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Resolving the phylogeny and population genetic structure of South African pollinating fig wasps

by

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Declaration

I, Johannes Christoffel Erasmus, hereby declare that the dissertation submitted herewith for the degree MSc. (genetics) at the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other institution.

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Summary

A distinct pattern of obligate mutualism exists between fig tree hosts and their pollinating fig wasps. Normally one section or subsection of fig tree hosts is associated with one fig wasp genus. In general, each species is pollinated by a specific fig wasp species. This led to the hypothesis that the fig wasp and fig tree lineages diverged simultaneously. African fig wasps pollinating hosts of the *Galoglychia* section frequently break the normal one fig wasp species-to-one host species ratio. The phylogeny for these species was reconstructed using three DNA segments and compared to the morphological classification of their *Ficus* hosts. Pollinator genera were monophyletic for all analyses, however, the relative positioning of genera was inconsistent. Analyses suggest frequent host jumps between fig trees and fig wasps. Fig wasps of the genus *Alfonsiella* that pollinate *Ficus craterostoma*, *Ficus stuhlmannii* and *Ficus petersii* are morphologically similar in South Africa. Based on host association, genetic differentiation for this group was investigated. Molecular data indicated that the pollinator of *F. craterostoma* is a good species, while the *F. stuhlmannii* and *F. petersii* pollinators were genetically indistinguishable. Based on molecular data and morphological re-evaluation, a new *Alfonsiella* species is described, *Alfonsiella pipithiensis* sp. n. A key to all described species of *Alfonsiella* is provided. In order to resolve the population genetic differentiation of pollinating fig wasp species in South Africa, *Platyscapa awekei* was used as a model species. A few studies indicate that pollinating fig wasps can disperse between 30 and 55 kilometers. However, a recent study on two *P. awekei* populations in South Africa reported an F_{ST} value of 0.011, indicating that pollinators disperse approximately ten times further. This study aims to confirm these results with more detailed sampling of populations. In addition, possible temporal differentiation was tested for the South African population. Six microsatellite loci were used to detect spatial and temporal genetic differentiation in seven populations (collected from 2004 to 2006) over a 340 kilometer range. Genetic differentiation between sampled populations was low (F_{ST} = 0.0055), however, the data suggest stronger temporal genetic isolation than spatial genetic isolation.

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I would also like to thank Jean-Yves Rasplus, Snowy Baijnath and Norbert Hahn for their enthusiastic contribution to the fig wasp specimens used in the study. My lab colleagues, Ronnie Nelson, Gerrit Jansen van Vuuren and Jason Pienaar provided many insights into this project and accompanied me on many fieldtrips in search of figs that were in the right phase. Gerrit isolated highly informative microsatellite loci from *Platyscapa awekei*, providing the tools needed for studying the population genetics of this species (third chapter). Ronnie was always available for lively scientific discussions and provided the photos in the appendix.

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Chapter 1: Introduction

Phylogenetic studies aim to clarify the formation and association among species. When gene flow is restricted between groups of individuals in a population and the number of migrants between groups is low, distinct breeding populations will form, leading to genetically distinct groups (Hedrick 2005). Various factors could guide a population to diversify into two or more genetically distinct groups, for example, geographic barriers, long distances or reproductive time differences between populations (Hedrick 2005). In the case of cospeciation, parasites or pollinators diverge with their hosts (Page & Charleston, 1998). When aiming to resolve the historical grouping of species, phylogenetics provides a useful tool to clarify the formation of lineages, while population genetics is useful to detect recent and present forces contributing to the genetic structure of populations. This study aims to investigate the historical patterns of fig wasp pollinator speciation with their fig tree hosts and also to resolve the genetic structure within a species due to geographic and temporal factors.

The best-known feature of pollinating fig wasps and their fig tree hosts is probably their obligate mutualism (Hill 1967; Ramírez 1970; Wiebes 1979a; Bronstein & McKey 1989; Anstett *et al.* 1997; Cook & Rasplus 2003). Fig wasps use fig fruit for a space to complete their life cycle and they are the exclusive carriers of pollen between fig trees (Ramírez 1970; Wiebes 1979a; Berg & Wiebes 1992). Two aspects of the evolution of fig wasps and fig trees point towards cospeciation between the two lineages. Firstly, one fig wasp genus is normally associated with one fig tree section, and secondly, the association is generally in a one pollinator species to one host species ratio (Wiebes 1979a, 1987; Berg & Wiebes 1992; Herre *et al*. 1996; Kerdelhue *et al*. 1999; Weiblen 2000, 2001, 2004; Cook & Lopez-Vaamonde 2001; Machado *et al*. 2001; Jousselin *et al*. 2003, Rønsted *et al*. 2005). The fig tree-fig wasp system is especially noteworthy due to the large number of distinct species in each of the lineages. Even though many of the pollinating fig wasp species have not yet been described (more than 300 (Weiblen 2002)), there are approximately 750 distinct fig tree species (Berg 1989; Rasplus 1996), building up to roughly 750 species-specific associations. The pollination of yucca plants by yucca moths is another example of obligate mutualism where specific insect species

exclusively pollinate specific host species (Pellmyr 2003; Riley 1892). Both the fig wasp-fig tree and yucca-yucca moth systems were shaped over a long time span, with approximately 60 and 40 million years for the fig and yucca systems, respectively (Pellmyr & Leebens-Mack 1999; Rønsted *et al*. 2005).

The cospeciation between fig wasps and fig trees is not as strict as once thought and in a few cases there are more than one pollinator species for one host, as well as more than one host for one pollinator species (Berg & Wiebes 1992; Rasplus 1996; Kerdelhue *et al.* 1999; Cook & Lopez-Vaamonde 2001; Jousselin *et al*. 2001b; Cook & Rasplus 2003; Molbo *et al*. 2003; Machado *et al.* 2005). African fig wasps and fig trees have the lowest specificity, while Australasian lineages have a higher degree of specificity and American species the highest degree of specificity (Rasplus 1996). In Africa, two or more pollinator species for one host species occur in approximately 17% of the cases and one pollinator for two or more host species in approximately 15% of the species, while for Australasian species the values are approximately 13% and 7%, respectively, and almost no exceptions for American species (Rasplus 1996). At least 80% of the associations between the two lineages are therefore species-specific.

The *Galoglychia* section fig trees occur mostly in Africa (Africa south of the Sahara, southern Arabian peninsula, including the Mascarene Islands and Madagascar) (Burrows & Burrows 2003). The relationship between these fig trees and their pollinators are less species-specific and this host section is pollinated by seven fig wasp genera (Berg & Wiebes 1992). Analysis of molecular characters is a valuable tool for estimating the phylogenetic association between species. Chapter 2 will focus on the molecular phylogeny of fig wasps associated with *Ficus* hosts from the *Galoglychia* section. This part will therefore focus on the broad resolution of fig wasps and will contribute to the understanding of cospeciation between the two lineages.

Alfonsiella binghami pollinates three hosts in South Africa and is thus an ideal example of possible deviation from the strict cospeciation between fig trees and fig wasps. The three hosts form part of two subsections of the *Galoglychia* section that are clearly defined as separate subsections based on morphological characters (Burrows & Burrows 2003). The pollinator for these three *Ficus*

species might form genetically distinct groups based on host associations. Based on the gene tree obtained for the *Alfonsiella* species, according to host association, morphological characters were re-evaluated for this species group and a new species is described. This part is included in chapter 2.

Another exceptional characteristic of fig wasps is their dispersal capability. Adult female pollinating wasps are tiny insects (approximately 2 millimetres in length) and have a short lifespan (approximately one to two days), yet they have the capacity to colonize and disperse over long distances. The 40 to 55 kilometres of ocean that separates islands from mainland fig tree host populations do not seem to act as a barrier to pollinating fig wasp dispersal (Shanahan *et al*. 2001; Zavodna *et al*. 2005). Various other studies also indicate that pollinating fig wasps disperse over long distances (Compton *et al*. 2000; Compton 2002; Nason *et al*. 1998). This indicates that isolation by distance will only act on continuous pollinator populations that occupy large geographical areas (in terms of thousands of kilometers). Fig wasps develop over a period of approximately two months (pers. com. J. Pienaar & R.M. Nelson) in the fig syconium and mature females have a life span of approximately one to two days (Kjellberg *et al*. 1988; pers. com. J. Pienaar & R.M. Nelson). This indicates that temporal effects (isolation by time) could shape pollinator populations into genetically distinct groups.

Platyscapa awekei serves as the pollinator of *Ficus salicifolia* and is distributed from the northern parts of South Africa to northern Africa and the Arabian Peninsula, Socotra, Egypt and Algeria (Berg & Wiebes 1992). The distribution of *P. awekei* in South Africa includes the northern parts of Gauteng and spreads to Kwazulu-Natal, north of Durban (figure 3.1) (Burrows & Burrows 2003). Jansen van Vuuren *et al*. (2006) analyzed genetic variation for two *P. awekei* populations, sampled during July 2003, that are approximately 450 kilometers apart. The F_{ST} value were 0.011, indicating that distant populations are genetically similar. The first aim was to verify this result with a larger sampling effort. In addition, temporal effects on population differentiation have been tested for P. awekei in South Africa. Chapter 3 will focus on the role that geographic and temporal factors play in genetic differentiation of *P. awekei* populations.

Chapter 2: Molecular phylogeny of fig wasp pollinators (Agaonidae; Hymenoptera) of *Ficus* section *Galoglychia*

This chapter was accepted for publication in the journal *Zoologica Scripta.* The co-authors include Simon van Noort, Emmanuelle Jousselin and Jaco Greeff. Dr. van Noort did all the taxonomic work, Dr. Jousselin helped with phylogenetic analyses and writing, Prof. Greeff helped with writing and submission of the manuscript and I collected many specimens, did the laboratory work, analysed data and wrote the first draft (excluding the taxonomy) for the manuscript.

Abstract

The obligate mutualism between fig trees and their fig wasp pollinators, together with the general trend that each host species is pollinated by one fig wasp species, led to the hypothesis that these two lineages have cospeciated. The pollinators of African figs of section *Galoglychia* form a diverse group of genera whose species seem to be less constrained to a specific host than other pollinating fig wasp genera. Various authors have suggested remarkably different phylogenetic relationships between the seven genera that are associated with section *Galoglychia*. These uncertainties concerning the classification make it difficult to understand the historical patterns of association between these wasps and their hosts. The phylogenetic tree for the pollinators was reconstructed with 28S, COI and ITS2 DNA sequence data and compared with morphological classification of the hosts. Pollinator genera were monophyletic in all analyses. However, the relative position of some genera remains unresolved. Investigation of host fig association suggests that there have been frequent host jumps between host subsections. This indicates that cospeciation between fig trees and fig wasps is not as stringent as previously assumed. In addition, pollinators of the genus *Alfonsiella* associated with three host figs (*Ficus craterostoma*, *Ficus stuhlmannii* and *Ficus petersii*) are morphologically very similar in South Africa. The possibility that these pollinators form a complex of species with host-based genetic differentiation was therefore tested. Molecular analyses supported the distinction of the pollinator of *F. craterostoma* as a good species, but the pollinators of *F. stuhlmannii* and *F. petersii* clustered within the same clade, suggesting that these two host species share a single pollinator, *Alfonsiella binghami*. Based on both molecular data and morphological re-evaluation, a new *Alfonsiella* species is

described, *Alfonsiella pipithiensis* sp. n., which is the pollinator of *F. craterostoma* in southern Africa. A key to both females and males of all described species of *Alfonsiella* is provided.

Introduction

The symbiosis between fig trees (Moraceae, *Ficus*) and their pollinating fig wasps (Hymenoptera: Chalcidoidea: Agaonidae) is considered as a model system for the study of mutualism and cospeciation between plants and insects (Hill 1967; Ramírez 1970; Wiebes 1979a; Bronstein & McKey 1989; Anstett *et al.* 1997; Cook & Rasplus 2003). There are few examples of such specialized interactions between plants and pollinating insects. The interaction between Yucca moths and Yuccas and the association between *Glochidion* tree and *Epicephala* moths (Kawakita *et al*. 2004) also form specific relationships in which the partners totally depend on each other for reproduction. These relationships can potentially lead to parallel diversification of the interacting lineages (Riley 1892; Hill 1967; Pellmyr 2003).

Fig trees are solely pollinated by fig wasps, which in turn only reproduce in the syconia of fig trees (Ramírez 1970; Wiebes 1979a; Berg & Wiebes 1992). Female pollinating fig wasps enter the fig through the ostiole and in section *Galoglychia*, are usually trapped inside the syconium (Ramírez 1974; Bronstein & McKey 1989; Berg & Wiebes 1992; Moore *et al*. 2003). Females pollinate some of the pistillate flowers, either actively or passively, and lay their eggs preferentially in the short-styled gall flowers (Ramírez 1969; Cook & Power 1996; Nefdt & Compton 1996; Anstett 2001; Jousselin *et al*. 2001a). After development of the larvae, male fig wasps emerge and copulate with female fig wasps (Bronstein & McKey 1989; Berg & Wiebes 1992). The males chew an exit hole through the fig wall and females disperse to a new receptive fig tree (Bronstein & McKey 1989; Berg & Wiebes 1992). A few male wasps sometimes disperse to other figs once the exit hole has been created (Greeff *et al*. 2003). Fig wasps detect receptive fig trees based on chemical cues that are specific to fig tree species (Barker 1985; van Noort *et al*. 1989; Gibernau *et al.* 1997; Grison-Pigé *et al*. 2001). Fig wasps therefore benefit from the mutualism through the production of progeny in the syconium of fig trees,

while fig trees are able to produce seeds and disperse their pollen to other fig trees (Herre & West 1997; Cook & Rasplus 2003).

The pollination of fig trees is in many cases highly host specific, with one fig wasp species pollinating only one species of fig tree (Ramírez 1970, 1974; Janzen 1979; Herre *et al*. 1997; but see Cook & Rasplus 2003). This is also known as the general one-to-one ratio rule between fig trees and fig wasps. Historical classification and recent progress on the reconstruction of phylogenies for fig trees and pollinating wasps suggest that fig tree sections or subsections are usually pollinated by only one fig wasp genus. This is consistent with the idea that there is cospeciation between the two lineages (Wiebes 1979a, 1987; Berg & Wiebes 1992; Herre *et al*. 1996; Kerdelhue *et al*. 1999; Weiblen 2000, 2001, 2004; Cook & Lopez-Vaamonde 2001; Machado *et al*. 2001; Jousselin *et al*. 2003, Rønsted *et al*. 2005). However, studies on the fit of pollinator and host phylogenies where one pollinator genus is compared with one host section show that the situation is not as simple as previously thought and host shifts do occur (Kerdelhue *et al.*1999; Jackson 2004; Machado *et al.* 2005). In addition, exceptions to the one-to-one ratio and the pollination of one fig tree section or subsection by one fig wasp genus have also been documented (Berg & Wiebes 1992; Rasplus 1996; Kerdelhue *et al.* 1999; Cook & Lopez-Vaamonde 2001; Jousselin *et al*. 2001b; Cook & Rasplus 2003; Molbo *et al*. 2003; Machado *et al.* 2005). For instance, in Africa, more than one pollinator per host and one pollinator for two or more hosts occur in respectively 17% and 15% of cases (Rasplus 1996).

