Molecular phylogeny of fig wasp pollinators (Agaonidae, Hymenoptera) of *Ficus* section *Galoglychia*

- *J. Christoff Erasmus,*
- *Simon Van Noort,*
- *Emmanuelle Jousselin &*
- *Jaco M. Greeff*

*J. Christoff Erasmus, Department of Genetics, **University** of **Pretoria**, **Pretoria** 0002, South Africa. E-mail: s9803257@tuks.co.za*
Simon van Noort, Natural History Division, South African Museum, Iziko Museums of Cape Town, PO Box 61, Cape Town 8000, South Africa. E-mail: svannoort@iziko.org.za
*Emmanuelle Jousselin, Department of Genetics, **University** of **Pretoria**, **Pretoria** 0002, South Africa and INRA — Centre de Biologie et de Gestion des Populations (CBGP), Campus International de Baillarguet — CS 30 016 34988 Montferrier-sur-Lez — France. E-mail: jousseli@ensam.inra.fr*
*Jaco M. Greeff, Department of Genetics, **University** of **Pretoria**, **Pretoria** 0002, South Africa. E-mail: jaco.greeff@up.ac.za*

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Abstract

The obligate mutualism between fig trees and their fig wasp pollinators, together with the general tendency for each host species to be 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 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*, *F. stuhlmannii* and *F. petersii*) are morphologically very similar in South Africa. We investigated the possibility that these pollinators form a complex of species with host-based genetic differentiation. 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. nov., 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 to be 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 species specific interaction between yucca moths and yuccas and the association between *Glochidion* tree and *Epicephala* moths (Kawakita *et al*. 2004) are additional example where 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, *Elisabethiella* and *Nigeriella* are closely related morphologically 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 by hand 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 section *Galoglychia* are reported to be pollinated by different wasp species, sometimes even species belonging to different genera (see Table 1 for specific associations; Wiebes 1979a, 1990; Berg 1986; Berg & Wiebes 1992; Compton & van Noort 1992; Rasplus 1996). The 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 agreeing that some of the discrepancies may be resolved through reappraisal of characters, Berg (1989) recognized that the 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 extinction been recognized 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 wasp 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*, *F. craterostoma* and *F. 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 *A. 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, semideciduous woodlands (Burrows & Burrows 2003). Until recently its pollinator had not been collected very often; however, *A. 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 colonized 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 paper has two objectives. First, we investigate the molecular phylogeny of *Galoglychia* pollinators using DNA sequence data, which allows a test of the monophyly of the different genera. By looking at host association, we then discuss whether our reconstruction is congruent with the existing fig tree classification. Second, we specifically focus on the *A. binghami* complex 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 discernible.

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 1). For a couple of wasp species that are known to be associated with several fig species (i.e. *Elisabethiella stuckenbergii*, *E. socotrensis*) we attempted to sample specimens reared from different hosts. Reciprocally, when fig species hosted different pollinator species we attempted to include these wasps. 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 *F. craterostoma*, *F. stuhlmannii*, and *F. petersii*), pollinators of each of the three host fig trees were collected from 4−6 different trees for each host (Table 1). Five *F. stuhlmannii* pollinator samples were collected at five different time intervals and two different locations, six *F. craterostoma* pollinators were collected at seven different locations and four *F. petersii* pollinators were collected at four different time intervals from one location.

DNA extractions were performed with one or several individuals for the phylogenetic analyses of the pollinator genera, and a single individual for the *A. binghami* species group. The protocols for DNA extractions included the DNeasy Tissue Extraction Kit (Qiagen) following the manufacturer's instructions as well as the 10% Chelex 100 DNA extraction method with proteinase K treatment described by Estoup *et al*. (1996). The Chelex extractions were performed with the 100/50–100 mesh instead of the 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 associations for the pollinators of section *Galoglychia*. 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 those of the 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 *A. binghami* group.

The 28S, COI and ITS DNA segments were amplified with 1.5 units *Taq* DNA polymerase (Promega) in 50 μL PCR reactions with 1 × PCR reaction buffer provided by the manufacturer (Promega), 2.5 mM $MgCl_2$, 0.2–0.5 pM 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; Harry *et al*. 1998; Lopez-Vaamonde *et al*. 2001). The PCR conditions for the amplification of the 28S gene segment were 3 min denaturing at 94 °C, followed by 35 cycles of 1 min at 94 °C, 30 s at 55 °C, and 1.5 min at 72 °C, with a final extension of 4 min at 72 °C.

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; Simon *et al*. 1994). The PCR conditions for the amplification of the Pat-Jerry COI DNA region were 3 min denaturing at 94 °C, with 35 cycles for 1 min at 94 °C, 1 min at 45–48 °C, and 1.5 min at 72 °C, with 4 min final extension at 72 °C. The ITS2 intergenic DNA sequences were amplified and sequenced with the ITSF forward and ITSR reverse primers (Table 2; Campbell *et al*. 1993; Lopez-Vaamonde *et al*. 2001). The PCR conditions for the amplification of the ITS2 sequences were 3 min denaturing at 94 °C, followed by 35 cycles for 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, with a final extension of 4 min at 72 °C.

PCR products were purified with a High Pure PCR Purification Kit (Roche). DNA fragments were cycle sequenced with BigDye ver. 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 (*Wolbachia* for instance).

Phylogenetic analyses

Sequence alignments were performed with ClustalW (Thompson *et al*. 1994). When necessary, we modified the default ClustalW alignments obtained by manually inserting/deleting gaps to minimize their numbers. COI sequences were individually checked by eye, verified for protein coding frame-shifts and nonsense codons to avoid pseudogenes (Zhang & Hewitt 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 (MCMC), as well as maximum parsimony (MP) analyses. Phylogenetic analyses were performed with PAUP* (Swofford 2000), for likelihood and parsimony, and MrBayes ver. 3 (Huelsenbeck & Ronquist 2001) for Bayesian analyses.

