

# Molecular phylogeny of fig wasps Agaonidae are not monophyletic

*Phylogénie moléculaire des Chalcidiens associés aux figues.  
Les Agaonidae ne sont pas monophylétiques*

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(Received 1st December 1997, accepted after revision 6 April 1998)

**Abstract** – According to the present classification, the family Agaonidae contains all fig pollinators as well as five subfamilies of non-pollinating fig wasps. The molecular phylogeny of the family was reconstructed using partial sequences of the 28S rRNA (D1 and D2 domains). Our results show that the family Agaonidae is not monophyletic. As a consequence, we restrict the family to the pollinator clade, and assign the non-pollinating subfamilies to various chalcid families. Sycoecinae, Otitesellinae and Sycoryctinae are included in Pteromalidae, whereas Sycophaginae and Epichrysomallinae are left unclassified and will require more in-depth morphological studies. Moreover, we proved that the fig pollination syndrome evolved only once, early in group history. The resource due to the fig–pollinator mutualism has secondarily been colonized independently by different Chalcid lineages. (© Académie des sciences / Elsevier, Paris.)

Agaonidae / phylogeny / parsimony / 28S / Hymenoptera / Chalcidoidea

**Résumé** – Suivant la classification actuelle, la famille des Agaonidae comprend tous les pollinisateurs de *Ficus* mondiaux et cinq sous-familles de Chalcidiens non-pollinisateurs strictement inféodés à la ressource « figue ». Nous avons reconstruit la phylogénie moléculaire de cette famille à partir de séquences partielles de l'ARNr 28S (domaines D1 et D2). Nos résultats montrent que la famille des Agaonidae n'est pas monophylétique. En conséquence, nous restreignons la famille au seul groupe des pollinisateurs. Les sous-familles de non-pollinisateurs sont déplacées dans d'autres familles de chalcidiens. Les Otitesellinae, Sycoecinae et Sycoryctinae font désormais partie des Pteromalidae, alors que les Sycophaginae et Epichrysomallinae ne sont pas classées, et nécessiteront de plus amples études. D'autre part, nous avons montré que le syndrome de pollinisation est apparu une seule fois, anciennement dans l'histoire du groupe. La ressource offerte par le mutualisme *Ficus*-pollinisateur a été secondairement colonisée indépendamment par plusieurs lignées de Chalcidiens. (© Académie des sciences / Elsevier, Paris.)

Agaonidae / phylogénie / parcimonie / 28S / Hymenoptera / Chalcidoidea

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## Version française abrégée

Pendant longtemps, la famille des Agaonidae a été restreinte aux pollinisateurs de *Ficus*, alors que la plupart des chalcidiens non-pollinisateurs associés aux figuiers étaient classés dans la famille des Torymidae. En 1988, Boucek a regroupé la plupart des insectes inféodés aux figuiers (pollinisateurs, galligènes, inquilines ou parasitoïdes), dans la famille des Agaonidae. Ainsi définie, cette dernière comprend donc une sous-famille de pollinisateurs (Agaoninae) et cinq sous-familles de non-pollinisateurs (Epichrysomallinae, Sycophaginae, Otitesellinae, Sycoecinae et Sycoryctinae). Cette nouvelle classification est essentiellement basée sur la présence d'un pont post-génal chez tous les Agaonidae, alors que l'absence de carène occipitale les sépare des Torymidae. L'étude morphologique des chalcidiens liés aux figues est cependant délicate, car les insectes sont très transformés à cause d'adaptations liées à l'exploitation de la ressource, et les convergences morphologiques sont difficiles à séparer des apomorphies. Nous avons voulu tester l'hypothèse de Boucek selon laquelle la famille des Agaonidae est monophylétique, et les Epichrysomallinae représentent le groupe frère ancestral des autres sous-familles. D'après cet auteur, les Sycoecinae (qui entrent dans la figue pour pondre de l'intérieur) seraient le groupe frère des pollinisateurs. Nous avons donc reconstruit la phylogénie moléculaire de la famille à partir de séquences partielles de l'ARNr 28S (domaines D1 et D2), en échantillonnant deux genres distincts par sous-famille, ainsi que des représentants des familles de chalcidiens proches des Agaonidae (Pteromalidae, Torymidae et Eurytomidae). Pour enraciner la phylogénie, les groupes externes ont été choisis

parmi les superfamilles Ichneumonoidea et Cynipoidea. Les séquences ont été alignées avec le logiciel Clustalw, et nous avons ensuite traité les séquences à la fois en maximum de parcimonie (Paup), en maximum de vraisemblance (Puzzle) et en neighbour-joining sur distances génétiques (Mega). La robustesse des phylogénies a été testée par la méthode du bootstrap. Les topologies des trois arbres obtenus sont cohérentes, et tous nos résultats concordent pour montrer que la famille des Agaonidae *sensu* Boucek n'est pas monophylétique. Les pollinisateurs ressortent en groupe frère de tous les autres chalcidiens, ce qui suggère une évolution ancienne de ce clade. Les non-pollinisateurs ne forment pas un groupe homogène; trois des sous-familles (Sycoecinae, Otitesellinae et Sycoryctinae) ont des affinités fortes avec les Pteromalidae, alors que les Epichrysomallinae et les Sycophaginae ne montrent d'affinités particulières avec aucune des familles représentées dans notre analyse. Nous proposons donc de restreindre à nouveau la famille des Agaonidae aux pollinisateurs, et d'assigner les Sycoecinae, Otitesellinae et Sycoryctinae à la famille des Pteromalidae. Nous ne sommes pas en mesure de proposer de classification familiale pour les Epichrysomallinae et les Sycophaginae. Notre étude prouve que les insectes liés aux figues n'ont pas évolué à partir d'un ancêtre commun, mais au contraire que la colonisation de la ressource figue a eu lieu en plusieurs vagues successives, chaque groupe évoluant indépendamment après la diversification des pollinisateurs. De plus, les Sycoecinae ne sont pas le groupe-frère des pollinisateurs, ce qui prouve que la capacité à entrer dans la figue pour pondre de l'intérieur a évolué indépendamment plusieurs fois.