Fig tree species belonging to section *Galoglychia* and their associated pollinators present exceptions to both the one-to-one ratio and the association between one fig tree section or subsection with one fig wasp genus (Wiebes 1979a, 1986a, 1987; Berg & Wiebes 1992; Compton & van Noort 1992). Section *Galoglychia* is restricted to the Afrotropical region (Africa south of the Sahara, southern Arabian peninsula and including the Mascarene Islands and Madagascar). The 77 described *Galoglychia* species are divided into six subsections (Berg 1986; Berg & Wiebes 1992; Burrows & Burrows 2003). Whereas most other fig sections are only pollinated by a single wasp genus, section *Galoglychia* is pollinated by seven fig wasp genera: *Alfonsiella, Elisabethiella*, *Nigeriella*, *Courtella*, *Agaon*, *Allotriozoon*, and *Paragaon*. Delimitation of these genera is based on morphological taxonomic

appraisal (Wiebes 1972,1974a, 1974b, 1986b, 1988, 1989a, 1989b; Wiebes & Compton 1990) and their monophyly has yet to be tested using rigorous morphological or molecular phylogenetic analyses. Generic limits are not always clear, for example morphologically *Elisabethiella* and *Nigeriella* are closely related in the female sex, but male morphology separates *Elisabethiella* from *Nigeriella* and *Alfonsiella* (Berg & Wiebes 1992). Elucidation of fig wasp phylogeny is challenging, and past tentative assessments based on morphological appraisal have suggested remarkably different phylogenetic placements for the genera associated with section *Galoglychia* (Ramírez 1978; Wiebes 1982). There are currently 53 described species for these genera (Berg & Wiebes 1992; Weiblen 2002). The genera *Courtella*, *Agaon* and *Allotriozoon* are specifically associated with, respectively, subsections *Caulocarpae*, *Cyathistipulae* and *Galoglychia*. On the other hand, species of *Alfonsiella*, *Nigeriella*, *Paragaon* and *Elisabethiella* are associated with three subsections: *Platyphyllae*, *Chlamydodorae*, and *Crassicostae*. Fig trees within subsection *Platyphyllae* are pollinated by *Elisabethiella*, *Nigeriella* and *Alfonsiella* species, those within subsection *Chlamydodorae* by *Alfonsiella* and *Elisabethiella* species, and those within subsection *Crassicostae* by *Elisabethiella*, *Nigeriella* and *Paragaon* species. Within *Elisabethiella* and *Alfonsiella* genera, some wasp species can sometimes be associated with several fig species. Reciprocally, some fig species in the *Galoglychia* section are reported to be pollinated by different wasp species, sometimes even wasp species belonging to different genera (see Table 2.1 for specific associations; Wiebes 1979a, 1990; Berg 1986; Berg & Wiebes 1992; Compton & van Noort 1992; Rasplus 1996). This lack of a strict association between fig wasp genera and host fig sections/subsections was suggested by Wiebes to be a result of inaccuracies in the classification of the figs and/or the fig wasps (Wiebes 1987, 1989a). While Berg (1989), although agreeing that some of the discrepancies may be resolved through reappraisal of characters, recognised that functional significance of taxonomic characters also needed to be taken into account. Only recently with the application of rigorous phylogenetic analyses, has host switching or duplication followed by extinctions been recognised as playing a significant role in the evolution of the associations between fig wasps and figs (Molbo *et al.* 2003). The lack of congruence between the classification of *Galoglychia* pollinators and their host taxonomy makes it an interesting group to investigate the validity of

taxonomic delineation of wasp genera. This will allow a test of whether mismatches between wasps and fig classification and reports of breakdowns of specificity are due to taxonomic mistakes or whether they are the reflection of a complex evolutionary history between the two lineages.

In addition, a few *Alfonsiella* fig wasps provide us with a possible example of a recent host-switching event. In southern Africa, the *Alfonsiella* pollinators for *Ficus stuhlmannii*, *Ficus craterostoma* and *Ficus petersii* are morphologically similar and are difficult to tell apart. Only the pollinator of *F. stuhlmannii* is described. This species, *Alfonsiella binghami*, occurs throughout its host distribution, from South Africa to Uganda. *Ficus craterostoma* occurs in evergreen forests from South Africa to Uganda, and westwards to Sierra Leone, and is pollinated by *Alfonsiella michaloudi* in the central and western areas of its distribution (Berg & Wiebes 1992; Burrows & Burrows 2003). In southern and eastern Africa, however, *F. craterostoma* is pollinated by an *Alfonsiella* species morphologically similar to *A. binghami*. *Ficus petersii* occurs in south-central Africa from Kenya and Angola (possibly also Democratic Republic of Congo) southwards to northern Namibia, northern Zimbabwe and northern Mozambique, with an outlying isolated population in north-eastern South Africa and Swaziland, in relatively dry, semi-deciduous woodlands (Burrows & Burrows 2003). Until recently its pollinator had not been collected very often, however, *Alfonsiella brongersmai* had been recorded from *F. petersii* in Zambia, although this may have been a misidentification of the host species (Bouček *et al.* 1981; Burrows & Burrows 2003). In Zambia, Malawi and South Africa, *F. petersii* is pollinated by an *Alfonsiella* species again morphologically very similar to *A. binghami.* Uncertainty exists regarding the species status of these three separate host-associated pollinator populations. An assumption that the three populations represent the same species would suggest that *A. binghami* has subsequently colonised two further host fig species, displacing *A. michaloudi* in *F. craterostoma* and *A. brongersmai* in *F. petersii*. This situation is thus in contradiction with the cospeciation hypothesis. Alternatively, each fig species hosts a specific pollinator species, but so far, no morphological differences have been found between the three host-associated populations.

This study has two objectives. First, the molecular phylogeny of *Galoglychia* pollinators was investigated by using DNA sequence data, which will allow a test of the monophyly of the different

genera. By looking at host association, congruence between the reconstruction of the pollinator phylogeny with the existing fig tree classification will be discussed. Second, the *A. binghami* complex was resolved with DNA sequence data to determine whether each host fig species has a different species of pollinator. We also re-evaluate morphological characters of the three *Alfonsiella* populations to determine whether species level diagnostic features were discernable.

Materials and Methods

Taxon sampling and DNA extraction

Fig wasp pollinators were reared from figs occurring in their natural distributional range in southern Africa and central Africa. Pollinator samples were collected from one or more figs and preserved in 96% ethanol. For the reconstruction of the pollinator phylogeny, species were sampled from all seven genera associated with section *Galoglychia*: *Nigeriella*, *Alfonsiella*, *Elisabethiella*, *Courtella*, *Agaon*, *Paragaon*, and *Allotriozoon* (Table 2.1). For a couple of wasp species that are known to be associated with several fig species (i.e *Elisabethiella stuckenbergii*, *E. socotrensis*) specimens reared from different hosts were sampled. Reciprocally, when fig species hosted different pollinator species, samples were taken based on host association. DNA sequence data for a few species were obtained from the GenBank database. In order to analyse the existence of several species in the *A. binghami* pollinator group (pollinators of *Ficus craterostoma*, *F. stuhlmannii*, and *F. petersii*), pollinators of each of the three host fig trees were collected from four to seven different trees for each host (Table 2.1). Five *F. stuhlmannii* pollinator samples were collected at five different time intervals and two different locations, seven *F. craterostoma* pollinators were collected at seven different locations and four *F. petersii* pollinators were collected at four different time intervals from one location.

Table 2.1 Collection site, species and subsection of host *Ficus*, DNA region amplified and EMBL accession numbers for pollinating wasps in this study

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
Alfonsiella pipithiensis sp. n.	1. Tanzania, Amani, 09-2000	*F. craterostoma*	Chlamydodorae	ITS2	AJ972638
				28S	AJ971635
	2. RSA, Soutpansberg, Hanglip, 03-2002			ITS2	AJ972623
	3. RSA, Soutpansberg, Piesanghoek, 03-2002			ITS2	AJ972624
	4. RSA, Soutpansberg, Entambeni, 03-2002			ITS2	AJ972625
	5. RSA, Saddleback Pass, Barberton, 02-2002			ITS2	AJ972626
				COI	AJ971649
	6. RSA, Lekgalameetse, Ofcoloco, 01-2003			ITS2	AJ972627
	7. RSA, Ngome Forest, Vryheid, 01-2003			ITS2	AJ972628
Alfonsiella longiscapa	GenBank	*F. natalensis*	Chlamydodorae	COI	AY014974
	GenBank			28S	AY616525

Table 2.1 (continued)

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
Alfonsiella binghami	1. RSA, Nelspruit, 1999	*F. petersii*	Chlamydodorae	ITS2	AJ972634
				COI	AJ971650
	2. RSA, Nelspruit, 09-2002			ITS2	AJ972635
	3. RSA, Nelspruit, 01-2003			ITS2	AJ972636
	4. RSA, Nelspruit, 01-2001			ITS2	AJ972637
Alfonsiella binghami	1. RSA, Nelspruit, 11-2002	*F. stuhlmannii*	Platyphyllae	ITS2	AJ972629
	2. RSA, Lekgalameetse, Ofcoloco, 01-2003			ITS2	AJ972630
	3. RSA, Nelspruit, 01-2001			ITS2	AJ972631
	4. RSA, Nelspruit, 01-2003			ITS2	AJ972632
				COI	AJ971648
	5. Tanzania			ITS2	AJ972633
	GenBank			28S	AY616526

Table 2.1 (continued)

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
Nigeriella excavata	RSA, Makhado, 12-1999	F. tettensis	Platyphyllae	ITS2	AJ972654
				COI	AJ971655
				28S	AJ971638
Nigeriella fusciceps	Burkino Faso, 2001	*F. abutilifolia*	Platyphyllae	ITS2	AJ972653
				28S	AJ971637
Elisabethiella bergi	RSA, Ballito, 01-2003	*F. trichopoda*	Platyphyllae	ITS2	AJ972643
				28S	AJ971642
Elisabethiella comptoni	RSA, Pretoria, 05-2001	*F. abutilifolia*	Platyphyllae	ITS2	AJ972645
				COI	AJ971652
Elisabethiella glumosae	RSA, Pretoria, 07-2001	*F. glumosa*	Platyphyllae	ITS2	AJ972647
				COI	AJ971654
				28S	AJ971639

Table 2.1 (continued)

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
Elisabethiella socotrensis	Tanzania, 09-2001	*F. vasta*	Platyphyllae	ITS2	AJ972648
				28S	AJ971641
	RSA, Durban, 11-2001	*F. natalensis*	Chlamydodorae	ITS2	AJ972650
				28S	AJ971640
				COI	AM260706
				COI	AM260707
	RSA, Durban, 11-2001	*F. burkei*	Chlamydodorae	ITS2	AJ972649
				COI	AM260705
Elisabethiella stuckenbergi	Tanzania, vallée de Mayo, 02-1995	*F. burkei*	Chlamydodorae	ITS2	AJ972640
				COI	AM260704
	RSA, Durban, 11-2001	*F. natalensis*	Chlamydodorae	ITS2	AJ972641
				COI	AJ971651
				28S	AJ971644

Table 2.1 (continued)

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
Elisabethiella enriquesi	Namibia, Namib-Nankluft National Park, 10-1997	*F. ilicina*	Chlamydodorae	ITS2	AJ972646
				28S	AJ971643
Elisabethiella baijnathi	RSA, Cape Town, 04-1997	*F. burtt-davyii*	Chlamydodorae	ITS2	AJ972639
				COI	AJ971653
	GenBank			28S	AY616557
Elisabethiella articulata	Gabon, 2000	*F. elasticoides*	Crassicostae	ITS2	AJ972642
Elisabethiella sp. n.	Tanzania, Pongwe, 03-1996	*F. usamberensis*	Crassicostae	ITS2	AJ972644
Paragaon josephi	Réserve des Monts Doudou, Gabon, 2000	*F. louisii*	Crassicostae	ITS2	AJ972658
Courtella michaloudi	Tanzania, 1995	*F. bubu*	Caulocarpe	ITS2	AJ972656
	GenBank			28S	AY616551
Courtella armata	RSA, Olifantskamp, 09-2003	*F. sansibarica*	Caulocarpe	ITS2	AJ972655
	GenBank			28S	AY616549
	GenBank			COI	AY014978

Table 2.1 (continued)

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
Courtella sp.	RSA, Mkambati Game Reserve, 09-1998	*F. bizanae*	Caulocarpe	ITS2	AJ972657
				28S	AJ971636
Courtella bekiliensis	GenBank	*F. polita*	Caulocarpe	28S	AY616550
Allotriozoon heterandromorphum	RSA, Durban, 2001	*F. lutea*	Galoglychia	ITS2	AJ972651
				28S	AJ971646
Allotriozoon nigeriense	Guinea, Sao Tome, 02-2001	*F. chlamydocarpa*	Galoglychia	ITS2	AJ972652
Agaon sp. n.	Ivory Coast, Lamto. 04-1994	*F. scott-elliotii*	Cyathistipulae	ITS2	AJ972659
				28S	AJ971647
Agaon taiense	GenBank	*F. tesselata*	Cyathistipulae	28S	AY616524
Pleistodontes froggatti	GenBank	*F. macrophylla*	outgroup	28S	AJ275085
Pleistodontes imperialis	GenBank	*F. rubiginosa*	outgroup	28S	AJ298405
Tetrapus americanus	GenBank	*F. maxima*	outgroup	COI	AY014971
Tetrapus costaricanus	GenBank	*F. insipida*	outgroup	COI	AY014973

DNA extractions were performed with one or several individuals for the phylogenetic analyses of the pollinator genera, and a single individual for the *Alfonsiella binghami* species group. The protocols for DNA extractions included the DNeasy Tissue Extraction Kit (Qiagen) according to manufacturer's instructions and the 10% Chelex 100 DNA extraction method with proteinase K treatment described by Estoup *et al*. (1996). The Chelex extractions were performed with Chelex 100/50-100 mesh instead of Chelex 100/100-200 mesh recommended by Estoup *et al*. (1996).

DNA amplification and sequencing

Phylogenetic relationships for the pollinating fig wasps were determined with partial cytochrome oxidase subunit I (COI), partial 28S rDNA and ITS2 (internal transcribed spacer 2 for rDNA) DNA sequences. Two additional mitochondrial DNA regions were tested (cytochrome B and another partial COI region), however, these two regions were not useful for resolving phylogenetic association for the pollinators of the *Galoglychia* section. The sequence data for these two regions will, however, be available in the GenBank database. The COI gene has been used in various phylogenetic studies on fig wasps (Weiblen 2001; Machado *et al*. 2001). The mutation rate of the 28S DNA region is remarkably lower than the mutation rates of COI and ITS2 DNA regions, which might be useful for the phylogenetic placement of the genera. The ITS2 region seems to be ideal for species, as well as genus level phylogenetic analyses in insects (Young & Coleman 2003). ITS2 DNA sequences were also used for the analysis of differentiation between the three different host associated populations within the *Alfonsiella binghami* group.

The 28S, COI and ITS DNA segments were amplified with 1.5 units of *Taq* DNA polymerase (Promega) in 50μl PCR reactions with 1 x PCR reaction buffer provided by the manufacturer (Promega), 2.5 mM $MgCl_2$, 0.2 to 0.5 pM of each primer and 0.2 mM of each of dCTP, dATP, dGTP, and dTTP (Promega). The 28S genes were amplified and sequenced with the D1F (forward) and D3R (reverse) primers (Table 2.2; Harry *et al*. 1998; Lopez-Vaamonde *et al*. 2001). The PCR conditions for the amplification of the 28S gene segment were 3 minutes denaturing at 94ºC, followed by 35 cycles

of 1 minute at 94ºC, 30 seconds at 55ºC, and 1.5 minutes at 72ºC, with a final extension of 4 minutes at 72ºC. The amplification and sequencing of the two cytochrome oxidase I DNA segments were performed with the C1-J-2183 (alias Jerry) forward and TL2-N-3014 (alias Pat) reverse primers (Table 2.2; Simon *et al*. 1994). The PCR conditions for the amplification of the Pat-Jerry COI DNA region were 3 minutes denaturing at 94ºC, with 35 cycles for 1 minute at 94ºC, 1 minute at 45-48ºC, and 1.5 minutes at 72ºC, with four minutes final extension at 72ºC. The ITS2 intergenic DNA sequences were amplified and sequenced with the ITSF forward and ITSR reverse primers (Table 2.2; Campbell *et al*. 1993; Lopez-Vaamonde *et al*. 2001). The PCR conditions for the amplification of the ITS2 sequences were 3 minutes denaturing at 94ºC, followed by 35 cycles for 1 minute at 94ºC, 1 minute at 55ºC, 1 minute at 72ºC, with a final extension of 4 minutes at 72ºC.

PCR products were purified with a High Pure PCR Purification Kit (Roche). DNA fragments were cycle sequenced with BigDye version 3.1 ready reaction mixture (Perkin Elmer) in the forward and reverse direction with the primers used for the PCR reactions. Cycle sequencing was performed according to the recommended method for the BigDye cycle sequencing procedure. Sequencing fragments were analysed on an ABI 3100 sequencer. Sequences obtained were subjected to a standard nucleotide BLAST search in the GenBank database in order to confirm that the sequence belonged to a wasp, rather than one of its parasites (for example, *Wolbachia*).

Table 2.2 Primer sequences used for PCR amplification and sequencing.

