Phylogeny of a taxonomically difficult group and evolution of host location mechanism

Nina Laurenne



Helsinki 2008

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Academic dissertation

To be presented, with the permission of the Faculty of the Biosciences of the University of Helsinki, for public criticism in Auditorium C204 at Economicum (Arkadiankatu 7) on May 9th 2008, at 12 o'clock noon.

Helsinki 2008

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Layout: Nina Laurenne
Cover: *Cryptus* sp., Nina Laurenne

ISBN 978-952-92-3715-9 (Paperback)
ISBN 978-952-10-4644-5 (PDF)
http://ethesis.helsinki.fi

Yliopistopaino
Helsinki 2008

This thesis is based on the following articles:

I Laurenne, N., Broad, G. & Quicke, D. (2002). Preliminary molecular phylogenetic analysis of Cryptinae and related taxa based on 28S D2+D3 rDNA analysed using POY. In: Melika, G. & Thuroczy, C. (Eds) Parasitic Hymenoptera: Taxonomy and Biological Control. Ministry of Agriculture and Regional Development of Hungary, pp. 229-233.

II Broad, G. R., Laurenne, N. M & Quicke, D. L. J. (2004). The genus *Nipponaetes* Uchida (Hymenoptera: Ichneumonidae: Cryptinae) in Costa Rica, with a reassessment of the generic limits. European Journal of Entomology, 101, 651-655.

III Quicke, D. L. J., Laurenne, N. M. & Barclay, M. (2003). Host location and a new host record for *Gabunia* aff. togoensis Krieger (Hymenoptera: Ichneumonidae: Cryptinae) in Kibale Forest National Park, West Uganda. African Entomology, 11, 308-310.

IV Laurenne, N. M., Broad, G. R. & Quicke, D. L. J. (2006). Direct optimization and multiple alignment of 28S D2-3 rDNA sequences: problems with indels on the way to a molecular phylogeny of the cryptine ichneumon wasps (Insecta: Hymenoptera). Cladistics 22, 442-473.

V Laurenne, N. M., Karatolos, N. & Quicke, D. L. J. (accepted). Hammering homoplasy (multiple gains and losses of vibrational sounding in cryptine wasps (Insecta, Hymenoptera, Ichneumonidae).

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1. Introduction

"Nothing in biology makes sense except in the light of evolution", Dobzhansky (1973) pointed out, and this is very true when it comes to understanding the life-history and biology of the ichneumonid parasitoids. Several biological features concerning morphology and behaviour are understandable only in a phylogenetic context. Morphology itself has been traditionally used in constructing classifications and phylogenies, but due to the high level of homoplasy in many groups, the ability of morphology to recover true relationships is debatable. Homoplastic characters pose real problems for systematics, and especially if there is a consistent (directed) bias. The amount of homoplasy is known to be exceptionally high in the ichneumonid and the related braconid wasps (Gauld and Mound, 1982) and identifying reliable characters for understanding their relationships is difficult. For example, only ten characters were used in the first phylogenetic study of ichneumonoids (Sharkey and Wahl, 1992), this reflects the lack of suitable characters, especially for resolving deeper nodes. Recognising monophyletic groups in higher taxa is important because it allows the construction of ground-plan biologies that are important when trying to understand evolutionary trends, such as forms of parasitism, host associations and host shifts. Molecular biology provides an efficient tool for approaching these problems and characters at the molecular level are essential when constructing evolutionary models. Biological information can be examined against a phylogenetic tree and hypotheses about the origin and development of traits can be constructed. A reliable tree with good taxonomic coverage and well supported branches is required to create an evolutionary story.

In this thesis the first phylogeny of the cryptine wasps is presented and behavioural observations and morphological adaptations are examined in the phylogenetic context providing a view to the evolution of the subfamily Cryptinae. The aims were to investigate and test: (1) the existing taxonomic classification of the group which is based on morphology, many groups being defined by combinations of characters rather than on clear synapomorphies; (2) to test the placements of several groups whose placement in the Cryptinae, or the related Ichneumoninae, has been subject to debate; (3) to assess whether antennal modifications that are similar to those known in other parasitic wasps to be associated with a form of echolocation for hosts, play a similar role in the Cryptinae; and (4) to determine whether the antennal modifications are statistically associated with attacking hosts of a particular type.

In a broader context the work provides insights into the high level of homoplasy in morphological characters in the traditionally taxonomically difficult family Ichneumonidae, and into the limits of using a single gene region with dense taxon sampling, in providing a robust phylogenetic hypothesis. In particular, variation in the widely used D2-D3 region of the nuclear ribosomal 28S gene is examined using two different methods of "alignment" (dynamic versus static), and while it is shown that neither is immune from problems of indel homoplasy, current implementations of the dynamic optimization programme POY are more susceptible than multiple alignments using Clustal.

1.1. Biology of ichneumonid wasps

Despite the fact that more species of Coleoptera have been described, there is growing evidence that the Hymenoptera is probably the most species rich of all insect orders, the number of species might reach more than half a million (Gaston and Gauld, 1993). The biggest hymenopteran family is the Ichneumonidae with some 42 generally recognized subfamilies and more than 21433 described species (Yu et al., 2005). The real number of species was esti-

mated by Townes (1969) to be far higher, with probably up to 60 000 species (Gauld, 1991). The number of species is underestimated as an extensive sampling in tropics reveals (Gaston and Gauld, 1993; Hanson and Gauld, 1995; Sääksjärvi et al., 2004).

Ichneumonids are cosmopolitan and most of its members are parasitoids of arthropods, principally other insects and less frequently of spiders and a few other groups (Austin, 1985; Finch, 2005). The biology of ichneumonids is very variable in general, and all forms of parasitism are represented, but common to all ichneumonids is that they kill their host. The most usual insect groups of hosts are Lepidoptera, Coleoptera and Diptera. Hosts are often at a larval or pupal stage when attacked, but some species are ovo-larval parasitoids.