First, separate analyses were performed on each data set. Unfortunately, we failed to obtain the sequences for the three markers for all specimens; some combination of DNA primers consistently failed to amplify the template. We thus could not attempt combined analyses for the three genes. However, we managed to obtain 28S sequences and ITS2 data for a substantial number of species. 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 MP 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 *A. binghami* species group analysis only.

The models of nucleotide substitution for ML 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 with 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 MCMC approach and sampling according to the Metropolis−Hastings algorithm were performed with one cold 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 using 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 species. Heuristic MP 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 1 depicts the Bayesian 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* (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 (Fig. 1), MP heuristic searches clustered the *E. socotrensis* specimen pollinating *Ficus vasta* as a sister species to a clade formed by *E. bajnathi* 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 bp in length. No frame shift or nonsense 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 (Fig. 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* is 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 that of the *Elisabethiella* species differed slightly between the ML/Mr Bayes 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.

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) suggests that these are due to a high divergence between wasp genera and not to 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 bp 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, Fig. 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); *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 Fig. 3.

Again, in this analysis, *E. stuckenbergi* pollinating different host species did not group together, and *E. socotrensis* pollinating *F. vasta* did not cluster with the rest of the *E. socotrensis* specimens. Bayesian and ML analyses clustered the *A. binghami*-like specimens (*Alfonsiella pipithiensis* sp. nov.) associated with *F. craterostoma* together and placed them as sister species to the other two *Alfonsiella* species.

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 sets 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 relativel 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. We thus proceeded to the combined analysis.

Allotriozoon was designated as an outgroup. The aligned 28S and ITS2 were 1296 bp. 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 (Fig. 4). To test more specifically the monophyly of wasp genera, we enforce the nonmonophyly of each genus by conducting reverse constraints searches in P AUP* under both MP and ML criteria. Each time, the trees obtained were longer/less likely than the best unconstrained trees. However, the topologies obtained were not significantly different according to the Wilcoxon rank tests and Shimodeira−Hasegawa test (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 *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.

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 (Fig. 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 appeared as a sister taxa to the *Alfonsiella* specimens reared from *F. stuhlmannii* and *F. petersii*. However, the *Alfonsiella* specimen collected from *F. craterostoma* from Tanzania had quite a different sequence from the rest of the *F. craterostoma* pollinators, but still 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 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 provided in the next section, with a revision of the key to the *Alfonsiella* species.

Taxonomic treatment

Genus Alfonsiella Waterston, 1920

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

Distribution and host relationships.
Summarized in Table 3.

Key to Alfonsiella species (modified from Wiebes 1988)

FEMALES

1

Head distinctly longer than wide (almost 1.5×). Antennal scape with an apical projection. Mandibular appendage with 35–40 hook-like ridges and fine crenulations.....2

—

Head approximately as long as wide (0.95–1.1×). Antennal scape without an apical projection. Mandibular appendage with 15–20 hook-like ridges.....3

2

Longitudinal diameter of the eye 1.3× the length of the cheek. Apical projection of the antennal scape wide; the pedicel rather slender: almost 3× as long as wide. Mandibular appendage with about 40 ridges, ex *F. 'natalensis'*..... *A. natalensis*

—Longitudinal diameter of the eye 1.5× 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 2 or 3× the length of the cheek.....5

—

Longitudinal diameter of the eye 5× 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× the length of the cheek. Mandibular appendage with 14–17 ridges.....6

6

Postgenal suture running parallel to postoccipital suture (Fig. 6B,C arrowed); mandible usually with two complete and 3–4 incomplete transverse lamellae; mandibular appendage with 13–16 ridged teeth (Fig. 7B,C), ex *F. stuhlmannii* & *F. petersii* (South African population)..... . *A. binghami* Wiebes

—

Postgenal suture converging towards postoccipital suture (Fig. 6A arrowed); mandible usually with three complete and 3–4 incomplete transverse lamellae; mandibular appendage with 15–18 ridged teeth (Fig. 6A), 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 1.15× longer than wide, ex *F. natalensis leprieurii* & *F. kamerunensis*..... *A. fimbriata*

—

Head 1.25× longer than wide.....
.....4

4

Lateral sulcus on head complete and well-defined, extending from eye to vertex (reaching under pronotal overlap on head) (Fig. 7B,C arrowed); no fovea on posterior eye margin (Fig. 7B,C); mandibles elongate 3.5× longer than medial width, inner margin smooth; posterior extensions sharp (Fig. 7E,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 (Fig. 7A); strong fovea situated on posterior eye margin (Fig. 7A, arrow); mandibles squatter, twice maximum width, inner margin basally with blunt tooth; posterior extensions blunt (Fig. 7D), ex *F. craterostoma*
..... *A. pipithiensis* sp. nov.

5

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

—

Head 1.25× as long as wide, narrows anteriorly (width across eyes 0.75–0.80× 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 15 conical spines, ex *F. n. natalensis* & *F.* cf. *burkei A. longiscapa*

—

Dorsal edge of the hind tibia with more than 15 conical spines, ex *F. 'natalensis'*..... *A. natalensis*

Alfonsiella pipithiensis sp. nov. (Figs 6A,D and 7A,D)