## 1. Introduction

Among plant-insect associations, *Ficus* (Moraceae) and their species-specific pollinator chalcid wasps constitute one of the most remarkable examples of plant-insect obligate mutualism. With few exceptions [1], each of the ca. 750 fig species world-wide [2] exclusively depends on its single mutualistic wasp for pollen dispersal and pollination, as the flowers are enclosed within the fig or syconium. The pollinator, whose female is shaped to enter the fig through the ostiolar bracts, in turn strictly depends on its single host *Ficus* for reproduction and larval development, as it lays eggs in some of the fig ovaries from the inner of the syconium while passively or actively pollinating some others. The larval development is entirely completed within the fig. Many authors consider the system as a case of strict sense coevolution [3–6]. However, in addition to its mutualistic partner, each fig species shelters a variety of so-called non-pollinating fig wasps that develop in the fig flowers just as the pollinator, but act as exploiters of the mutualism without providing any benefit [7–13]. They are all strictly dependent on the fig resource for their reproduction and larval development. Some of

them mimic the pollinator, as they also enter the fig through the ostiole and oviposit in the ovaries through the style from the fig cavity, but most species oviposit from the outside of the fig, through the syconial wall thanks to a long ovipositor. These numerous wasps are still mostly undescribed and biologically poorly known, but it is now clear that some of them are gall-makers and oviposit in the fig ovules, while others are parasitoids or inquilines and lay eggs in gall-transformed ovaries that already contain a pollinator or another gall-maker larva. Their role in the mutualism functioning and evolution has been studied in a few recent works [9, 11–14].

The taxonomy of fig wasps has varied greatly over the last decades. The family Agaonidae has long been restricted to the fig pollinators, while most non-pollinating wasps were classified as Torymidae (Chalcidoidea) in the subfamily Sycophaginae, and considered to be parasites of the pollinators [3, 15]. They were then assigned to various tribes [16, 17]. In 1964, Joseph [18] restricted the subfamily Sycophaginae to the known non-pollinating fig wasps that enter the fig through the ostiole to oviposit from the fig cavity (= the present Sycoecinae and the genus *Sycophaga*), and placed all other species (= those

ovipositing through the fig wall) in the subfamily Idarninae. The Otitesellini were then named and placed as a tribe of Idarninae [18]. Later, Hill and Riek [19, 20] described the Epichrysomallinae as a subfamily of Torymidae, and Hill defined the Sycoecini as a tribe of Sycophaginae [20]. In 1981, Bouček [21] modified this classification. The subfamily Idarninae was synonymized with Sycophaginae, which was kept within Torymidae and contained the tribes Sycophagini and Apocryptini. At the same time, the tribes Sycoecini and Otitesellini were raised to subfamily ranks (Sycoecinae and Otitesellinae). The subfamilies Epichrysomallinae and Otitesellinae were shifted to family Pteromalidae. The author could not decide whether Sycoecinae (including Sycoecini and Philotrypini) should be placed in Pteromalidae or in Torymidae. He also left the subfamily Sycoecinae unclassified, while considering it as the sister group of fig pollinators.

More recently, most non-pollinating fig wasps have been reclassified by Bouček [7, 8] in the Agaonidae family, which consequently includes both pollinating and non-pollinating wasps, and consists of six subfamilies: the Agaoninae groups all the pollinator species, whereas Sycoecinae, Sycophaginae, Sycoecinae (with tribes Sycoecini and Apocryptini), Otitesellinae and Epichrysomallinae are non-pollinating subfamilies. The latter was considered as the family stem group. The six subfamilies are presented in table 1. Except for Otitesellinae and Sycoecinae, which appear to group morphologically heterogeneous taxa, each subfamily is clearly monophyletic [7]. This classification was based on occipital structure characteristics. The species included in such an extended Agaonidae family possess a post-genal bridge that closes the back of the head below the foramen magnum, and lack an occipital carina. The first characteristic separates Agaonidae from Pteromalidae, while the second separates it from Torymidae [7], even though some Epichrysomallinae (considered by Bouček as the ancestral group of Agaonidae) do have an occipital carina. Interestingly,

previous studies showed similarities of gastral anatomy among Agaonidae sensu Bouček [22]. In addition, some chalcid wasps that do not belong to the Agaonidae family also occur within the fig fauna. These insects have been non-ambiguously assigned to the families Ormyridae, Eurytomidae, Pteromalidae and Torymidae.

This view opens up an interesting field of research, especially with regards the evolution of figs and wasps. Whether the six pollinating and non-pollinating subfamilies of agaonid fig wasps are truly monophyletic or not is still a matter of debate. An objective answer would shed light on many evolutionary questions that have been asked about the fig–pollinator mutualism, such as understanding the origin and maintenance of the pollinating syndrome in the Agaoninae subfamily. It would also allow us to test the hypothesis of Bouček and collaborators [21] following which the pollinators and the non-pollinating species that have developed the ability to enter the fig through the ostiole could be sister taxa. Some authors have also suggested that non-pollinating fig wasps have had a coevolutionary history with pollinators and *Ficus* [23, 24]. Determining whether or not pollinating and non-pollinating agaonids have a common ancestor would be relevant before studying the evolutionary scenario of the whole system.

