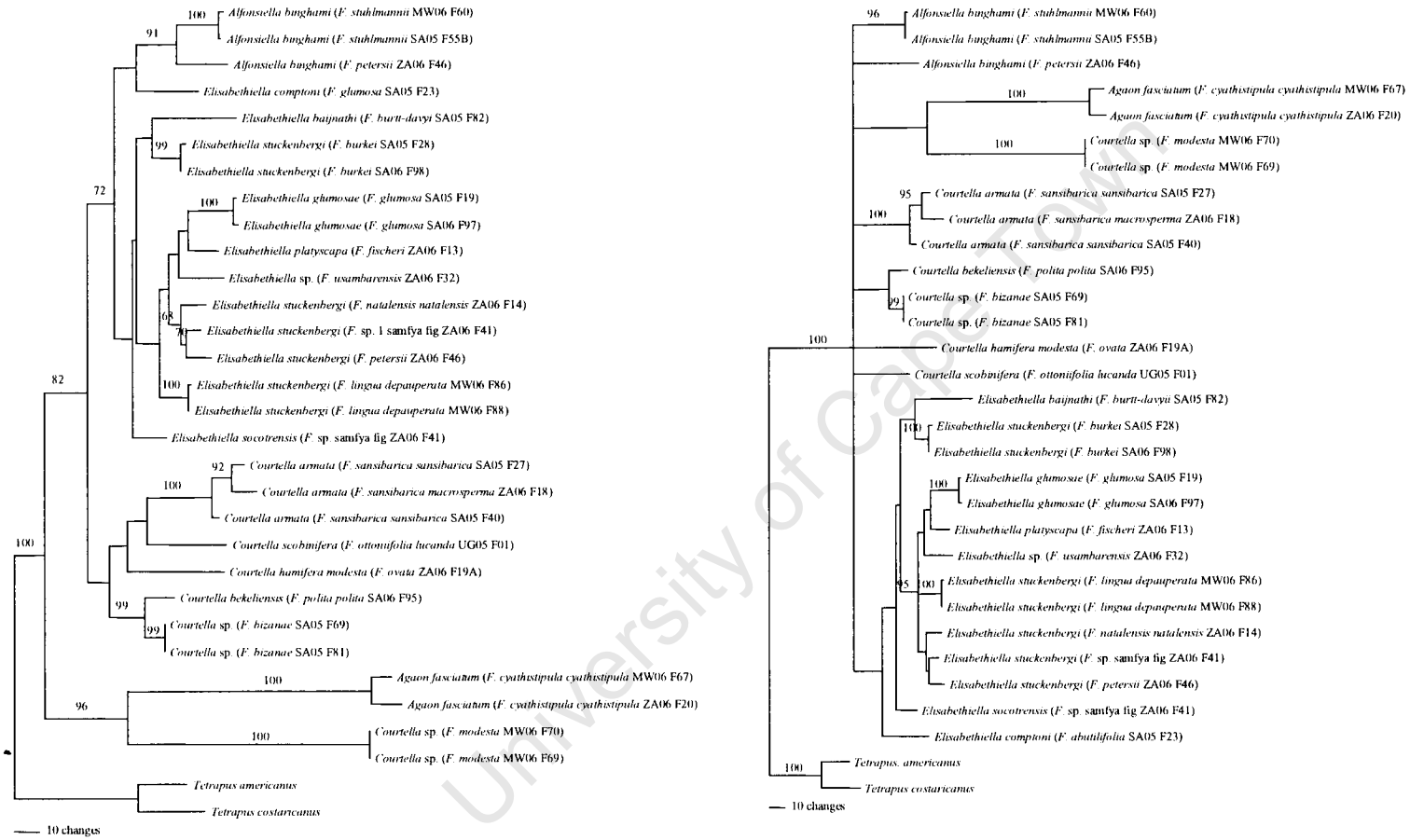
Appendix 2.3: Maximum parsimony phylogram (a) and Bayesian consensus tree (b) constructed from the sycoecine cytochrome oxidase subunit II (COII) dataset. Host associations and collection numbers of the taxa are indicated in parentheses. Bootstrap support values ≥50% are indicated above the nodes. Posterior probabilities ≥95% are indicated on the Bayesian consensus tree; five million generations of the Bayesian runs were discarded as burn-in.