Holotype.
♀(slide mounted): SOUTH AFRICA, *Limpopo Province*, Pipithi waterfalls, 22°52.5'S 30°22.5'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, 23°50'S 30°01'E, 1.ix.1989, S. van Noort & A.B. Ware, C144, ex *F. craterostoma*; series ♀♀, ♂♂: Pipithi waterfalls, 22°52.5'S 30°22.5'E, 19.xi.1991, A.B. Ware, C361, ex *F. craterostoma*; series foundress ♀♀, *Gauteng*, **Pretoria**, **University** of **Pretoria** campus, 25°45.19'S 28°13.82'E, 28.xi.1999, planted tree; 1♀: *Mpumalanga*, 10 km E of Barberton, rd. to Shiyalongubo dam, 1500 m, 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, 1523 m, iii.2002, J.C. Erasmus & J.M. Greeff, SPB11-32002, ex *F. craterostoma*; series ♀♀: Soutpansberg, Piesanghoek, 23°02.852'S 30°02.852'E, 1142 m, iii.2002, J.C. Erasmus & J. Greeff, SPB29-32002, ex *F. craterostoma*; series ♀♀, ♂♂: Soutpansberg, Entambeni, 23°00.831'S 30°14.668'E, 1250 m, iii.2002, J. C. Erasmus & J. Greeff, SPB47-32002, ex *F. craterostoma*; series ♀♀ ditto, except 1255 m, SPB49-32002; series ♀♀: Lekgalameetse, 24°09.580'S 30°13.419'E, 1109 m, 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, 25°17.875'S 30°30.595'E, 1400 m, 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.
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 nonpollinating wasp faunas associated with the two populations are also different.

Diagnosis.
Female. Morphologically very similar to *A. binghami* Wiebes, but mandible usually with three complete and 3–4 incomplete transverse lamellae and mandibular appendage with 15–18 ridged teeth. *Alfonsiella binghami* usually only has two complete transverse lamellae and 13–16 ridged teeth. The postgenal suture converges towards the postoccipital suture, whereas in *A. binghami* the suture runs parallel to the postoccipital 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* (3.5× 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, 1.02× wider than long. Eye 2.67× longer than cheek length. Two lateral ocelli. Antenna with 11 segments, the fifth to eleventh segments each with a single row of elongate sensilla, 3–4× 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 (three apical ridges transversely complete; 3–4 basal ridges short, incomplete); the mandibular appendage with 15–18 hook-like ridges, 6× longer than wide (Fig. 6A). Postgenal suture converges towards the postoccipital suture (Fig. 6A arrowed).

Mesosoma: pronotum with deep, medial, smoothly concave posterior invagination. Mesonotum 1.5× 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 0.6× length of fore wing. Propodeal peritremata narrow and long, 0.57× as long as the propodeum. Fore femur 3.75× longer than wide; fore tibia with two teeth on dorso-apical margin and one on ventro-apical margin; fore coxa with pollen pocket; hind femur 1.5× 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 1.23× longer than wide, narrowing anteriorly. Lateral sulcus extending posteriorly from eye a third of distance between eye and anterior edge of pronotum (Fig. 7A). Distinct foveal pit present on dorso-posterior margin of eye (Fig. 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 (Fig. 7D).

Mesonotum.
Pronotum 1.2× wider than long. Fused mesonotum, metanotum and propodeum 1.3× wider than long. Propodeal spiracles transversely oval, 1.5× wider than long. Fore femur 1.3× longer than wide; fore tibia with two teeth on dorso-apical margin and one on ventro-apical margin; hind femur 2.7× 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, 12°57.7'S 34°18'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 rd., 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, 25°34.36'S 31°10.90'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, 24°09.239'S 30°25.917'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*.

Discussion

Validity of fig wasp genera

Our study represents the first molecular approach to 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 our molecular reconstructions that group the *Courtella* species together. ITS2 and 28S data, indicating that *Nigeriella* and *Agaon* both form monophyletic clades; however, our sampling is limited to two species for each genus. As a result we cannot draw firm conclusions concerning the monophyly of these genera. Nevertheless, to a

large extent our molecular data validate the generic classification based on morphological appraisal.

Phylogenetic positions of the genera

Though most genera form well-defined groups, there is disagreement concerning their placement 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 suggest that *Allotriozoon* forms the sister group of the rest of the pollinators of section *Galoglychia*.

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 divide the two species groups, with *Nigeriella* clustering between them.

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 analysis 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 analysis: (((((*Elisabethiella* & *Nigeriella*) *Agaon*) *Allotriozoon*) *Paragaon*) *Alfonsiella*). *Courtella* was synonymized 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, while *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 it. 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 suggest 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 & Wiebes 1992). Our results neither 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 more comprehensive 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. Host associations were mapped on the phylogenetic trees obtained from the ITS2 and 28S sequence data (Figs 1–4). 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 analyses. The classification of subsection *Crassicostae* 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. stuhlmanii* appeared to belong to subsection *Chlamydodorae* rather than to subsection *Platyphyllae* (Rønsted *et al*. 2005). This recent phylogeny also sustains the monophyly of sections *Cyathistipulae* and *Caulocarpae*.

The genus *Courtella* is restricted to one subsection (*Caulocarpae*) but *Nigeriella*, *Alfonsiella* and *Elisabethiella* species are not constrained to a specific host subsection (*Elisabethiella* species pollinate hosts from subsections *Crassicostae*, *Chlamydodorae* and *Platyphyllae*; *Alfonsiella* species pollinate hosts of subsections *Chlamydodorae* and *Platyphyllae*; *Nigeriella* species pollinate hosts of subsections *Crassicostae* and *Platyphyllae*). This poses the question as to 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; Ware & Compton 1992; 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. We show here that at a lower taxonomic level, within one section (*Galoglychia*), pollinator phylogeny does not reflect host taxonomy.

If we assume that fig subsections form monophyletic clades, this suggests that switches between subsections have been frequent during the course of evolution. This suggests in turn 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 break-down 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. Our 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.

We conducted this kind of approach on *Alfonsiella binghami*. The *F. craterostoma* pollinators occurring in South Africa were *a priori* identified as *A. binghami* specimens. Our molecular analyses showed that these wasps did not group with the morphologically similar *Alfonsiella* species pollinating *F. petersii* and *F. stuhlmannii* but formed a monophyletic 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. nov..

Ficus craterostoma is thus pollinated by two pollinating species. These two pollinators may specialize in different habitats. *Alfonsiella michaloudi* pollinates *F. craterostoma* at low altitudes in lowland rainforest (central and west African population), whereas *A. pipithiensis* sp. nov. pollinates *F. craterostoma* populations occurring at higher altitudes in Afromontane forest (southern African population).

