Phylogeny of the subfamily Braconinae (Hymenoptera: Braconidae)

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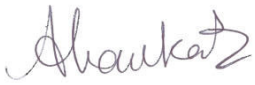
Abstract

The phylogeny of the parasitic wasp subfamily Braconinae was estimated using both nuclear (28S rDNA) and mitochondrial (CO1 and NADH1) genes along with morphological characters. The phylogenetic tree obtained from the nuclear 28S rDNA failed to resolve this subfamily as monophyletic and divided into two monophyletic tribes, the Aphrastobraconini and Braconini, with the Coeloidini in the middle as was established by previous studies. The *Compsobraconides*, *Virgulibracon*, *Myosoma*, *Shelfordia* and *Atanycolus* genus-groups were recovered as monophyletic. However, some tribes and genus groups, based on traditional classification, were not recovered as expected. Therefore, a character re-weighting criterion, devised by Goloboff, was used to downweight any noise in the data, though this procedure also revealed almost similar results. Both of the mitochondrial genes, CO1 and NADH1, were also unable to recover a monophyletic Braconinae on their own. Although two different methods of phylogeny reconstruction, maximum parsimony and Bayesian analysis were used, similar results were obtained. However, both of the mitochondrial genes divided the genus *Bracon* into meaningful groups based on the geographic origin of the species sequenced. The combined analysis of nuclear (28S rDNA) and mitochondrial (NADH1) genes revealed Braconinae to be monophyletic and comprised two monophyletic tribes, Aphrastobraconini and Braconini. This combined analysis has recovered the genus *Digonogastra* and the *Atanycolus*-group of genera as monophyletic. The rooting of the phylogenetic tree was different from the one obtained from the analysis of nuclear gene (28Sr DNA) alone and this is discussed in chapter 7.

Declaration

The work presented in this thesis is entirely my own and it has not been submitted for any other academic qualification.

People who provided less formal help and advice are listed in the acknowledgements.



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Chapter 1: General Introduction

1.1. Phylogenetics of Braconidae

The Ichneumonoidea is the largest superfamily in the order Hymenoptera, is further divided into two extant families, the Ichneumonidae, with 40 subfamilies and 23,331 valid species (Quicke et al., 2009), and the Braconidae with 48 subfamilies and 17,605 valid species (Yu *et al*., 2004). Thus the superfamily comprises approximately 2% of Earth's described species (Dolphin, 2001). Almost all ichneumonoids are parasitoids of other arthropods, principally Coleoptera, Lepidoptera, Diptera and Hymenoptera. Ichneumonoids show a variety of parasitoid strategies as they can be ectoparasitic, when the larva feeds from the host externally or endoparasitic, when larva feeds inside from the host, also from another view point depending upon the effects of parasitoids on their host, they can be idiobionts, arresting the host activity after attack or koinobionts where the host can carry on usual life activities for some time after being oviposited by the parasitoid (Shaw & Huddleston, 1991; Haeselbarth, 1979; Askew & Shaw, 1986). These specializations make them ideal tools in biological control and model organisms for evolutionary biology (Godfray, 1994; Quicke, 1997; Dowton *et al*., 2000).

Traditionally, the Braconidae has been divided into two major groups, the cyclostomes and the non-cyclostomes, based on morphology and biology (Shaw & Huddleston, 1991), especially considering the hypoclypeal depression above the mandibles. The cyclostomes typically have the lower part of the clypeus sharply recessed and concave exposing a concave and largely glabrous labrum (Wharton, 1997) while the non-cyclostomes lack this character. Some groups now known with high certainty to belong to the cyclostome

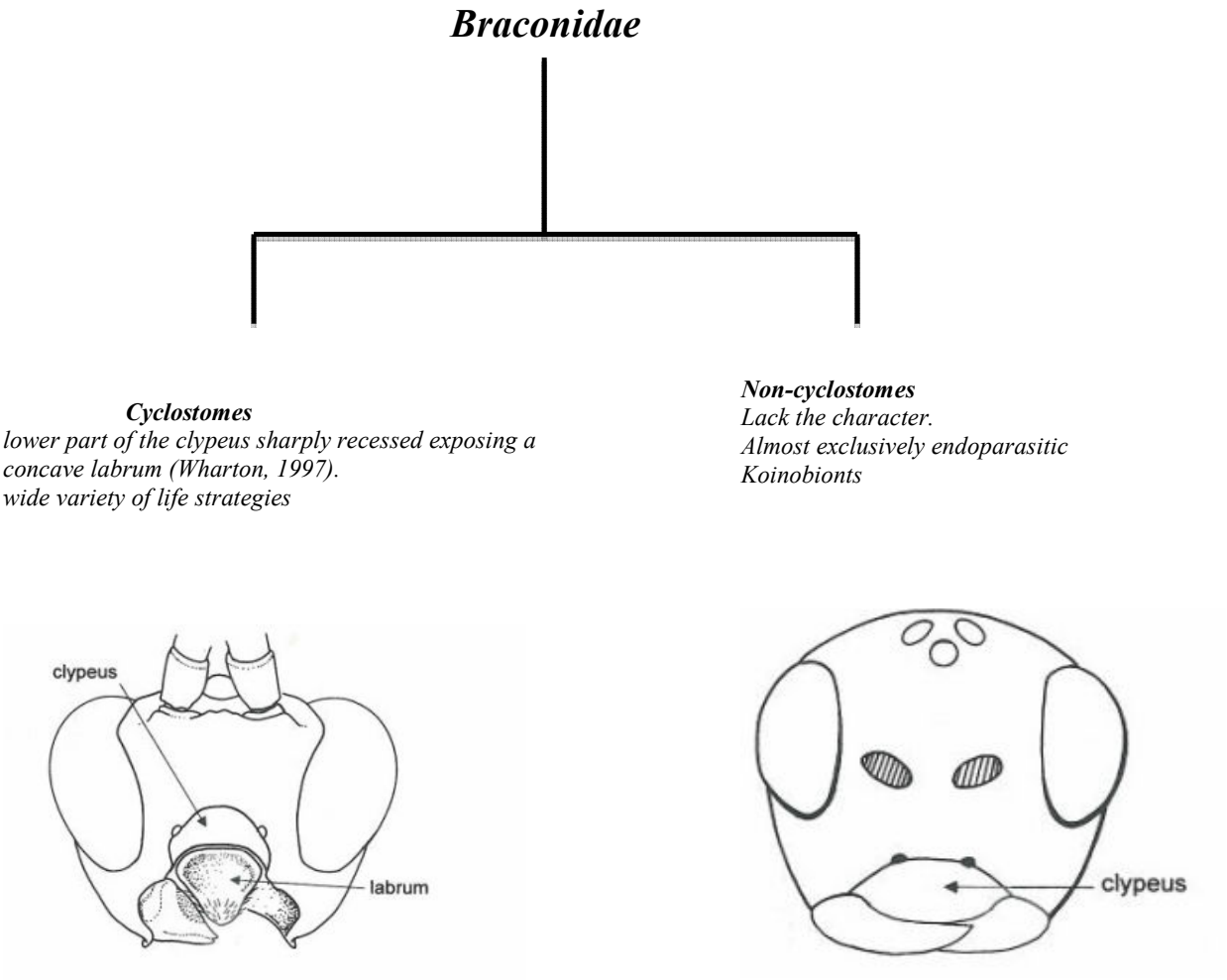


Figure 1.1 Difference between the cyclostome and non-cyclostome condition in Braconidae.

lineage have apparently secondarily lost their cyclostome morphological condition. These include some Betylobraconinae, most Opiinae, Aphidiinae and all Alysiinae. The non-cyclostomes are also subdivided into the microgastroid complex and a poorly supported helconoid complex (Wharton, 1993). Secondly the biologies also differ as non-cyclostomes are almost exclusively endoparasitic koinobionts while cyclostomes are mostly ectoparasitoids but also exhibit a wide variety of life strategies as they attack not only a range

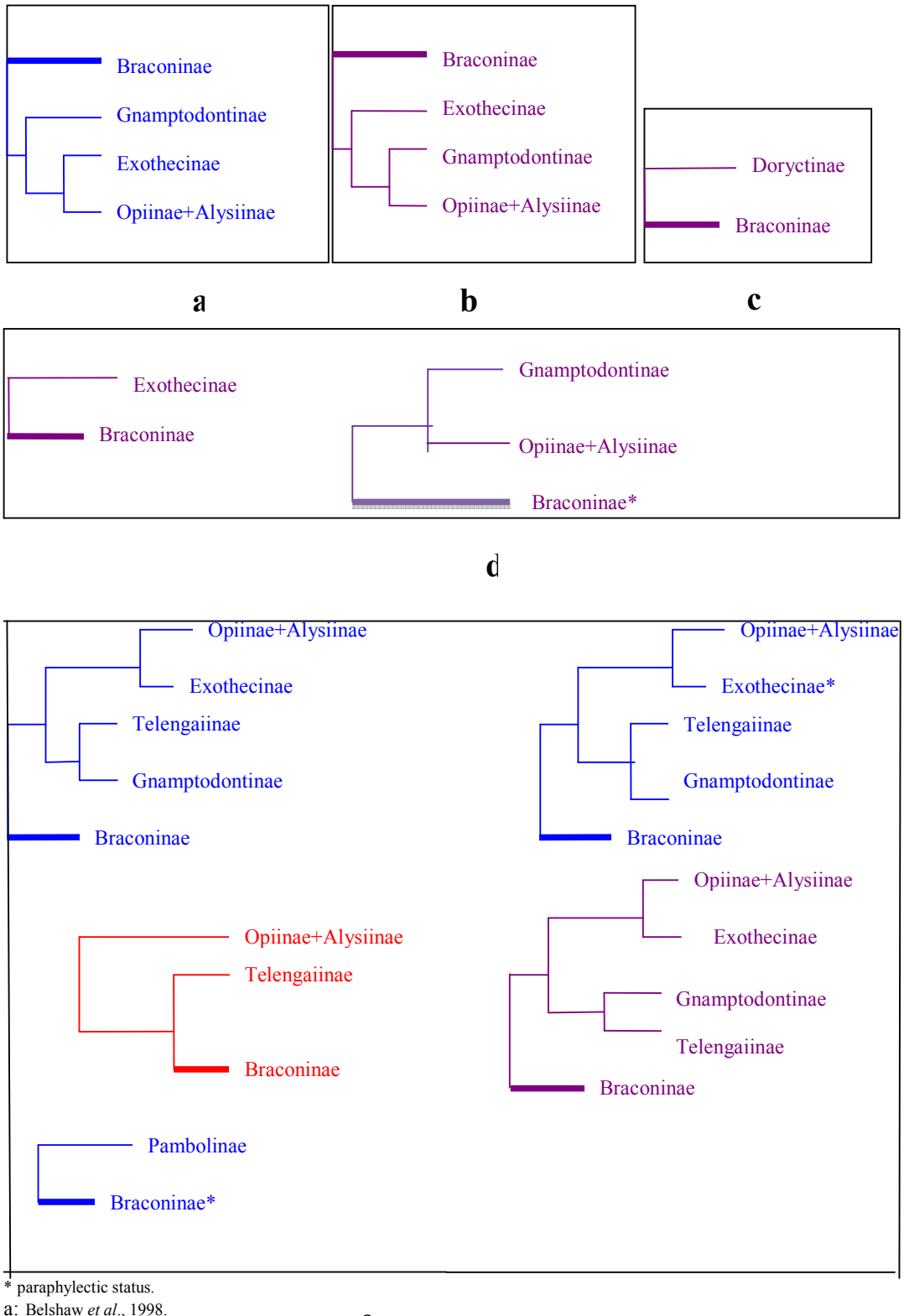
of holometabolous larvae (Belshaw *et al*., 1998; Dowton *et al*., 2002) but also hemimetabolous insect juvenile and adults as in Euphorinae and Aphidiinae (Belshaw & Quicke, 1997). Phytophagy is also found in the Braconidae as shown in the form of seed-associated gall forming Doryctinae and gall forming Mesostoinae (Wharton & Hanson, 2005) plus the suspected inquilines that feed on plant tissues of galls induced by cynipids or cecidomyiids and one species from Braconinae, *Bracon phytophagus* which is also a specialist seed feeder (Flores *et al.,* 2005).