The particular ways of life of both pollinating and non-pollinating fig wasps are responsible for very strong morphological adaptations within the agaonid family [25] due to host–fig anatomy and oviposition strategy. Morphological data are therefore likely to be prone to adaptive convergence and to homoplasy. Furthermore, our knowledge about chalcid evolution is still limited, and new characteristic systems are clearly needed to undoubtedly resolve the relationships among families and subfamilies of chalcids [26–28]. Molecular tools are thus of great interest for studying the evolution of such a system. In 1996, Machado et al. [23] proposed the first molecular reconstruction of Agaonidae phylogeny, and proved that the pollinators (subfamily Agaoninae) form a mono-

**Table 1.** Diversity, biology, host association and distribution of Agaonidae subfamilies.

Subfamily	Numbers of genera and species	Biology	Host <i>Ficus</i>	Distribution
Agaonidae	20 genera 320 species	Pollinators. Oviposit from the fig cavity and induce galls	All subgenera and sections	World-wide
Epichrysomallinae	20 genera 40 species	Gall-makers. Most oviposit through the fig wall (few species from the fig cavity, or on leaves)	All paleotropical <i>Ficus</i> sections	Old world regions
Sycophaginae	6 genera 50 species	Gall-makers. Most oviposit through the fig wall. The genus <i>Sycophaga</i> oviposits from the fig cavity.	Subgenus <i>Sycomor</i> sensu lato, and sections <i>Malvanthera</i> and <i>Americana</i>	World-wide
Sycoecinae	6 genera 67 species	Gall-makers. Oviposit from the fig cavity	Sections <i>Galoglychia</i> , <i>Conosycea</i> , and <i>Malvanthera</i>	Old world regions (mainly Africa)
Otitesellinae	14 genera 69 species	Gall-makers. Most oviposit through the fig wall (few species from the fig cavity)	Subgenus <i>Urostigma</i>	World-wide
Sycoecinae	7–9 genera 140 species	Gall-makers, parasitoids or inquilines. Oviposit through the fig wall	All subgenera and sections	World-wide

phyletic clade. However, their data set did not enable them to test the monophyly of the whole Agaonidae family, mainly because of the lack of non-agaonid chalcid sequences in the proposed phylogeny.

The purpose of the present paper is to examine the supposed monophyly of the Agaonidae family, by using sequences of the first and second expansion segments of the 28S rRNA (D1 and D2 domains), which are known to evolve at relatively low rates, and are thus useful to reconstruct phylogenies of higher taxa [29, 30]. In order to determine the main features of fig wasp evolution, we studied representatives of the six Agaonidae subfamilies as well as non-ambiguous representatives of Eurytomidae, Torymidae and Pteromalidae, and we reconstructed the evolutionary history of the Agaonidae family (sensu Bouček) within the Chalcidoidea. In addition, we conducted observations of post-occipital structures to obtain

a better understanding of the morphological characteristics used by Bouček to revise the Agaonidae classification.

## 2. Material and methods

### 2.1. Sampling and collections

All the specimens sampled are listed in *table II*. We analysed representatives of all the six subfamilies of Agaonidae. Whenever possible, we used two distinct genera for each subfamily occurring in separate biogeographical regions. To adequately test the monophyly of Agaonidae, we furthermore sequenced species from three chalcid families (Torymidae, Eurytomidae and Pteromalidae, respectively) supposedly close to (but distinct from) Agaonidae. We selected species that had non-ambiguously been assigned to their families, and whose classification