Ichneumonids and other parasitoid wasps can be divided into two groups on the basis of their life-strategy: koinobionts and idiobionts (Askew and Shaw, 1986; Godfray, 1994; Quicke, 1997). These two strategies are biologically very different, but the defining difference between them is that idiobionts stop their host's further development when a female lays eggs, and koinobionts let their host's development continue after an oviposition. In idiobiosis, a paralyzed host is vulnerable to predators, thus they are usually concealed; they can be inside a leaf-roll or buried deep into wood. Idiobiont parasitoids are often ectoparasitoids who have a wide host-range. Koinobionts, instead, are often endoparasitoids whose host range is narrow due to the host's immunological defence. Several biological features are connected to idiobiosis and koinobionsis, such as generalism-specialism and the degree of concealment of hosts.

Exploiting concealed hosts is regarded to be an ancestral trait in the superfamily Ichneumonoidea. The proposition is supported by the Aculeata being a sister-group to the ichneumonoids on the basis of morphological evidence (Hanson and Gauld, 1995; Ronquist et al., 1999). Idiobiosis is suggested to be the ancestral trait in a molecular study of hymenopteran relationships, although the aculeates are not a sister-group to the ichneumon wasps (Dowton and Austin, 2001).

The ability to adapt to a host's development in host utilization is a remarkable event in the life-history of parasitoids. This allows exposed hosts at larval stage to be attacked without the risk of predators to the parasitoids, and when hosts have moved to less open places they are utilized (Shaw, 1983; Gauld, 1988). The transition from idiobionts using concealed hosts to koinobionts using exposed hosts on plants has happened multiple times in the Ichneumonidae (Belshaw et al., 1998; Whitfield, 1998).

However, there is no agreement about the mode of origin of koinobionts that have evolved to exploit concealed hosts: such instances could be the result of reversals from the more common biology of exposed hosts (Quicke et al., 1999) or they might represent direct shifts from idiobionts using concealed hosts to koinobionts also using concealed hosts (Shaw, 1983; Gauld, 1988).

Most of the species richness of insects is concentrated in tropical areas (Gaston, 1991), but the number of described species of the Ichneumonidae has for some while been thought to increase less towards the tropics and it has even been suggested that they are most diverse in temperate regions (Owen and Owen, 1974; Janzen, 1981). Janzen (1981) proposed that due to the very high species richness of plants and specialised herbivores in the tropics, their population sizes are low and more scattered and thus they cannot sustain viable parasitoid populations (RFH, resource fragmentation hypothesis). Therefore parasitoids have to either have a broader host range or be better at finding hosts (Janzen, 1981; Gauld and Gaston, 1994). Another theory put forward to explain this apparently less tropicocentric distribution pattern of ichneumonids is the "nasty host hypothesis" (NHH) introduced by Gauld et al. (1992). It is based on the idea that a larger proportion of tropical plants are defended by toxins and that hosts feeding on these poisonous plants are harmful to parasitoids, because

the parasitoids have to cope with the sequestered plant toxins as well as host defenses. This would lead to a decline in species richness towards the tropics where the parasitoids would have to specialise to cope with the host toxicity. Gauld et al. (1992) showed that the diversity of ichneumonid parasitoids attacking hosts at larval stage in the tropics is lower due to the chemical defense and respectively, the species richness is higher at pupal stages due to the lack of chemical defense. In ichneumonids, pupal parasitoids such as the Pimplinae and Cryptinae have the most species in the tropics (Gauld, 1991).

1.2. Cryptine wasps and their biology

The subfamily Cryptinae is the largest within the Ichneumonidae with 4661 described species belonging to 395 genera (Yu et al., 2005). Collectively, they are cosmopolitan and occur in all terrestrial habitats. Based on the number of described species, the species richness of the tribe Cryptini is relatively higher in the tropics and the number of Phygadeuontini and Hemigasterini species are higher in temperate regions (Hanson and Gauld, 1995) (fig. 1).

The RFH and NHH theories can both explain the differences in species richness between temperate and tropical areas. However, the former suggests that the effect will be apparent in all species whereas the latter suggests that the effect will differ between parasitoids with different biologies. Therefore, neither can be applied to the situation in the Cryptinae as members of all three tribes are (almost) entirely idiobiont ectoparasitoids (Janzen, 1981; Gauld and Gaston, 1994; Gauld, 1992). One possibility is that the relative number of described species is not representative of the true number of species in the different tribes and, therefore, their apparent distributional differences might be an artefact. A likely source of such a bias is body size, since the mean sizes of species in each tribe differs substantially, and smaller species tend to be less well known especially in the tropics (Rathcke and Price, 1976; Janzen, 1981; Gauld et al., 1992; Hawkins et al., 1992). Of the Cryptini, 1724 species have been described in tropical regions and of these only 410 are phygadeuontines and 66 hemigasterines. In the Palearctic and Nearctic regions the number of described cryptines species is 1044, phygadeuontines 520 and hemigasterines 2836 (fig 1). The body size of members of the Cryptini ranges from 2.8 to 27 mm and between 2.8 and 20 mm in hemigasterines whereas phyga-