The early studies on the evolution of Braconidae by Tobias (1967) based on adult morphology showed cyclostomes to form a grade independent of non-cyclostomes but when Čapek (1970) used larval morphology the cyclostomes seemed to form a basal grade leading to non-cyclostomes. This conflict continues even later as van Achterberg (1984a) found a result similar to Tobias (1967) with morphological and biological characters followed by Quicke & van Achterberg's (1990) cladistic analysis of a larger set of morphological and biological characters, which also found cyclostomes to form a basal grade. However, this result was highly controversial and disliked by many other braconid workers. Wharton *et al*. (1992) reassessed Quicke's study but admitted that in their reanalyses of the data they obtained similar results. Van Achterberg & Quicke (1992) defended many of the details of their analysis.

The addition of molecular data and use of combined analysis is improving the situation for Braconidae as it is constantly reveal to be divided into cyclostome and non-cyclostome groups and these appear as sister-groups (Belshaw *et al*., 1998; Dowton *et al*., 2002; Shi *et al*., 2005; Pitz *et al*., 2005; Zaldivar *et al*., 2006). The study by Belshaw *et al*. (1998) was based on molecular data alone and their unrooted tree defined a grade with cyclostomes and another with non-cyclostomes with Aphidiinae in the middle. This was followed by a combined molecular and morphological analysis by Dowton *et al*. (2002) that

also divided the cyclostomes and noncyclostomes in two groups separately with the Aphidiinae as a sister-group to cyclostomes. A subsequent study of Shi *et al*., (2005) was very poor as discussed by Pitz *et al*. (2005) who pointed out that they used a reverse compliment for one of the gene sequences, their morphological data was not good enough, their results were not always supported by their data, and they did not explain the model of evolution chosen nor the basis for choosing that particular model for their Bayesian analysis. When data of Shi *et al*. (2005) were reanalysed in Pitz *et al*., (2005) and they pointed out the above stated problems they also made the same mistake of data not supported by results when they mentioned that the cyclostomes formed a basal grade as it was not supported by the trees they published because the noncyclostomes *Urosigalpus* and *Blacus* were found nested among cyclostomes. In the study by Zaldivar Riveron *et al*. (2006), several small subfamilies e.g. Pambolinae, Rhysipolinae, Hormiinae, Lysiterminae and Betylobraconinae were not recovered as monophyletic. Also most of the data sets recovered Mesostoinae + Aphidiinae as a 'Gondwanan' basal clade that also confirms the results from (Belshaw *et al*., 2000; Belshaw & Quicke, 2002; Dowton *et al*., 2002) followed by a clade by members of Rhyssalinae that was found paraphylectic with respect to *Histeromerus* (previously in its own subfamily). Zaldivar Riveron *et al*. (2006) recovered Opiinae + Alysiinae + Exothecinae + Braconinae + Gnamptodontinae + Telengaiinae clade with last two families appearing as sister taxa in all combined analysis.

The sister group relations of the Braconinae found in all the above studies are summarized in figure 1.2. It is apparent that a small subset of subfamilies is routinely found as sister groups that include Alysiinae, Doryctinae, Exothecinae, Gnamptodontinae, Telengaiinae, Lysiterminae, Opiinae and Rogadinae.



* paraphylectic status.
a: Belshaw *et al*., 1998.
b: Dowton *et al*., 2002.
c: Shi *et al*., 2005.
d: Pitz *et al*., 2005.
e: Zaldivar *et al*., 2006.

Figure 1.2. Previous studies showing the relationship of other subfamilies of Braconidae with Braconinae.

1.2. The subfamily Braconinae

The Braconinae is the largest subfamily in the family Braconidae with 180 valid genera and more than 2,893 valid species (Yu *et al*., 2005) and these numbers are constantly increasing. Its great diversity is seen in Old World tropical and subtropical regions (Quicke, 1987b). Members of the Braconinae are usually ectoparasitoids and, as such, tend to be less host specific compared to endoparasitoids (Wharton, 1993) thus the host range of the subfamily includes more than one insect order. The hosts of Braconinae may belong to Coleoptera, Diptera, Lepidoptera, Hymenoptera and can even be plants as shown by phytophagous species *Bracon phytophagus* (Ryan & Rudinsky, 1962; Quicke, 1983a; Quicke, 1987c; van Achterberg, 1988a; Wharton *et al*., 1989; van Achterberg, 1993; Kenis, 1997; Yu & Quicke, 1997; Achterberg & Weiblen, 2000; Quicke & Polaszek, 2000; Quicke *et al*., 2000; Pettersson *et al*., 2001; Santos *et al*., 2004; Quicke *et al*., 2005; Quicke & Stanton; 2005; Wharton & Hanson, 2005; Flores *et al.*, 2005; Wang *et al*., 2006). These wasps are largely ectoparasitoids but some members of the Aspidobraconina (*Philomacroploea, Hyboteles* and *Aspidobracon*) are endoparasitic on pupae of Nymphalidae (Danainae), Pieridae and Hesperiidae (van Achterberg, 1984b; Quicke, 1987a). There is a publication suggesting that other hosts of *Philomacroploea* may include stem-boring moths but this is almost certainly erroneous (Quicke, 1987b).

1.2.1. Biology of the subfamily Braconinae

The members of the subfamily Braconinae cover a vast host range and ecological niches as their hosts also cover a large number of habitats. The subfamily Braconinae seems to have evolved with the evolution of Tropical forests as a result their great abundance is seen in these tropical areas. The tropical forests are rich in all kind of biological activity. These

forests have dead trees forming the logs that support a lot of wood boring insects belonging to Coleoptera specially the families like Scolytidae, Curculionidae, Buprestidae, Cerambycidae, Bruchidae and Anthribidae. In case of Braconini, Physaraia parasitizes the gall forming curculionid beetle larvae (Donaldson, 1989), members of genus *Aneuradesha* are specialist ectoparasitoids on the hispine larvae (van Achterberg, 1984b: Quicke, 1987). A new species, L. keijua Laurenne & Quicke, belonging to the genus *Latana* Cameron was found parasitic on lamiine cerambycid beetle larvae in western Uganda (Laurenne & Quicke, 2004).

In Aphrastobraconini, most members attack coleopterous wood boring beetles belonging to families Cerambycidae, Buprestidae, Scoltyidae and Anthribidae. Genus *Shelfordia* attacks fungus weevils of the family Anthribidae (Wang *et al*., 2003). Some species of *Bathyaulax* attack wood boring beetles belonging to Cerambycidae attacking 'forest mahogony' tree, *Trichilia dregeana* Sond. (Meliaceae) (Quicke *et al*., 2005). The genus *Euurobracon* parasitizes members of Cerambycidae (Shenefelt, 1975, 1976, 1978) and long horn beetles of this family are also attacked by genus *Megalommum* Szépligeti (van Achterberg & Mehrnejad, 2011). The genus *Coeloides* parasitize larvae of pests attacking coniferous and broadleaved trees belonging to the families Scolytidae, Curculionidae and Buprestidae (Kenis, 1997; Pettersson *et al*., 2001; Wang *et al*., 2006). The *Coeloides filiformis*, was found parasitizing Coleoptera (particularly *Leperisinus* spp. [Hylesinus]) developing in *Fraxinus* sp. bark, and C. *sordidator*, parasitizing Coleoptera feeding in pine bark (especially *Pissodes* spp.). In United Kingdom, *C. abdominalis* (a parasitoid of Scolytidae in pine bark), *C. melanotus* (a parasitoid of *Leperisinus* spp. in Fraxinus bark) and *C. scolyticida* (a parasitoid of Scolytidae in Ulmus bark) were found (Shaw, 2000). The genus *Cyclaulacidea* was found attacking bruchid and curculionid beetle pests of Palm trees in Central America (Qucike & Delobel, 1995; Leathers *et al*., 2005).

The order Lepidoptera is another large insect order serving as host to the Braconinae

parasitoids. The Lepidopterous families Crambidae, Limacodidae, Noctuidae, Oecophoridae, Pyralidae, Tortricidae are commonly found parasitized by Braconinae genera. In the tribe Braconini, *Bracon hebetor* SAY., *B. picticornis* WESM., *B. variator* NEES, *B. variegator* SPIN were found as larval ectoparasitoids of leaf rollers belonging to the family Tortricidae (Diaconu & Lozan, 2000). These leaf rollers are important insect pests of fruit trees. *Calcaribracon camaraphilus* was found a larval ectoparasitoid of *Casmara patrona* Meyrick (Lep.: Oecophoridae) while *Myosoma yanoi* was also recorded in China (You *et al*., 1994; Quicke & You, 1996). *Stenobracon rufus* Szépligeti was found attacking the stem borer pests belonging to Noctuidae, Crambidae while *Bracon sesamiae* Cameron on stem borers from Pyralidae and Noctuidae in Africa (Milner, 1967; Achterberg &Walker, 1998; Achterberg & Polaszek, 1996; Kfir, 1997; Chinwada & Overholt, 2001). *Bracon albolineatus*, *Bracon alaquei*, *Bracon chinensis* [*Amyosoma chinensis*], *Bracon gelechiae*, *Bracon onukii*, *Stenobracon hispae*, *Stenobracon deesae*, *Tropobracon luteus*, *Tropobracon indicus* and *Tropobracon schoenobii* were found parasitizing rice borer insect pests belonging to family Pyralidae (Ahmad & Haris, 2002).

The genus *Glyptomorpha* was found parasitizing the stem borer belonging to the family Noctuidae in Turkey (Beyarslan *et al*., 2006). *Acrocerilia* has been reported to parasitize the cocoa moth in Philippines while *Cordibracon* parasite of Limacodidae on palms from Malyasia (van Achterberg, 1989). Quicke (1983) reported Braconinae in various countries including *Euvipio* sp. from *Chilo partellus* (Swinh.) (*C. zonellus* (Swinh.)) (a pest of maize and sorghum) in Sudan, *Merinotus* sp. from *Busseola fusca* (Fuller) (a pest of maize and sorghum) in Nigeria, *Curriea* sp. from *Pansepta teleturga* Meyr. on cacao in Papua New Guinea, *Tropobracon* sp. from a rice stem-borer in Hong Kong, and *Zaglyptogastra* sp. possibly from *Neonitocris princeps* (Jordan) (*Dirphya princeps*) (a pest of coffee) in Uganda.

In case of order Diptera, *Bracon celer* is reported to be a potential biocontrol agent for

olive fruit fly belonging to the family Tephritidae (Daane & Marshall, 2010). *Bracon exhilarator* is reported to parasitize *Amaurosoma* sp. (Scatophagidae), *Bracon luteator* attacks on *Urophora solsititialis* L. (Tephritidae), *Bracon minutator* on *Lipera lucens* MG. (Chloropidae), *Bracon nigratus* on *Chaetostomella cylindrica* R. D. (Tephritidae), *Bracon (Glabrobracon) anthracinus* on *Urophora cuspidate* MG. (Tephritidae), *Bracon (Glabrobracon) atrator* on *Tephritis separata* RD. *T. conura* LW. (Tephritidae), *Phytobia flavifrons* MG. (Agromyzidae) and several other species of *Bracon* attack Diptera but also Coleoptera, Lepidoptera, Hymenoptera as well (Beyarslan *et al*., 2005).

The Braconinae parasitoid wasps have strong and well developed mandibles The mandibles in parasitoids can be used to tear off puparium in order to emarginate, mating, searching for hosts, handling of hosts, nest excavation and construction and feeding but their function in the Braconinae is still not confirmed (Jervis *et al*., 1996; Jervis, 1998; Jervis & Kidd, 1998). Braconinae wasps also show variation in their ovipositor structure depending upon their hosts and host localities. As they may have long serrated ovipositor as in *Zayglaptogastra* or some have small ovipositor like *Soter* (Quicke, 1987b).

1.2.2. History of the Subfamily Braconinae

The Braconinae was erected by Nees (1811), and was not further subdivided by him into tribes. Recognition of the group largely corresponds to current subfamily except that Braconinae has been used in the past in a broader sense by combining Braconinae, Doryctinae, Exothecinae and Rogadinae (Shenefelt 1975, 1976, 1978). Previously several genera of Braconinae have been placed in other subfamilies for example *Archibracon* Saussure and *Mesobracon* Szépligeti were placed in the Exothecinae by scientists in early Twentieth Century because of the importance they attributed to the position of fore wing vein cu-a.