**Table II.** List of the taxa used in the analysis.

Superfamily	Family	Subfamily	Species	Biology
Ichneumonoidea	Ichneumonidae	Orthopelmatinae	<i>Orthopelma mediator</i>	Endoparasitoid of <i>Diplolepis</i> larvae
Cynipoidea	Figitidae	Eucoilinae	<i>Leptopilina boulardi</i>	Endoparasitoid of <i>Drosophilidae</i> species
Chalcidoidea	Agaonidae	Agaoninae	<i>Courtella armata</i>	Pollinator of <i>Ficus ovata</i> in Africa
Chalcidoidea	Agaonidae	Agaoninae	<i>Pegoscapus franki</i>	Pollinator of <i>Ficus citrifolia</i> in Florida
Chalcidoidea	Agaonidae	Sycophaginae	<i>Idarnes carme</i>	Gall-maker in figs of <i>Ficus aurea</i> in Florida (oviposit from outside)
Chalcidoidea	Agaonidae	Sycophaginae	<i>Sycophaga silvestrii</i>	Gall-maker in figs of <i>Ficus sur</i> in Africa (oviposit from the cavity)
Chalcidoidea	Agaonidae	Epichrysomallinae	<i>Lachaisea</i> n.sp. (nom. provis)	Gall-maker in figs of <i>Ficus ovata</i> in Guinea (oviposit from outside)
Chalcidoidea	Agaonidae	Epichrysomallinae	<i>Herodotia subatriventris</i>	Gall-maker in figs of <i>Ficus rubiginosa</i> in Australia (oviposit from outside)
Chalcidoidea	Agaonidae	Sycoryctinae	<i>Sycoscapter</i> n.sp1	Parasitoids of gall-makers in figs of <i>Ficus ovata</i> in Africa
Chalcidoidea	Agaonidae	Sycoryctinae	<i>Sycoscapter</i> n.sp2	Parasitoids of gall-makers in figs of <i>Ficus ovata</i> in Africa
Chalcidoidea	Agaonidae	Sycoryctinae	<i>Watshamiella</i> n.sp.	Parasitoids of gall-makers in figs of <i>Ficus ovata</i> in Africa
Chalcidoidea	Agaonidae	Sycoryctinae	<i>Apocrypta guineensis</i>	Parasitoids of gall-makers in figs of <i>Ficus sur</i> in Africa
Chalcidoidea	Agaonidae	Otitesellinae	<i>Phylosycus monstruosus</i>	Gall-maker in figs of <i>Ficus ovata</i> in Africa (oviposit from outside)
Chalcidoidea	Agaonidae	Otitesellinae	<i>Otitesella</i> n.sp.	Gall-maker in figs of <i>Ficus ovata</i> in Africa (oviposit from outside)
Chalcidoidea	Agaonidae	Otitesellinae	<i>Aepocerus nadelae</i>	Gall-maker in figs of <i>Ficus citrifolia</i> in Florida (oviposit from outside)
Chalcidoidea	Agaonidae	Sycoecinae	<i>Seres armata</i>	Gall-maker in figs of <i>Ficus ovata</i> in Africa (oviposit from the cavity)
Chalcidoidea	Agaonidae	Sycoecinae	<i>Philocaenus bifurcus</i>	Gall-maker in figs of <i>Ficus ottoniifolia ulugurensis</i> in Africa (oviposit from the cavity)
Chalcidoidea	Pteromalidae	Pteromalinae	<i>Dinarmus basalis</i>	Ectoparasitoid of <i>Bruchidae</i> (Coleoptera) larvae
Chalcidoidea	Pteromalidae	Pteromalinae	<i>Pachycrepoideus vindemiae</i>	Endoparasitoid of <i>Drosophilidae</i> pupae
Chalcidoidea	Eurytomidae	Eurytominae	<i>Eurytoma rosae</i>	Ectoparasitoid of <i>Diplolepis</i> (Cynipidae) larvae
Chalcidoidea	Eurytomidae	Eurytominae	<i>Sycophila</i> sp.	Inquiline of <i>Epichrysomallinae</i> galls in figs of <i>Ficus ovata</i> in Africa
Chalcidoidea	Torymidae	Toryminae	<i>Glyphomerus stigma</i>	Ectoparasitoid of <i>Diplolepis</i> (Cynipidae) larvae
Chalcidoidea	Torymidae	Toryminae	<i>Podagrion</i> n.sp.	Endoparasitoid of <i>Mantidae</i> (Mantodea) larvae in Africa

has never been contested [31, 32]. Finally, two outgroup taxa belonging to the superfamilies Ichneumonoidea and Cynipoidea (Hymenoptera: Parasitica) were sequenced to root the phylogeny. The superfamily Cynipoidea is supposed to be ancestral compared to Chalcidoidea. It is sometimes considered to be closely related to Chalcidoidea [33]. However, Gibson [26] found no support for such a hypothesis. Rasnitsyn [34] classified it within Proctotrupomorpha as sister group of all other superfamilies, and more recently, Dowdon et al. [35] placed it as sister group of all other Parasitica.

The fig insects were collected directly in the fig in which they developed. Mature figs were collected once soft, but prior to the formation of the exit hole chewed by the pollinator males, that is, before the adult insects escaped. Each fig was then opened and put in a box where insect emergence was allowed. Adult wasps were then killed and conserved in absolute alcohol. The other chalcid, cynipid and ichneumonid wasps included in our study were either caught by sweeping or reared from their host, killed by ethyl acetate and immediately conserved in absolute ethanol.

## 2.2. DNA protocols

DNA was extracted from five individual wasps for each species using standard phenol-chloroform extractions [36]. The polymerase chain reaction (PCR: [37]) was used to amplify the D1 and D2 domains of the 28S rRNA. The primers used for both amplification and sequencing are listed in *table III*. We performed 30 cycles of 1 min dena-

**Table III.** List of the PCR primers used for amplification and sequencing of the D1 and D2 regions.

Primer	Sequence (5' à 3')
D1	ACCCGCTGAATTTAAGCATAT
D1b (REV)	GCTGCATTCCCAAGCAACCCGACT
D2	TCCGTGTTTCAAGACGGG
D2' (REV)	AAAGTTGAAAAGAAGCTTTGAA

REV = reverse primer.

turation at 95 °C, 1 min annealing at 50 °C and 1 min extension at 72 °C preceded by an initial denaturation of 5 min at 95 °C and ended by a final extension of 5 min. DNA strands were then purified and sequenced following the dideoxynucleotide chain-termination method [38].

## 2.3. Sequence analysis

Sequences were aligned using the CLUSTALW software [39]. Due to indels, the alignment of the D2 domain was problematic, and final alignments were made by eye. The aligned sequences were analysed using the neighbour-joining method [40] on uncorrected genetic distances using the 'complete deletion' option of the program MEGA 1.0 [41]. PAUP 3.1.1 [42] was used to complete parsimony analyses, and gaps were then treated as fifth

characteristic state. Transition and transversion were equally weighted. Most parsimonious trees were determined by heuristic search, using the 'branch swapping' option. In both neighbour-joining and maximum parsimony analyses, the reliability of the nodes was examined using bootstrap resampling procedures [43]. Finally, the maximum likelihood method was completed with the PUZZLE 3.1 software [44], following the model of Hasegawa et al. [45], in which the parameters were estimated directly from the data, with 1 000 re-iterations.

## 2.4. Morphological observations

A thorough observation of the bridge that separates the foramen magnum from the oral fossa was conducted under a dissecting microscope in order to obtain a clear interpretation of the morphological characteristics used by Bouček in his revision.