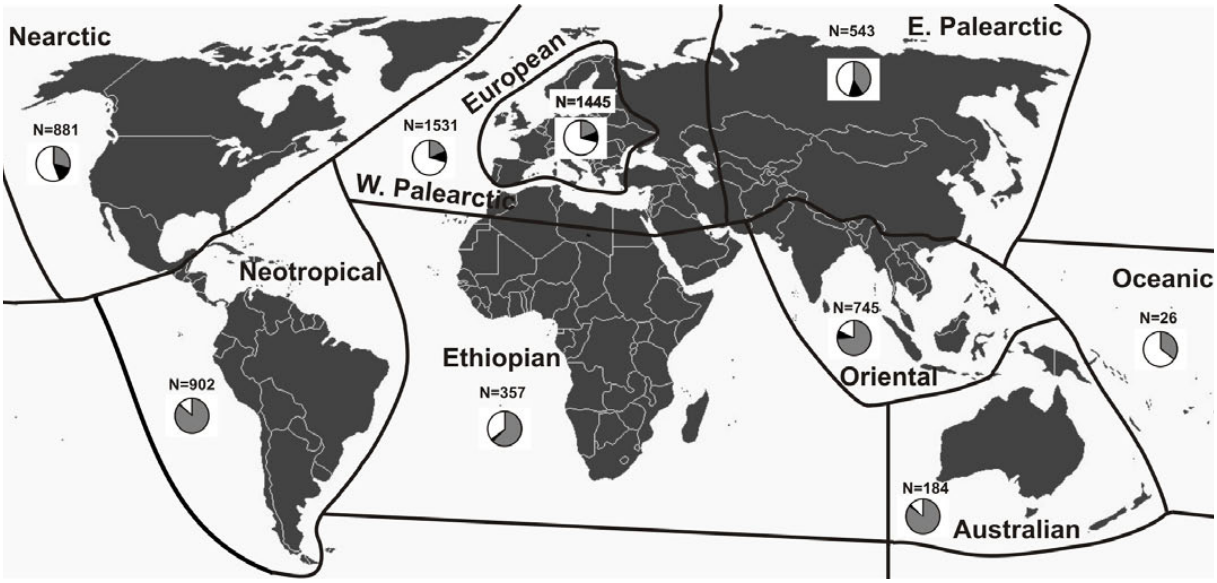

Figure 1. Proportions of the subtribes Cryptini (grey), Hemigasterini (black) and Phygaduontini (white) in each biogeographical region.

deuontines are markedly smaller ranging from 2.8 to 11 mm. In general, bigger species are better known than smaller ones, which might explain the differences in species richness in temperate and tropical areas.

The life-history strategies and biologies of cryptines are remarkably varied making them a particularly interesting group in which to study shifts in biology. Although most cryptines are idiobiont ectoparasitoids that mainly exploit Lepidoptera, Coleoptera and Hymenoptera as hosts, parasitism of Diptera occurs amongst the phygadeuontines and there are spider egg sac predators known within all three tribes (Townes, 1970; Austin, 1985; Fitton et al., 1987). Many of the smaller cryptine species are either obligately or facultatively pseudohyperparasitoids of other ichneumonoids which they attack in their cocooned stage (Hanson and Gauld, 1995; Harvey et al., 2006). Although the host range of cryptines as a whole is broad, individual species can be strictly host specific or have a narrow host range (Askew and Shaw, 1986; Gauld, 1988; Schwarz and Shaw, 1998, 2000). For example, the genus *Phygadeuon* has 109 recorded hosts, but the majority of species with host records are known only to attack 1-2 hosts (30 of 49 species with host records) (Yu et al., 2005), though their actual host ranges may well be larger.

In general, the published host records of parasitoid wasps are sparse in comparison with the number of described species, and they are very often unreliable through the misidentification of the wasp, the host, or both (Noyes, 1994) or their wrong association. This is a major problem when trying to interpret evolutionary trends or macroecological patterns (Pybus and Harvey, 2000). Further, even when the host is known reliably, very few details have generally been published about the precise developmental stages of the host attacked, or its situation on or within the host substrate, again hindering the development of broad theories. Detailed field observations of parasitoid host searching behaviour are virtually non-existent and even when some observations have been published they usually contain very vague terms such as "the wasp antennated" without describing what the wasps were actually doing with their antennae (Ohara et al., 2003; Ueno and Ueno, 2005).

1.3. Finding a host

Finding a host is a process that consists of several stages. A parasitoid must first find a right habitat, then locate a host there, and finally confirm that the species of the host is correct (Vinson, 1985). Several cues can lead a parasitoid to a host, for instance volatile chemicals from host trail, host frass or damaged plants caused by a host larva (Dutton et al., 2000; Rogers and Potter, 2002; Gohole, et al., 2003; Bukovinszky et al. 2005). Visual cues are also important, since wasps can recognise colour, shape and a movement of a host (Fischer et al., 2001, 2004). Pheromones and other kairomones are shown to play an important role in insect behaviour in general, but there are also other species that have developed the ability to detect them and use them for host searching (Wertheim et al., 2003; Jumean et al., 2005). Amongst the other cues, the silk of spiders and insect cocoons can guide parasitoids to the host (Gauld, 1988; van Baarlen et al., 1996; Thibout, 2005). It was postulated that the use of silk in host location might have played an important role in mediating shifts between lepidopteran cocoons and spider egg sacks among the cryptines (Gauld, 1988) but there is so little detail available on the actual cues involved, that this is still just a tempting hypothesis. Multiple cues are used in host searching and their role and efficiency is dependent on environmental factors, such as temperature (Fischer et al., 2001; Kröder et al., 2007a,b). Before an oviposition can occur, females examine the potential host with her antenna and/or ovipositor, and after the host is recognised and accepted, she lays her eggs in/on it (Rogers and Potter, 2004).