Primer name	Primer Sequence	DNA region
D1F	ACC CGC TGA ATT TAA GCA TAT	28S
D3R	TAG TTC ACC ATC TTT CGG GTC	28S
LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	COI
HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	COI
TL2-N-3014 (Pat)	TTC AAT GCA CTT ATT CTG CCA TAT TA	COI
C1-J-2183 (Jerry)	CAA CAT TTA TTT TGA TTT TTT GG	COI
ITSR	CGC CTG ATC TGA GGT CGT GA	ITS2
ITSF	ATT CCG CAC CAC GCC TGG CTG A	ITS2

Phylogenetic analyses

Sequence alignments were performed with Clustal W (Thompson *et al*. 1994). When necessary, the default ClustalW alignments obtained were modified by manually inserting or deleting gaps to minimize their numbers. COI sequences were individually checked and verified for protein coding frame-shifts and non-sense codons to avoid pseudogenes (Zhang & Hewit 1996) using Mega3 (Kumar *et al*. 2004). The phylogenies for the 28S, COI, and ITS2 genes were reconstructed using maximum likelihood (ML) analyses, Bayesian analyses with Markov Chain Monte Carlo simulation, as well as maximum parsimony (MP) analyses. Phylogenetic analyses were performed with PAUP* (Swofford 2000), for likelihood and parsimony, and MrBayes version 3 (Huelsenbeck & Ronquist 2001) for Bayesian analyses. First, separate analyses were performed on each data set. Unfortunately, all the sequences for the three markers were not obtained for all specimens, because some combination of DNA primers consistently failed to amplify the template. Combined analyses for the three genes could therefore not be performed. However, 28S and ITS2 sequences data for a substantial number of species were obtained. The congruence of the two data sets was checked using the Incongruence Length Difference (ILD) test (Cunningham 1997) before conducting combined analyses. The ILD test compares the difference in the numbers of steps required by individual and combined analysis of the original partitions with the value obtained for a series of randomised partitions. The test was run with 1000 replicates and 50 random additions of taxa with all constant characters excluded.

For maximum parsimony analyses, heuristic searches with 1000 random sequence additions and TBR branch swapping were performed. Bootstrapping was performed with 1000 replicates with 10 random sequence additions and tree-bisection-reconnection (TBR) branch swapping to determine internal branch support. Branch support for nodes indicating the monophyly of wasp genera was also assessed by Decay index values using reverse constraints searches in PAUP*. Gaps were treated as a fifth character for the *Alfonsiella binghami* species group analysis only.

The models of nucleotide substitution for maximum likelihood analyses were chosen by comparing nested models with likelihood ratio tests (Posada & Crandall 1998). The general time

reversible model (GTR) (Yang 1994) with estimated rate heterogeneity (Γ) (Yang 1994) and a proportion of invariable sites (I) fitted the data best. Phylogenies were obtained with heuristic searches with 100 random sequence additions, using the TBR branch swapping method.

For Bayesian analyses the posterior probability analyses with the Markov chain Monte Carlo approach and sampling according to the Metropolis-Hastings algorithm were performed with one cold chain and three hot chains. The nucleotide substitution model chosen for the sequence data was the GTR + Γ +I. Starting trees were random for the chains and the analyses were run for 10^6 generations with tree sampling every 100 generations. The 'burn in' values were set to 500 and the posterior probabilities were summarized accordingly.

Results

Phylogenetic analyses of 28S sequences

The aligned 28S sequences were 946 base pairs in length. Two *Pleistodontes*, the pollinators of the Australian subsection *Malvanthera* (Berg 2003, 2004) were chosen as outgroup. Heuristic maximum parsimony searches gave three most parsimonious trees, based on 121 parsimony informative characters (L= 420, CI= 0.729, RI= 0.688). The –ln likelihood score of the tree inferred from 28S data was 3223.70653. MP, ML and Bayesian analyses gave very similar trees. Figure 2.1 depicts the preferred ML tree. *Alfonsiella*, *Courtella*, *Nigeriella*, *Elisabethiella* and *Agaon* formed strongly supported monophyletic clades in all analyses, even though decay index values were low. *Allotriozoon* grouped as the sister species to the rest of the sampled species. *Paragaon* grouped as the sister species to the two *Agaon* species. *Alfonsiella* and *Elisabethiella* clustered together in all the analyses (MP bootstrap = 58, Bayesian pp = 99). The clustering of *Nigeriella* with *Courtella* was strongly supported in the Bayesian analysis, but the MP bootstrap support for this node was very low. The placement of these three groups (*Alfonsiella*/*Elisabethiella*, *Nigeriella*/*Courtella* and *Paragaon*/*Agaon*)

relative to each other differed between Bayesian and MP/ML analyses. The Bayesian analysis supported the clustering of *Paragaon*/*Agaon* with *Alfonsiella*/*Elisabethiella* (Bayesian pp = 54), while the MP and ML analyses placed the *Paragaon*/*Agaon* group with the *Nigeriella*/*Courtella* group but with a very low bootstrap support.

The finer placement of species was mostly the same for the different analyses, except for the placement of a few *Elisabethiella* species. Contrary to ML and Bayesian analyses (Figure 2.1), MP heuristic searches clustered the *E*. *socotrensis* specimen pollinating *Ficus vasta* as sister species to a clade formed by *E*. *baijnathi* and *E*. *socotrensis* pollinating *F*. *natalensis*. The placement of the three species was not well supported in any of the analyses. In none of the analyses did *E. socotrensis* specimens pollinating different fig tree species form a monophyletic group, which casts doubt on the validity of this species.

Phylogenetic analyses of COI sequences

The aligned COI sequences were 797 base pairs in length. No frame-shift or non-sense codons were identified in any of the sequences. Two *Tetrapus* species, the pollinators of the New World section *Pharmacosycea*, were chosen as outgroup species. The heuristic MP search yielded one most parsimonious tree based on 162 parsimony informative characters (L = 502, CI = 0.606, RI = 0.605). The –ln likelihood score of the tree obtained for the COI data was 3217.865 (Figure 2.2). For all analyses, *Alfonsiella* and *Elisabethiella* species formed monophyletic clades (MP bootstrap = 63 & low, respectively, Bayesian pp = 100 & 75, respectively). According to the MP analyses, *Nigeriella* forms the sister species to the *Alfonsiella*/*Elisabethiella* group, while ML and Bayesian searches suggested that *Nigeriella* was more closely related to the *Alfonsiella* species. *Courtella* grouped as the sister genus to the rest of the sampled species for all analyses.

The finer placement of the *Alfonsiella* species was the same for all analyses, while the finer placement of the *Elisabethiella* species differed slightly between the ML/Bayesian and MP analyses. In all analyses, however, *Elisabethiella socotrensis* specimens pollinating *F. burkei* and *F. natalensis*

formed a clade, while the two specimens of *E. stuckenbergi* pollinating different host species did not cluster together.

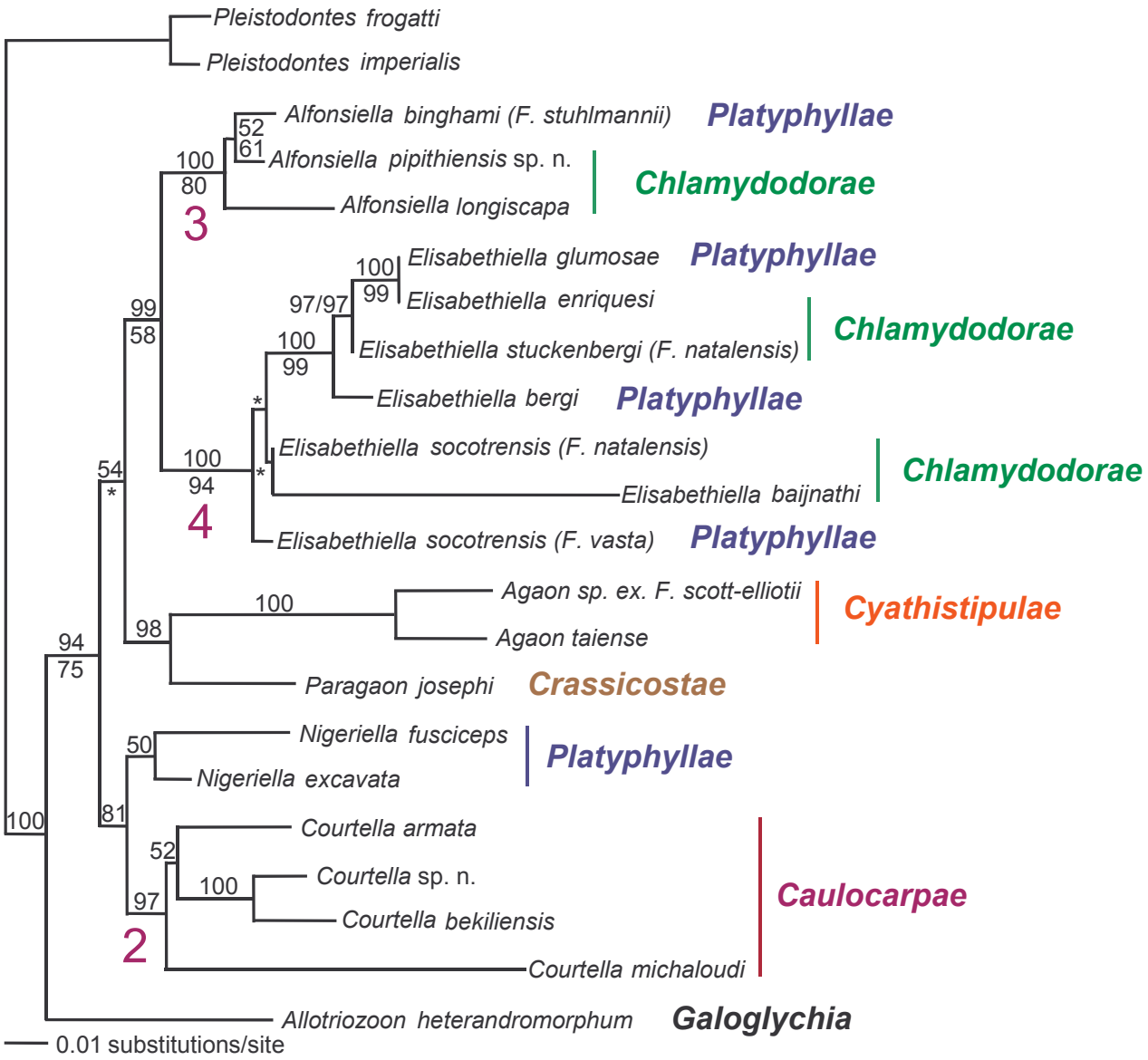

Figure 2.1 Tree obtained with ML analysis of 28S data.

Values above branches are Bayesian posterior probabilities (pp), while values below branches indicate maximum parsimony bootstrap support. * indicates nodes for which MP and Bayesian analyses were different (see text for details). Bootstrap or pp values below 50 are not indicated. Numbers in bold under the nodes supporting the monophyly of wasp genera indicate the decay index. Host fig species for new undescribed pollinator species of wasps and wasps with two hosts are indicated in brackets.

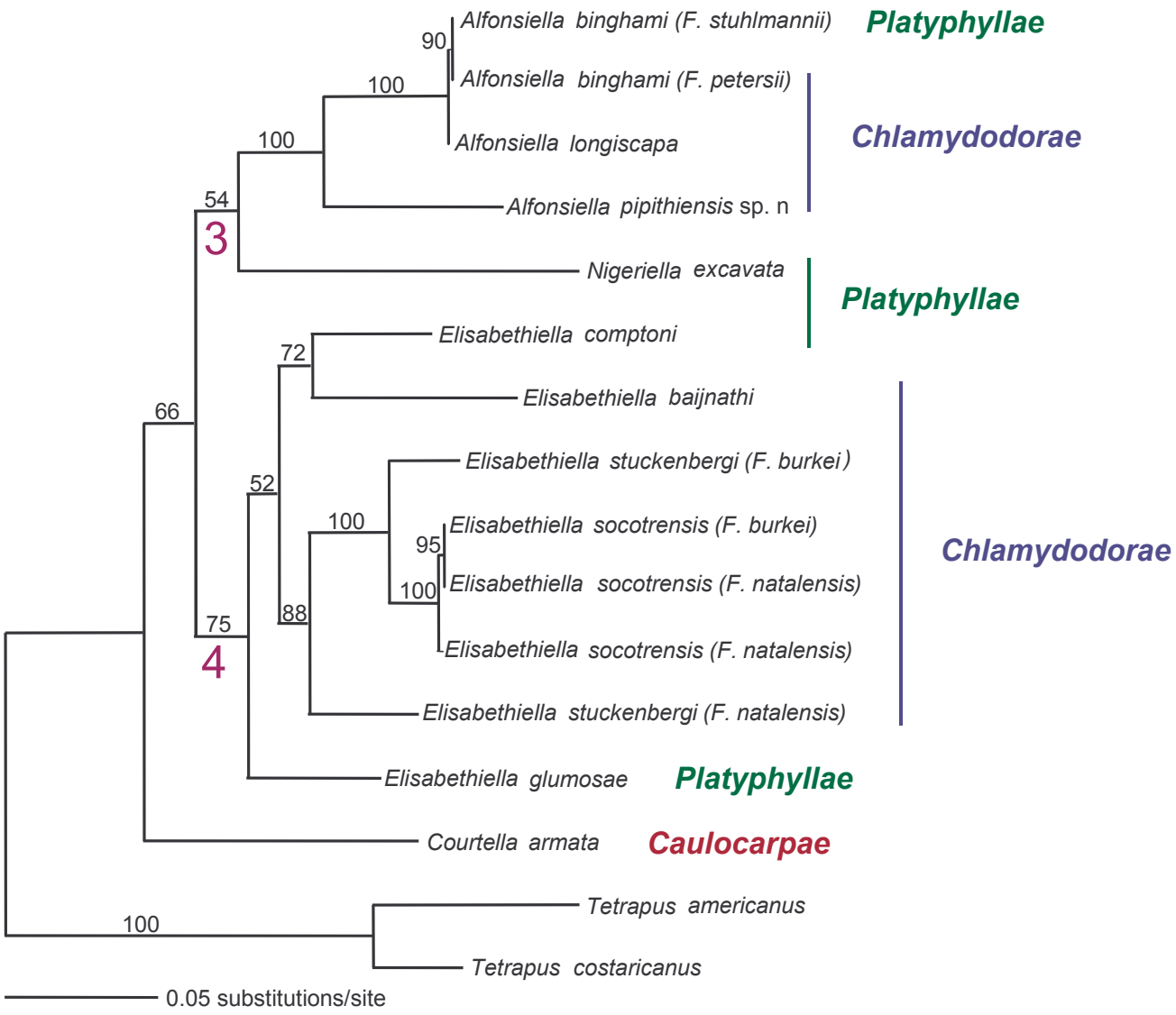

Figure 2.2 Tree inferred from ML analysis of COI.

Two *Tetrapus* species were chosen as the outgroups. The values above the branches are posterior probabilities for identical nodes obtained via Bayesian analysis. Supports below 0.5 are not indicated. Numbers in bold under the nodes supporting the monophyly of wasp genera indicate the decay index.

Phylogenetic analyses of ITS2 sequences

The ITS2 DNA segments proved difficult to align between wasps belonging to different genera and very ambiguous when non-*Galoglychia* pollinators were included. Hence, only species within the *Galoglychia* pollinator group were included for effective alignment. Sequences varied from 346 to 554 bp in length. In a few cases, several ITS2 sequences from the same individual were compared to check for paralogy. Sequences were always identical, which suggests that paralogues were not divergent or not amplified. The fact that alignment problems occurred only between species groups and not within genera (i.e. closely related species) suggest that these are due to high divergence between wasp genera and not having sequenced different paralogues. Approximately 150 bp regions (depending on the species) were excluded from phylogenetic analyses, due to the difficulty of assessing sequence homology. The aligned ITS2 sequences were 391 base pairs in length, with 126 parsimony informative characters. Based on the results of the 28S data, two *Allotriozoon* species were chosen as outgroup. MP heuristic searches resulted in four most parsimonious trees (L = 486, CI = 0.6379, RI = 0.6009, Figure 2.3). The –ln likelihood score of the tree obtained for the ITS2 data was 2723.57809. Again, the pollinator genera *Courtella*, *Elisabethiella*, *Nigeriella* and *Alfonsiella* appeared monophyletic in all analyses. All analyses clustered *Paragaon* with *Agaon* (MP bootstrap = low, Bayesian pp = 81) and *Elisabethiella* formed a sister group to this clade though this node was not strongly supported. *Nigeriella* formed the sister genus to the (*Elisabethiella* (*Agaon, Paragaon*)) group with *Alfonsiella* positioned as a sister genus to this group. *Courtella* formed the sister genus to the rest of the sampled species.

The finer placement of species differed mainly within the *Elisabethiella* group. The consensus of all methods for the placement of *Elisabethiella* species is given in figure 2.3. Again in this analysis *E. stuckenbergi* pollinating different host species did not group together, and *E. socotrensis* pollinating *F. vasta* does not cluster with the rest of the *E. socotrensis* specimens. Bayesian and ML analyses clustered the *A. binghami*-like specimens (*Alfonsiella pipithiensis* sp. n.) associated with *F. craterostoma* together and placed them as sister species to the other two *Alfonsiella* species.