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 of *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 the savanna habitat. The fact that the nonpollinating 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 unique wasp communities.

The *Alfonsiella* species pollinating *F. stuhlmannii* and *F. petersii* clustered together in a very shallow tree (Fig. 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 our current resolution, both morphologically and genetically, it is most parsimonious to conclude that one species pollinates both *F. stuhlmanii* and *F. petersii*.

In conclusion, 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 certain genera in the phylogeny. We confirmed some of the taxonomic studies that suggest that African figs can often be pollinated by several 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. Therefore, African fig wasps could be more host-specific than previously assumed and reports of lack of specificity can sometimes be due to taxonomic mistakes. For instance,

the *Alfonsiella* pollinator for *F. craterostoma* from South Africa, *A. pipithiensis* sp. nov., is genetically and morphologically distinct from *A. binghami*, the pollinator of *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. Our findings are also in line with other studies (Rasplus 1996; Cook & Rasplus 2003; Jackson 2004; Machado *et al*. 2005) that suggest that the legendary pattern of cospeciation observed in deep nodes breaks down when one looks at fine scale phylogenies of closely related species.

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Figures and Tables

Fig. 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 differed (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. Association with host subsection are indicated on the right-hand side.

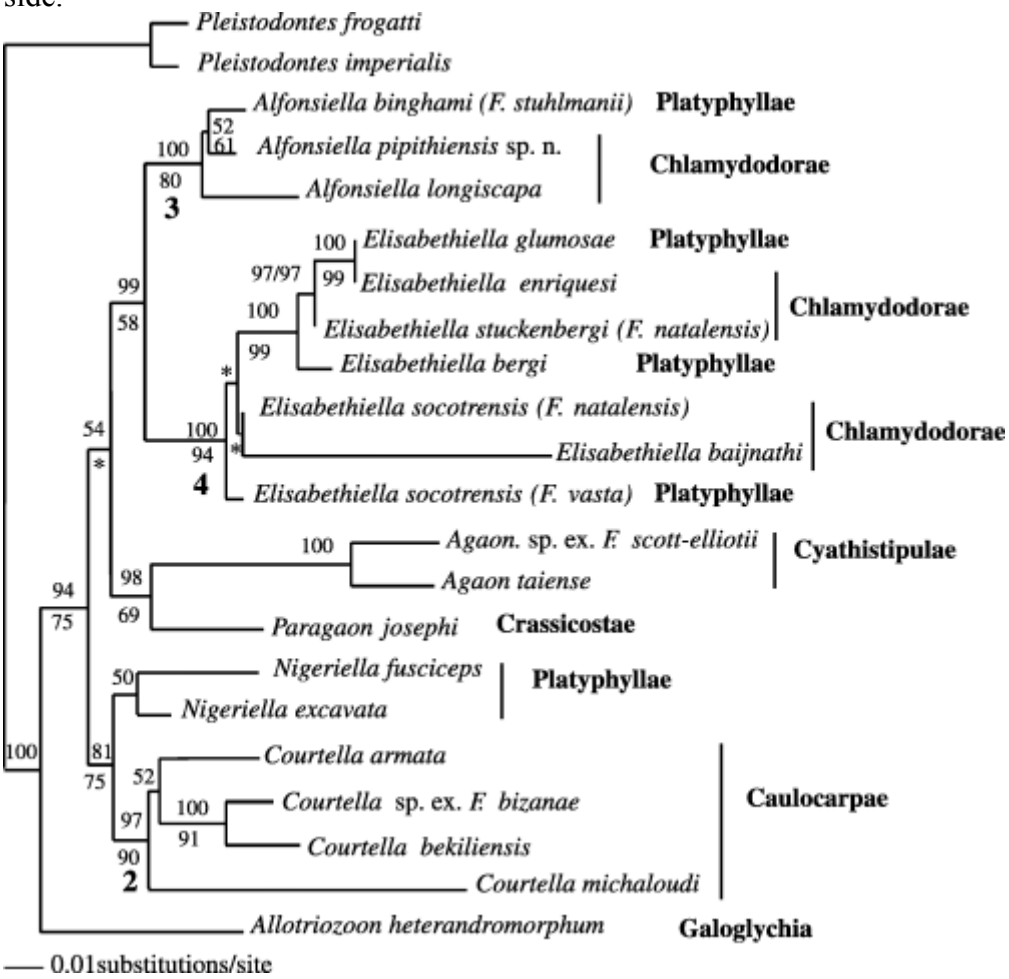

Fig. 2 Tree inferred from ML analysis of COI. Two *Tetrapus* species were chosen as outgroups. The values above the branches are posterior probabilities for identical nodes obtained via Bayesian analysis. Supports below 0.5 are not indicated. Numbers in bold under the nodes supporting the monophyly of wasp genera indicate the decay index. Association with host subsection are indicated on the right-hand side.