1.4. Role of echo-location

Many mobile hosts live in concealed conditions, such as in leaf rolls, plant stems and inside wooden material. Female parasitoids can detect the vibration of the substrate caused by larvae; they usually have enlarged subgenual organs in their fore or hind legs for this (Vilhelmsen et al., 2001; Otten et al., 2003). Finding a deeply concealed host that is immobile and silent, such as a pre-pupa or pupa, is much harder for parasitoids. However, alternative ways have evolved; females can produce sound by tapping the substrate using their antennae and detect echoes with subgenual organs (Broad and Quicke, 2000; Otten et al., 2003). In these wasps, the tip of the female antenna has evolved to form a hammer-like structure which is used to "hammer" potential host substrate so as to create vibrations that can, in turn, be detected by their enlarged subgenual organs. The success of vibrational sounding is affected by several factors, for example Otten et al. (2001) show how females with increased size are better at finding concealed hosts in comparison with smaller ones. The vibrations are better transmitted through increased body mass. Ambient temperature has a remarkable effect on the efficiency on echo-location, as the reliability of it decreases in cooler conditions, where vision becomes more important (Kröder et al., 2006; Kröder et al., 2007a). Parasitoids in a temperal region show adaptation to environmental conditions by adjusting the intensity of vibrational sounding according to the temperature (Kröder et al., 2007b). There is a complicated interaction between the environmental factors of the microhabitat and parasitoids' evolutionary adjustment to them.

1.5. Phylogenetic relationships

1.5.1. Sister group relationship of the Cryptinae and status of the Alomyinae

The family Ichneumonidae is considered monophyletic, although at the subfamily level there is no agreement of monophyly or limits of most of its subfamilies (Sharkey and Wahl, 1992; Quicke et al., 1999, 2000). Several changes in subfamilies and in their names reflect the general difficulty of the group and high level of homoplasy, which may be caused by the convergent evolution of morphology as first really expressed by Gauld and Mound (1982) More recently, much of this has been shown to result from major morphological parallelisms associated with being either endo- or ectoparasitoids, or being idiobiont versus koinobiont (Belshaw and Quicke, 2002; Belshaw et al., 2003). A similar massive set of adaptations has confounded attempts to resolve subfamily level relationships within the Braconidae, the sister group of the extant ichneumonids (Quicke and Belshaw, 1999).

Gauld (1997) introduced an informal classification of the Ichneumonidae where the family is divided into four groups: Labeniformes (= Ichneumoniformes *sensu* Wahl, 1993), Pimpliformes, Ophioniformes and Tryphoniformes, leaving nine subfamilies unplaced (Collyriinae, Lycorininae, Metopiinae, Microleptinae, Neorhacodinae, Oxytorinae, Paxylommatinae, Phrudinae and Stilbopinae). Further studies based on molecular and morphological data (Belshaw et al., 1998; Quicke et al., 2000) recognised Labeniformes (incl. only Labeninae), Pimpliformes, Ophioniformes (incl. Tryphoninae from Gauld's Tryphoniformes and previously unplaced Lycorininae, Neorhacodinae, Phrudinae and Stilbopinae), Orthopelmatiformes (incl. only Orthopelmatinae), Xoridiformes (only Xoridinae) and Ichneumoniformes. In Quicke et al.'s (2000) classification Ichneumoniformes included Cryptinae, Ichneumoninae, Brachycyrtinae, Claseinae, Pedunculinae, Adelognathinae and Eucerotinae (two latter subfamilies from Gauld's Tryphoniformes). The five latter subfamilies have undergone several changes, their statuses have varied and they have been related to the Cryptinae in various ways (Gauld, 1983, 2000; Townes, 1970; Gokhman 1996; Porter, 1998).

The exact borders of these groups and the placements within this structure of several subfamilies have remained elusive. And not surprisingly, not all genera can be placed satisfactorily in subfamilies due to the frequent lack of clear synapomorphies in addition to the rampant homoplasy mentioned above.

The subfamily Alomyinae traditionally has only two genera, Palearctic *Alomya* and *Megalomya*, and the position of the subfamily is unclear. It has been regarded as a separate subfamily (Perkins, 1959, 1960) or as a tribe inside the Ichneumoninae (Diller, 1981; Rasnitsyn and Sijan, 1981; Selfa and Diller, 1994). According to Wahl and Mason (1995) the Alomyinae is synonymous with the tribe Phaeogenini of the Ichneumoninae. The biology of the koinobiont genus *Alomya* is special, because it mummifies its hosts which are larvae of ghost moths living subterreaneally in turf (Lepidoptera: Hepialidae) (Hinz and Short, 1983). The Ichneumoninae genus *Colpognathus* Wesmael, which is placed in the Phaeogenini, is also known to mummify its hosts (Shaw and Bennet, 2001), and superficially its head, as suggested by the generic name, is considerably modified, and superficially similar to that of *Alomya*, especially in females.

Gokhman (1996) used several biological conditions as characters in his phylogenetic study and showed ectoparasitism to be a plesiomorphic condition in the cryptines and endoparasitism to have arisen independently in the Stilpnina-group, Orthopelmatinae and Ichneumoninae. Another plesiomorphic feature is the use of coleopterous hosts and a transition to the Diptera in Stilpnina-group and some other cryptine groups. A transition to the Aranei has occurred in *Adelphion+Pedunculus* clade. Gokhman (1996) continued that attacking hosts in plant stems and trunks is a plesiomorphic condition and that there is a transition to hosts living exposed cocoons in Brachycyrtinae, Cryptinae and Ichneumoninae. Furthermore, oviposition through a thick substrate is widely believed to be an ancestral state for the Ichneumonidae, and a switch to direct contact with the host during an oviposition has evolved in the same three subfamilies.

1.5.2. Classification of the Cryptinae

The classification of the Cryptinae has been based on Townes and Townes (1966) intuitional division into three tribes: Cryptini, Hemigasterini and Phygadeuontini. Further, these tribes have been subdivided into numerous subtribes most of which lack clear synapomorphic characters and this led Gauld (1984) to abandon the subtribal divisions in his classification.