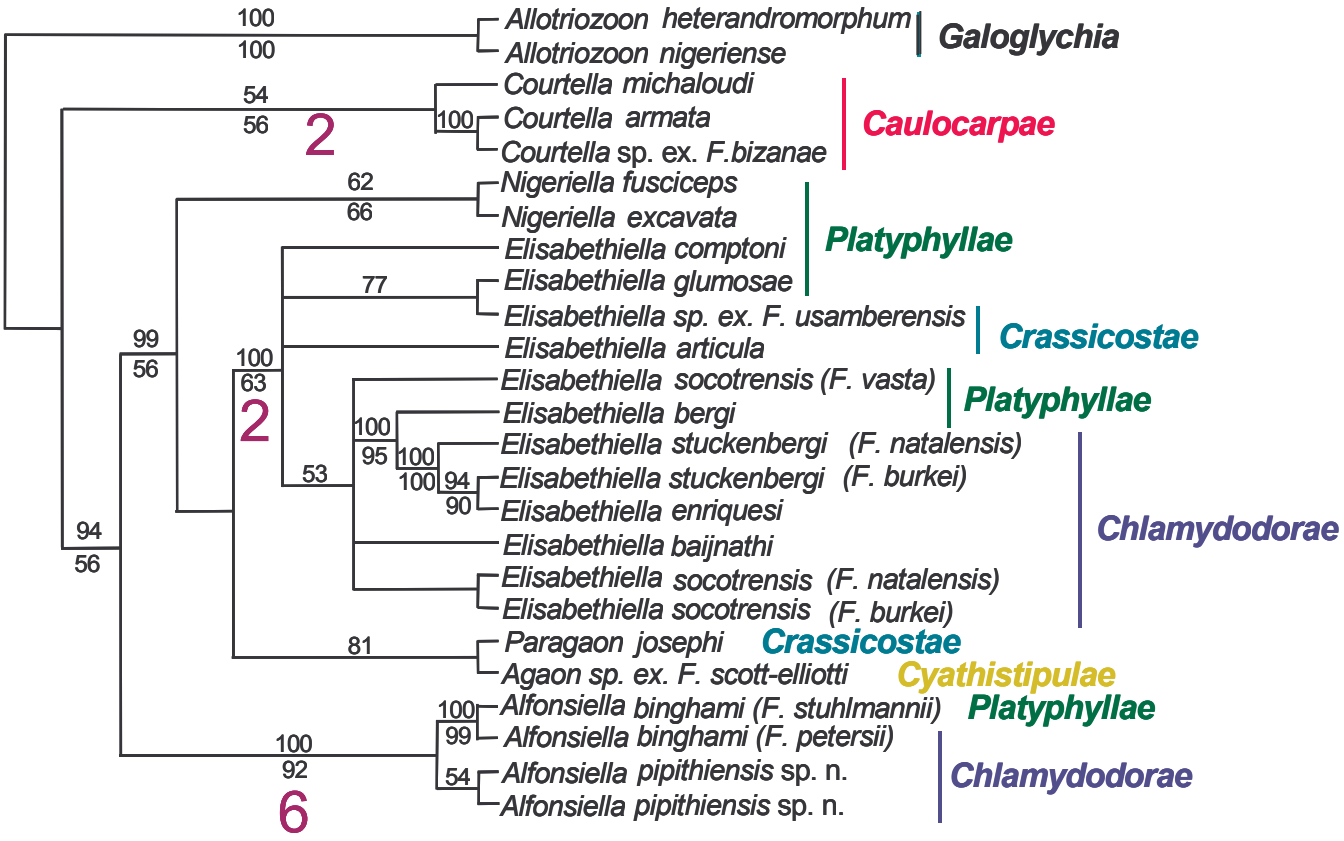

Figure 2.3 Consensus tree of MP, ML and Bayesian analyses of ITS2 data.

Values above branches indicate Bayesian support and values below the branches show MP bootstrap support. Numbers in bold under the nodes supporting the monophyly of wasp genera indicate the decay index. Two *Allotriozoon* species were chosen as outgroup. Association with *F. chlamydocarpa*, *F. bizanae, F. scassellatii, F. natalensis, F. burkei* and *F. vasta* are indicated for the respective pollinators. Associations with host subsections are indicated on the right-hand side.

Combined phylogenetic analysis of 28S and ITS2 sequences

The ILD test detected significant incongruence between ITS2 and 28S data sets (P = 0.001). The ILD test, however, does not distinguish whether incongruence between data set results from different phylogenetic histories or different rates of evolution (de Queiroz *et al*. 1995), it has also been shown that it is not a good measure of incongruence when data sets differ in size (Dowton & Austin 2002). The difference in tree topology seemed to be limited to a few nodes (phylogenetic positions of the genera relatively to each other). ITS2 sequences seem to evolve more rapidly than 28S sequences, a feature which is sufficient to explain the lack of congruence detected by the ILD test. Combined analysis was therefore performed.

Allotriozoon was designated as the outgroup. The aligned 28S and ITS2 were 1296 base pairs. MP heuristic searches gave four most parsimonious trees based on 193 parsimony informative characters (L = 754, CI = 0.684, RI = 0.561). The -ln likelihood score of the ML tree obtained via heuristic searches was 5454.06081. *Nigeriella*, *Alfonsiella*, *Elisabethiella* and *Courtella* formed strongly supported monophyletic clades and *Agaon* and *Paragaon* clustered together in all analyses (Figure 2.4). To test more specifically the monophyly of wasp genera, the non-monophyly of each genus was enforced by conducting reverse constraints searches in PAUP* under both MP and ML criteria. Each time, the trees obtained were longer or less likely than the best unconstrained trees. However, the topologies obtained were not significantly different according to Wilcoxon rank tests and Shimodeira-Hasegawa tests (Shimodaira & Hasegawa 1999). However, these tests are known to be overly conservative, i.e. prone to type II errors.

The Bayesian, MP bootstrap and ML analyses grouped the *Agaon* and *Paragaon* as a sister group of the *Elisabethiella* clade. Bayesian and MP analyses clustered *Nigeriella* and *Alfonsiella* together, as a sister clade to the group containing *Elisabethiella*, *Agaon* and *Paragaon*. However, according to ML analyses, *Nigeriella* grouped as the sister species to a clade formed by *Elisabethiella*, *Agaon* and *Paragaon*. *Courtella* formed the sister genus to the rest of the sampled species for all analyses, excluding the specified outgroup.

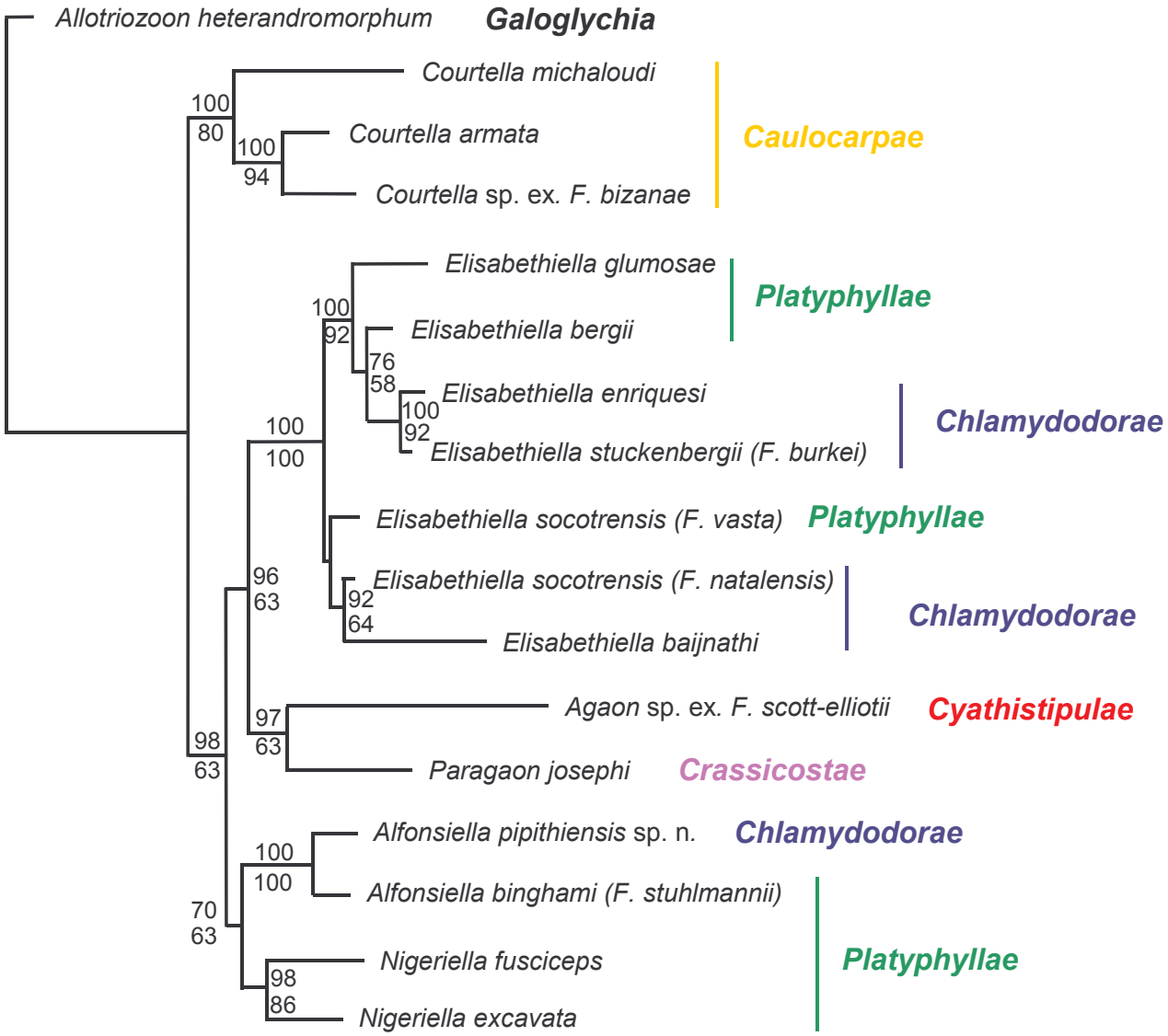

Figure 2.4 Tree inferred from Bayesian analysis of 28S/ITS2 combine data set. *Allotriozoon heterandromorphum* was chosen as outgroup. The values above the branches are Bayesian posterior probabilities. *Ficus* subsections are given on the right-hand side.

***Alfonsiella binghami* species analysis**

The phylogenetic relationships among the *Alfonsiella* pollinators of *F. stuhlmannii*, *F. petersii*, and *F. craterostoma* were investigated with ITS2 intergenic spacer sequences. Bayesian, MP and ML analyses all gave similar results (Figure 2.5). The MP heuristic search retained 2162 trees based on 182 informative characters (L = 149, CI = 0.970, RI = 0.988).

Ficus craterostoma pollinators formed a well-supported clade, which formed the sister taxa to the *Alfonsiella* specimens reared from *F. stuhlmannii* and *F. petersii*. The *Alfonsiella* specimen collected from *F. craterostoma* from Tanzania had quite a different sequence compared to the rest of the *F. craterostoma* pollinators, but clustered with them. The *F. stuhlmannii* and *F. petersii* pollinators clustered together. The *F. stuhlmannii-F. petersii* pollinators were divided into two clades, the *F. stuhlmannii* samples from Tanzania and Lekgalameetse (South Africa) clustered together and the rest of the *F. stuhlmannii* pollinators and the four *F. petersii* pollinators formed a clade. The three *F. stuhlmannii* and four *F. petersii* samples that clustered together were collected in Nelspruit at different time intervals. The morphological characters for these wasps were re-evaluated *a posteriori*, based on the clustering of *F. craterostoma* pollinators. Morphological characters validated the results obtained with ITS2 DNA sequences. The description of a new *Alfonsiella* species is extended in the next section, with a revision of the key to the *Alfonsiella* species.

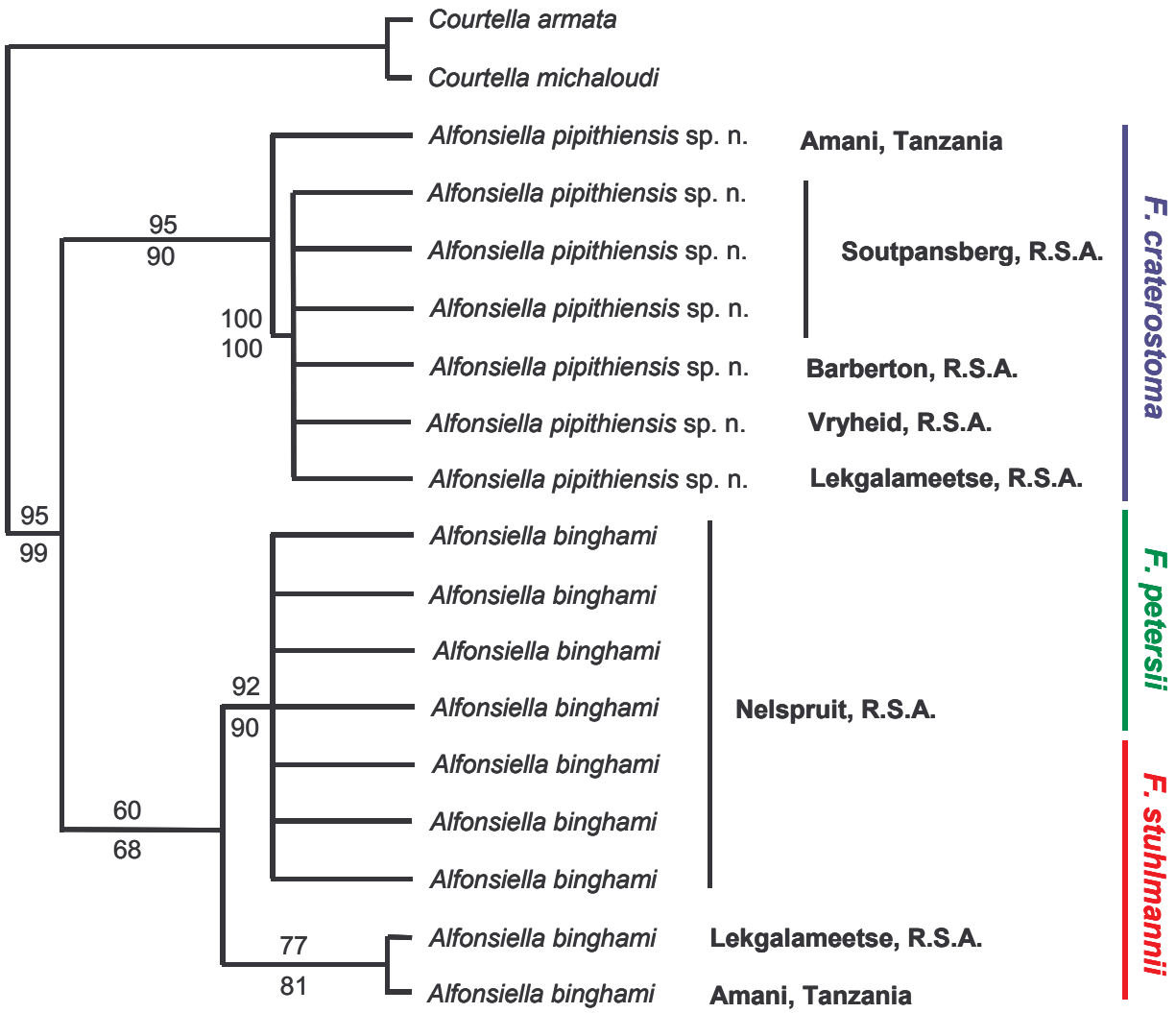

Figure 2.5 Phylogenetic relationships of *Alfonsiella binghami* specimens inferred from Bayesian analyses of ITS2 data.

Two *Courtella* species were chosen as outgroup species. Values above the branches are Bayesian posterior probabilities, while values below branches indicate MP bootstrap support. The localities where the samples were collected, as well as host associations are indicated on the right-hand side.

Taxonomic Treatment

Genus *Alfonsiella* Waterston, 1920

Alfonsiella Waterston, 1920: 198. Type species *Alfonsiella fimbriata* Waterston, by monotypy.

Distribution and host relationships. Summarized in Table 2.3.

Table 2.3 Host relationships and distribution of *Alfonsiella* species.
Host names between commas indicate uncertainty regarding their identification.