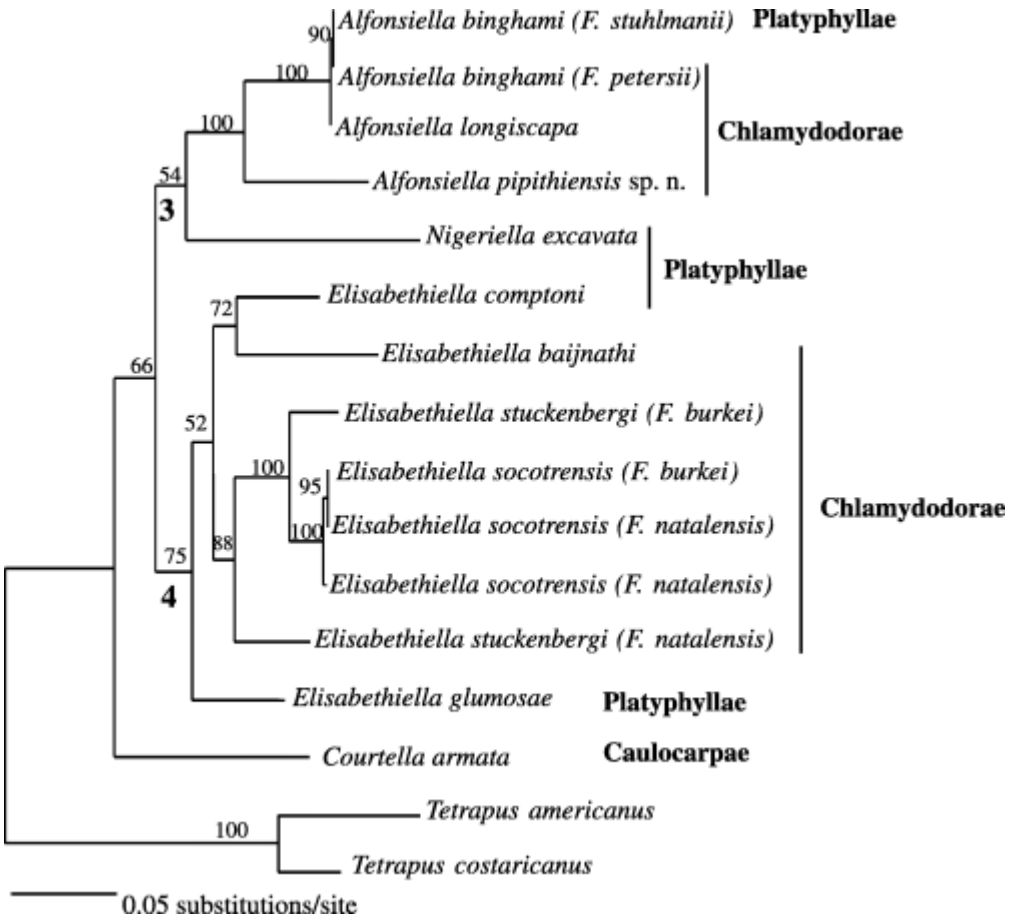

Fig. 3 Consensus tree of MP, ML and Bayesian analyses of ITS2 data. Values above branches indicate Bayesian support and values below the branches show MP bootstrap support. Numbers in bold under the nodes supporting the monophyly of wasp genera indicate the decay index. Two *Allotriozoon* species were 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 subsection are indicated on the right-hand side.

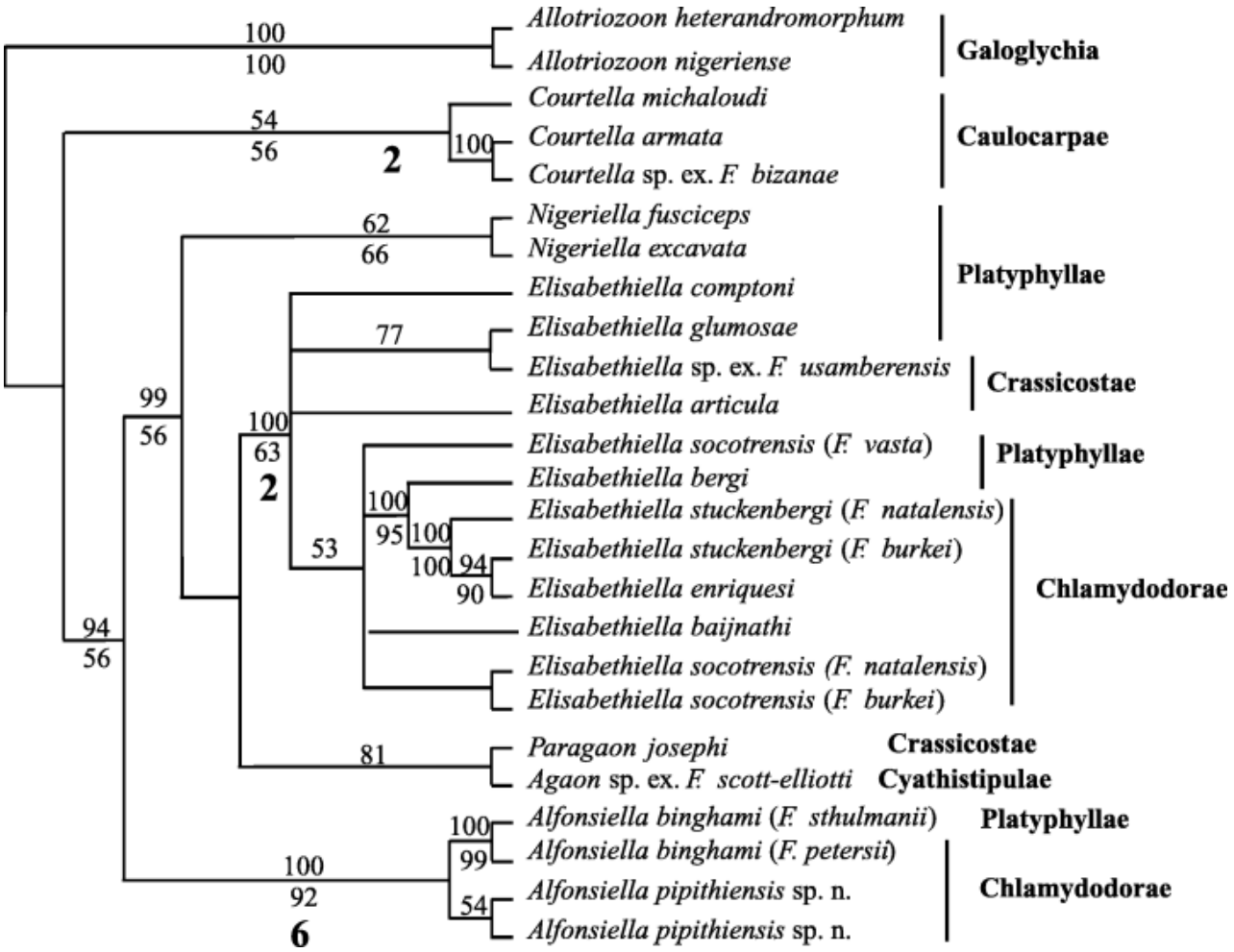

Fig. 4 Tree inferred from Bayesian analysis of 28S/ITS2 combined data set. Two *Tetrapus* species were chosen as outgroup. The values above the branches are Bayesian posterior probabilities. *Ficus* subsections are given on the right-hand side.