Only one cladistic study has been carried out to date, which was based entirely on morphological characters and included only seven cryptines (Gokhman, 1996). In Gokhman's (1988, 1996) analysis the final phylogeny was not resolved and the relationship between the Cryptinae and the Ichneumoninae was ambiguous and the cryptines could be paraphyletic with this respect to the Ichneumoninae. Gokhman's taxon sampling consisted of only 29 taxa, collectively representing 6 subfamilies, and in addition to an analysis where all characters were equally weighted, he carried out an analysis in which characters correlating positively with biology were downweighted, though the results obtained were broadly similar.

1.6. Phylogenetic methods

Molecular characters provide a powerful tool for solving phylogenetic relationships. Many morphological characters are homoplastic, and therefore not useful in the construction of a phylogeny. Convergent evolution and adaptation to similar environments increase the level of homoplasy, and morphological characters can also be open to various interpretations which make coding of them subjective. Although molecular methods are an incontestable part of systematics, some problems are encountered. The most serious weakness is how to handle length variable sequences. The

result of an analysis can be completely dependent on sequence alignment that can be carried out in several ways. Commonly, sequences are aligned by programs based on the criteria of similarity or a model of secondary structure of a molecule. The choice of parameters is crucial as the result can be entirely caused by, for example, a gap:substitution ratio. Also, big indels in sequences may lead to long-branch attraction where non-related taxa come next to each other although the only link between them is a deletion or an insertion of a gene fragment.

One option to solve the problem is to use a dynamic alignment instead of a static one. In dynamic alignment characters are optimised directly to a tree without an aligning *a priori*. The method is computationally very demanding and a supercomputer or a computer grid is needed when analysing larger data sets. Even a complicated one-gene data set can be impossible to analyse using a single PC.

Evolutionarily different assumptions are often applied when data is aligned and analysed separately. For example, similarity is used in a sequence alignment, but when constructing a tree, the criteria are changed to parsimony or likelihood. The obvious lack of suitable methods available has led to a situation where a pragmatic approach is applied despite the logical conflict between the methods.

1.7. The aim of the study

This thesis focuses on the phylogenetic relationships of the ichneumoniform ichneumonids, with particular reference to the subfamily Cryptinae, based on molecular data. It then uses the recovered molecular phylogenies to assess the evolution of a particular morphological and behavioural adaptation to host searching behaviour, *viz*. the evolution of a particular type of echolocation, termed vibrational sounding, to locate deeply concealed and inactive hosts such as pupae or pre-pupae of wood-boring beetles.

The goals are following:

1. To test the monophyly of the subfamily Cryptinae and to solve its relationship to the Ichneumoninae.
2. To resolve the position of the subfamily Alomyinae in relation to the two subfamilies above.
3. To resolve internal relationships within the Cryptinae and to test the monophyly of the currently accepted tribes and subtribes.
4. To describe the host searching behaviour of the genera *Gabunia* and *Echthrus*, and interpret this in a phylogenetic context.
5. To investigate the role of inferred vibrational sounding (echolocation) in the cryptine wasps based on the distribution of hammer-like modifications of the setae/sensillae at the apex of the terminal antennal flagellomere.

2. Material and Methods

2.1. Taxon selection and molecular methods

As many genera as possible were included and more than one species was selected from several large genera (figs. 2-3). Figure 2 shows the number of currently valid genera (Yu et al., 2005) in the tribes of subfamily of Cryptinae and the number of sequenced genera in this study. Fig. 3 presents more detailed the sequenced cryptine genera per each subtribe. Also, taxa closely related to the cryptines were included to solve their association with cryptine wasps. Altogether at least 298 species belonging to 236 genera were included in this thesis. The material is collected from all over the world representing every continent to ensure comprehensive coverage. The taxon sampling was limited by the suitability of DNA. In the case of several specimens, it was possible to obtain only one leg, therefore equally wide sampling could not be applied in part V.

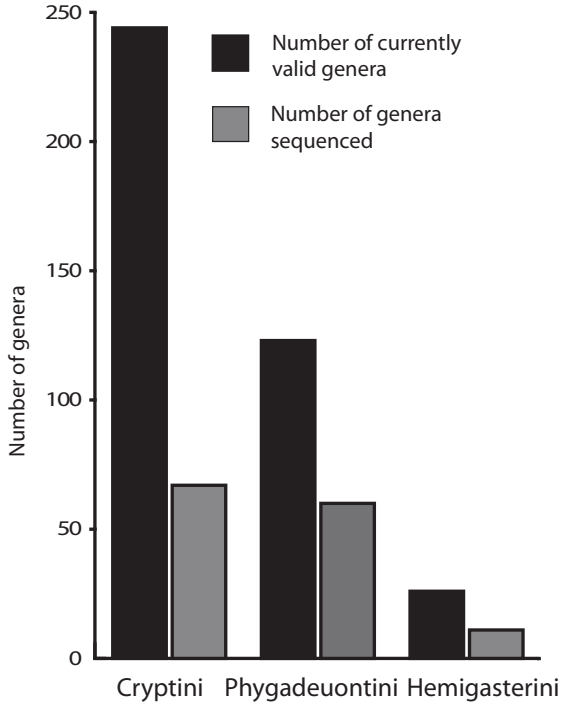

Figure 2. The number of currently valid genera (Yu et al., 2005) in the subfamily of Cryptinae tribes and the number of sequenced genera in this study.

Molecular techniques of DNA extraction, PCR and sequencing are described in detail in parts I-IV. The nuclear gene rDNA28S D2-3 was used to construct the phylogenies.