***Alfonsiella* species**	***Ficus* species (recorded localities)**
A. binghami	*F. stuhlmannii* (South Africa, Zambia, Tanzania)
	F. petersii (South Africa, Zambia, Malawi)
A. pipithiensis	*F. craterostoma* (South Africa)
A. michaloudi	*F. craterostoma* (Gabon)
	F. lingua lingua (Gabon)
A. bergi	*F. nigropunctata* (Zambia)
A. fimbriata	*F. natalensis leprieurii* (Gabon, Cameroon, Ivory Coast)
	F. kamerunensis (Gabon, Ivory Coast, Guinea, Tanzania)
	F. "dekdekena" (Uganda)
A. brongersmai	*F. burkei* (Zimbabwe)
	F. petersii (Zambia)
	Ficus sp. (Kenya)
	At light (Nigeria)
A. longiscapa	*F. natalensis natalensis* (Malawi, Zambia)
	F. cf. *burkei* (Zambia)
	F. "natalensis leprieurii" (Guinea, Nigeria, Ivory Coast)
A. natalensis	*F. "natalensis"* (Uganda, Kenya)

Key to *Alfonsiella* species (modified from Wiebes 1988)

FEMALES

1. Head distinctly longer than wide (almost 1.5X). Antennal scape with an apical projection. Mandibular appendage with 35-40 hook-like ridges and fine crenulations 2
- Head approximately as long as wide (0.95-1.1X). Antennal scape without an apical projection. Mandibular appendage with 15-20 hook-like ridges..... 3
2. Longitudinal diameter of the eye 1.3X the length of the cheek. Apical projection of the antennal scape wide; the pedicel rather slender: almost 3X as long as wide. Mandibular appendage with about 40 ridges, ex *F. "natalensis"* ..... *A. natalensis*
- Longitudinal diameter of the eye 1.5X the length of the cheek. Apical projection of the antennal scape narrow; the pedicel wider: about twice as long as wide. Mandibular appendage with about 35 ridges, ex *F. n. natalensis* & *F.* cf. *burkei* ..... *A. longiscapa*
3. Lateral ocelli present 4
- Ocelli absent 7
4. Longitudinal diameter of the eye two or three times the length of the cheek 5
- Longitudinal diameter of the eye five times the length of the cheek. Mandibular appendage with about 17 ridges, ex *F. nigropunctata* ..... *A. bergi*
5. Longitudinal diameter of the eye twice the length of the cheek. Mandibular appendage with 20 ridges, ex *F. burkei* & *F. petersii* (Zambian population) ..... *A. brongersmai*
- Longitudinal diameter of the eye 2.7-3 times the length of the cheek. Mandibular appendage with 14-17 ridges 6
6. Postgenal suture running parallel to post occipital suture (Figures 2.6 B,C arrowed); mandible usually with 2 complete and 3-4 incomplete transverse lamellae; mandibular appendage with 13-16 ridged teeth (Figures 2.6 B,C), ex *F. stuhlmannii* & *F. petersii* (South African population) ..... *A. binghami* Wiebes

- Postgenal suture converging towards post occipital suture (Figure 2.6 A arrowed); mandible usually with 3 complete and 3-4 incomplete transverse lamellae; mandibular appendage with 15-18 ridged teeth (Figure 2.6 A), ex *F. craterostoma* (South African population) .... *A. pipithiensis* sp. nov.

7. Mandibular appendage with about 15-18 hook-like ridges consisting of one tooth, ex *F. natalensis leprieurii* & *F. kamerunensis* ..... *A. fimbriata*

- Mandibular appendage with more than one tooth (or crenulation) in the 18 ridges, ex *F. craterostoma* (West African population) & *F. l. lingua* .....*A. michaloudi*

MALES

1. Antennal scape relatively slender 2

- Antennal scape more robust, distinctly club-like 5

2. Head quadrate, as wide as long, ex *F. nigropunctata* ..... *A. bergi*

- Head longer than wide 3

3. Head 1.15X longer than wide, ex *F. natalensis leprieurii* & *F. kamerunensis* .... *A. fimbriata*

- Head 1.25X longer than wide 4

4. Lateral sulcus on head complete and well-defined, extending from eye to vertex (reaching under pronotal overlap on head) (Figures 2.7 B,C arrowed); no fovea on posterior eye margin (Figures 2.7 B,C); mandibles elongate 3.5X longer than medial width, inner margin smooth; posterior extensions sharp (Figures 2.7 E,F), ex *F. stuhlmannii* and *F. petersii* (South African population)

.....*A. binghami* Wiebes

- Lateral sulcus on head incomplete, only extending a third of the distance to vertex (Figure 2.8 A); strong fovea situated on posterior eye margin (Figure 2.7 A arrow); mandibles squatter, twice maximum width, inner margin basally with blunt tooth; posterior extensions blunt (Figure 2.7 D), ex *F. craterostoma* .....*A. pipithiensis* sp. nov.

5. Head 1.15X as long as wide, almost as broad (0.90X) anteriorly as posteriorly, ex *F. burkei* & *F. petersii* (Zambian population) ..... *A. brongersmai*

- Head 1.25X as long as wide, narrows anteriorly (width across eyes 0.75-0.80X posterior head width) 6

6. ex *F. craterostoma* & *F. l. lingua* ..... *A. michaloudi*

- ex *F. n. natalensis* & *F.* cf. *burkei* 7

7. Dorsal edge of the hind tibia with less than fifteen conical spines, ex *F. n. natalensis* & *F.* cf. *burkei* ..... *A. longiscapa*

- Dorsal edge of the hind tibia with more than fifteen conical spines, ex *F. "natalensis"* .... *A. natalensis*

***Alfonsiella pipithiensis* sp. n.**

(Figures 2.6 A,D; 1.7 A,D)

Holotype

♀ (slide mounted): SOUTH AFRICA, *Limpopo Province*, Pipithi waterfalls, 22º52.5'S 30º22.5'E, 20.i.1987, S.G. Compton & V.K. Rashbrook, C12, ex *F. craterostoma* (SAMC).

Paratypes

(SAMC; SANC). Series ♀♀, ♂♂: same data as holotype; series ♀♀, ♂♂: SOUTH AFRICA, *Limpopo Province*, Magaboeskloof, De Hoek State Forest, 23º50'S 30º01'E, 1.ix.1989, S. van Noort & A.B. Ware, C144, ex *F. craterostoma*; series ♀♀, ♂♂: Pipithi waterfalls, 22º52.5'S 30º22.5'E, 19.xi.1991, A.B. Ware, C361, ex *F. craterostoma*; series foundress ♀♀, *Gauteng*, Pretoria, University of Pretoria campus, 25º45.19'S 28º13.82'E, 28.xi.1999, planted tree; 1♀: *Mpumalanga*, 10km E of Barberton, rd. to Shiyalongubo dam, 1500m, 25˚47.90′S 31˚08.51′E, 10.xii.1999, S. van Noort, J.M. Greeff & F. Kjellberg, KW99-F44, ex *Ficus craterostoma*; series ♀♀, ♂♂: ditto, but 25˚47.84′S 31˚08.74′E, KW99-F45; series ♀♀: Soutpansberg, Hangklip, 22˚59.962′S 29˚52.940′E, 1523m,

iii.2002, J.C. Erasmus & J.M. Greeff, SPB11-32002, ex *F. craterostoma*; series ♀♀: Soutpansberg, Piesanghoek, 23˚02.852′S 30˚02.852′E, 1142m, iii.2002, J.C. Erasmus & J. Greeff, SPB29-32002, ex *F. craterostoma*; series ♀♀, ♂♂: Soutpansberg, Entambeni, 23˚00.831′S 30˚14.668′E, 1250m, iii.2002, J. C. Erasmus & J. Greeff, SPB47-32002, ex *F. craterostoma*; series ♀♀: ditto, except 1255m, SPB49-32002; series ♀♀: Lekgalameetse, 24˚09.580′S 30˚13.419′E, 1109m, i.2003, J.C. Erasmus, E. Jousselin, J. Pienaar & R.M. Nelson, LGMS2-12003, ex *F. craterostoma*; series ♀♀, *Mpumalanga*, Buffelskloof Private Nature Reserve, Calodendrum Kloof, 25º17.875'S 30º30.595'E, 1400m, 6.x.2001, S. van Noort & J.M. Greeff, PT01-F12, ex *Ficus craterostoma*, Afromontane forest (foundress female remnants extracted from C-phase figs).

Etymology

Named after the type locality.

Distribution and host affinities

Alfonsiella pipithiensis is only known from South Africa. The host fig is *Ficus craterostoma* Mildbr. & Burret. In southern Africa *F. craterostoma* is restricted to remnant patches of Afromontane forest. A different pollinator species, *A. michaloudi* is associated with *F. craterostoma* in Gabon. It is possible that these two host fig populations are distinct at species level, as the non-pollinating wasp faunas associated with the two populations are also different.

Diagnosis

Female

Morphologically very similar to *A. binghami* Wiebes, but mandible usually with 3 complete and 3-4 incomplete transverse lamellae and mandibular appendage with 15-18 ridged teeth. *Alfonsiella binghami* usually only has 2 complete transverse lamellae and 13-16 ridged teeth. The postgenal suture converges towards the post occipital suture, whereas in *A. binghami* the suture runs parallel to the post occipital suture.

Male

The lateral sulcus that extends posteriorly from the eye does not reach the vertex of the head as it does in *A. binghami*. Two strong fovea are situated on the posterior eye margin; the ventral fovea forms part of the beginning of the sulcus (*A. binghami* has a slight fovea at start of the sulcus, but no fovea on the dorso-posterior margin of the eye); mandibles are squatter (twice maximum width) than in *A. binghami* (3.5X longer than medial width), inner margin has a blunt tooth basally (absent in *A. binghami*). Head usually dark brown as opposed to light yellow in *A. binghami*.

Description

Female (holotype).

Head and mandibles dark brown; dorsal half of mesosoma and metasoma, as well as the hypopygium, ovipositor valves and antennal flagellum lighter brown. Rest of body including legs and antennal scape pale.

Head quadrate, 1.02X wider than long. Eye 2.67X longer than cheek length. Two lateral ocelli. Antenna with eleven segments, the fifth to eleventh segments each with a single row of elongate sensilla, 3-4X the length of the segment from which they arise; the last three segments not forming a club; scape elongate, with a bluntly produced tooth medially on the ventral edge; scape not prolonged beyond the base of the pedicel. Mandible with a single strong apical tooth and 6-7 ventral ridges (3 apical ridges transversely complete; 3-4 basal ridges short, incomplete); the mandibular appendage with 15-18 hook-like ridges, 6X longer than wide (Figure 2.6 A). Postgenal suture converges towards the post occipital suture (Figure 2.6 A arrowed).

Mesosoma: pronotum with deep, medial, smoothly concave posterior invagination. Mesonotum 1.5X wider than long. Fore wing twice as long as wide; postmarginal vein shorter than stigmal vein; submarginal, marginal, stigmal and postmarginal veins in the ratio 28:6:7:5. Hind wing 0.6X length of fore wing. Propodeal peritremata narrow and long, 0.57X as long as the propodeum. Fore femur 3.75X longer than wide; fore tibia with 2 teeth on dorso-apical margin and one on ventro-apical margin; fore coxa with

pollen pocket; hind femur 1.5X longer than wide; hind tibia with a single small tooth on dorso-apical margin and two spurs (one twice the length of the other) on ventro-apical margin. Mesosternum with pollen pockets.

Metasoma with medium-sized spiracular peritremata, ovipositor sheaths as long as the metosoma.

Male (paratype).

Head dark brown, body usually very pale yellow, but can be as dark as the head.

Head 1.23X longer than wide, narrowing anteriorly. Lateral sulcus extending posteriorly from eye a third of distance between eye and anterior edge of pronotum (Figure 2.7A). Distinct foveal pit present on dorso-posterior margin of eye (Figure 2.7A arrowed). Antennal toruli separated by slightly less than the width of a torulus. Scape robust, twice as long as wide, club-like. Antenna with six segments, the pedicel as long as the first two flagellar segments combined; club large. Mandible robust (twice maximum length) with single apical tooth, small blunt tooth on posterior inner margin; posterior projection of mandible blunt, a third of mandible length (Figure 2.7 D).

Mesonotum. Pronotum 1.2X wider than long. Fused mesonotum, metanotum and propodeum 1.3X wider than long. Propodeal spiracles transversely oval, 1.5X wider than long. Fore femur 1.3X longer than wide; fore tibia with 2 teeth on dorso-apical margin and one on ventro-apical margin; hind femur 2.7X longer than wide; hind tibia with two spurs (one twice the length of the other) on ventro-apical margin.

Metanotum in unexpanded form shorter than mesonotum.

***Alfonsiella binghami* Wiebes**

Alfonsiella binghami Wiebes 1988: 432-434; Berg & Wiebes, 1992: 249-250 (summary, key).

Material examined.

Alfonsiella binghami: Holotype ♀ (RMNH); series ♀♀, ♂♂: MALAWI, Mt Mulanje, Forestry Station, 7.vii.1990, S.G. Compton, C322, ex [*F. thonningii* B] = *F. petersii*; series ♀♀, ♂♂: 5 km S. of Nkhotakota, 12º57.7'S 34º18'E, 18.ii.1995, J.E. & S.M. Burrows, JEB 5825, foundress ex *F. petersii*; series ♀♀, ♂♂: SOUTH AFRICA, *KwaZulu-Natal*, Hluhluwe Game reserve, 2832AA, 5.xii.1986, S.G.

Compton & A.J. Gardiner, C38, ex *F. stuhlmannii*; series ♀♀, ♂♂: Mbaswana, 2732DA, 9.xii.1986, SGC, C39, *F. stuhlmannii*; series ♀♀, ♂♂: Pongola, 6.ix.1989, S. van Noort & A.B. Ware, C148, *F. stuhlmannii*; series ♀♀, ♂♂: Ingwavuma, 23.i.1990, S. van Noort & A.B. Ware, C244, *F. stuhlmannii*; series ♀♀, ♂♂: Tshongwe, 23.i.1990, S. van Noort & A.B. Ware, C248, *F. stuhlmannii*; series ♀♀, ♂♂: Tshongwe, 23.i.1990, S. van Noort & A.B. Ware, C249, *F. stuhlmannii*; Mbazwana, 26.i.1990, S. van Noort & A.B. Ware, C254, *F. stuhlmannii*; series ♀♀, ♂♂: Mselini, 26.i.1990, S. van Noort & A.B. Ware, C256, *F. stuhlmannii*; series ♀♀, ♂♂: Hluhluwe Nature Reserve, 2832AA, 30.xi.1991, A.B. Ware, C398, *F. stuhlmannii*; series ♀♀, ♂♂: Mselini- Sibaya rd., Riverine, 2732BC, 4.xii.1991, A.B. Ware, C415, *F. stuhlmannii*; series ♀♀, ♂♂: Mbazwana-Hluhluwe road, 2732CD, 7.xii.1991, A.B. Ware, C421, *F. stuhlmannii*; series ♀♀, ♂♂: *Limpopo Province*, Duiwelskloof Pietersburg, 1.ix.1989, S. van Noort & A.B. Ware, C169, *F. stuhlmannii*; series ♀♀, ♂♂: Giyani, 2330BC, 21.xi.1991, A.B. Ware, C373, *F. stuhlmannii*; series ♀♀, ♂♂: Tzaneen, Letaba Estates, 2330CD, 22.xi.1991, A.B. Ware, C378, *F. stuhlmannii*; series ♀♀, ♂♂: *Mpumalanga*, Weltevreden (Pullen's) Farm, 25º34.36'S 31º10.90'E, 1.v.1989, P. Hawks, C72, *F. stuhlmannii*; series ♀♀, ♂♂: Nelspruit, Outspan Citrus Center, 25˚28.82′S 30˚59.66′E, 9.xii.1999, S. van Noort, J.M. Greeff & F. Kjellberg, KW99-F36, ex *Ficus petersii*; series ♀♀, ♂♂: Louw's Creek, 25˚37.72′S 31˚18.23′E, 10.xii.1999, S. van Noort, J.M. Greeff & F. Kjellberg, KW99-F48, ex *Ficus petersii*; S. van Noort, J.M. Greeff & F. Kjellberg, KW99-F47: *Ficus petersii*; 2♀♀, Nelspruit, JC2001-11, J.C. Erasmus ex *F. stuhlmannii*; 2♀♀, Nelspruit, JC2001-36, J.C. Erasmus, ex *F. stuhlmannii*; 1♀, Ofcoloco, 24º09.239'S 30º25.917'E, 575m, iii.2003, J.C. Erasmus, J. Pienaar, E. Jousselin, R.M. Nelson, ex *F. stuhlmannii*; series ♀♀, ♂♂: Nelspruit, c/o Louis Trichardt and Aurora streets, v.2002, J.C. Erasmus, ex *F. petersii*; series ♀♀, ♂♂: SWAZILAND, road to Big bend, 2631DD, 29.xi.1991, A.B. Ware, C393, *F. stuhlmannii*; series ♀♀, ♂♂: TANZANIA, Kisiwani, 4 08.23S 37 57.54E, 8.xii.1995, S. van Noort, ex *F. stuhlmannii*; series ♀♀, ♂♂: Mkomazi Game Reserve, near Kikola Plot, 4 06.72S 38 01.37E, 16.iv.1996, S. van Noort, ex *F. stuhlmannii*; series ♀♀, ♂♂: ZAMBIA, Cathedral, Lusaka, 25.i.1988, R.J. Nefdt, C62, *F. stuhlmannii*; series ♀♀, ♂♂: Lusaka East, Xanadu Farm, 29.xii.1987, R.J. Nefdt, C68, *F. stuhlmannii*; series ♀♀, ♂♂: road to

Luangwa Game Reserve, 11.vii.1990, S.G. Compton, C323, ex [*F. thonningii* B] = *F. petersii* (all SAMC).