The first record of dividing the Braconinae into tribes goes back to Ashmead (1900)

who erected two tribes each based on a single genus, the Aphrastobraconini and Euurobraconini, based on the forewing vein and its junctions migrating towards wing base which is very common in Braconidae. In so doing he also effectively placed all other genera in the Braconini. Van Achterberg (1993) massively expanded the concept of the Aphrastobraconini by treating it as senior synonym of Fahringer's Iphiaulacini based, it seems, on the ventrally elongate scapus that was not discussed in the literature but was vaguely presented in the illustrations.

The tribe Gnathobraconini was erected for a strange South American genus, *Gnathobracon* Costa (1864), as it has massive, rather sickle-shaped mandibles, which was first described by Costa (1864). Szépligeti (1904) erected a new family, the Gnathobraconinae, based on this genus then van Achterberg (1976) placed this genus in the Rogadinae without studying the specimen or its illustrations. Shenefelt (1979) studied the holotype and suggested it might be closely related to the Braconinae on the basis of its wing venation. Quicke & Huddleston (1991) proposed it to be closely related to predominantly Neotropical genus *Digonogastra* Viereck (Quicke, 1988d) as they share the characters of large scapus, longer ventrally than dorsally in lateral aspect, frons largely setose, forewing with 1-SR+M moderately curved basally and 1st metasomal tergite with a pair of sub-median pits just behind the middle.

Fahringer (1928) erected the Gastrothecini, equivalent to a subfamily in his scheme, having equal status to Braconini (defined by him) on the basis of type genus *Gastrotheca* Guerin-Meneville which is primarily Afrotropical genus having the first three metasomal tergites fused and forming a complete carapace. The genus name *Gastrotheca* was however, preoccupied and it was replaced by *Physaraia* Shenefelt. The Physaraiina was recognized by van Achterberg (1984b) as a subtribe within the Braconini and as a sister group to Aspidobraconina because both have immovably joined first and second metasomal tergites

and a complete medial carina on the metanotum. Fahringer (1928) further proposed the tribe Vipionini (type genus *Vipio* Authors), Longiradialii (no type genus was given) and Habrobr-aconini (type genus *Habrobracon* Ashmead, 1985) within Braconinae, but these have been almost entirely ignored by subsequent workers.

Shestakov (1932) erected the Chiviniini to accept his new genus *Chivinia* from the former U. S. S. R. but Tobias (1971) regarded it no more than a species of *Bracon* Fabricius, which has been generally accepted so presently Chiviniini is a synonym of Braconini.

Telenga (1952) separated two tribes from the Braconini based on his more extensive study of Palaearctic Braconinae and structured his tribes on the basis of a number of new characters. These were the Iphiaulacini (type genus *Iphiaulax* Foerster) and Vipionini (type genus *Vipio* Authors) based on wing venation (short submarginal cell) and antennal features, but their boundaries were not clearly defined.

Tobias (1957) based his tribe Glyptomorphini on the blunt and laterally compressed terminal flagellomeres of *Glyptomorpha* Holmgren and *Teraturus* Kokoujev Glyptomorphini but placed *Vipio* Authors in the Braconini. *Glyptomorpha* had been previously placed in the Vipionini on the basis presumably of its elongate labio-maxillary complex and short forewing marginal cell. He further, believed that the flared basal flagellomeres and remarkably setose parameral processes of the male genitalia as plesiomorphous character states in the Holarctic genus *Coeloides* Wesmael and separated it into the tribe Coeloidini on this basis.

Van Achterberg (1976) erected two tribes, the Pseudodicrogeniini and the Histeromerini. The Pseudodicrogeniini was based on the unusual genus, *Pseudodicrogenium* Fahringer, which had previously been considered as a separate subfamily of Braconidae (Fahringer, 1936), and was similarly considered again by Shenefelt (1979) but is now considered as a synonym of the Euurobraconini (Quicke, 1988a). The Histeromerini (van Achterberg, 1976) was erected to accommodate the puzzling genus *Histeromerus* Wesmael

1838, which had previously moved between Doryctinae (e.g. Shenefelt & Marsh, 1976; Belokobylskij & Tobias, 1986) and Braconinae (van Achterberg, 1976; Tobias, 1976). It has now been placed in its own subfamily the Histeromerinae (van Achterberg, 1984 & 1988a; Quicke & van Achterberg, 1990; Shaw & Huddleston, 1991) as is supported by more recent molecular studies. In 1981, van Achterberg also placed Afrotropical genus *Rhamnura* Enderlein in his own tribe, the Rhamnurini, as the two fore tibial spurs appeared to him to be potentially plesiomorphous condition when compared with other Braconinae and Braconidae. In 1983, van Achterberg erected another tribe, the Adeshini, to provide placement for the genus *Adesha* Cameron and his new Afrotropical genus, *Adeshoides* based on wing venation, i.e. fore wing vein CU1 arising at the same level as 2-CU1, and 3-CU1 being absent or very short and longitudinal.

Tobias (1986) erected another tribe, the Victoroviellini, to place his genus *Victoroviella* based on two remarkable specimens from Central Asia that he suggested on being closely related to *Odontoscapus* Kriechbaumer (a member of the *Atanycolus* Foerster group of genera within Aphrastobraconini: Quicke, 1987b). More recently, the Victoroviellini was suggested to be closely related to Glyptomorphini based on a preliminary analysis of phylogenetic relationships within Braconinae by Chisti & Quicke (1995). Characters suppo-rting this result are *Victoroviella's* short marginal cell, blunt and laterally compressed terminal flagellomeres and the basally nearly glabrous fore wings (Sarhan & Quicke, 1989).
Quicke (1988a) erected the Bathyaulacini, based on the Afrotropical genus *Bathy-aulax* Szépligeti and including a number of apparently related taxa, separating it from the Glyptomorphini. Both tribes have a short marginal cell of fore wing with distally expanding 2nd sub-marginal cell (so too does the Vipionini). Quicke (1988a) also synonymised the Pseudodicrogeniini with the Euurobaconini as they differ only in the enlarged mandibles and associated facial modifications of the former.

The genus *Argamania* Papp (1989) was initially placed in the subfamily Exothecinae without providing any argument. The genus sometimes has a faintly indicated occipital carina, a comparatively long vein M+CU of the hind wing and the position of the metasomal spiracles (the latter character state was not mentioned by Papp) that can be misleading. Van Achterberg (1991) transferred *Argamania* to the Braconinae based on thirteen character states. At the same time he erected the tribe Argamaniini for it, and defined it by the position of metasomal spiracles of the second-seventh tergites in their epipleura, the enlarged upper valve of the ovipositor, vein M+CU of hind wing longer than vein 1-M, antennal sockets situated near middle of the eyes, comparatively long pronotum, forewing nearly as long as hind wing, occasionally faintly indicated occipital carina laterally, the clypeus not differentiated from the face and anterior ocellus on frontal plane of the head. *Argamania* is considered to be basally derived in the Braconinae because of its (putative retained) occipital carina, and a venom gland dissection done by Quicke (unpublished) reveals swollen venom glands, but still essentially Braconina like.

A new genus *Vaepellis* Quicke (1987a) was described on the basis of unique apomorphies such as comparatively long, clubbed scapus, densely short setose body, very short palps and hind tibial spurs, very small eyes and ocelli and massive legs. It also had long hind wing vein M+CU and hind wing vein 1-M was not basally thickened thus lacking two synapomorphies of the Braconinae and placed in a separate subfamily potentially also derived basally on the stem budding to crown-group Braconinae. Tobias (1988) moved it into the Braconinae because of the presence of inverted Y-shaped groves on the first metasomal tergite. As it is thus far only known from two rather old specimens from Ghana, it has not been available for molecular investigations.

The latest catalogue providing information on the Braconinae is Taxapad (Yu *et al*., 2005) that divides this subfamily into twelve tribes Adeshini, Aphrastobraconini,

Argamaniini, Bathyaulacini, Braconini, Coeloidini, Euurobraconini, Glyptomorphini, Gnathobraconini, Physaraiini, Rhammurini and Vaepellini.

As can be seen from the above, most tribes have been erected based on one or just a few genera as with the Argamaniini, Coeloidini, Gnathobraconini, Physaraiini, Rhamnurini and Vaepellini while others are based on only one or few characters such as the Aphrastobraconini, members of which have the scapus ventrally elongate, thus leading to a mess where a lot of genera are left without sensible classification and most of them ending into Braconini without any specific reasons based on affinities in characters or biologies.

In addition to the above-mentioned tribes, a number of informal genus groups have been recognized by Quicke (1987b). He described eleven different groups of genera. Within Aphrastobraconini he recognized the *Atanycolus*-group, based on basally, sharp and concavely narrowed scapus and more or less strongly petiolate pedicellus (fig. 1.3a) (van Achterberg, 1983), *Merinotus*-group, with blunt terminal flagellomere and hypopygium large and extending well beyond the apex of the metasomal tergites (fig. 1.3b), *Nesaulax*-group, having forewing vein 2-CU1 often strongly curved (fig. 1.3c) and facial protuberances and purple coloration of metasoma. *Plaxopsis*-group of genera, fore wing vein cu-a interstitial or slightly postfurcal (fig 1.3f, g), 2nd metasomal suture and furrows demarking the antero-lateral areas of the 3rd tergite crenulated and *Shelfordia*-group, face shiny with some punctures and scutellar sulcus crenulate.

In Braconini Quicke described five genus groups comprising the *Bracon*-group, based on fore wing vein C+SC+R and 1-SR forming an angle of more than 50º, base of hind wing usually densely setose, 1st metasomal tergites with the lateral areas usually inclined upwards away from the midline, the *Chelonogastra*-group, notauli distinct, metasoma robust, short, broad, rather oval (fig. 1.3d), at least tergites 2 and 3 with coarse sculpture, 3rd to 5th tergites

Genus Groups

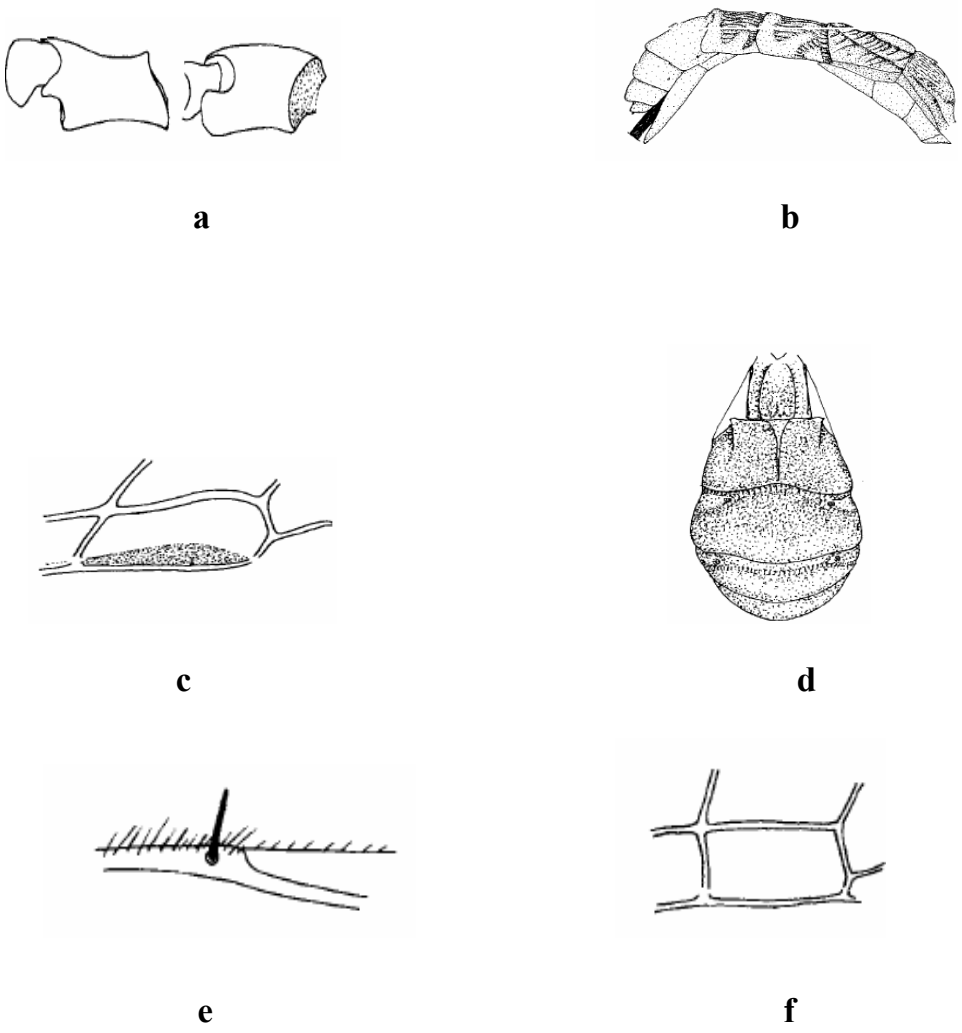


Figure 1.3. Genus groups: **a**, Antennal features of *Atanycolus*-group; **b**, Hypopygium in *Merinotus*-group; **c**, Strongly curved forewing vein 2-CU1 in *Nesaulax*-group; **d**, metasoma of *Chelonogastra*-group; **e**, Bristle in *Mesobracon*-group; **f**, forewing vein cu-a in *Plaxopsis*-group (Illustrations from Quicke, 1987b).