2.2. Choice of the phylogenetic method

The molecular data were analysed using the cladistic parsimony program POY that optimizes characters directly onto a tree without aligning *a priori* (Wheeler, 1996). POY was chosen because the gene fragment, 28S rDNA D2-D3 region is highly length-variable, and I wanted to explore the potential phylogenetic signal of this variation. A strength of POY was thought to be that it simultaneously aligns and builds trees and so more closely mirrors the evolutionary process. All other methods available require separate alignment which means that the same criterion is not used consistently through the analysis.

Sequences were first aligned by eye and then split into smaller parts, each block starting and ending in a conservative region, to speed up the analysis. (see II-IV). A supercomputer of the Finnish IT center for science (CSC) was used to carry out the analysis. In part IV ClustalW (Thompson, et al., 1994) was used for creating a static alignment that was then analysed using a PAUP (Swafford, 1999). The results of dynamic optimization were compared with those obtained by static alignment + PAUP (IV). A sensitivity analysis was carried out with a range of gap:substitution costs in both dynamic and static alignments. The support values for the clades were estimated in two ways (Goloboff and Farris, 2001). First, the implied alignment from POY was analysed with PAUP and bootstrapped. Second, a rough POY search was carried out and then the majority consensus tree was built from the resulting trees in order to get the confidence values for the nodes.

2.3. Field observations of host searching

The observations were made in August of 2002 in Kibale Forest National Park in Western Uganda. The area was lightly logged in 1969 and it had many fallen trees and standing dead trees. Three different females of *Gabunia* aff. *togoensis* Krieger were observed. One of the

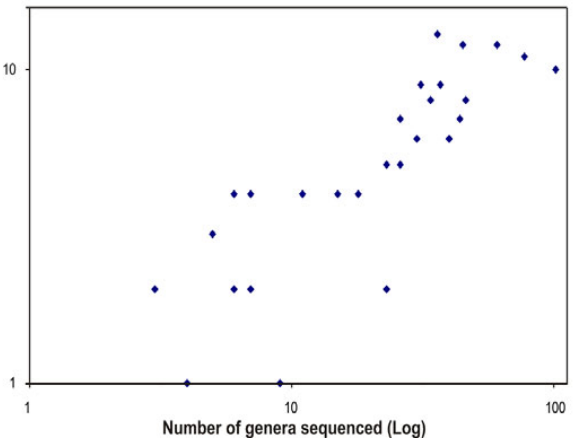

Figure 3. The number of sequenced Cryptine genera per subtribes.

observed female specimens was collected for the identification and the log beneath her was dissected to find a host.

2.4. SEM pictures, morphological coding and host records

The antennae of dissected specimens were photographed using a scanning electronic microscope (LEO 1455VP Electron Microscope with OXFORD Instruments INCA Energy Dispersive X-Ray Analysis System) or the specimens were examined under a light microscope. The taxa examined were classified into five different categories according to the morphological modification on their antennal tips. The host records of each taxon were gathered from literature and the association of buprestid and cerambycid beetle larvae to the antennal modifications were tested using a comparative method using independent contrasts (CAIC) (Felsenstein, 1985; Purvis and Rambaut, 1995).

3. Summary of results and discussion

3.1. Phylogenetic relationships of the cryptine wasps and related taxa (Part I and IV)

The informal grouping of Ichneumoniformes, Ophioniformes and Pimpliformes was mainly supported in all analyses.

Most of the Ichneumoninae tribes with multiple representatives were recovered as paraphyletic. The ichneumon genus *Alomya* was never inside the Ichneumoninae and it appeared to be basal to the Cryptinae. Also, inspecting the sequence data showed *Alomya* not sharing synapomorphies of the Ichneumoninae. The position of *Adelognathus* was not resolved, but in most analysis it came inside the cryptines sharing only one unique synapomorphy with them. The genera *Agriotypys* and *Brachycyrtus* went deeply inside the Ichneumoninae which could be due to their anomalous sequence length. The "Gondwanan" *Clasis* + *Adelphion* + *Euceros* clade was constantly coming out most commonly base of the clade of Ichneumoniformes + Pimpliformes. Its exact placement was sensitive to the gap:substitution ratio.

The relationship between the Ichneumoninae and Cryptinae was not resolved consistently. With lower gap costs both these two groups were sister groups to each other and with high gap cost the Phygadeuontini was a sister group to the Ichneumoninae.

The cryptine tribes Cryptini, Phygadeuontini and Hemigasterini came out largely as monophyletic groups thus mostly agreeing with the Townes's (1969) classification, though the positions of a few taxa were unstable. Townes's (1969) tribal classification had no support, but most of the genera included formed stable genus groups. The genera *Ateleute* (+*Austriteles*), *Bathythrix*, *Endasys* Förster + *Idiolispa* and *Surculus* were basal cryptines in all analyses, but their positions varied leaving the question of the basal taxa of the subfamily open. The position of the genus *Chrysocryptus* is an obvious mistake, due to a mix-up of samples or a mis-identification. The genus *Pleurogyrus* was a mis-identification which turned out to be *Zoophorus* after a re-examination.

3.1.1. Role of the methods applied.
Despite the dense taxon sampling, several misplacements of taxa appeared to be explained by sequence length variation. POY was clearly unable to handle homoplastic indels and to recognise synapomorphies. This led to ambiguous placements of taxa with sequence lengths differing from the average length. One option is to cut off the length variable loop regions, which however means losing information, when in fact, the informative parts of the sequences are in variable regions. The taxa with indels were obviously sensitive to the gap:substitution ratio, thus the placement of these jumping taxa cannot be regarded as reliable.

The results of the POY and ClustalW + PAUP analyses were basically the same. The

placement of a few taxa (*Brachycyrtus*, "Gondwanan" clade and *Agriotypus*) was considered more reliable in the ClustalW + PAUP analysis, but ClustalW does not escape the problems of homoplastic sequence variation. For example, the ichneumonine genera *Dagoo* and *Macrojoppa* both had inserts and they did not recover with the Ichneumoninae.