Distribution and host relationships.

Alfonsiella binghami has been collected from South Africa, Zambia, Malawi and Tanzania and is recorded as the pollinator of two host fig tree species: *Ficus stuhlmannii* and *F. petersii.*

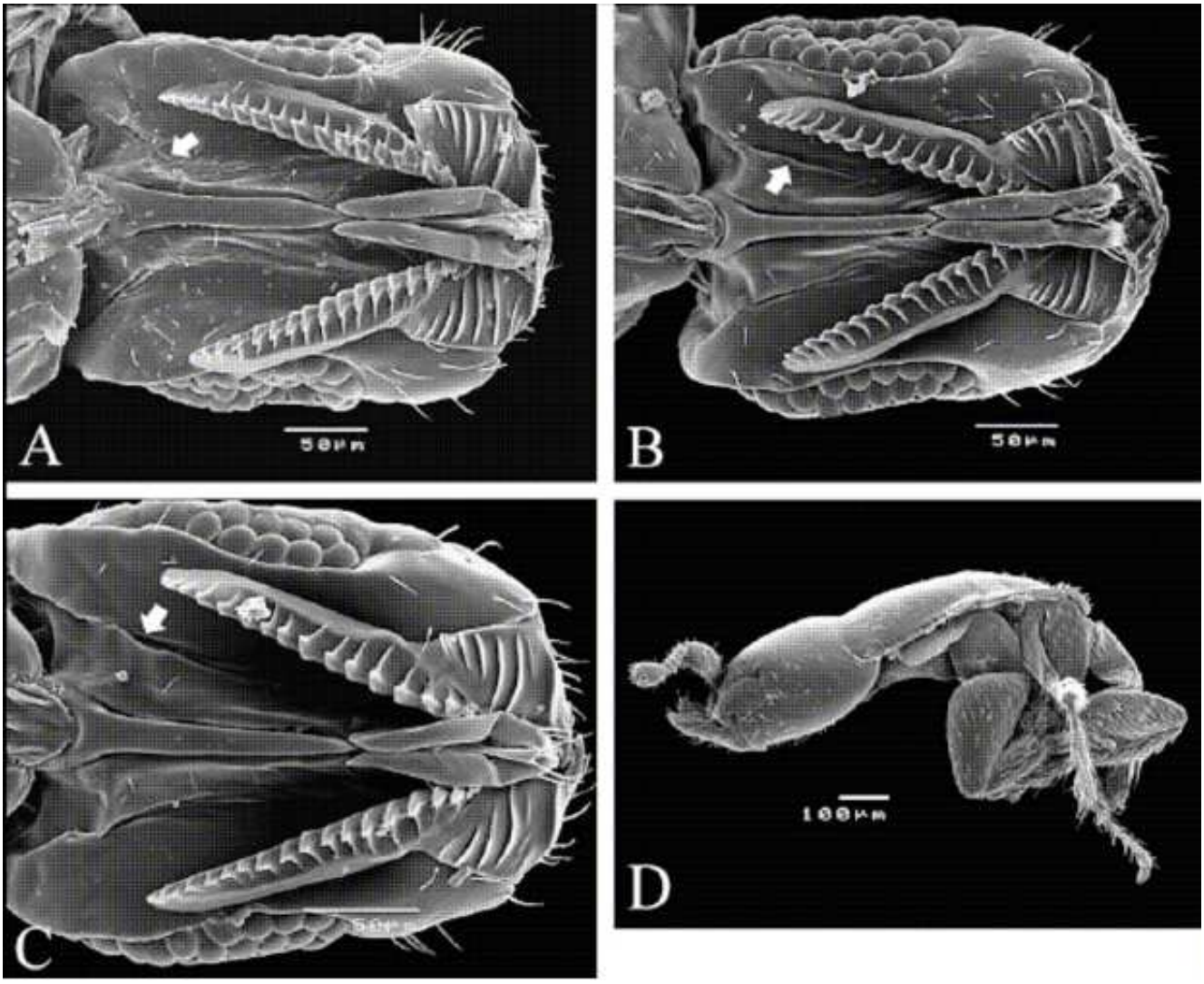

Figure 2.6 Head ventral views of female *Alfonsiella binghami* species group and male *Alfonsiella pipithiensis* sp. n.

A-C: *Alfonsiella* species ♀ head ventral view. A: *Alfonsiella pipithiensis*; B: *Alfonsiella binghami* (ex *Ficus stuhlmannii*); C: *Alfonsiella binghami* (ex *Ficus petersii*). D: ♂ *Alfonsiella pipithiensis* habitus.

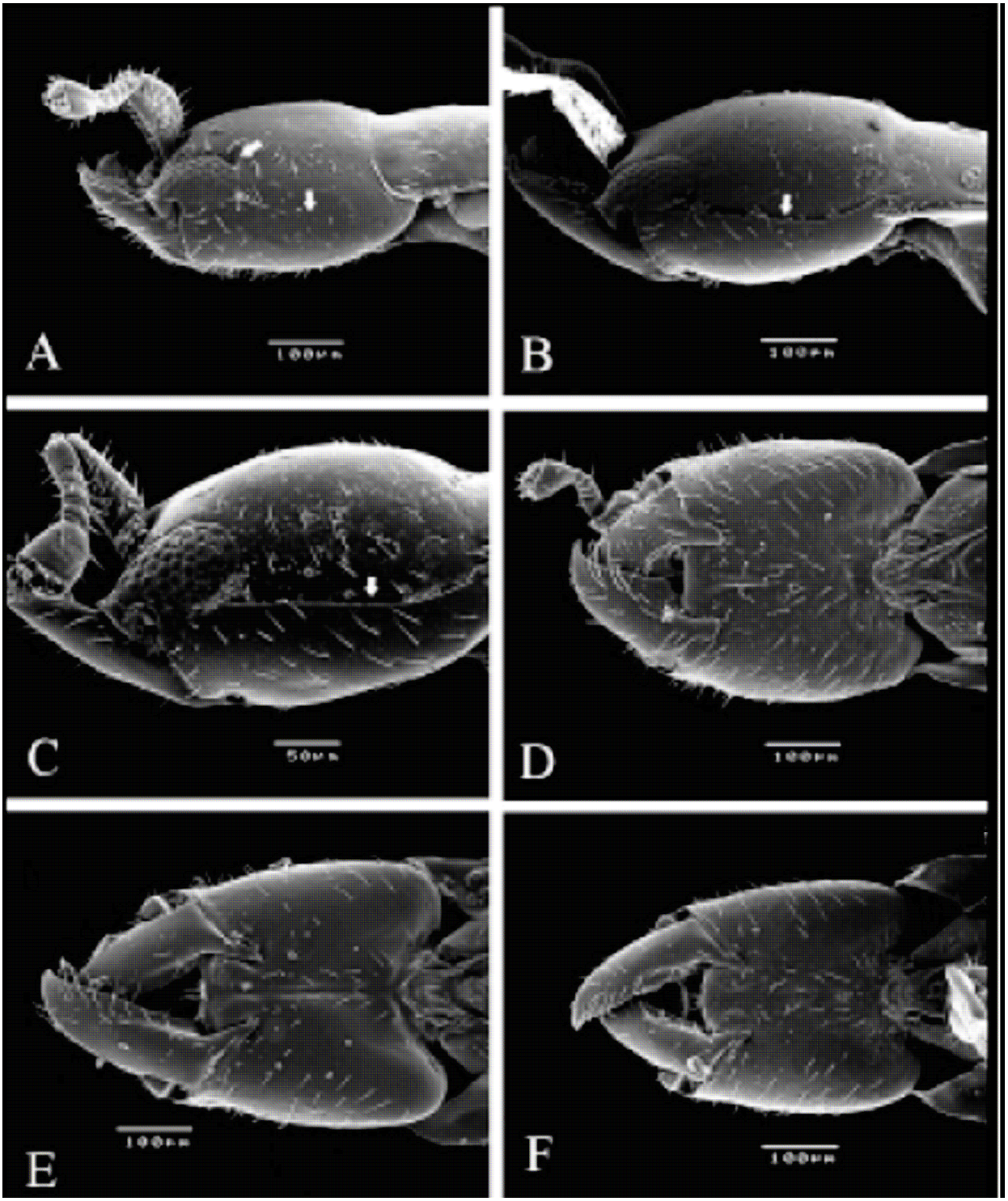

Figure 2.7 Head lateral and ventral views of *Alfonsiella binghami* species
A-C: *Alfonsiella* species ♂ head lateral view. A: *Alfonsiella pipithiensis*; B: *Alfonsiella binghami* (ex *Ficus petersii*); C: *Alfonsiella binghami* (ex *Ficus stuhlmannii*). D-F: ♂ head ventral view. A: *Alfonsiella pipithiensis*; B: *Alfonsiella binghami* (ex *Ficus petersii*); C: *Alfonsiella binghami* (ex *Ficus stuhlmannii*).

Discussion

Validity of fig wasp genera

This study represents the first molecular approach of the phylogenetic relationships of the pollinators associated with *Galoglychia* figs. The phylogenies are in agreement with the delimitation of fig pollinator genera associated with section *Galoglychia*. All analyses of 28S, COI and ITS2 data indicated good support for the clustering of *Elisabethiella*, *Alfonsiella* and *Courtella* species into monophyletic clades. *Courtella* comprises two species-groups delimited by Wiebes (1979b, 1986b). Historically these two groups have been moved in and out of *Agaon* (Michaloud *et al*. 1985; Wiebes 1986b). *Courtella armata* and *C. bekiliensis* belong to the same species-group and are morphologically similar. The undescribed *Courtella* species pollinating *F. bizanae* also belongs to this species-group and is very similar to *C. bekiliensis* (pers. obs). *Courtella michaloudi* belongs to the second and basal species-group (based on morphological characters) and this fits with the molecular reconstructions that group the *Courtella* species together. ITS2 and 28S data indicated that *Nigeriella* and *Agaon* both form monophyletic clades, however, the sampling is limited to two species for each genus. As a result firm conclusions concerning the monophyly of these genera cannot drawn. Nevertheless, to a large extent the molecular data validate the generic classification based on morphological appraisal.

Phylogenetic positions of the genera

Though most genera formed well-defined groups, conflict exists concerning the placement of these groups in the phylogeny. Even though the 28S data were initially used to determine deeper nodes of the *Galoglychia* pollinator phylogeny, support for basal relationships was quite low. However, 28S data suggests that *Allotriozoon* forms the sister group of the rest of the pollinators of the *Galoglychia* section.

All genes support the position of *Courtella* as a sister genus to the rest of the pollinators, excluding *Allotriozoon*. The 28S data strongly support the cluster of *Alfonsiella* and *Elisabethiella* as sister genera, while the ITS2 analyses suggest that *Nigeriella*, *Agaon* and *Paragaon* are more closely related to *Elisabethiella* than to *Alfonsiella.* The COI data divided the two species groups with *Nigeriella* clustering between the two species groups. These uncertainties concerning the relationships between *Nigeriella*, *Alfonsiella* and *Elisabethiella* are in line with the conflict existing in phylogenetic appraisal using morphological characters. Ramírez (1978) suggested that *Alfonsiella* and *Elisabethiella* should form sister genera, while Wiebes (1982) suggested that *Elisabethiella* should be the sister genus of *Nigeriella,* together forming the most derived clade (based on the presence of a derived state for the antennal pedicel, which is somewhat expanded, circular or ovoid in outline and bears axial spines in these two genera) and that *Alfonsiella* was basal to the other *Galoglychia* pollinators. Ramirez's analyses was based largely on pollen pocket morphology, which is a character under strong selection imposed by the mutualistic relationship with figs (Kjellberg *et al.* 2001), whereas Wiebes (1982) took into account 21 characters and suggested the following cladogram based on a manual analyses: (((((*Elisabethiella* & *Nigeriella*) *Agaon*) *Allotriozoon*) *Paragaon*) *Alfonsiella*). *Courtella* was synonymised with *Agaon* at that point in time. The *Elisabethiella*, *Nigeriella*, *Agaon* and *Allotriozoon* clade is supported by the longitudinal division of the pronotum by a sulcus or fine groove (*Alfonsiella* has a whole pronotum; *Paragaon* has a pronotum that is emarginated at the base suggesting a transformation between the whole and divided state). The ITS2 analyses support the existence of the *Elisabethiella*/ *Nigeriella*/*Agaon* clade but exclude *Allotriozoon* from this clade. Hence, none of our analyses (28S, ITS2 or combined) support the phylogenetic hypothesis of Wiebes (1982).

The phylogenetic placement of *Courtella*, *Paragaon*, *Agaon* and *Nigeriella* genera also differed for the 28S and ITS2 data. The 28S data suggests that *Courtella* and *Nigeriella* form closely related genera, while the ITS2 data indicate that these two groups are distantly related. Thus, there is no consensus for the positions of these genera in the global tree. From a morphological perspective *Courtella* is closely related to *Agaon* (Wiebes 1979b, 1986b) and is basal to *Nigeriella* (Wiebes 1982). *Nigeriella* is more closely related to *Elisabethiella* (Wiebes 1982) at least in the female sex (Berg &

Wiebes1992). The results obtained for this study cannot validate nor contradict these assumptions due to a lack of resolution and conflicts between data sets. The relative positioning of *Nigeriella, Courtella* and *Agaon* could probably be improved with a bigger sampling effort in these groups and/or additional genes.

Patterns of association between fig wasps and their hosts: cospeciation and host specificity

Even though the placement of genera in the fig wasp phylogeny is not conclusive, host associations among and within genera could be evaluated (Figures 2.1 to 2.4). It should be noted, however, that the division of *Galoglychia* into six subsections is based on morphological characters and this subdivision has so far not been validated by any thorough phylogenetic analysis. The classification of the *Crassicostae* subsection is especially uncertain (Burrows & Burrows 2003). Nevertheless, *Platyphyllae* and *Chlamydodorae* seem to form two valid subsections (Burrows & Burrows 2003). Recent molecular work (Rønsted *et al*. 2005; Rønsted unp. data) seems to confirm this suggestion, though *F. stuhlmannii* appeared to belong to subsection *Chlamydodorae*, rather than subsection *Platyphyllae* (Rønsted *et al*. 2005). This recent phylogeny also supports the monophyly of sections *Cyathistipulae* and *Caulocarpae*.

The genus *Courtella* is restricted to one subsection (subsection *Caulocarpae*) but *Nigeriella*, *Alfonsiella* and *Elisabethiella* species are not constrained to a specific host subsection (*Elisabethiella* species pollinate hosts from the *Crassicostae*, *Chlamydodorae* and *Platyphyllae* subsections; *Alfonsiella* species pollinate hosts of subsections *Chlamydodorae* and *Platyphyllae*; *Nigeriella* species pollinate hosts from subsections *Crassicostae* and *Platyphyllae*). This questions whether there has been strict cospeciation between *Galoglychia* figs and their pollinators. The higher-level phylogenies suggested that host jumps between different host sections occurred only a few times (Machado *et al*. 2001; Weiblen & Bush 2002). There are probably physical constraints to successfully enter, lay eggs and hatch in the syconium of fig species that are phylogenetically distantly related (Compton 1990;

van Noort & Compton 1996). Additional constraints might include the ability of male wasps to chew a tunnel through the syconium, and the capacity of females to find their hosts. All these factors supposedly limit host switches between unrelated hosts. This study shows that at a lower taxonomic level, within one section (*Galoglychia*), pollinator phylogeny does not reflect host taxonomy. If one assume that fig subsections form monophyletic clades, this suggests that switches between subsections have been frequent during the course of evolution. This suggests that the pollinator/fig tree associations for *Elisabethiella*, *Alfonsiella* and *Nigeriella* do not result from cospeciation but probably from several host shifts. An alternative but not mutually exclusive scenario explaining this incongruous pattern of association could be multiple radiations of several pollinating wasp genera onto *Galoglychia* figs followed by asymmetrical extinctions. A well-resolved molecular phylogeny at the section level with appropriate dating will be necessary to test these alternative scenarios.