with clearly defined antero-lateral areas, the *Mesobracon*-group with apex of hind wing vein C+SC+R with only a single hamular bristle apically (fig. 1.3e), metasoma with at least 6 visible tergites, metasomal tergites 2-6 with fairly coarse, foveate to rugose sculpture, metasomal tergites 3-6 without transverse sub-posterior grooves, the *Myosoma*-group, hypoclypeal depression deep, strongly rounded dorsally, mesoscutum smooth and shiny, 2nd

metasomal suture smooth sometimes shallow; the *Plesiobracon*-group, mesoscutum densely and evenly setose, hind wing vein without any trace of vein 2-1A (Quicke, 1988c) while *Virgulibracon*-group of genera show affinities with *Myosoma*-group (*Calcaribracon* and *Mollibracon*, it was postulated that the latter belongs to *Virgulibracon*-group of genera) of Braconini on basis of form of scapus, posterior angulation of forewing and reduced lateral sclerotization of the 1st and 2nd metasomal tergites at the same time partial fusion of the terminal two flagellomeres, thickened bristles of the fore tibia in *Vomeribracon* and the presence of antero-lateral areas on the 3rd and 4rth metasomal tergites are collectively indicative of a relationship with *Archibracon* thus to Euurobraconini.

1.2.3. Phylogenetics of the Braconinae

Quicke (1988a) followed Telenga's (1952) and Tobias's (1957) discoveries of potentially informative male genitalia characters by surveying a number of features across a wide range of genera of Braconinae. As a result he proposed a number of possible relationships as well as pointing out a number of general trends. It was suggested to synonymise the Rhamnurini with the Euurobaconini as they share a number of apomorphous characters such as a flattened face and very long ovipositors. The Euurobaconini still faces the problem of defining boundaries (Quicke, 1988a) and may need further division before anything certain can be said about it. The other finding of the study was that *Aphrastobracon* -group of genera being constantly revealed as a sister group to *Nesaulax*-group of genera, which additionally include *Sylvibracon* and *Paranesaulax* (Quicke, 1988a). The *Nesaulax*-group shares a number of morphological characters with *Aphrastobracon*-group such as coarse facial sculpture, 2nd submarginal cell of fore wing long and narrow, vein 1-SR+M strongly curved, oval 1st subdiscal cell and ovipositor without a dorsal nodus. However, *Nesaulax* itself also appeared to be related to other groups of Old World genera including the

Merinotus, Atanycolus and *Shelfordia*-groups thus showing seemingly two different evolutionary routes. Although the cladogram (Quicke, 1988a) showed *Iphiaulax* to be close to the Bathyaulacini, it does not look like a true relationship as only one character, a ventrally produced scapus, link them while, as shown in Quicke (1988a), *Iphiaulax*, on the basis of digital tooth of the male genitalia, is closely related to the Afrotropical genera *Plaxopsis* Szépligeti and *Zanzopsis* van Achterberg (Quicke, 1988a), it also appears to be closely related to *Zaglyptogastra* Ashmead and perhaps also to the *Aphrastobracon*-group. *Victoroviella* appeared as sister-group to Bathyaulacini + Glyptomorphini but still needs additional characters to confirm this relationship.

The Adeshini was proposed to be sunk within the Braconini by Quicke (1988a) and a new subtribe within Braconini should be raised to accept the *Plesiobracon*-group genera + *Braconella* + *Cratocnema* + *Kimavu* which would then be a sister-group to the Aspidobraconina + Physaraiina of the latter relationship was upheld. The Coeloidini was found not close to the Braconini due to its apomorphous character of flared basal flagellomeres plus characters like forewing vein 1-SR+M straight or very gently curving posteriorly (fig. 1.4a,b) and hind wing without vein 2-1A (fig.1.4c) etc.

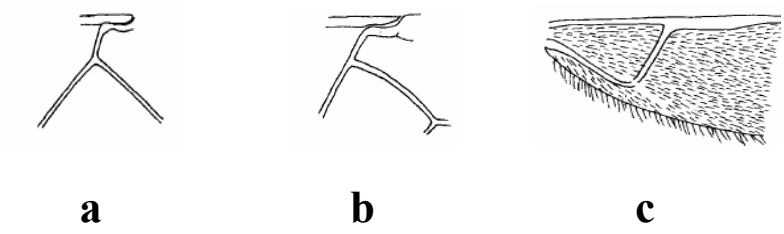


Figure 1.4. Apomorphous characters of Coeloidini: **a**, fore wing vein straight; **b**, forewing vein slightly curving posteriorly; **c**, hind wing (Figures from Quicke, 1987b).

Chishti and Quicke (1995) in their phylogenetic analysis of 55 morphological characters, for a limited number of genera, putatively related to the Bathyaulacini and the

Glyptomorphini found these two groups to be monophyletic with *Vipiomorpha* and *Rhytimorpha* coming together within Glyptomorphini in agreement to Quicke (1988a). The Bathyaulacini was further divided into two clades, i.e., *Annectobracon* + *Ischnobracon* and *Bathyaulax*+*Bicentra* van Achterberg & Sigwalt (1987). Subsequently, Kaartinen & Quicke (2007) synonymised *Bicentra* with *Bathyaulax* as they differ only in number of spurs on the fore tibia.

The first molecular phylogeny for the Braconinae was presented by Belshaw *et al*., (2001) who used sequence data from the 28S D2-D3 rDNA gene region in combination with a morphological data set, largely derived from Quicke, (1988a), to divide the Braconinae into meaningful groups with special emphasis on the genus *Bracon*. They proposed that the Braconini and Aphrastobraconini should be retained as tribes along with a monotypic Coeloidini while all other monotypic tribes should be collapsed into the Braconini and Aphrastobraconini and genera that do not come under specific tribes should be called genus group rather than tribes or subtribes due to relatively poor resolution of their phylogenetic analysis. Coeloidini on the tribal level can be used on the basal position in the subfamily due to its plesiomorphic character state described earlier. But the more recently described genus *Megacoeloides* Quicke was not recovered with *Coeloides* despite the fact that it possess the same apomorphous modification of the basal flagellomeres and the putatively plesiomorphic highly setose male parameres. For *Bracon* they suggest it should be upresented in nomenclature by using asterisk or quotation marks to highlight its doubted paraphyletic nature (see Schulte *et al*., 1998).

1.3. Thesis aims and outline

The general aim of this thesis is to study the phylogenetic relationship of the subfamily Braconinae (Braconidae: Hymenoptera). Until now, a few attempts have been made to

resolve the phylogenetic relationships among different Braconinae genera (Telenga, 1952; Tobias, 1957; Quicke, 1988a; Chishti & Quicke, 1995; Belshaw *et al*., 2001). The first two studies by Telenga (1952) and Tobias (1957) were mainly based on the Russian material, so lacking the tropical regions of the world where this subfamily is found to be most diverse. The studies of Quicke (1988a) and Chishti & Quicke (1995) were based on the morphological characters while Belshaw *et al*. (2001) was a combination of morphology and 28SrDNA. All these studies shed some light on one or the other part of the Braconinae phylogeny but failed to fully resolve the phylogenetic relationship between different genera. Based on these studies, this work will test the following hypothesis:

Hypothesis: The subfamily Braconinae is monophyletic and divided into three monophyletic tribes, the Aphrastobraconini, the Braconini and the Ceoloidini.

This thesis has three main parts, the first part constitutes the description of new genera and species that were discovered during this study. The second part of the thesis is based on a combination of single genes, both nuclear and mitochondrial, and morphology, while the third part of the thesis comprises a combined analysis of nuclear and mitochondrial gene along with morphology to not only understand phylogenetic relationship of the subfamily but also to date the diversification of its taxa.

Chapter 2: Two new genera, *Synvipio* Shaukat & Quicke and *Combibobracon* Shaukat & Quicke, along with a new species *Serraulax hassani* of the genus *Serraulax*, are described following the keys by Quicke (1987b) and Papp (2011).

The type species *Synvipio fakhari* Shaukat & Quicke and *Combibobracon ishtiaqi* are also described for genus *Synvipio* and *Combibobracon,* respectively. Thus, inclusion of these genera would lead the total number of valid taxa in Braconinae to be 185 (Yu *et al*., 2005; Jones *et al*., 2009: Mahmood *et al*., 2010) making it really important to sort a reasonable

division of Braconinae into tribes and subtribes. In order to achieve this classification:

Chapter 3: The expansion segments D2 and partial D3 of the large ribosomal subunit 28S rDNA gene fragment along with morphological characters (Quicke, 1988a: Belshaw *et al*., 2001) are used to analyze the phylogenetic relationships in the subfamily Braconinae.

The alignment of this gene fragment is very problematic due to its length-variable regions usually referred as loops. Their inclusion may reduce phylogenetic signal and their exclusion leads to loss of important evolutionary information. To overcome this situation the model of sequence alignment proposed by Gillespie *et al*. (2005) based on the secondary structure of the molecule was used. In addition, modifications proposed by Zaldivar *et al*. (2006) were employed in order to include maximum evolutionary information hidden in the length variable region of the 28S rDNA gene fragment. Further more the Goloboff's method of character weighting (Goloboff, 1993; Turner & Zandee, 1995) was used to find a tree fitted best to taxonomy.

Chapter 4: The mitochondrial gene cytochrome oxidase subunit 1 (CO1) was selected as this gene has no gaps and no length variable regions.

CO1 has gained special importance in recent years as a barcoding gene (Hebert *et al*., 2003; Stoeckle, 2003). This work has also utilized the universal primers LCO and HCO (Folmer *et al*., 1994) to understand the inter- and intra-phylogenetic relationship of the Braconinae taxa. Two different methods of tree construction are utilized, maximum parsimony (by using PAUP* version 4.0b10) and Bayesian analysis (implemented in the programme MrBayes). The effects of including and excluding 3rd codon position on the phylogenetic relations of the taxa are also studied.

Chapter 5: The chapter 5 is based on Nicotinamide adenine dinucleotide hydrogenease (NADH) gene fragment from mitochondria. This gene plays an important role in electron transport chain along with CO1.

This gene fragment has previously been used in phylogenetic studies in Braconidae, especially in Aphidiinae. In the case of the Aphidiinae, use of NADH1 by Smith *et al*., (1999) resulted in a tree similar to the 28S rRNA tree found by Belshaw and Quicke (1997). Thus this study showed this gene to be a good choice for the confirmation of results obtained by the 28S rRNA gene in case of Braconinae as well. So, NADH1 is utilized to obtain maximum parsimony and Bayesian phylogenetic trees that are further compared to identify similar clades with good support (bootstrap for MP and posterior probabilities for Bayesian).

Chapter 6: Following the lead from Smith *et al*. (1999), a combined data set of 28S rRNA and NADH 1 is put to test to see if addition of both these genes with morphology can improve the situation and we can get more reliable trees that can divide Braconinae in main three tribes and shed some light on the genera within these tribes with a better resolution. Also this data set is utilized to understand the divergence age of Braconinae. Molecular dating is applied to estimate the time of origin of the subfamily Braconinae.

Chapter 7: Presents an overall discussion about each chapter and overall conclusions are drawn so that future studies can benefit from the work.

Each chapter is treated as a separate entity for the ease of exposition.

Chapter 2: Two new genera and one new species of the genus *Serraulax* Quicke Braconidae (Hymenoptera: Braconinae).

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2.1. Abstract

Two new genera, *Synvipio* and *Combibobracon,* (Braconinae, Braconidae) are erected to include two new species from Africa. These species are *Synvipio fakhari* **sp. nov.** and *Combibobracon ishtiaqi* **sp. nov.** from Benin and an unknown country in West Africa respectively. The two genera were found closely related to previously defined genus *Vipio* Latreille. A new species of the genus *Serraulax* is also described, *Serraulax hassani* **sp. nov.** from Ghana.