# 3. Results

## 3.1. Sequences and phylogenetic reconstructions

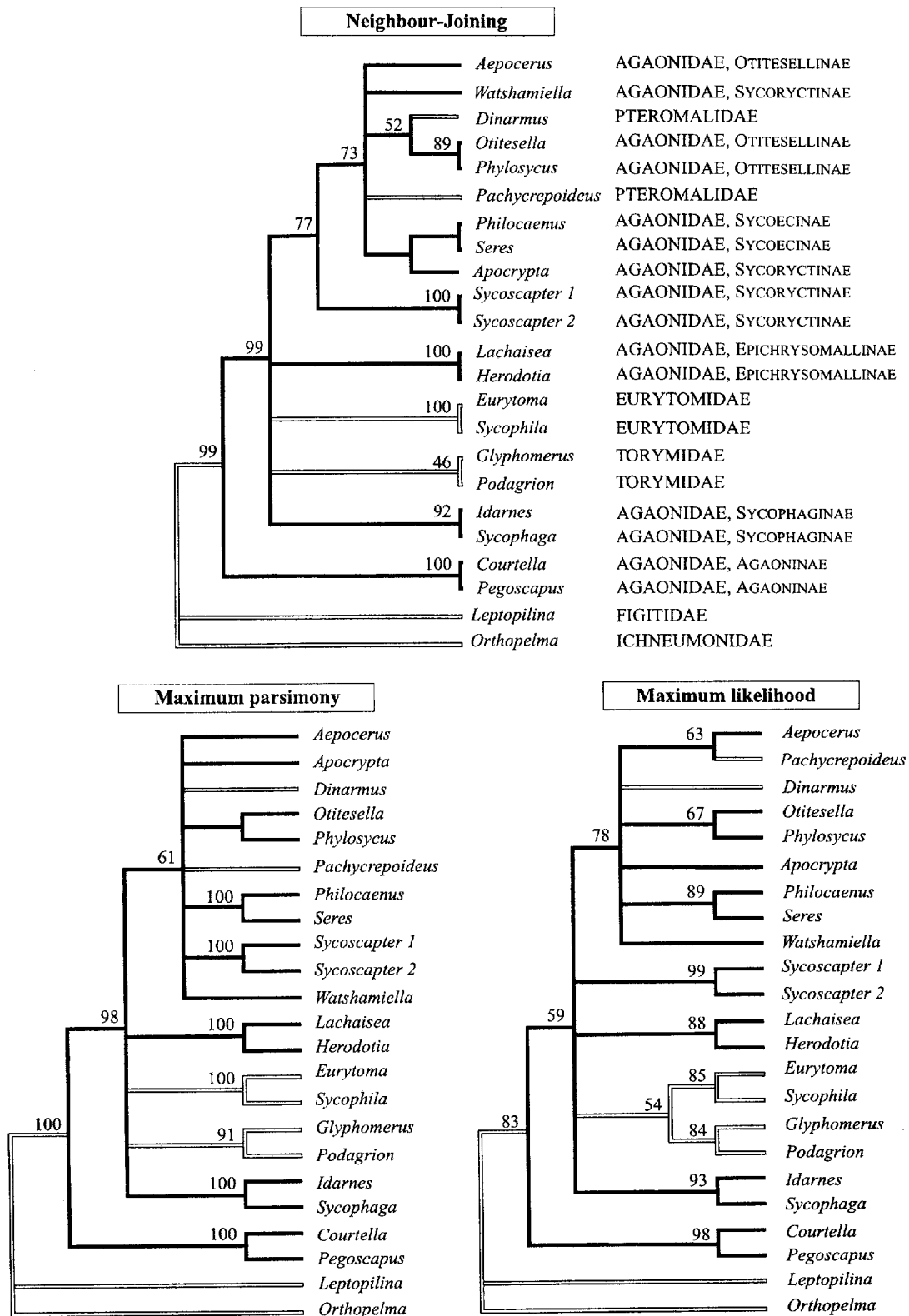
We obtained sequences of 237 base pairs on the D1 domain, and of 482 base pairs on the D2 domain (including gaps). The analyses were thus conducted on 719-base-pair-long total sequences, including 386 variable sites.

The phylogenetic trees reconstructed with the three algorithms (i.e. neighbour-joining, maximum parsimony and maximum likelihood) have very similar topologies, and are represented on *figure 1*. One single most-parsimonious tree was found, which was 1 208 steps long, with a CI of 0.637. In all reconstructed trees, the pollinator genera are strongly grouped together, and appear as the sister group of all other sampled chalcids. Among these, the species of Pteromalidae and of the subfamilies Sycoryctinae, Otitesellinae and Sycoecinae form a robust clade, supported by high bootstrap values. With regards the families Torymidae and Eurytomidae, and the subfamilies Sycophaginae and Epichrysomallinae, the species belonging to one taxonomic group are strongly associated, but the relative position of the four groups is poorly resolved.

## 3.2. Morphological observations

Our observations of the post-occipital structures show that the characteristics are not homologous in the different clades. *Figure 2* shows the back of the head for several chalcid groups. Three clearly different types of 'post-occipital' bridge structure have been identified:

– In Epichrysomallinae, Sycophaginae and Eurytomidae, the posterior tentorial pits are inserted close to the foramen magnum (*figure 2b, c and i*). Consequently, only the subgenal suture is lengthened. The bridge is mainly due to the approximation of the postgenae and could therefore be called a postgenal bridge.



**Figure 1.** Trees estimating the phylogenetic relationships of the major groups of pollinating and non-pollinating fig wasps, generated by analysis of the 28S rRNA D1 and D2 domains.