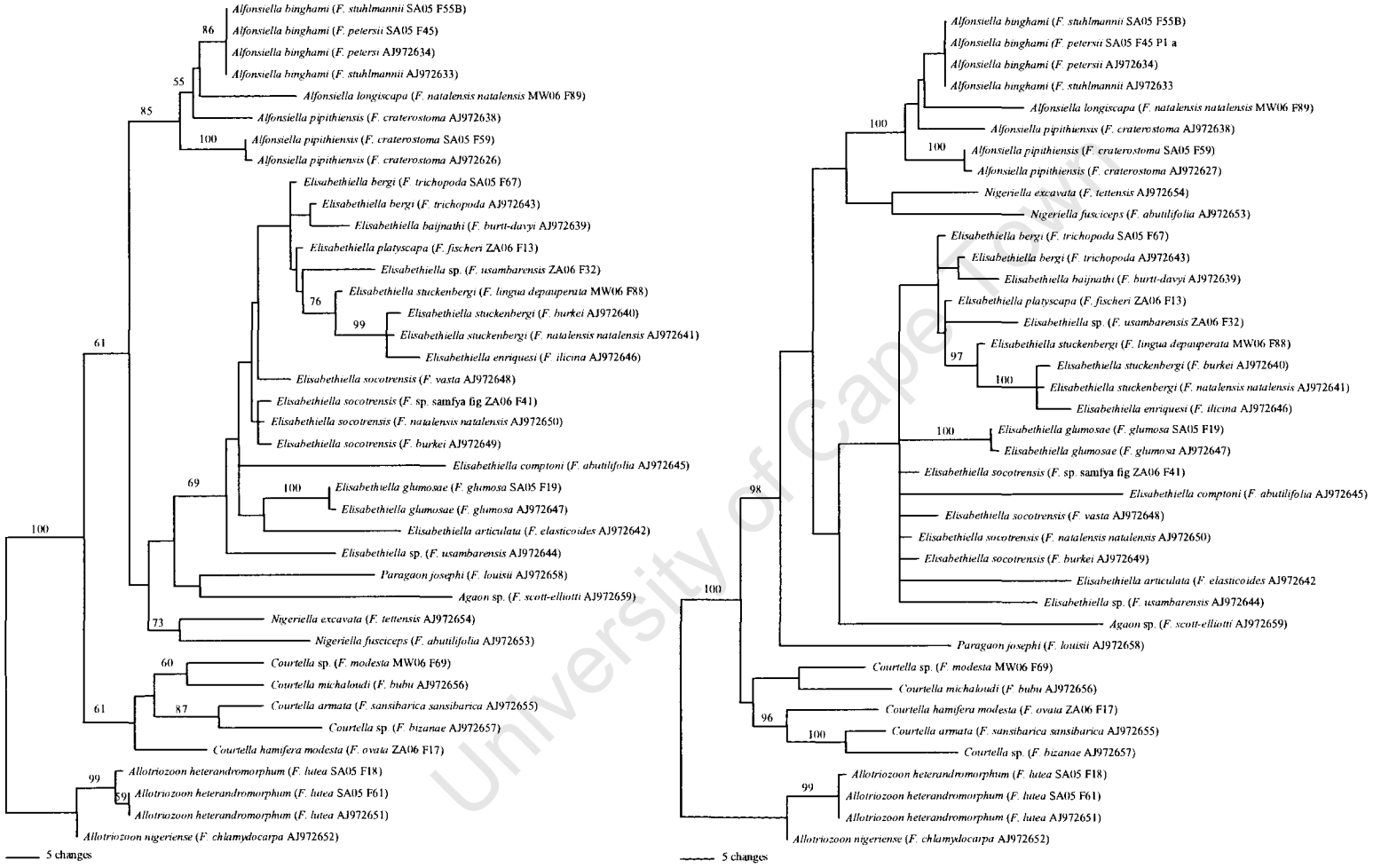
Appendix 2.4: Maximum parsimony phylogram (a) and Bayesian consensus tree (b) of the Sycoecinae using partial sequences of sycoecine internal transcribed spacer (ITS2). Host associations and collection numbers of the taxa are indicated in parentheses. Bootstrap support values ≥50% are indicated above the nodes. Posterior probabilities ≥95% are indicated on the Bayesian consensus tree; five million generations of the Bayesian runs were discarded as burn-in.



Appendix 3.1: One of 28200 maximum parsimony trees from the combined analyses of all the ITS and ETS sequence data of *Ficus* section *Galoglychia*. The outgroup and the taxa belonging to *Ficus* section *Americana* (see text) are labeled. Taxa marked with an asterisk are new taxa for which both ITS and ETS sequences were amplified, and which were added to the sequence data analysed by Rønsted *et al.* (2007). Taxa for which either ITS or ETS were amplified are shown in gray. Bootstrap support values ≥50% and Bayesian posterior probabilities ≥95% are indicated on the parsimony phylogram and the Bayesian consensus tree respectively.