Including more genes would certainly bring information, but there is clearly a trade-off between fewer genes with multiple taxa and multiple genes and fewer taxa. The choice of the gene is also fundamental, for instance adding a rapidly mutating gene raises the level of "noise" and the results are less resolved than those obtained by using only one gene (Laurenne, unpublished).

Bootstrapping the POY implied alignment exceeded 50% in 75 branches, but the quick POY estimation supported only 39 branches, which reflects the difficulty of the computation with direct optimization. The most supported groups were located on the tips. The quick consensus estimation runs for assessing branch supports did not give the same groupings as the runs for the actual analysis because of "jumping taxa". Therefore, giving a support value was not possible if the clade did not have exactly the same taxa in both runs. This had an effect on the clade support values of the higher level grouping especially. Using jackknifing was not possible in the version 2.7-02-08-01-p2-beta of POY that was used.

The 28S gene was informative when investigating the genus level relationships and the utility of it in hymenopteran research has been pointed out by other researchers as well (Belshaw and Quicke, 1997; Mardulyn and Whitfield, 1998). The alignment of the gene is problematic due to the length variation which affects the result as discovered in part IV. Alignment based on secondary structure was not used it is only very labour-intensive and it is not fool-proof in its assessment of homology. Also, in its basic form, a lot of potentially useful phylogenetic information is excluded.

3.2. The genus *Nipponaetes* (Hymenoptera: Ichneumonidae: Cryptinae) in Costa Rica, with a reassessment of the generic limits (Part II)

The endemic Costa Rican genus *Zurquilla* Gauld, 1997 (Tryphoninae: Oedemopsini) was synonymised with *Nipponaetes* and transferred from the Tryphoninae to the Phygadeuontini on the basis of morphological and molecular evidence. A phygadeuontine female specimen collected in Thailand was identified as *Nipponaetes haeusleri* (Uchida, 1933). It is a small genus of old world tropics which Townes (1970) placed in the tribe Phygadeuontini. The identification of the specimen led to a tryphonine genus *Zurquilla* indicating a possible synonymy with *Nipponaetes*. Two alternative hypotheses were tested: firstly, *Zurquilla* was mistakenly described as a tryphonine and it actually belongs to the Cryptinae, secondly *Nipponaetes* is an aberrant tryphonine and does not belong to the Cryptinae. In the phylogenetic analysis consisting of a wide range of tryphonines and cryptines *Nipponaetes haeusleri* recovered as a phygadeuontine. In the majority of gap:substitution costs, it emerges as the sister group to the genus *Hyparcha*. Also, the morphological characters support the molecular evidence that *Nipponaetes* is a phygadeuontine.

Apomorphies of *Zurquilla*, such as the features of the forewing and the structure of mesosternum (Gauld, 1997), are found also in phygadeuontines. The genus *Zurquilla* lacks of features of oedemopsines, such as the membranous area on the lower valve of the ovipositor. These findings reflect the high level of homoplasy amongst the ichneumonids discussed earlier.

3.3. Host location behaviour and a new host record for *Gabunia* aff. *togoensis* Krieger (Hymenoptera: Ichneumonidae: Cryptinae) in Kibale Forest National Park, West Uganda (Part III)

The large females of the Afrotropical cryptine genus *Gabunia* use vibrational sounding for finding their wood-boring beetle hosts. The host searching behaviour of three different females was observed in Kibale Forest National Park in western Uganda. The behaviour observed in these females was essentially identical and the searching behaviour of one of them is described in detail. The *Gabunia* specimen was active only in sunshine in the afternoons, performing a slow to-and-fro walk along the dead *Celtis africana* Burm. F (Celtidaceae) tree, tapping the substrate with her antenna. After finding a potential host, she examined the tree surface with her ovipositor sheath tips, which probably means that she was specifically searching for existing cracks or holes to access the host. The ovipositor was penetrated into a hole very quickly and no "drilling" was involved. This minimizes the time the wasp was exposed to predators. The *Gabunia* specimen was collected and the tree was dissected to find the host. No larva was found beneath the ovipositor penetration point within a potential reach of the wasp's ovipositor, which could be due to the wood being riddled with frass-filled beetle larvae galleries. On the opposite side of the 10 cm diameter log there was a large buprestid (Chalcophorinae) beetle larva in the depth of 1 cm from the surface. Dissecting the remainder of the log revealed no other buprestids, but there was a cerambycid that had pupated very recently. This was identified as *Phryneta leprosa* (Lamiinae: Phrynetini). As it was a similar size to the buprestid larva, it might have been a potential host for the *Gabunia*. The female described here failed to find the exact position using vibrational sounding, which means that this wide-spread strategy used by woodborer parasitoids is not completely efficient. The heavily-eaten substrate might not produce echoes to the tapping sufficiently. Vibrational sounding can also be attenuated by thick substrate, such as hard wood. Thus, females showing host-searching behaviour, do not necessarily start cost-effective drilling (Fischer et al., 2003). The response of substrate tapping helps a parasitoid, not only to evaluate the success of finding, but also reaching a host.

The hemigasterine genus *Echthrus* is shown to have a similar behaviour (video ref.). Also, the morphology of antennal structure supports the behaviour; the tips of antennae have modified into a hammer-like structure.

3.4. Hammering homoplasy: multiple gains and losses of vibrational sounding in cryptine wasps (Insecta: Hymenoptera: Ichneumonidae) (Part V)

Within the Cryptinae the tips of the antennae have been modified into a hammer-like structure that is suitable for knocking the substrate for locating hosts. Host searching using vibrational sounding is typical to the tribe Cryptini and antennal modification is shown to be associated with the exploitation of wood-boring beetle larvae.