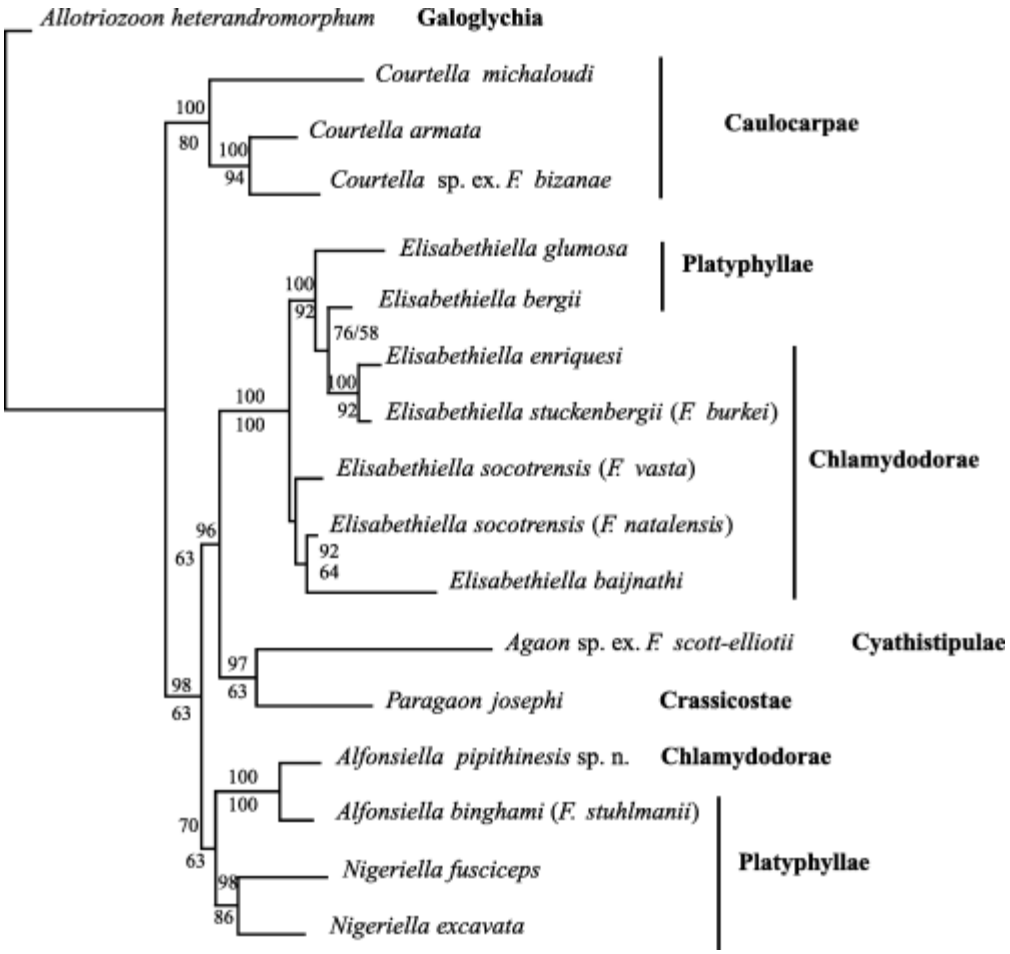

Fig. 5 Phylogenetic relationships of *Alfonsiella binghami* specimens inferred from Bayesian analyses of ITS2 data. Two *Courtella* species were chosen as outgroup species. Values above branches are Bayesian posterior probabilities, while values below branches indicate maximum parsimony bootstrap support.The localities where the samples were collected, as well as host associations, are indicated on the right-hand side.