Appendix 3.2: Cladogram resulting from parsimony analysis (a) and the Bayesian consensus tree constructed from COII gene region of the agaonid pollinators of *Ficus* section *Galoglychia*. Bootstrap support values (≥50%) and Bayesian posterior probabilities (≥95%), are indicated above the nodes. Species names are followed by host association and collection number (Table 3.2).



Appendix 3.3: One of 128 most parsimonius phylograms (a) and the Bayesian consensus tree (b) from the analysis of ITS2 gene region of the agaonid pollinators of *Ficus* section *Galoglychia*. *Ficus* host associations and collection numbers of the taxa are indicated in parentheses. Bootstrap support values (≥50%) and Bayesian posterior probabilities (≥95%) are indicated above the nodes

Appendix 3.4: Raw head width measurements and head shape ratios (head shape = average head length to head width ratio) used in the ancestral character optimization.

***Ficus* host**	**Sycoecinae**	**Head width**	**Head shape**	**Agaonidae**	**Head Width**	**Head shape**
F. abutilifolia	*Philocaenus rotundus*	0.545	1.00	*Elisabethiella comptoni*	0.503	0.95
F. bizanae	*Crossogaster* sp.	0.543*	1.12	*Courtella* sp.	0.544*	1.40
F. bubu				*Courtella michaloudi*	0.603	1.47
F. burkei (F. thonningii)	*Crossogaster odorans*	0.356	1.00			
	Philocaenus barbarus	0.399	1.03			
F. burtt-davyi	*Philocaenus liodontus*	0.353	1.02	*Elisabethiella baijnathi*	0.355	0.8
F. c. cyathistipula	*Sycoecus* sp.	0.449*	1.50			
F. c. pringsheimiana	*Sycoecus taylori*	0.484*	1.30			
F. chirindensis	*Crossogaster* sp.	0.430*	1.22			
F. chlamydodcarpa				*Allotriozoon nigeriense*	0.488	1.33
F. craterostoma	*Philocaenus quatuordentatus*	0.479*	0.95	*Alfonsiella pipithiensis*	0.534*	1.12
F. curtipes	*Diaziella yangi*	0.456*	0.94			
F. fischeri				*Elisabethiella platyscapa*	0.381*	1.15
F. glaberrima	*Diaziella bizarrea*	0.368*	1.03			
F. glumosa	*Crossogaster quadrata*	0.409	0.97	*Elisabethiella glumosae*	0.342	0.96
	Crossogaster stigma	0.365	1.03			
	Philocaenus warei	0.414	1.03			
F. louisii	*Crossogaster* sp.	0.303*	0.99	*Paragaon josephi*	0.283*	1.24
F. lutea	*Philocaenus silvestrii*	0.570	0.95	*Allotriozoon heterandromorphum*	0.505	1.06
F. natalensis natalensis	*Philocaenus medius*	0.426	1.33	*Alfonisiella longiscapa*	0.369	1.06
				Elisabethiella stuckenbergi	0.317*	1.01
F. ottoniifolia lucanda	*Philocaenus levis*	0.442*	1.37			
F. ovata	*Seres solweziensis*	0.475*	1.68	*Courtella hamifera_modesta*	0.511*	1.52
F. petersii	*Crossogaster odorans*	0.393	0.94	*Alfonisiella binghami*	0.353	1.22
	Philocaenus liodontus	0.418	1.02			
F. polita polita				*Courtella bekiliensis*	0.607*	1.44
F. sansibarica sansibarica	*Seres solweziensis*	0.498	1.85			
F. sansibarica macrosperma	*Crossogaster inusitata*	0.464*	1.58	*Courtella armata*	0.676*	1.52
	Seres sp.	0.524*	1.78			
F. scassellatii	*Sycoecus* sp.	0.390*	1.67			
F. sp. samfya fig				*Elisabethiella socotrensis*	0.346*	1.09

Appendix 3.4: *Continued*

F. stuhlmannii	*Crossogaster odorans*	0.326	0.96	*Alfonisiella binghami*	0.384	1.08
	Philocaenus liodontus	0.442	1.07			
	Philocaenus barbarus	0.472	0.99			
F. tesselata				*Agaon taiense*	0.520	1.35
F. tettensis				*Nigeriella excavata*	0.330*	1.52
F. trichopoda	*Crossogaster robertsoni*	0.455	1.16	*Elisabethiella bergi*	0.447	1.08
	Philocaenus hippopotamus	0.433	1.34			
F. usambarensis	*Philocaenus* sp.	0.407*	1.17	*Elisabethiella* sp.	0.334*	1.14

*Denotes head width measurements and head shape ratios obtained from either one measurement, or the average of two measurements. All other head width measurements and head shape ratios are mean values calculated from the measurements of approximately ten individuals