2.2. Introduction

The Braconinae is the largest subfamily in the family Braconidae with 183 valid genera and more than 2,893 valid species (Yu *et al*., 2005; Jones *et al*., 2009; Mahmood *et al*., 2010) and these numbers are constantly increasing. Its great diversity is seen in Old World tropical and subtropical regions (Quicke, 1987b). After the publication of the Old World key a number of new Afrotropical genera have been defined (Quicke 1987b, Achterberg and Sigwalt 1987, Quicke 1988, Braet, 1999, Achterberg 2003; Mahmood *et al*., 2010) but the diversity of the fauna in this region remains far from completely known. In this article we describe three new genera from Africa along with their type species.

2.3. Material and Methods

The morphological terminology used in this paper follows that of van Achterberg (1979, 1988b) and Quicke (1987b).

2.4. Descriptions

2.4.1. Genus *Synvipio*, Shaukat & Quicke (Figs. 2.1-2.2)

Head **(Figs. 2.1b, c, d)**. Antenna slender; 1st, 2nd and 3rd segments of flagellum longer than wide; median segments as long as wide; scapus modified, large subglobose, longer ventrally than dorsally, apico-laterally emarginate. Head wider than long, smooth and shiny dorsally; labiomaxillary complex normal; malar suture present; dorsal carina separating the clypeus from hypoclypeal depression, which is bordered dorsally by 10 separated long setae; face

smooth and shiny, largely setose; eye moderately large, glabrous; frons slightly depressed with midlongitudinal groove.

***Mesosoma* (Fig. 2.2c).** Mesosoma smooth and sparsely hairy; mesoscutum smooth with well developed notauli; scutellar sulcus narrow and crenulated; median area of metanotum with a mid-longitudinal carina anteriorly; propodeum smooth but densely setose.

***Wings* (Figs. 2.1e, f).** Fore wing: length of fore wing 3 times its width; vein 1-SR+M straight; pterostigma wide; vein 1-SR distinct; vein 1-M curved; marginal cell long and narrow; vein 2-SR+M well developed; 2nd submarginal cell long; vein cu-a slightly postfurcal; hind wing vein SC+R longitudinal; wing base glabrous; vein 2-1A absent.

Legs. Apex of fore tibia with transverse row of spines; tarsal claw with rounded basal lobe; hind femur robust; hind tibia slender with two apical spurs. Claw simple.

***Metasoma* (Figs. 2.2a, b, d, e, f).** Metasoma with moveably joined 1st and 2nd metasomal tergites; 1st metasomal tergite with small striate with no distinct carina and poorly defined mid basal area; 2nd tergite with mid basal triangular area and antero-lateral triangular areas; 3rd tergite with antero-lateral triangular areas; 4th tergite with grooves in the middle; posterior margin of 5-8 metasomal tergites smooth; hypopygium long; ovipositor longer than the body, with pre-apical dorsal nodus and apico-ventral serrations.

Male. Unknown.

Etymology. Based on close similarities to genus *Vipio*.

Type species: *Synvipio fakhari* sp. nov.

Diagnosis. The new genus runs to the couplet 112 of the Old World genera key by Quicke (1987b) but fails there due to lack of a well developed midlongitudinal carina and well developed dorsal carina on the 1st metasomal tergite, as it is striate with no distinct carina (fig. 2.2d).

Figure 2.1. *Synvipio fakhari* Shaukat & Quicke, sp. n. ♀, holotype. **a**, *habitus,* lateral view; **b**, head, frontal view; **c**, head, dorsal view; **d**, head, lateral view; **e**, Fore wing; **f**, hind wing.

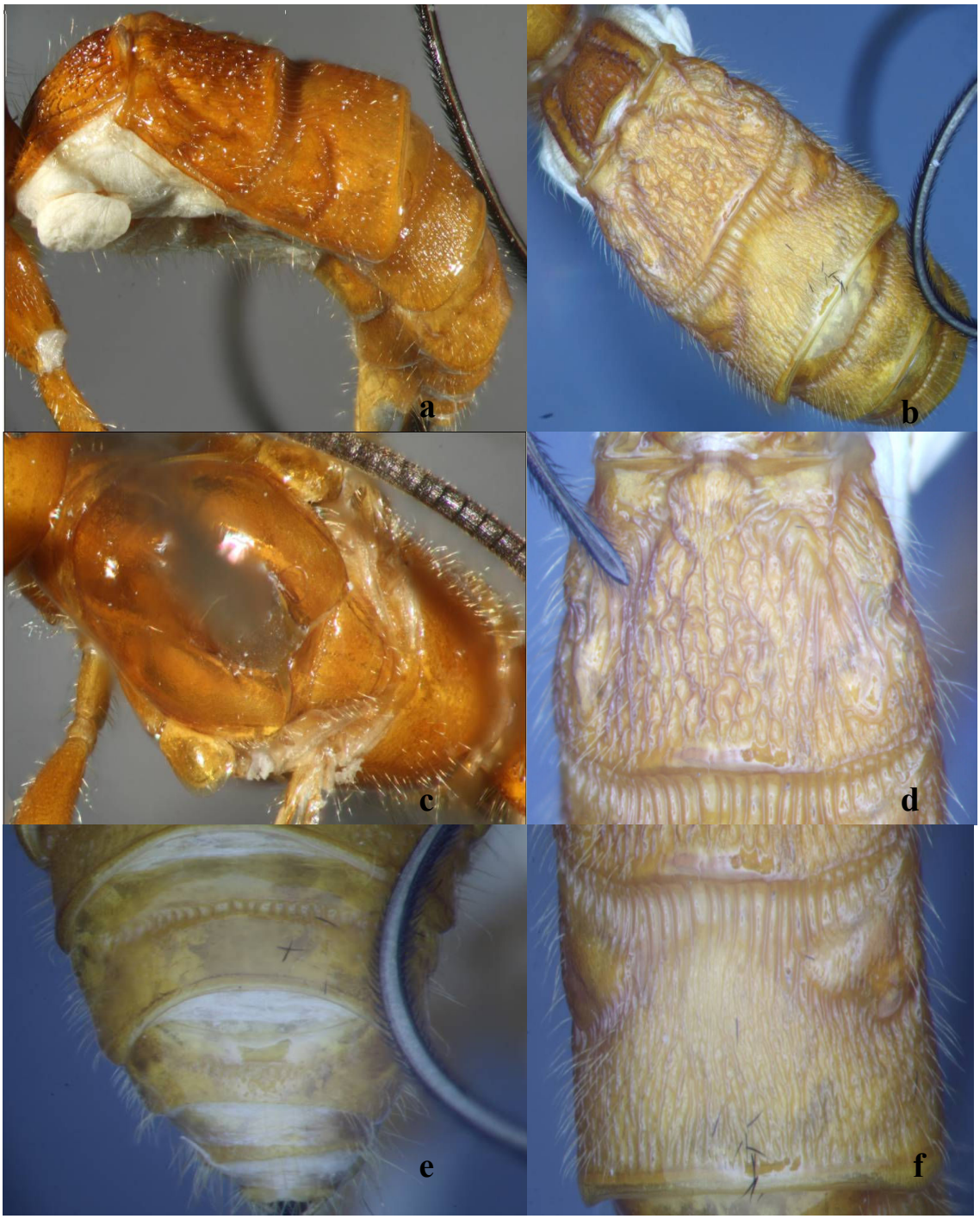

Figure 2.2. *Synvipio fakhari* Shaukat & Quicke, sp. n. ♀, holotype. **a**, abdomen, lateral view; **b**, abdomen, dorsal view; **c**, mesosoma, dorsal view; **d**, tergite 1, dorsal view; **e**, tergite 4-8, dorsal view; **f**, tergite 2, dorsal view.

***Synvipio fakhari* sp. nov. Shaukat & Quicke.**

Female. Length of body 10 mm, of fore wing 8 mm, and of ovipositor 13mm.

Head. Antenna with more than 34 flagellomeres (damaged), 1st flagellomere 1.3 times longer than second and third. Scapus 3.75 times length of pedicle. Face setose, micro sculptured with weak median longitudinal carina. Face 1.5 times wider than long. Eye in lateral view 1.4 times as high as wide, glabrous in frontal view. Stemmaticum with round edges but triangular and yellow in colour, posterior ocellar line: transverse diameter of posterior ocellus: shortest distance between posterior ocellus to eye= 1.125:1.0:1.25.

Mesosoma. Shiny, sparsely hairy and brownish yellow in colour. Mesosoma 1.5 times longer than deep, almost 3 times longer than wide. Scutellar sulcus narrow and crenulated. Propodeum smooth and shiny but densely setose.

Wings. Fore wing: Ratio of length of veins SR1:3-SR:r = 0.5:5.3:12. Ratio of length of veins 2-SR:3-SR:r-m = 12:53:20. Veins 2-M and 3-SR, almost 3 times and 2.4 times as long as r-m, respectively. Vein SR1 reaching wing margin more than 0.8 of the distance from apex of the pterostigma to the wing tip. Ratio of length of vein 1-M relative to m-cu = 1.25. Vein cu-a postfurcal. Veins 1-M and m-cu equal in length. Veins 1-M and m-cu slightly curving towards anterior wing margin. Vein 3-CU1 straight. Hind wing: vein 1SC+R 1.3 times longer than vein 1 r-m.

Legs. Ratio of hind femur:tibia:basitarsus = 1.6:1.7:1. Hind femur (excluding trochantellus) 5 times longer than deep, broadest at the middle. Rather flattened laterally, sparsely setose. Hind basitarsus 7.5 times longer than deep. Hind tibial spur reaching approximately 2.5 of distance along basitarsus.

Metasoma. Sparsely setose and foveolate. First tergite with short dorsal carinae, 1.6 times longer than apically wide. 2nd tergite with mid basal triangular area and antero-lateral triangular areas, 3rd tergite with antero-lateral triangular areas. 4th tergite with lateral grooves

in the middle. Posterior margin of 5-8 metasomal tergites smooth. Ovipositor 1.3 times longer than the body, with pre-apical dorsal nodus and 3 serrations.

Colour. Yellow-brown except antennae, apex of mandibles, tarsal claw, telotarsus and ovipositor sheath that are black; wings with black pattern; apex of pterostigma black; veins brown.

Etymology. Named after Mrs Fakhar Sultana, mother of AS.

Type material. Holotype female, Benin, Natural History Museum, London, UK (BMNH), collector G. Georgen.

2.4.2. Genus *Combibobracon*, Shaukat & Quicke (Figs. 2.3-2.5)

Head **(Figs. 2.3b, c, d).** Antenna densely setose; apex of antenna highly modified with last 8 segments apically emarginated; 1st segment of flagellum longer than wide; median segments of flagellum as long as wide; scapus globose, strongly hairy, longer dorsally than ventrally. Head wider than long, smooth, shiny and setose; labio-maxillary complex elongate to form proboscis; malar suture present; eyes slightly emarginate dorsally; groove running from eye to antennal socket; groove on frons behind the antennal sockets with black coloration on both sides of the groove running to the middle of vertex behind ocelli.

Figure 2.3. *Combibobracon ishtiaqi* Shaukat & Quicke, sp. n. ♀, holotype. **a**, *habitus,* lateral view; **b**, head, lateral view; **c**, head, dorsal view; **d**, mouthparts frontal view; **e**, Fore wing; **f**, hind wing.

Figure 2.4. *Combibobracon ishtiaqi* Shaukat & Quicke, sp. n. ♀, holotype. **a**, abdomen, dorsal view; **b**, abdomen, lateral view; **c**, mesosoma, dorsal view; **d**, mesosoma, lateral view.

Mesosoma **(Fig. 2.4c, d).** Mesosoma smooth and sparsely setose; noutali weakly developed; mesoscutum smooth without setae; scutellar sulcus narrow and crenulated; scutellum anteriorly emarginate; median area of metanotum with three carinae anteriorly; propodeum with a deep depression at the mid-posterior margin bordered by a pair of anteriorly diverging carinae.