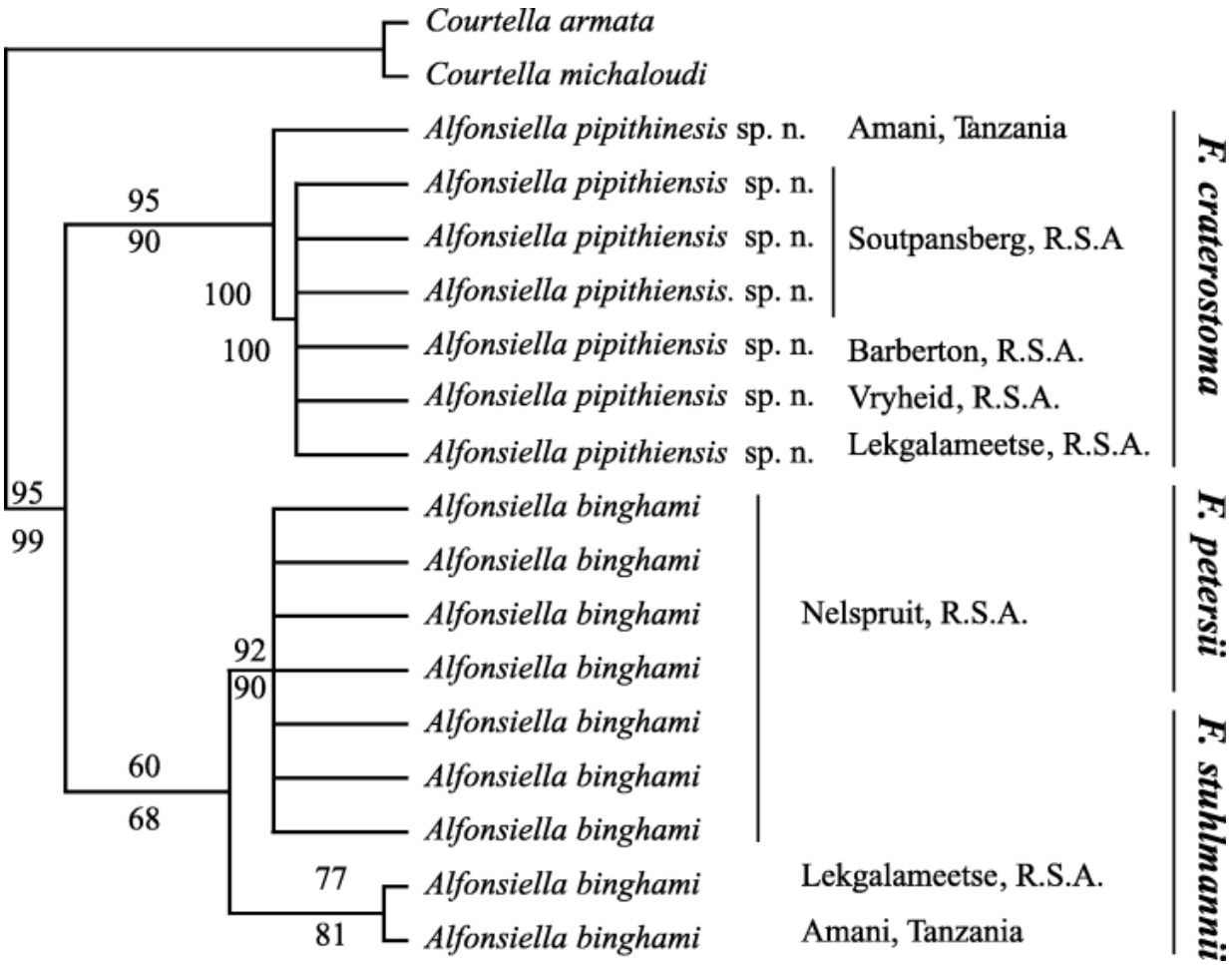

Fig. 6 A–D. *Alfonsiella* species ♀ head ventral view. —A. *A. pipithiensis*; —B. *A. binghami* (ex *Ficus stuhlmannii*); —C. *A. binghami* (ex *Ficus petersii*). —D. ♂ *A. pipithiensis* habitus.

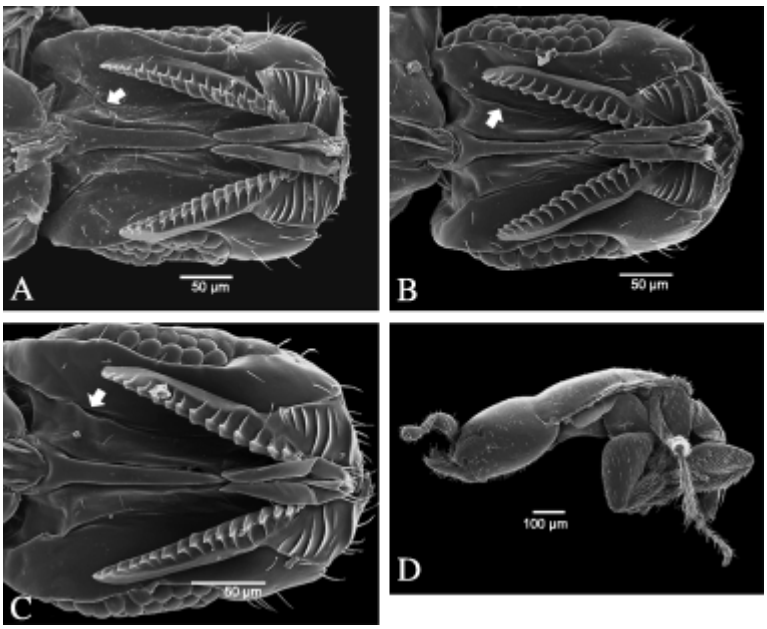

Fig. 7 A–F. *Alfonsiella* species ♂ head lateral view. —A. *A. pipithiensis*; —B. *A. binghami* (ex *Ficus petersii*); —C. *A. binghami* (ex *F. stuhlmannii*). D–F: ♂ head ventral view. —D. *A. pipithiensis*; —E. *A. binghami* (ex *F. petersii*); —F. *A. binghami* (ex *F. stuhlmannii*).