Black lines symbolize Agaonidae sensu Bouček (1988), white lines represent putative non-agaonid outgroups. Numbers are bootstrap values for maximum parsimony and neighbour-joining trees and percentage of support for the unrooted quartet puzzling tree based on maximum likelihood.

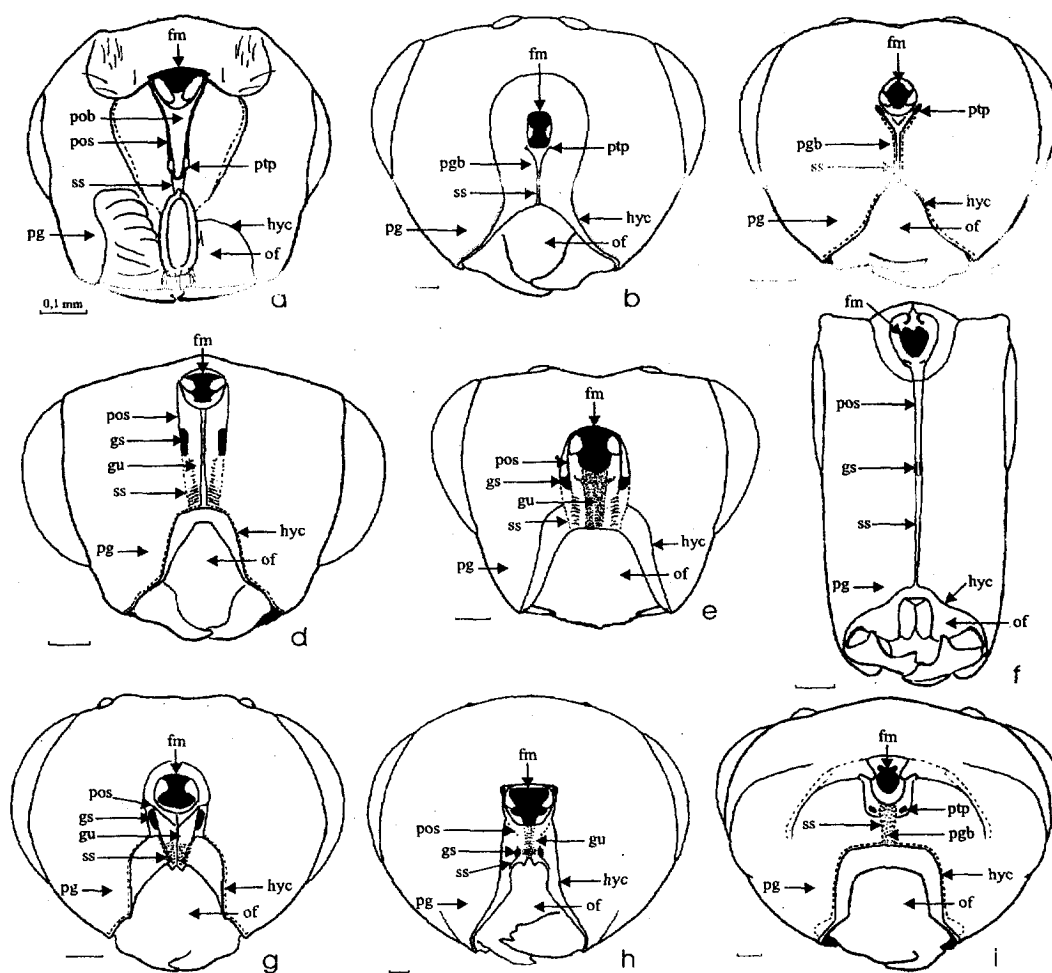
– In Otitesellinae and Sycoryctinae (figure 2d, e and g), the posterior tentorial pits are concealed in a longitudinal deep groove of variable size, which is called gular sulcus. The bridge is thus mainly constituted of a subrectangular sclerite which is situated between the foramen magnum and the oral fossa, and is delimited by the postoccipital suture, the gular sulcus and the subgenal suture. This sclerite is sometime called gula [24]. The gula is longitudinally divided by a median suture in Otitesellinae and Sycoryctini but not in Pteromalinae and Apocryptini. In Pteromalinae the structure of the back of the head is somehow the same (figure 2h) but the lengthening of the hypostomal carina (that reaches the foramen magnum) induces a sinking of the gula. This characteristic could be apomorphic for Pteromalinae. In Sycoecinae the posterior tentorial pits are centrally situated between the foramen magnum and the oral fossa (figure 2f) probably because

of the prognathous position of the head and its lengthening. Consequently the bridge is due to a closeness of the postgenae both above and below the posterior tentorial pits, while the gula is lengthened and sometimes absent.

– In Agaoninae (figure 2a), the structure is quite different. The bridge is mainly composed of a carinated sclerite, probably of postoccipital origin, situated between deep grooves that contain the posterior tentorial pits. In that case alone the bridge could be called a postoccipital bridge.