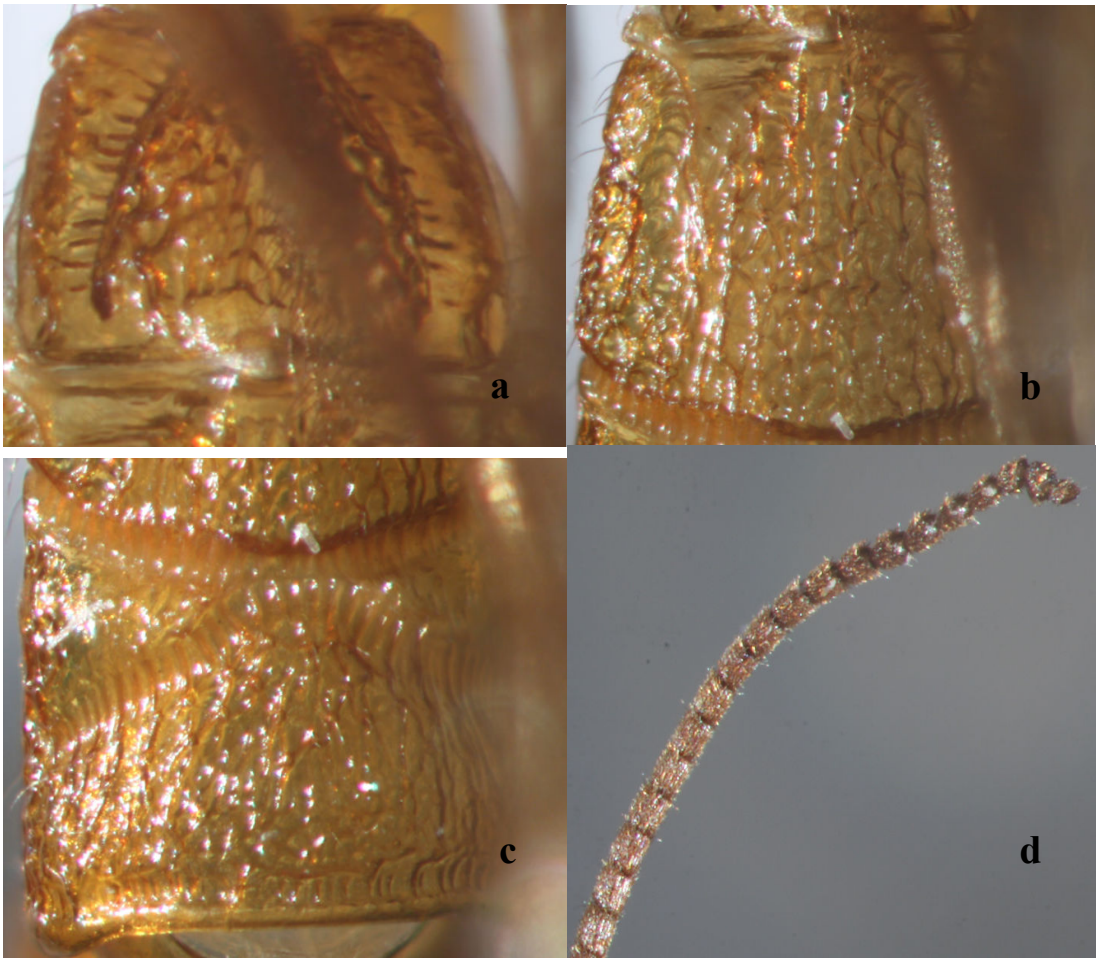

Figure 2.5. *Combibobracon ishtiaqi* Shaukat & Quicke, sp. n. ♀, holotype. **a**, 1st tergite dorsal view; **b**, 2nd tergite dorsal; **c**, 3rd tergite dorsal view; **d**, antennal tip.

***Wings* (Figs. 2.3e, f).** Fore wing: length of fore wing 3 times its width; vein 1-SR+M curving anteriorly after arising from 1-M; pterostigma wide; vein 1-SR distinctly swollen; vein 1-M curved; marginal cell short and wide; vein 2-SR+M present; 2nd submarginal cell long; vein cu-a interstitial; vein 3-CU1 thickened posteriorly, longer than CU-1; wing base sparsely setose; vein 2-1A present. Modified hamuli; 1 basal hamulus present, dark and thickened. The basal hamuli of the hind wing are less modified than that in the Glyptomorphini (Quicke, 1987b; Sarhan & Quicke, 1989).

Legs. Fore tibia with a transvere row of pegs laterally; tarsal claw simple, with rounded basal

lobe; hind femur robust.

***Metasoma* (Figs. 2.5a, b, c).** Metasoma with movably joined 1st and 2nd tergites; 1st tergite without carinae, 2nd tergite with antero-medial triangular areas; 3rd tergite with antero-lateral triangular areas separated by grooves; 4th tergite with transverse basal groove; tergites 1-4 rugose, tergites5-7 smooth.

Female. Unknown.

Etymology. Based on the sucking mouth parts, *combibo* is Latin for suck in/ drink in, and the genus name *Bracon*.

Type species. *Combibobracon ishtiaqi* **sp. nov.**

Diagnosis. This genus runs to the couplet 87 of the Old World key to genera by Quicke (1987b), and so is close to genus *Liomorpha* Szépligeti. It differs from the genus *Vipio* as it lacks the pair of clypeal hair pencils (fig. 2.3b,c,d); propodeum coarsely sculptured posteriorly (fig. 2.4c); Vein 1-M strongly curved (fig. 2.3c), angle between C+SC+R and 1-SR more than 55° (fig. 2.3f). Last nine flagellomeres are modified but the terminal flagellomere is blunt (fig. 2.5d). Scapus short and robust (fig. 2.3c).

***Combibobracon ishtiaqi* sp. nov. Shaukat & Quicke.**

Male. Length of body 0.7 mm, of fore wing 0.6mm.

Head. Antenna with 41 flagellomeres, apical 9 terminal flagellomeres modified, triangular and apically flared; terminal flagellomere pointed not blunt; 1st flagellomere 1.18 times longer than 2nd and 1.3 times longer than 3rd flagellomer; Scapus 2.28 times longer than pedicle. Eye 2.1 as high as wide; Face 2 times wider than long; Face coarsely sculptured, shiny sparsely hairy. Groove running between the antennal sockets. Groove on frons behind the antennal sockets with black coloration on both sides of the groove running to the middle of vertex behind ocelli. Height of clypeus; inter-tentorial distance: tentorio-ocular distance =

1.0:2.5:1.5. POL: diameter of posterior ocellar line: shortest distance between posterior ocellus and eye: transverse diameter of posterior ocellus = 1.0:2.0:2.2.

Mesosoma: Mesosoma 1.75 times longer than deep and 2 times longer than wide. Smooth and very sparsely setose with weakly developed notauli. Scutellar sulcus narrow and crenulated.

Wings: Ratio of length of fore wing veins SR1: 3-SR:r = 7.5:11:1.0. Forewing vein SR1 curving anteriorly towards wing margin. Forewing vein m-cu 6.5 times longer than r. Vein m-cu slightly curved. Vein 1-M 1.3 times longer than m-cu. Hind wing vein 1r-m 0.5x length of SC+R. Wing base sparsely hairy.

Legs: Ratio of length of fore femur:tibia:tarsus = 1.0:1.1:1.5. Ratio of length of hind femur:tibia:basitarsus = 1.0:1.6:1.1. Inner hind tibial spur reaching approximately 0.5 of the way along the basitarsus.

Metasoma: 1st tergite 1.8 times longer than apically wide. 2nd tergite 1.13 times wider than long; 3rd tergite 1.6 with strongly crenulated transverse basal suture between dorso-lateral triangular areas. Tergites 1-4 rugose, tergites 5-7 smooth

Colour. Yellow-brown except antennae, apex of mandibles, Stematicum, tarsal claw, arolium black, wing veins, membrane and terostigma brown

Female. Unknown.

Etymology. Named after Mr Ishtiaq Salim, husband of AS.

Type material. Holotype, West Africa, BMNH.

2.4.3. *Serraulax hassani* Shaukat & Quicke (Figs. 2.6-2.7)

Female. Length of body 13 mm, of fore wing 12 mm, and of ovipositor 7mm.

Head **(2.6b, c, d)**. Antenna with more than 38 flagellomeres (damaged), scapus ventrally

produced and flared on one side with a ridge. 1st flagellomere 1.6 times longer than 2nd and

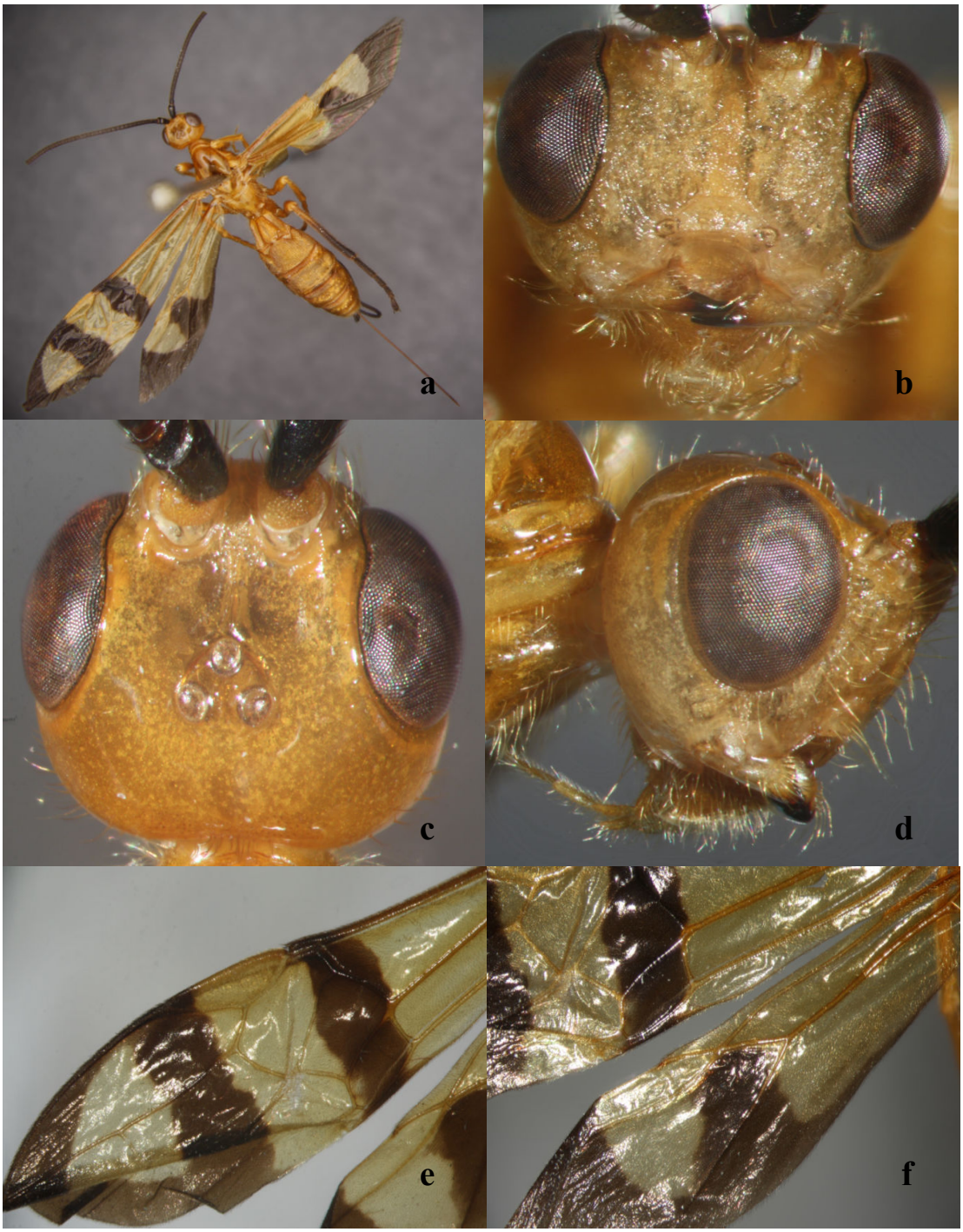

Figure 2.6. *Serraulax hassani* Shaukat & Quicke, sp. n. ♀, holotype. **a**, *habitus,* lateral view; **b**, head, frontal view; **c**, head, dorsal view; **d**, head, lateral view; **e**, Fore wing; **f**, hind wing.