In the Cryptini, 70 of 79 genera examined had antennal modification, in Phygadeuontini and Hemigasterini the occurrence of the hammer was uncommon. The modification was from the acuminate with simple setae on tip to being truncate with a large and flat or even concave hammer area. The antennal structures were clearly associated with the parasitasion of wood-boring buprestid or cerambycid beetles. Nineteen genera of the Cryptinae exploit buprestid or cerambycid larvae as a host, from which 13 are genera of the Cryptini, two of Hemigasterini and five genera of Phygadeuontini.

Also, other ichneumonid subfamilies (Labeninae, Xoridinae and Pimplinae) have hammers and amongst the other Hymenoptera, they occur in the sawfly family Orussidae (Broad and Quicke, 2000; Vilhelmsen, et al.,

2001). Thus, the feature must be clearly homoplastic and has evolved several times amongst the ichneumon wasps (Broad and Quicke, 2000). There are no field observations in the literature, but Quicke et al. (2003, part III) link the antennal morphology and searching behaviour in a natural environment. The morphological structures indicate an adaptation to finding concealed hosts.

The members of the Cryptinae subfamily are mostly idiobiont ectoparasitoids that have a wide host range and the Phygadeuontini are usually considered represent a ground-biology for the cryptines. Phygadeuontine wasps typically parasitise exposed or weakly concealed hosts such as various Lepidoptera, Coleoptera, Hymenoptera and Diptera (Gokhman, 1996). In large genera, such as *Phygadeuon* and *Gelis*, different forms of parasitism occur. For example, *P. clotho* is an endoparasitoid while most of the species of this genus are ectoparasitoids (Rotheray, 1988). The hosts of the Cryptini are typically more concealed and the association between wood-boring hosts and antennal hammers suggests that vibrational sounding has evolved in the Cryptini. Silent hosts, such as pupae, do not chew wood and are also suitable victims for echo-locating parasitoids. Silent hosts that can be found using vibrational sounding are larvae that are fed by adults, for example aculeatan larvae nesting in beetle-borings. The occurrence of antennal hammers amongst the Aculeata parasitoids might be explained by the shift of exploitation of Aculeata to Coleoptera (Gauld, 1988).

There are insufficient published observations of what part of the antenna is used for host-location. The term "antennation" covers the use of different parts of the antennae which can be from the mid-ventral part to the pre-apical dorsal surface of the antenna with the tip curled ventrally. Also, other parts can be used for vibrational sounding, for instance females of *Xorides* (Xoridinae) have T-shaped cuticle pegs at the elbowed points of the antennae (Quicke, unpublished observation).

4. Conclusions

1. The three subfamilies of the Cryptinae form stable groups and the subtribal division should be abandoned. Claseinae and Pedunculinae are separate subfamilies and not part of the Cryptinae. The Alomyinae is removed from synonymy with the Phaeogenini and is regarded as constituting a separate subfamily from the Ichneumoninae and Cryptinae. The relationship of the Cryptinae and the Ichneumoninae was not solved definitely.

2. The genus *Zurquilla* was synonymised with *Nipponaetes* and transformed from Tryphoninae to Cryptinae.

3. The females of the genus *Gabunia* use echolocation for searching buprestid or cerambycid hosts.

4. The antennal hammers are a typical feature for the tribe Cryptini that show adaptation to searching immobile hosts (pupae) and hosts living in concealed conditions (inside wooden material).

Future prospects

In order to get a solid phylogeny for the cryptine wasps, rare taxa should be included in the analysis. Work using molecular methods is often restricted by the unsuitability of material because museum specimens tend to be too old to yield a DNA work. Also, more genes are needed for constructing the evolutionary models and this needs the development of the laboratory methods, such as primer design. Using traditional morphological characters is important in order to respond to the criticism of not including them. If the morphology was in a key role, then there would probably be a tree based on it already, but the homoplasy problem is not easy to overcome.

Echolocation by parasitic wasps should be investigated more thoroughly using a laser vi-

brometer to determine the correlation between the type and size of antennal hammers and the volume of vibration. Also, using high speed cinematography to record host searching and antennation across a range of cryptines would hopefully determine what the precise roles of intermediate stages of antennal hammer development are. Another interesting question is whether the size of the subgenual organ is correlated with that of the hammer or to the substrate or both. The role of antennal pegs in males is also an open question which needs studying.

To understand the biology in a meaningful way, more host records are needed. Currently the hosts of most of the taxa are unknown and this makes it virtually impossible to study host shifts and the evolution of parasitism. Another problem is the lack of field observations on behaviour. Knowing what insects actually do in their natural environment allows for better interpretation of the function of morphological characters. Therefore, the importance of fieldwork cannot be overestimated.

Acknowledgements

I want to thank Prof. Donald Quicke for supervising this thesis, his enormous enthusiasm for parasitic wasps, his endless imagination and ideas have done this process rich and fruitful. I also thank my other supervisor Jyrki Muona; without Jyrki's remarkable help I would have never started this work in the first place. I thank the co-authors and those tens of people who kindly supplied material for this study. The following foundation supported me financially: Academy of Finland (FIBRE), Ella and Georg Ehrnrooth foundation, Helsingin Hyönteistieteellinen Yhdistys, Oskar Öflund foundation, Societas Pro Fauna et Flora Fennica and Suomen Hyönteistieteellinen Seura, their concrete help was essentially important. I want to thank Gunilla Ståhls-Mäkelä for teaching me the secrets of lab work, and the MES-lab technicians Tupu Kuismin and Anne Aronta. Marianna Teräväinen kindly helped my with the language. All the necessary facilities needed for this work were provided by Finnish Museum of Natural History.

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