## 4. Discussion

All our results challenge the monophyly of the family Agaonidae as defined by Bouček [7]. The six subfamilies are never grouped together in the phylogenetic recon-



**Figure 2.** Structure of the back of the head in different groups of chalcid wasps.

**a.** *Wiebesia* (Agaoninae). **b.** *Lachaisea* (Epichrysomallinae). **c.** *Anidarnes* (Sycophaginae). **d.** *Philotrypesis* (Sycoryctinae, Sycoryctini). **e.** *Apocrypta* (Sycoryctinae, Apocryptini). **f.** *Seres* (Sycoecinae). **g.** *Phyllosycus* (Otitesellinae). **h.** *Dinarmus* (Pteromalinae). **i.** *Sycophila* (Eurytomidae).

fm = foramen magnum; gs = gular sulcus; gu = gula; hyc = hypostomal carina; of = oral fossa; pg = postgena; pgb = postgenal bridge; pob = postoccipital bridge; pos = postoccipital suture; ptp = posterior tentorial pits; ss = subgenal suture.

structions we obtained by either algorithm, and they do not consist of a robust clade separated from the representatives of the chalcid families Torymidae, Eurytomidae and Pteromalidae. In contrast, the pollinator clade appears to be the sister group of all other sampled chalcids, which are placed in different groups whose relative positions are poorly resolved. Torymidae, Eurytomidae, Sycophaginae and Epichrysomallinae form four of these groups, while the fifth is composed of 'actual' Pteromalidae and representatives of three subfamilies of the former Agaonidae family (Otitesellinae, Sycoecinae and Sycoryctinae). This fifth group will henceforth be called 'Pteromalid complex'. We will now discuss the taxonomic status of the family Agaonidae sensu Bouček, which will be contested, and the origin of the 'Pteromalid complex'.

#### 4.1. Rejection of the Agaonidae monophyly hypothesis

The main consequence of our result is that we proved the family Agaonidae to be non-monophyletic. The family as defined by Bouček cannot be valid anymore, and we now need to re-consider the subfamilies that were included in it. As a first step, we will try to find all reasons why Bouček considered this group as monophyletic and discuss the apomorphies on which his study was based. We will then try to understand why the recent phylogenetic study of Machado and collaborators failed to prove the non-monophyly of the family [23].

Bouček [7] recently revised the family and included in it a majority of the non-pollinating fig wasps because most of the genera have a postgenal bridge and lack an occipital carina. These morphological characteristics are unlikely to be adaptive, and were thus possibly of interest in fig wasp morphological classification. However, our preliminary observations clearly show that these structures are not homologous in the various chalcid groups that were included in the Agaonidae family. We identified three origins for the bridge formation, which is actually due to the transformation of different morphological parts according to the subfamily. A reassessment of the post-occipital structure is thus greatly needed to reach conclusions concerning its phylogenetic signification. Furthermore, a recent study of ovipositor structure in various groups of microhymenoptera also suggests the non-monophyly of Agaonidae sensu Bouček [46]: Agaonidae exhibit great variation in ovipositor structure, whereas all other chalcid families are quite homogeneous for these characteristics.

On the other hand, a molecular phylogeny of the non-pollinating fig wasps based on the 12S rRNA gene sequences has recently been proposed by Machado and colleagues [23], who did not reject the hypothesis of Agaonidae monophyly. The reason for this must be sought for in the sampling of sequenced species. Their analysis actually included representatives of the Agaonidae subfamilies (except for Epichrysomallinae) and one non-chalcid outgroup, but lacked species of non-agaonid chalcid families, except for the genus *Physothorax* (Torymidae).

As the phylogenetic position of this genus was not satisfactorily resolved, this study did not enable them to draw any conclusion about the status of the Agaonidae family. The topology of their phylogenetic tree was nonetheless very similar to our own results and by no means contradicted our interpretations.

Consequently, we deny the validity of the family Agaonidae as defined by Bouček, and we raise the subfamily Agaoninae back to a family rank. This upgrading re-establishes the family status as understood during the last 40 years [3]. The non-pollinating fig wasp subfamilies must further be re-assigned to other chalcid families. We are not currently able to give any assignation for the Epichrysomallinae and the Sycophaginae, but we propose Sycoryctinae, Sycoecinae and Otitesellinae to be included in Pteromalidae. Noteworthy is that Bouček previously assigned Otitesellinae to the family Pteromalidae [21]. The 'Pteromalid complex' is discussed below.

#### 4.2. The 'Pteromalid complex': non-monophyly of the subfamilies Sycoryctinae and Otitesellinae

The subfamilies Sycoryctinae, Sycoecinae and Otitesellinae are hence grouped together with 'true' Pteromalidae in what we call a 'Pteromalid complex'. The family Pteromalidae sensu stricto (even prior to the adjunction of the pre-cited fig wasps) is a quite complex group that includes over 20 subfamilies world-wide, and its monophyly is highly questionable. The group may actually be a composite assemblage of modern and ancestral groups that share plesiomorphic characteristics. The heterogeneity of the Pteromalidae family is illustrated by the variability of labrum morphology in the family [47]. More data, as well as a better definition of the family, are definitely needed. Despite the need for a phylogenetic framework to better define Pteromalidae and in the light of our results, we nonetheless consider that including Sycoecinae, Otitesellinae and Sycoryctinae within Pteromalidae could be the best stopgap measure as long as a precise morphological study of the Pteromalid complex is not available. Our observations suggest that the presence of a gular sulcus could be apomorphic for the Pteromalid complex.

Our analysis strongly suggests that Sycoryctinae and Otitesellinae as currently defined are not monophyletic. Concerning Sycoryctinae, the reconstructed trees never group together the different genera sequenced (*Sycoscapter*, *Watshamiella*, *Apocrypta*). The non-monophyly of Sycoryctinae is relatively well supported by biological and morphological characteristics that are not congruent among the Sycoryctinae tribes. For example, the structure of the female 'gastral tail' greatly varies among groups. In *Sycoscapter* and *Watshamiella*, the ovipositor is protected by the tubularly lengthened last or two last tergites, and the cerci are then situated at the end of the narrowed last tergite. In contrast, in *Apocrypta*, the last tergite is very short and bears the cerci ventrally, while the ovipositor is protected by the third valvulae (sheaths) as in Torymidae. The structure of metasomal segments is furthermore



highly modified to enable oviposition (ploughshare-like metasomal sterna: [24]). Such modified sterna are also encountered in *Critogaster*, a Neotropical genus of Sycoryctinae, but homology of these structures has never been studied. All these structural differences in the female metasoma are highly correlated with great differences in the male structure of the recognized genera of Sycoryctinae (*Apocrypta*, *Sycoscapter*, *Critogaster*, *Philotrypesis* and *Watshamiella*).