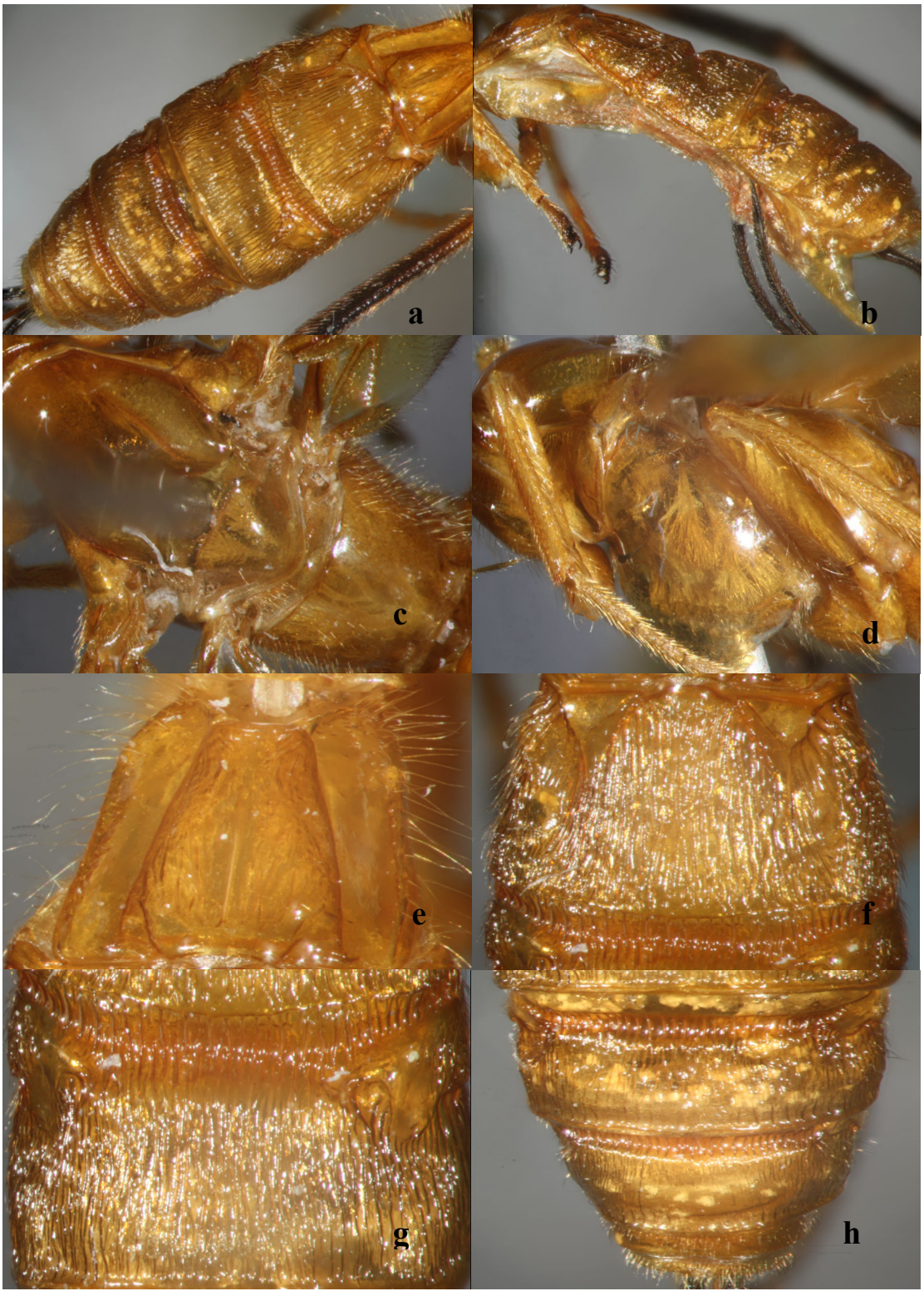

Figure 2.7. *Serraulax hassani* Shaukat & Quicke, sp. n. ♀, holotype. **a**, abdomen, dorsal view; **b**, abdomen, lateral view; **c**, mesosoma, dorsal view; **d**, lateral view; **e**, tergite 1, dorsal view; **f**, tergite 2, dorsal view; **g**, tergite 3, dorsal view; **h**, tergite4-8, dorsal view.

3rd flagellomere. Scapus 3.5 times longer than pedicle. Face coarsely sculptured, shiny and sparsely setose. Face 1.7 times wider than long. Groove running between the antennal sockets. Height of clypeus; inter-tentorial distance: tentorio-ocular distance = 1:1.5:1.5. Eye 1.3 as high as wide. POL: diameter of posterior ocellar line: shortest distance between posterior ocellus and eye: transverse diameter of posterior ocellus = 1.25:3.75:1.

***Mesosoma* (2.7c, d)**. Mesosoma 1.7 times longer than deep and 1.9 times longer than wide. Smooth and very densely setose with well developed notauli. Scutellar sulcus narrow and crenaulated. Median area of metanotum large and swollen. Propodeum smooth but densely hairy laterally. Metanotum smooth and setose.

***Wings* (2.6e, f).** Ratio of length of forewing veins SR1:3-SR:r = 1:7.0:16.6. Forewing vein SR1 straight. Forewing vein r 1.7 times shorter than m-cu. Vein m-cu slightly curved. Ratio of vein 1-M relative to m-cu 1= 1.5. Hind wing vein 1r-m 1.12 times larger than SC+R. 5 hamuli present. Hind wing base glabrous. Vein 2-1A absent.

Legs. Ratio of length of fore femur:tibia:tarsus = 1:1.12:2. Ratio of length of hind femur:tibia:basitarsus = 2:2.6:1. Hind tibia long and slender. Inner hind tibial spur reaching approximately 0.6 of the way along the basitarsus.

***Metasoma* (2.7a, b, e, f, g, h).** Metasoma approximately 1.15 times longer than head and mesosoma united. 1st metasomal tergite 1.4 times longer than apically wide, with well developed longitudinal dorsal carinae. 2nd metasomal tergite 1.7 times wider than long with dorsal triangular areas. 3rd metasomal tergite 2.25 times wider than long with dorsal row of grooves apically and dorso-lateral triangular areas. 4rth and 5th tergite with dorsal row of grooves apically and lateral triangular areas. Posterior margin of 7th metasomal tergite with a medial emargination. Hypopygium long. Ovipositor 1.8 times shorter than the body with pre-apical dorsal nodus and with 3 serrations on apex of lower valve.

Colour. Yellow-brown except antennae, apex of mandibles, tarsal claw, telotarsus in middle leg, more than half of tibia of hind leg, tarsus of hind leg and ovipositor sheath that are black in colour; wings yellow with dark brown pattern; less than ¼ pterostigma towards the apex, black; veins brown.

Male. Unknown.

Etymology. Named after Muhammad Ali Hassan, son of AS.

Type material. Holotype female, Ghana, BMNH.

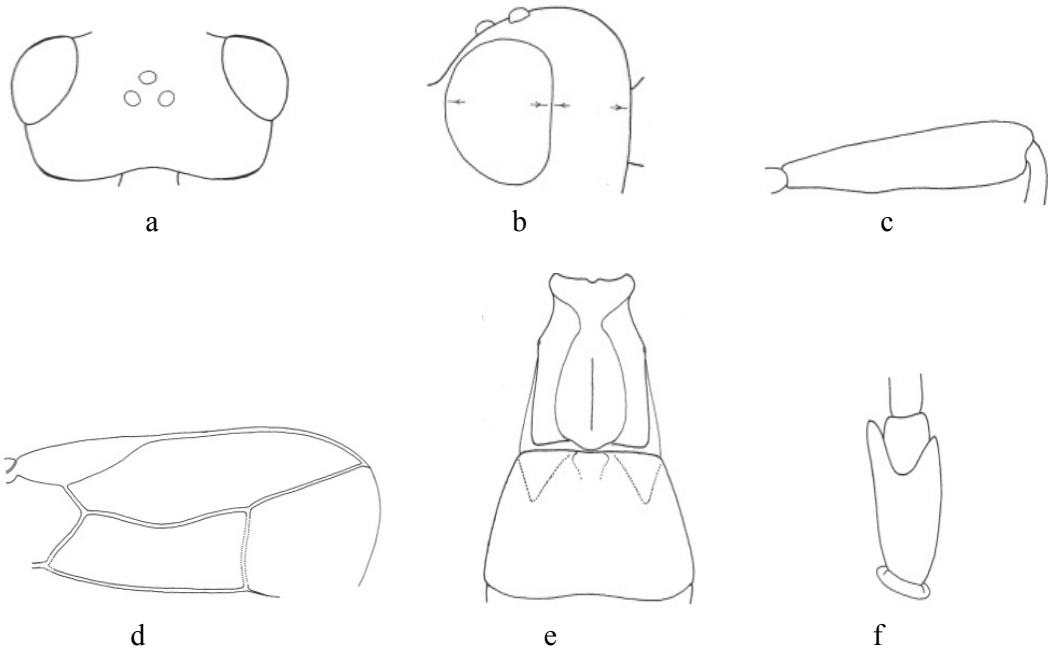

Figure 2.8 *Serraulax orientalis* (SZÉPLIGETI) (female lectotype): **a**, head in dorsal view; **b**, head in lateral view; **c**, hind femur; **d**, distal part of right fore wing; **e**, tergites 1–2; **f**, scape and pedicel in outer-lateral view (Illustrations from Papp, 2011).

Discussion: This specimen keys to the couplet 22 of the key to *Serraulax* species described by Szépligeti 1905-1914 (Papp, 2011) and found to be very close to *Serraulax orientalis* as they both have 3-SR distinctly bisinuate (fig. 2.6e, 2.8a) although it is not as distinctly in the new species as it is in *S. orientalis*. Both have the eye 1.4 times longer than temple and head in dorsal view 1.6 times as broad as long (fig. 2.6c,d; fig 2.8a,b). *S. hassani* **sp. nov.** differs from *S. orientalis* as the ratio between midlongitudinal length of tergite 2+3 and width of

tergite 3 is more in *S. hassani* than *S. orientalis* plus the suture in tergite 3 is more distinct in *S. hasani* than *S. orientalis*. Also the colouration of pterostigma, legs and wings is different.

Chapter 3: Use of 28S rDNA gene fragment with D2-3 regions along with morphology to resolve the phylogeny of Braconinae.

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3.1. Abstract

The 28S rDNA gene fragment was utilized to resolve the phylogenetic relationships in the Braconinae. Besides the phylogeny, particular emphasis was given to the method of alignment so as to include as much phylogenetic information as possible. Alignment was done on the basis of the secondary structure of the molecule plus some modifications. This gene seems to be very promising for resolving the phylogeny of different Braconinaidae

subfamilies but was unable to divide the Braconinae into two main clades based on tribes Aphrastobraconini and Braconini, separated by the Coelodini. Rooting of the tree is also problematic.

3.2. Introduction

With the advances in molecular studies over the last two decades phylogenetics is no longer entirely dependent upon the morphology and biology of organisms. The use of molecular information in phylogeny reconstruction has improved the level of understanding for the relationships among a huge range of taxa (Nadri *et al.*, 2003; Choudhury & Règagnon, 2005; Hemmrich *et al*., 2006; Erpenbeck & Wörheide, 2007). It has also increased the support for already described relations where possible, at the same time raising questions about previously defined relations (Zakharova *et al.*, 2004; Schaap *et al*., 2006; James *et al*., 2006; Wheat & Watt, 2008) which depended exclusively on morphological characters that often show high levels of homoplasy (Hedges & Poling, 1999; Grande *et al*., 2003; Tabuce *et al*., 2007). But molecular analyses have shown a disadvantage of poor resolution in many parts of the tree (Belshaw *et al.* 1998, Dowton *et al.* 1998; Bellocq *et al*., 2001; Yabsley *et al*., 2006) while supporting at least some traditional views.

Out of the order Hymenoptera, the Ichneumonoidea is one of largest superfamilies, comprising the Ichneumonidae and Braconidae. To date only a few molecular phylogenetic studies have been carried out on the Ichneumonidae (Quicke *et al*., 1999a; Quicke *et al*., 2005; Laurenne *et al*., 2006; Klopfstein *et al*., 2009; Quicke *et al*., 2009). In contrast, the Braconidae has formed the basis for describing new methods in phylogenetics, as it provides a system of evolutionary transitions among various parasitoid biologies as shown by Belshaw & Quicke, 1997; Dowton & Austin, 1998; Quicke & Belshaw, 1999b; Sanchis *et al*., 2000; Kankare & Shaw, 2004; Quicke *et al*., 2004; Shi *et al*., 2005; Murphy *et al*., 2006; Zaldivar-

Riverón *et al*., 2006; Zaldivar-Riverón *et al*., 2008. In the Braconidae phylogenetic reconstruction has focused mainly on two gene fragments: the expansion segments D2 and partial D3 of the large ribosomal subunit 28S rRNA (28S) and the 5' region of the mitochondrial cytochrome oxidase subunit 1 (CO1) (Quicke *et al*., 1999b; Whitfield *et al*., 2002; Quicke *et al*., 2005; Shi *et al*., 2005; Laurenne *et al*., 2006; Zaldivar- Riverón *et al*., 2006; Zaldivar- Riverón *et al*., 2008).