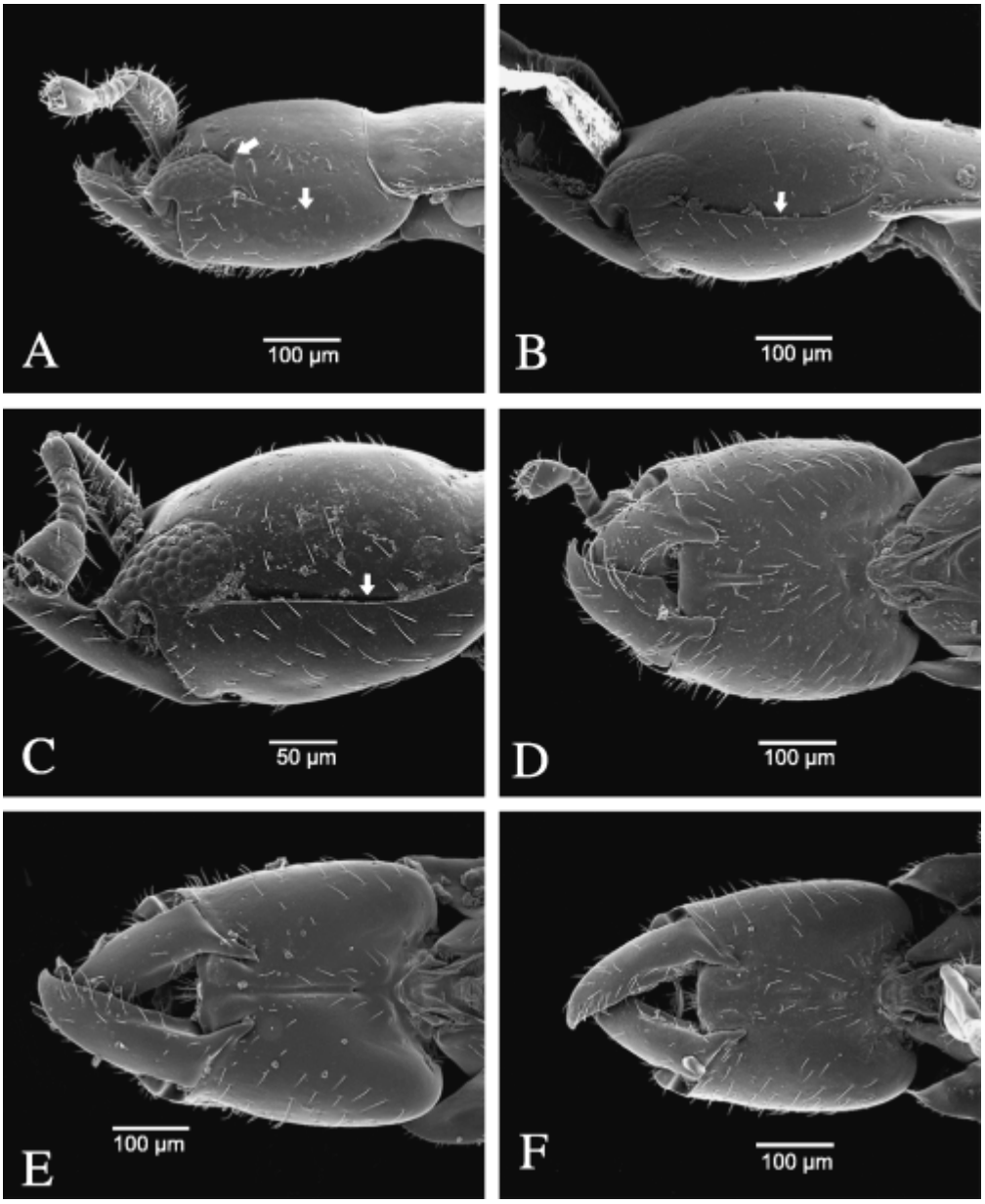

Table 1 Collection site, species and subsection of host *Ficus*, DNA region amplified and EMBL accession numbers for pollinating wasps in this study.

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
Alfonsiella pipithiensis sp. nov.	1. Tanzania, Amani, 09-2000	*F. craterostoma*	Chlamydodorae	ITS2 28S	AJ972638 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 COI	AJ972626 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
Alfonsiella binghami	1. RSA, Nelspruit, 1999	*F. petersii*	Chlamydodorae	ITS2 COI	AJ972634 AJ971650
	2. RSA, Nelspruit, 09-2002			ITS2	AJ972635
	3. RSA, Nelspruit, 01-2003			ITS2	AJ972636
	4. RSA, Nelspruit, 01-2001			ITS2	AJ972637

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
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 COI	AJ972632 AJ971648
	5. Tanzania			ITS2	AJ972633
	GenBank			28S	AY616526
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
Elisabethiella	Tanzania, Yemen, 09-2001	*F. vasta*	Platyphyllae	ITS2	AJ972648

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
socotrensis				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
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.	Tanzania, Pongwe, 03-1996	*F. usamberensis*	Crassicostae	ITS2	AJ972644

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
Paragaon josephi	Réserve des Monts Doudou, Gabon, 2000	*F. louisii*	Crassicostae	ITS2	AJ972658
Courtella michaloudi	Tanzania, 1995	*F. bubu*	Caulocarpae	ITS2	AJ972656
	GenBank			28S	AY616551
Courtella armata	RSA, Olifantskamp, 09-2003	*F. sansibarica*	Caulocarpae	ITS2	AJ972655
	GenBank			28S	AY616549
	GenBank			COI	AY014978
Courtella sp.	RSA, Mkambati Game Reserve, 09-1998	*F. bizanae*	Caulocarpae	ITS2	AJ972657
				28S	AJ971636
Courtella bekiliensis	GenBank	*F. polita*	Caulocarpae	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.	Ivory Coast, Lamto, 04-1994	*F. scott-elliotii*	Cyathistipulae	ITS2	AJ972659
				28S	AJ971647
Agaon taiense	Genbank	*F. tesselata*	Cyathistipulae	28S	gb|AY616524
Pleistodontes froggatti	GenBank	*F. macrophylla*	outgroup	28S	AJ275085
Pleistodontes imperialis	GenBank	*F. rubiginosa*	outgroup	28S	AJ298405

Pollinator	Collection locality and date	*Ficus* host	*Galoglychia* subsection	DNA region	EMBL accession #
Tetrapus americanus	GenBank	*F. maxima*	outgroup	COI	AY014971
Tetrapus costaricanus	GenBank	*F. insipida*	outgroup	COI	AY014973

Table 2 Primer sequences used for PCR amplification and sequencing.

Primer name	Sequence	DNA region
D1F	ACCCGCTGAATTTAAGCATAT	28S
D3R	TAGTTCACCATCTTTCGGGTC	28S
LCO1490	GGTCAACAAATCATAAAGATATTGG	COI
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	COI
TL2-N-3014 (Pat)	TTCAATGCACTTATTCTGCCATATTA	COI
C1-J-2183 (Jerry)	CAACATTTATTTTGATTTTTTGG	COI
ITSR	CGCCTGATCTGAGGTCGTGA	ITS2
ITSF	ATTCCGCACCACGCCTGGCTGA	ITS2

Table 3 Host relationships and distribution of *Alfonsiella* species. Host names in apostrophes 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, Tanzania)
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)