Concerning Otitesellinae, *Aepocerus* appears clearly distinct from the African genera *Phylosycus* and *Otitesella*. Interestingly, the placement of the Neotropical genera *Aepocerus* and *Heterandrium* in the family Otitesellinae has already been contested [8]. Morphological discrepancies actually exist between the two groups. African Otitesellinae males are all apterous and have large mandibles, whereas both Neotropical genera (*Heterandrium* and *Aepocerus*) show male polymorphism and have wingless, brachypterous and fully winged males [8]. Another argument suggesting the misplacement of these two genera is the presence of long exerted ovipositors in females *Heterandrium*. The extreme morphological similarity of two *Aepocerus* species to the newly described pteromaline genus *Nadelaia* [48] will deserve further attention. Our results strongly suggest that at least *Aepocerus* cannot be included within the Otitesellinae.

## 5. Conclusion

This study shows that the family Agaonidae sensu Bouček is not an homogeneous entity. Such a result involves a great taxonomical change in pollinating and non-pollinating fig wasp groups, as family Agaonidae is now restricted to the pollinator clade, the other subfamilies being assigned to various chalcid families. Our conclusions also affect the understanding we have of the *Ficus*-pollinator mutualism evolutionary history.

In all our phylogenetic reconstructions, as well as in those obtained by Machado et al. [23], Agaoninae appears to be the basal sister clade of the other Chalcidoidea included in the study. These results strongly suggest the ancestry and monophyly of the pollinator subfamily and weaken the theory following which Epichrysomallinae could be the stem group of fig wasps [7]. The ancient origin of the pollinator clade is confirmed by the distribution of contemporary Agaoninae. Actually, the supposed most primitive genera (*Tetrapus*, New Guinean *Pleistodontes* and *Courtella*) are distributed in South America, Australia–New Guinea and Africa, respectively, which suggests an ancient Gondwanian origin of the subfamily that could be seen as an old chalcid lineage of Cretaceous age [49, 50]. The oldest fossil that can definitely be assigned to the Agaonidae is very probably a *Pegoscapus* species from Oligocene–Miocene Dominican

amber [51], the age of which has been estimated at 23–30 million years (Myrs). This observation shows that the genus *Pegoscapus* has remained mostly unchanged for about 25 Myrs. As in the parasitic Strepsiptera [52], this could be seen as a consequence of a very ancient insect–host association of the Cretaceous age, which experienced a stasis. Agaonidae sensu stricto (i.e. pollinators) is the most ancient clade of fig wasps, which suggests both a long history of the fig–pollinator symbiosis and the ancestry of the pollinating syndrome that apparently only appeared once in fig history.

The ability of entering the fig through the ostiole has evolved several times independently in non-pollinating fig wasps. The convergent shape of the mandible found in Agaoninae and some Sycoecinae species is thus more likely to reflect parallel evolution than true phylogenetic relationships between these groups [25]. The Sycoecinae subfamily, whose species enter the fig through the ostiole just as the Agaoninae, cannot be regarded as the sister group of pollinating wasps [23], as has previously been proposed [7, 26]. The ability to enter the fig to oviposit from the cavity has actually evolved several times independently in Agaonidae sensu stricto, in Sycoecinae, in Sycophaginae (genus *Sycophaga*), in Otitesellinae (genera *Eujacobsonia* and *Grasseiana*: [7, 53, 54]) as well as in Epichrysomallinae (pers. obs.).

In the same way, adaptations that enable the wasps to oviposit through the wall in the fig flowers have evolved several times, and the structures covering and protecting the lengthened ovipositors are quite different among groups. In *Apocrypta* and Sycophaginae, the valvulae entirely cover the ovipositor and act like sheaths. The metasoma of *Apocrypta* are moreover transformed in relation to the exceptional oviposition behaviour exhibited in this genus. In Sycoryctini, the ovipositor is completely covered by the lengthened last tergite (8), while the two last tergites only partially cover it in Philotrypesini. In Epichrysomallinae, as well as in the eurytomid species that parasitize them, the ovipositor is coiled within the gaster and does not protrude [55].

Our results suggest that each non-pollinating fig wasp group has evolved separately from independent lineages to exploit the fig–pollinator mutualism. Fig colonization even occurred several times among the ‘Pteromalid complex’ (at least twice within Sycoryctinae, once in the fig-entering Sycoecinae, once in Otitesellinae and probably once in the Neotropical genus *Aepocerus*). All seem to have become secondarily adapted to this particular resource after the pollinator radiation. Contrarily to what was previously admitted, they did not evolve from a common ancestor, and the selective pressures the non-pollinating fig wasps impose on the fig–pollinator mutualism appeared at different periods of the mutualism evolutionary history.

**Acknowledgements:** The sequence data used in this study were deposited in EMBL GenBank, under accession numbers XXX to XXX. We thank D. Vautrin and E. Barrau for technical help during this work. We are grateful to J. Bronstein, J. Cook, Y. Carton and M. Rojas-Rousse for sending material. Thanks to M. Solignac and D. Lachaise for helpful discussions in the first developments of this project. The manuscript has been greatly improved thanks to an anonymous reviewer.

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