The fact that strict cospeciation does not seem to be the rule when looking at patterns of association at the subsection level is not surprising. Indeed, for cospeciation to occur in an interspecific interaction, one of the prerequisites is that the partners are specific. Several cases of breakdown of specificity in *Galoglychia* figs have been observed (Michaloud *et al.* 1985; Rasplus 1996), with the same host fig sometimes being pollinated by different pollinators and the same pollinator species pollinating different fig species. However, these observations can easily be due to identification mistakes. The molecular results confirmed that the same host fig could be pollinated by several pollinators. For instance *F. natalensis* pollinators (*E. socotrensis*, *E. stuckenbergii*) did not cluster together, nor did the two pollinators of *F. abutilifolia* (*Nigeriella fusciceps* and *Elisabethiella comptoni*), which confirms that there are different species pollinating these two hosts. On the other hand *E. socotrensis* pollinating different host species do not always cluster together, which suggests that specimens identified *a priori* as *E. socotrensis* could encompass different species, each being specific to a particular host fig. Similar results were found on *E. stuckenbergi*. However, these results on *E. stuckenbergi* and *E. socotrensis* are preliminary and more extensive sampling and morphological studies would be necessary to conclude the status of these species. This kind of approach was conducted on *Alfonsiella binghami*. The *F. craterostoma* pollinators occurring in South

Africa were *a priori* identified as *Alfonsiella binghami* specimens. The molecular analyses actually showed that these wasps did not group with the morphologically similar *Alfonsiella* species pollinating *F. petersii* and *F. stuhlmannii* but formed a distinct clade. The morphological characters for these *Alfonsiella* species were re-evaluated. The *Alfonsiella* species pollinating *F. craterostoma* in South Africa showed distinct characters and a separate species is described, *Alfonsiella pipithiensis* sp. n. *Alfonsiella michaloudi* pollinates *F. craterostoma* at low altitudes in lowland rainforest (central and west African population), whereas *A. pipithiensis* sp. n. pollinates *F. craterostoma* populations occurring at higher altitudes in afromontane forest (southern African population). This suggests that similar to *F. ottonifolia* (Michaloud *et al.* 1985) and several Panamanian fig trees (Molbo *et al.* 2003), *F. craterostoma* is pollinated by two closely related wasp species. The two pollinators of *F. craterostoma* could therefore specialize in different habitats. It is not clear whether these populations are completely allopatric, but if there is some sympatry then they may still be separated through occupying different ecological niches determined by altitude. This scenario resembles that in *F. ottonifolia ottonifolia* in Gabon (Michaloud *et al*. 1985) and *F. sur* in west Africa (Kerdelhue *et al*. 1999) where some niche separation occurs: one pollinator species is more prevalent in the forest habitat and the other is dominant in savanna habitat. The fact that the non-pollinating wasps from western and southern Africa are also distinct (pers. obs.) suggests that the currently described *F. craterostoma* may in fact be two species supporting two different wasp communities.

The *Alfonsiella* species pollinating *F. stuhlmannii* and *F. petersii* clustered together in a very shallow tree (Figure 2.5). Two explanations seem equally probable given the current resolution. First, since the *F. stuhlmannii* pollinators are paraphyletic containing the *F. petersii* pollinators within the clade, *A. binghami* may have spread onto *F. petersii*, either displacing its original pollinator or filling an empty niche. Second, since the branch leading to the Nelspruit wasps from both hosts shows no internal structure (i.e. just a single polytomy), it may be that this clade is the *bona fide* pollinator of *F. petersii*. In this case, due to *F. petersii*'s dominance in the Nelspruit (South Africa) area (pers. obs.), its pollinator may have colonized the *F. stuhlmannii* population, displacing its original pollinator. The re-evaluation of morphological characters did not detect reliable diagnostic characters to discriminate *F.*

stuhlmannii and *F. petersii* pollinators. With the current resolution, both morphologically and genetically, it is most parsimonious to conclude that one species pollinates both *F. stuhlmanii* and *F. petersii*.

Chapter 3: Temporal, but not geographic genetic differentiation among fig tree pollinator populations in South Africa

Abstract

Several studies have suggested that pollinating fig wasps could disperse between 30 and 55 kilometres. Recently, a population genetic study on the pollinating fig wasp, *Platyscapa awekei*, was conducted on two South Africa populations that are approximately 450 kilometres apart. The F_{ST} value obtained was very low (F_{ST} = 0.011), suggesting that pollinators could travel 10 times further than previously reported. The aim of this study is to verify these results by performing a more detailed sampling effort and include more populations. Seven populations that are up to 348 kilometres apart were sampled in South Africa, over a two-year period. Six highly variable microsatellite loci were used to determine whether genetic differentiation exists within the South African population. Pollinator dispersal to receptive figs takes place within one or two days and pollinators have an incubation period of approximately two months. Breeding populations of *P. awekei* could therefore be genetically structured based on time. Temporal variation within the South African population was therefore determined. Population differentiation estimates indicated that populations are genetically very similar and gene flow between populations is high (F_{ST} = 0.0055). Mantel tests were used to detect correlation between pair-wise population F_{ST} values ($F_{ST}/(1- F_{ST})$) and the corresponding pair-wise time and distance differences. The data indicate that there is a stronger temporal genetic isolation than spatial genetic isolation.

Introduction

When limited gene flow occurs between geographically distant groups of a species, local genetic drift within the different subpopulations will result in population genetic structure (isolation by distance, IBD)(Wright 1943). In a similar way, gene flow between subpopulations could be limited on a temporal scale, with different breeding populations occupying the same geographical area (isolation by time, IBT) (Henry & Day 2005). Various examples of genetic differentiation on a temporal and geographic scale have been recorded among subpopulations (Costa-Ribeiro *et al*. 2006; Jensen *et al*. 2005; Maes *et al*. 2006). European eel (Maes *et al*. 2006) and brown trout (Jensen *et al*. 2005) form two marine examples of subpopulations that show genetic differentiation on temporal level, caused by migration events that result in different breeding populations. Mosquitoes from Brazil form a good example of species that show temporal and geographic genetic differentiation among groups in the population (Costa-Ribeiro *et al*. 2006), while tsetse fly from Kenya and Tanzania have strong genetic differentiation based on geographical level (Ouma *et al*. 2006).

Fig trees produce fig fruit asynchronously and will stay receptive for pollination for approximately two to three weeks (Khadari *et al*. 1995), while minute pollinating fig wasps have a life span of approximately one to two days (Kjellberg *et al*. 1988). Even though pollinators have a short time-window to disperse to receptive fig trees, they are capable of relatively long-distance dispersal (Nason *et al*. 1998; Shanahan *et al*. 2001; Harrison 2003; Zavodna *et al*. 2005). Female pollinators use air currents high above ground level for dispersal and descent once they detect chemical volatiles released by receptive fig trees, and once they are closer to the ground, they actively fly to their host (Ware & Compton 1994a, b; Compton *et al*. 2000; Compton 2002). Pollen studies indicate that the female fig wasps regularly pollinate hosts that are 5.8 to 14.2 km from their natal fig (Nason *et al*. 1998). Harrison (2003) confirmed that fig wasps pollinating monoecious fig trees could disperse 30 kilometres in search of a receptive host. Fig wasp are also known for their capacity to colonize islands that are relatively far from the mainland. Long Island is approximately 55 kilometres from Papua New Guinea and was effectively populated by fig wasps from the mainland after extinction of fig trees on

the island (Shanahan *et al*. 2001), while Zavodna *et al*. (2005) compared microsatellite data for island and inland populations of two pollinator species that occur in Indonesia and discovered that 40 km of sea did not form a dispersal barrier for fig wasps.

Ficus salicifolia serves as the host for *Platyscapa awekei* and normally occurs in deciduous or semi-deciduous woodland in the eastern parts of Africa from North Africa to South Africa (Burrows & Burrows 2003). In South Africa, *F. salicifolia* occurs from the northern regions into Zimbabwe and Botswana and stretch to the northern regions of Kwazulu-Natal almost to Durban (Fig 3.1) (Burrows & Burrows 2003). A preliminary study by Jansen van Vuuren *et al*. (2006) concluded that two distant *P. awekei* populations (approximately 450 km apart) sampled during early July 2003 show low levels of genetic differentiation between them (F_{ST} = 0.011). This indicates that pollinators might have a tenfold increase in effective dispersal, compared to published results. Given the short life span of pollinators and their approximate two-month incubation period, female pollinators that disperse from their natal figs at the same time might form distinct breeding populations. If this dispersal cycle is consistent over time, population genetic structure could be influenced according to the isolation by time (IBT) pattern in South Africa. To date, temporal effects on genetic differentiation have not been tested for fig wasps.

The first aim of this study is to confirm the low level of spatial genetic differentiation for *P. awekei* in South Africa through more detailed population sampling. In this study, six variable microsatellite loci were used to analyse genetic differentiation for *P. awekei* collected from five locations in South Africa. In addition, temporal genetic differentiation within the population was investigated by sampling a few populations repeatedly at specific times.

Materials and Methods

Fig wasps biology

Fig wasps (Hymenoptera; Chalcidodae) are the only pollinators of fig trees (Moracea, *Ficus*) and they complete their life cycle in their fig tree hosts (Ramírez 1970; Wiebes 1979; Berg & Wiebes 1992). The

obligate mutualism between these two lineages is in the majority of cases even more specific, with one fig wasp species pollinating only one fig tree species (Ramírez 1970, 1974; Janzen 1979; Herre *et al*. 1997; but see Cook & Rasplus 2003). Female fig wasps, known as foundresses, enter the fig syconium and deposit their eggs mostly in the short-styled gall flowers. Depending on the species, foundresses pollinate the flowers actively or passively (Ramírez 1969; Bronstein & McKey 1989; Berg & Wiebes 1992; Jousselin *et al*. 2001b; Cook & Rasplus 2003). After the development of the larvae, the male pollinators emerge from the galls and copulate with females while still in the galls or shortly after the females emerged (depending on the species) and chew an exit hole through the fig wall (Bronstein & McKey 1989; Berg & Wiebes 1992). The winged females escape through the exit hole and carry pollen to receptive figs, where they in turn will lay their eggs (Bronstein & McKey 1989; Berg & Wiebes 1992). Female pollinators identify species-specific volatiles released by receptive fig trees in order to locate their host (Barker 1985; van Noort *et al*. 1989; Ware *et al*. 1993; Gibernau *et al*. 1997; Grison-Pigé *et al*. 2001). Since pollinating females lay all their eggs in only one fig, inbreeding often occurs between her offspring (Hamilton 1979; Janzen 1979).

Sample collection

Platyscapa awekei females were collected from February 2004 to February 2006 at five different locations in South Africa (table 3.1, Fig 3.1). The pair-wise population distances are given in table 3.2. For each of the locations, 32 to 60 fig fruit were collected from each tree. Depending on the development phase of the hosts, the two types of specimens collected were foundresses that entered the fig to lay eggs or offspring ready to disperse to receptive figs. All foundresses were collected from fig syconia and stored in 96% ethanol. In cases where more than one foundress occupied the fig syconium, all the pollinators were used for analyses. Fig fruit for the fig trees that were in the release phase were placed in containers with a mesh lid and fig wasps were allowed to release. The offspring were collected in separate vials for each of the fig fruit and stored in 96% ethanol. One pollinator from each fig syconium was used for DNA extractions.

Table 3.1 *Platyscapa awekei* collection information

Population	Date of Collection	Collection material
1. UP Campus, Pretoria, South Africa	February, 2004	Offspring
2. UP Campus, Pretoria, South Africa	August, 2004	Foundresses
3. UP Campus, Pretoria, South Africa	January, 2005	Foundresses
4. Botanical Gardens, Pretoria, South Africa	February, 2006	Offspring
5. Abel Erasmus Pass, South Africa	August, 2004	Foundresses
6. Nelspruit, South Africa	January, 2004	Foundresses
7. Skukuza, South Africa	October, 2004	Foundresses

DNA procedures

Total genomic DNA was extracted from one pollinator according to the Chelex with proteinase K treatment method described by Estoup *et al*. (1996). PCR reactions were performed with six fluorescent-labeled microsatellite primers designed for *Platyscapa awekei* (Jansen van Vuuren *et al*. 2006). Primers *Pa 1*, *Pa 4* and *Pa 32* were amplified with single reactions, while primers *Pa 4*, *Pa 7* and *Pa 21* were amplified in a multiplex reaction. PCR reactions consisted of approximately 5ng genomic DNA, 1x PCR buffer with 1.5 mM Mg_2Cl (Roche), 160 µM of each of the dNTPs (Promega), 6 µM each of the forward and reverse primers and 0.5 units High Fidelity Expand *Taq* polymerase (Roche) in 10µl reaction volumes. These reactions were subjected to an initial denaturing step at 95°C for 3 minutes, followed by 30 cycles at 95°C for 1 minute, 60°C for 40 seconds and 72°C for 1 minute. Microsatellite sizes were determined with an ABI 3100 sequencer (Applied Biosystems) using the G5 filter set and scored with Genemapper software (Applied Biosystems).

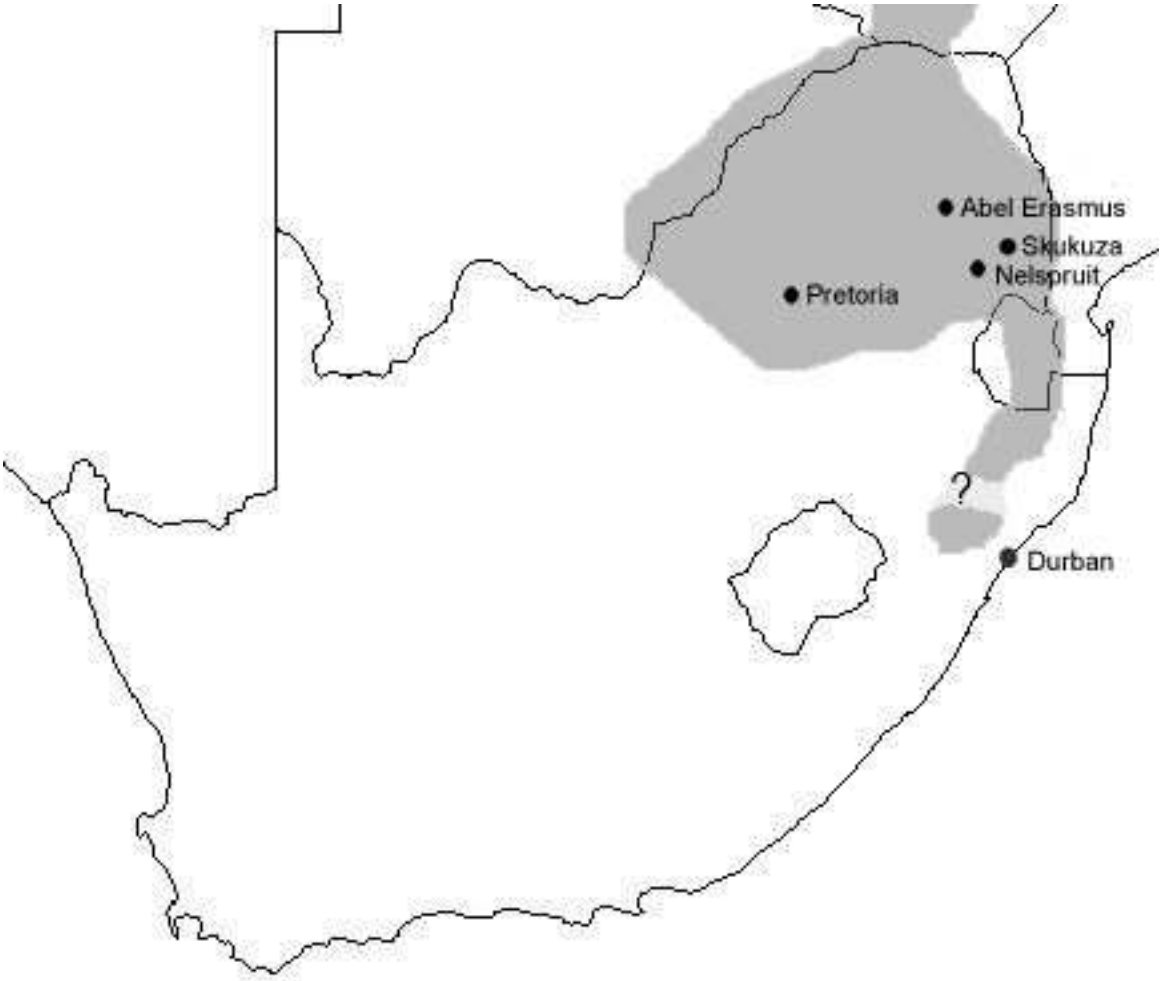

Figure 3.1 Distribution of *Ficus salicifolia* in South Africa.

The gray areas indicate the distribution of *F. salicifolia* in South Africa. Samples were collected at Nelspruit, Pretoria (University of Pretoria Campus and National Botanical Gardens), Skukuza and Abel Erasmus Pass. The distribution was adapted from Burrows & Burrows (2003).

Data analysis

Inbreeding, as well as loci that are in close proximity in the genome, could have an effect on the genotypic disequilibrium of loci in the genome. Many studies indicate that fig wasps have high levels of inbreeding. Genotypic disequilibrium was tested with the log likelihood ratio G statistic incorporated in the software package Fstat version 2.9.3.2 (Goudet 2001). The exact Hardy-Weinberg test was performed with Genepop version 3.3 (Raymond & Rousset 1995). In addition, the score test (U test) was used to detect significant heterozygote deficiency (Rousset & Raymond 1995). The Markov chain method with 500 batches and 5000 iterations per batch was followed for these tests. The number of private alleles for each of the sampled groups, gene diversity, number of migrants (Barton & Slatkin 1986) and expected and observed heterozygosities, as well as F_{IS} and F_{ST} values according to Weir & Cockerham (1984) were determined with Fstat version 2.9.3.2, Genetix version 4.02 (Belkhir *et al*. (2001) and Genepop version 3.3.

In order to estimate the gene flow between populations, pair-wise F_{ST} values were calculated according to Weir & Cockerham (1984). Pair-wise population distance and time matrices are given in table 3.2. The Mantel test, as implemented in Fstat version 2.9.3.2, were used in order to determine the effect of distance and time differences on pair-wise F_{ST} values. Distance matrices were calculated based on linear distances between samples and time matrices were calculated based on time differences, in weeks, between population pairs and correlated with $F_{ST}/(1-F_{ST})$. The incubation period for *P. awekei* is approximately two months; therefore foundresses would precede the offspring by the corresponding incubation period (pers. communication, R. M. Nelson). Population assignment tests and detection of first generation migrants were performed with GeneClass version 2 (Piry *et al*. 2004). Individuals were assigned to populations with Bayesian (Rannala & Mountain 1997; Baudouin & Lebrun 2001) and frequency (Paetkau *et al*. 1995) based methods, using all loci. The frequency for missing alleles was set to 0.01 for the frequency-based method. Probability computation was performed by simulating 10 000 individuals, according to Paetkau *et al*. (2004).

Table 3.2 Pair-wise time and distance differences for sampled *P. awekei* populations

	Nelspruit 01-2004	Campus (Pretoria) 02-2004	Abel Erasmus 08-2004	Campus (Pretoria) 08-2004	Skukuza 10-2004	Campus (Pretoria) 01-2005	Bot. Gardens (Pretoria) 02-2006
Nelspruit 01-2004	–	4 weeks	32 weeks	32 weeks	39 weeks	52 weeks	100 weeks
Campus (Pretoria) 02-2004	275 km	–	36 weeks	36 weeks	43 weeks	56 weeks	104 weeks
Abel Erasmus 08-2004	55 km	258 km	–	0 weeks	7 weeks	20 weeks	68 weeks
Campus (Pretoria) 08-2004	275 km	0 km	258 km	–	7 weeks	20 weeks	68 weeks
Skukuza 10-2004	84 km	348 km	108 km	348 km	–	13 weeks	61 weeks
Campus (Pretoria) 01-2005	275 km	0 km	258 km	0 km	348 km	–	48 weeks
Bot. Gardens (Pretoria) 02-2006	271 km	4 km	254 km	4 km	344 km	4 km	–

Results

Estimates of Genetic Diversity

Based on 2100 permutations for individual populations, no significant linkage disequilibrium was detected at 5% nominal level (Bonferonni-adjusted p = 0.000476) for pair-wise locus comparisons. The pollinator population sampled during August 2004 at the University of Pretoria showed marginal linkage disequilibrium between loci *Pa 8* and *Pa 21*. Comparing individuals from all populations, two of the pair-wise locus tests indicated marginal linkage disequilibrium (p = 0.00048). The populations differed significantly from Hardy-Weinberg equilibrium (p = 0.0000). Significant heterozygote deficiency was detected (p = 0.0000) with the score test. Table 3.3 gives the population statistics over all populations for each locus and table 3.4 the statistics for all loci for each population. The inbreeding coefficient estimate over all populations was 0.362 ± 0.024 with jackknifing over loci. The mean sample size was 42.6. The number of migrants was 10.61 per generation. Compared to the preliminary study of Jansen van Vuuren *et al*. (2006), a few additional alleles were detected for each of the microsatellite loci (table 3.3). The F_{IT} estimate was 0.3636 ± 0.025 for jackknife analysis over all loci.

Population differentiation

F_{ST} values were used to estimate the gene flow between populations. Over all loci, the F_{ST} value was 0.0055. Pair-wise population F_{ST} values are given in table 3.5. Comparing the pair-wise population F_{ST} values ($F_{ST}/(1-F_{ST})$) to pair-wise population distance and time matrices explained 27.89% of the variation in F_{ST} values according to the Mantel test (for ($F_{ST}/(1-F_{ST})$) vs. time, see figure 3.2). The distance component correlation was 0.380326 (p = 0.0896), while the time component correlation was 0.360326 (p = 0.0441). However, when distance was deleted from the model, time on its own explained only 13.43% of the variation and was not significant (p = 0.1034). The number of individuals assigned to the correct population according to the frequency-based criterion (Paetkau *et al*. 1995)

and the two Bayesian methods (Rannala & Mountain 1997; Baudouin & Lebrun 2001) were 26.6%, 26.9% and 20.3%, respectively.

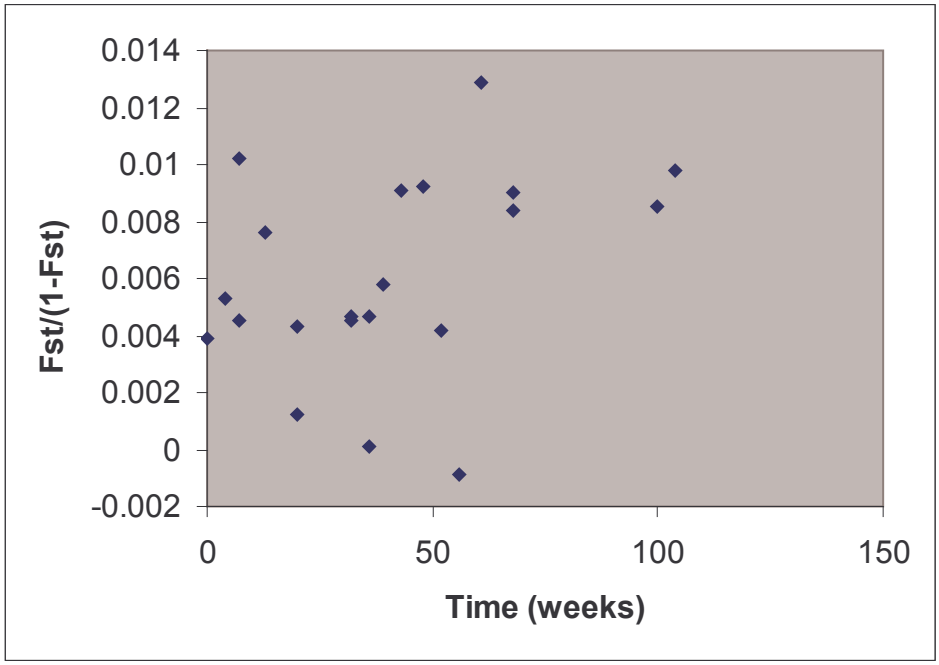

Figure 3.2 $F_{ST}/(1-F_{ST})$ vs. time

Pair-wise population F_{ST} values were used to calculate $F_{ST}/(1-F_{ST})$ and were plotted against the pair-wise population differences for the time component in weeks.

Table 3.3 Population statistics per locus across all populations

Locus	# of alleles	Observed heterozygosity	Expected heterozygosity	Gene diversity	F_{ST}	F_{IS}	F_{IT}
Pa 1	44	0.522	0.940	0.915 - 0.956	0.0083	0.4263	0.4310
Pa 4	42	0.608	0.952	0.940 - 0.968	0.0063	0.3509	0.3550
Pa 7	41	0.528	0.947	0.915 - 0.962	0.0063	0.4152	0.4189
Pa 8	29	0.639	0.870	0.840 - 0.888	0.0005	0.2491	0.2495
Pa 21	45	0.605	0.953	0.934 - 0.972	0.0051	0.3562	0.3595
Pa 32	49	0.587	0.951	0.936 - 0.964	0.0062	0.3657	0.3696
Overall	41.67 (average)	0.582	0.935	0.840 – 0.972	0.0055	0.3602	0.3636

Table 3.4 Population statistics per population for all loci

Population	F_{IS}		Private alleles	N	Expected heterozygosity	Observed heterozygosity
	Value	95% CI				
Nelspruit 01-2004	0.48477	0.35352 - 0.57255	0.50	32	0.9103	0.4841
Campus (Pretoria) 02-2004	0.20370	0.13838 - 0.27352	2.17	46	0.9266	0.7478
Abel Erasmus 08-2004	0.26104	0.18370 - 0.32049	1.67	50	0.9201	0.6888
Campus (Pretoria) 08-2004	0.27134	0.16949 - 0.33004	1.17	47	0.9201	0.6799
Skukuza 10-2004	0.54227	0.44928 - 0.63186	1.00	39	0.9115	0.4268
Campus (Pretoria) 01-2005	0.37718	0.29373 - 0.45130	1.67	60	0.9221	0.5813
Bot. Gard. (Pretoria) 02-2006	0.50884	0.42787 - 0.56775	1.83	46	0.9226	0.4617

Table 3.5 Pair-wise F_{ST} values for sampled *P. awekei* populations

	Nelspruit 01-2004	Campus (Pretoria) 02-2004	Abel Erasmus 08-2004	Campus (Pretoria) 08-2004	Skukuza 10-2004	Campus (Pretoria) 01-2005	Bot. Gardens (Pretoria) 02-2006
Nelspruit 01-2004	–						
Campus (Pretoria) 02-2004	0.0053	–					
Abel Erasmus 08-2004	0.0045	0.0047	–				
Campus (Pretoria) 08-2004	0.0047	0.0001	0.0039	–			
Skukuza 10-2004	0.0058	0.0091	0.0102	0.0045	–		
Campus (Pretoria) 01-2005	0.0042	-0.0009	0.0043	0.0012	0.0076	–	
Bot. Gardens (Pretoria) 02-2006	0.0085	0.0098	0.009	0.0084	0.0129	0.0092	–

Discussion

The populations were not in Hardy-Weinberg equilibrium due to heterozygote deficiency. Heterozygote deficiency is probably due to the large proportion of inbreeding between offspring in the fig syconium. The inbreeding coefficient was high (F_{IS} = 0.36) and corresponded to the high estimates reported by Jansen van Vuuren *et al*. (2006) (F_{IS} = 0.42) for *P. awekei*. Inbreeding coefficients for different pollinator species vary notably, e.g. Zavodna *et al*. (2005) reported F_{IS} = 0.15 - 0.41 for *Ceratosolen bisulcatus* and F_{IS} = 0.30 – 0.58 for *Liporrhopalum tentacularis*, while Molbo *et al*. (2004) reported F_{IS} values of 0.812 – 0.867 for *Pegoscapus hoffmeyeri* sp. A, 0.853 – 1.000 for *Pegoscapus hoffmeyeri* sp. B, 0.231 – 0.709 for *Pegoscapus gemellus* sp. A and 0.127 – 0.212 for *Pegoscapus gemellus* sp. B. These relatively low values for *P. awekei* stems, in part, from the fact that males disperse between figs (Greeff *et al*. 2003).

F_{ST} values lower than 0.05 indicate high levels of gene flow and low levels of genetic differentiation between populations. The very low F_{ST} estimate (F_{ST} = 0.0055) for the *P. awekei* populations indicates very low levels of genetic differentiation among populations. This is corroborated by the low number of private alleles in the population.

Even though the pair-wise population F_{ST} values were low, there was variation in the estimates of F_{ST}. The Mantel test indicates that there is a positive correlation between F_{ST} estimates and time and distance. However, the distance component was not significant and when it was deleted from the model, time lost its significance. This loss is probably due to the non-orthogonal nature of the sample collections. Presently, our data suggest that there is stronger temporal genetic isolation than spatial genetic isolation, but more samples and a more balanced design would be required for a conclusive answer.

The population assignment tests also indicated that temporal and geographic factors do not influence the genetic structure of *P. awekei* populations to a large extent, with only 20.3 – 26.9 % of the samples assigned to the correct population. This is only slightly higher than the random expected value of 14 %.

Chapter 4: Conclusion

According to the phylogenetic analyses of pollinators of the *Galoglychia* section fig trees, we found that fig wasp genera associated with section *Galoglychia* are valid monophyletic clades, which implies a high degree of host switching in the history of the association. We reached the same limits as classical taxonomy concerning the relative placement of genera in the phylogeny. We confirmed some of the taxonomic studies that suggest that African figs can often be pollinated by several host wasps. However, some of our results suggest that some wasps that were *a priori* thought to pollinate several host figs could actually show host-based genetic differentiation and therefore, African fig wasps could be more host-specific than previously assumed. For instance, the *Alfonsiella* pollinator for *F. craterostoma* from South Africa, *A. pipithiensis* sp. n., is genetically and morphologically distinct from the *F. craterostoma* pollinator from west and central Africa, *A. michaloudi*, but is also clearly distinct from *A. binghami*. Morphology and current genetic resolution suggests that *A. binghami* pollinates both *F. stuhlmannii* and *F. petersii*. This study underlines once more the synergism between molecular investigation and thorough morphological systematics to assess the specificity of the fig/ fig wasp relationships. These findings are in line with other studies (Rasplus 1996; Cook & Rasplus 2003; Jackson 2004; Machado *et al.* 2005) that also suggest that the legendary pattern of cospeciation observed in deep nodes breaks down when one looks at fine scale phylogenies of closely related species.

The lack of genetic differentiation in the South African *P. awekei* population based on geographic distance adds to the conclusions of other studies, namely that pollinators have a capacity to disperse over long distances. The populations sampled were all within 348-kilometre range in South Africa. Isolation by distance could be possible should samples be taken from more distant locations, e.g. northern Africa and the Arabian Peninsula. Temporal genetic isolation seems to play a larger role than spatial genetic isolation for the population genetic differentiation, however, a better sampling design is necessary to resolve the scenario.

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Appendix

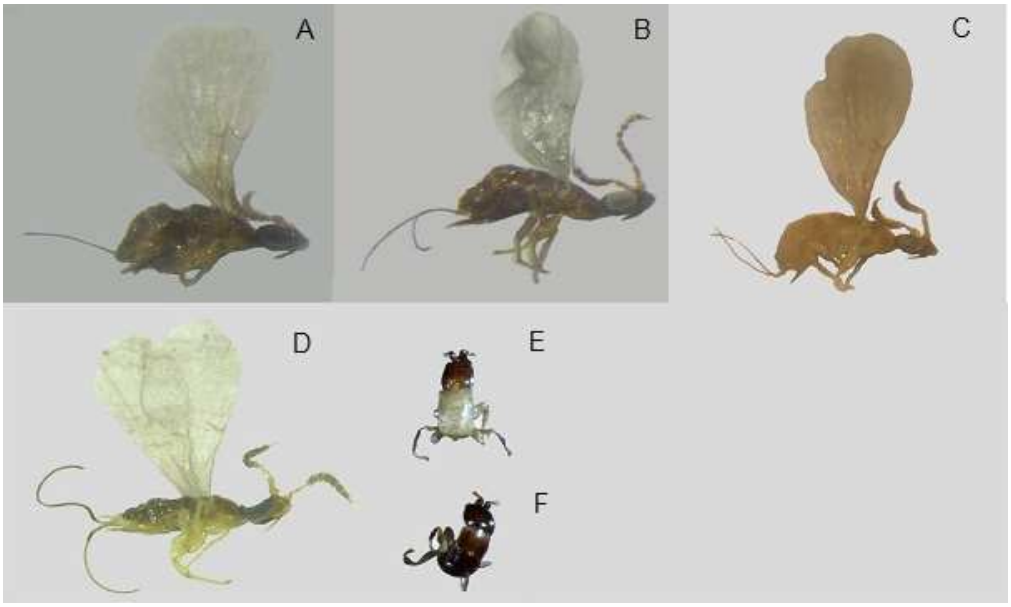

Figure A.1 Pictures of a few *Alfonsiella* species

A: *A. binghami* (♀) pollinating *F. petersii*, **B**: *A. binghami* (♀) pollinating *F. stuhlmannii*, **C**: *A. longiscapa* (♀) pollinating *F. natalensis natalensis* and *F. burkei*, **D**: *A. pipithiensis* sp. n. (♀) pollinating *F. craterostoma*, **E** & **F**: *A. pipithiensis* sp. n. (♂) pollinating *F. craterostoma*. **E** and **F** are the two male morphs for *A. pipithiensis* sp. n.

Figure A.2 Pictures of *Platyscapa awekei*

A: *P. awekei* (♀), **B**: *P. awekei* (♂)