3.2.1. Use of 28S rDNA gene in molecular phylogenetics

The 28S rDNA gene fragment has been widely used to understand the phylogenetic relationships among different organisms (e.g. Gonzalez *et al*., 1990: human & chimpanzee; Campbell *et al*., 1993: wasps; Pelandakis & Solignac, 1993: *Drosophila*; Littlewood & Johnston, 1995: parasitic worms; Porter & Collins, 1996: mosquito; Mallett *et al*., 2003: ecdysozoan taxa; Sauxa *et al*., 2004: Ants; Taylor *et al*., 2007: sponges; Rugman-Jones *et al*., 2010: scale insects) especially with respect to insects. The main points of interest are the expansion segments that are considered phylogenetically informative for a range of evolutionary processes as explained by Gillespie *et al*. (2005).

A considerable problem in using the 28S gene fragment lies with sequence alignment due to its often, considerable length variation, even between some closely related taxa. This length variation can be understood in terms of the molecule's secondary structure, which is characterized by the presence of stems and loops usually referred as hairpin-stem loops (fig. 3.1). As more sequences are combined for a phylogenetic analysis the number of length variable sites become numerous and if all are included, they are likely to violate basic assumptions of homology and may reduce the phylogenetic accuracy, while on the other hand, if excluded, they may reduce the resolving power and branch length estimates (Lutzoni *et al*., 2000).

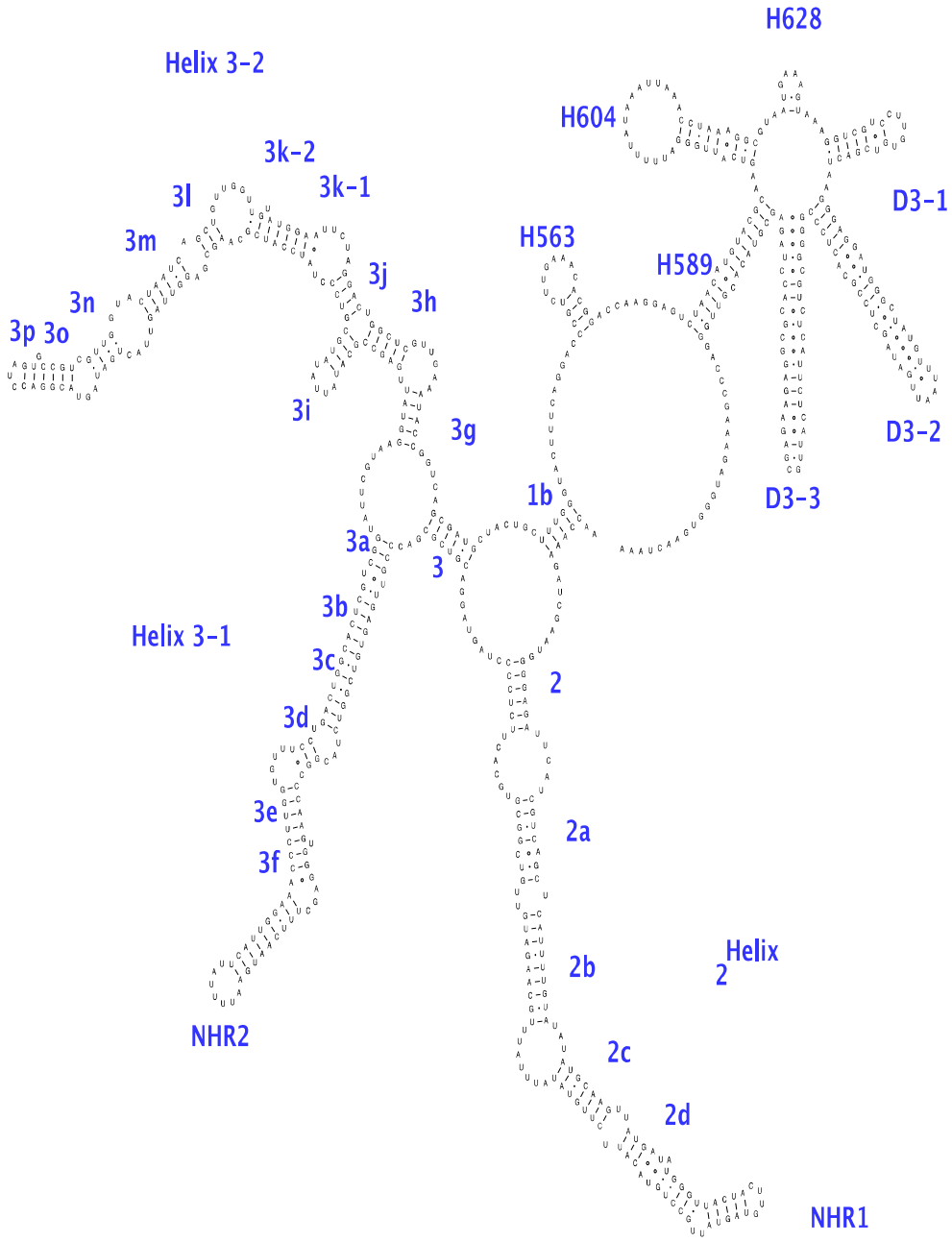

Figure 3.1. Secondary structure model of the expansion segments D2 and D3, the variable region H604, and related core sequence of the 28S rDNA from the sample ASW248, i.e. *Bracon* sp from Brazil

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3.2.2. Alignment methods for length variable genes

There are two main approaches to deal with length variable alignments, classical approach and optimization alignment

The classical approach is based on the Needleman & Wunsch (1970) algorithm where sequences are aligned by use of pairwise or multiple alignments (Feng & Doolittle, 1987, 1990; Higgins & Sharp, 1988, 1989). The CLUSTAL (Des Higgins, 1988, 1992; Thompson *et al*., 1994; Ramu *et al*., 2003) series of programs are based on this principle. First, pair-wise alignments/distance matrices are made by aligning two sequences using default or user picked gap penalties both for gap opening (GO) and gap extension (GE) where GO penalty is higher than GE penalty. Next the scores are calculated by using dynamic algorithm. A neighbour joining tree is constructed from the distance matrix and is used as a guide tree for subsequent improvement of the alignment (Saitou & Nei, 1987). Sequence weights are also calculated from these trees (Thompson *et al*., 1994). This is followed by progressive alignment where larger and larger groups of sequences are aligned following the branching order of guide tree. The programmes T-Coffee (Notredame *et al*., 2000) and MUSCLE (Edgar, 2004) are based on the same underlying method but MUSCLE is a much faster implementation. The drawback of these methods is the acquisitiveness towards the tree of choice, i.e. the guide tree so they may ignore the important components of the sequences integrated later in the alignment.

A different type of method was proposed by Wheeler (1996), based on initial work of Sankoff & Cederdren (1983), Feng & Doolittle (1987, 1990), Hein (1990) and others, known as optimization alignment where sequences are taken directly to the level of phylogenetic reconstruction and gaps are treated as indels (insertion/deletion events) rather than character states. These transformations link the ancestral and descendant nucleotide sequences rather than acting as non-observable nucleotide change (Giribet & Wheeler, 1999). The drawback of

not using any alignment is that one may not be able to detect site-to-site variation in terms of parameters such as transition:transversion ratios, rates of nucleotide substitution, base frequency bias, incorrect topologies due to loss of phylogenetic signal, inclusion of ambiguously aligned sites to resulting phylogenetic tree and divergent sequences that can easily be detected in the alignment step (Lutzoni *et al*., 2000). A recently introduced method that combines alignment, phylogeny reconstruction and model parameters is StatAlign (Nóvak *et al*., 2008) that has removed the problem of the guide tree effect on the real phylogenetic tree as was present in the previous methods.

The method used in this study followed Gillespie *et al*. (2005) that was predicated on the chemical secondary structure approach of Kjer (1995) where the length variable regions were further characterized (Gillespie, 2004). The Zaldivar-Riverón *et al*. (2006, 2008) coding approach was followed to preserve the potential phylogenetic information in the length variable regions of a sequence.

3.3. Material and Methods

3.3.1. Taxon Sampling

A total of 97 Braconinae genera, out of the possible 180 Braconinae genera are used to construct a sub-family level phylogeny for Braconinae. The genus *Bracon,* which includes more than 20 % described species of Braconinae, was represented by 90 species to investigate its monophyly.

A total of 376 sequences from D2-3 region of 28S were used for this study, out of which 25 belong to the subfamilies used as outgroups (3 species of Alysiinae, 7 species of Doryctinae, 6 species of Exothecinae, 5 species of Gnamptodontinae, 3 species of Rogadinae

and 1 species of Lysiterminae). The list of specimens with their collection localities and voucher codes is given in Table 3.1.

Table 3.1. Taxa with unique codes and their countries of origin

Taxon	Country	Voucher no.	Accession no
Adesha albolineata	NA	NA	AJ231511
Adesha sp.	Malaysia	NA	AJ296030
Adeshoides sp.	NA	CNIN174	NA
Africadesha sp.	Tanzania	NA	NA
Amyosoma chinense	China	NA	AY167647
Antiolcus sp.	Uganda	NA	NA
Archibracon deliberator	NA	NA	AY529702
Archibracon sp.	Madagascar	NA	670943
Archibracon sp.	Madagascar	NL493	AY296644
Aspidobracon sp.	NA	CNIN196	NA
Aspidobracon sp.	Malaysia	ASW725	NA
Aspidobracon sp.	Malaysia	ASW727	NA
Aspidobracon sp.	Malaysia	ASW730	NA
Aspidobracon sp.	Malaysia	ASW746	NA
Aspidobracon sp.	Sulawesi	NA	AJ296031
Aspidobracon sp.	Taiwan	RMNH|Jo791	AY935437
Atanycolus sp.	France	NA	AJ231531
Atanycolus sp.	USA	ASW298	NA
Atanycolus sp.	USA	NA	NA
Atanycolus sp.	USA	ASW292	NA
Atanycolus sp.	USA	ASW294	NA
Atanycolus sp.	NA	RAW|2006	DQ538402
Bathyaulax cyanogaster	Ghana	NA	AY527194
Bathyaulax sp.	Kenya	NA	AJ231501
Bathyaulax sp.	NA	NA	NA
Bathyaulax sp.	Uganda	NL844	AY527195
Iphiaulax sp.	Madagascar	NA	670951
Iphiaulax sp.	Madagascar	NA	670963
Bracon sp.	NA	1AZ0	NA
Bracon sp.	NA	138	NA

Bracon sp.	NA	208	NA
Bracon annuli	NA	NA	NA
Bracon expod	Belize	NA	NA
Bracon sp.	Tanzania	NL519	NA
Bracon sp.	Taiwan	NA	NA
Bracon mauritanicus	Israel	NA	AJ296060
Bracon nigripedator	Turkey	NA	AJ296032
Bracon phylacteophagus	Australia	NA	AF173222
Bracon phytophagus	Venezuela	NA	DQ167438
Bracon sp.	Africa	ASW655	NA
Bracon sp.	Brazil	ASW592	NA
Bracon sp.	Brazil	ASW593	NA
Bracon sp.	Brazil	ASW595	NA
Bracon sp.	Brazil	ASW596	NA
Bracon sp.	Brazil	ASW732	NA
Bracon sp.	Brazil	ASW733	NA
Bracon sp.	Brazil	ASW734	NA
Bracon sp.	Brazil	ASW736	NA
Bracon sp.	Brazil	ASW737	NA
Bracon sp.	Brazil	ASW738	NA
Bracon sp.	Colombia	ASW566	NA
Bracon sp.	Colombia	ASW567	NA
Bracon sp.	NA	ASW299	NA
Bracon sp.	NA	NA	NA
Bracon sp.	England	ASW751	NA
Bracon sp.	England	ASW752	NA
Bracon sp.	England	ASW753	NA
Bracon sp.	England	ASW754	NA
Bracon sp.	England	ASW755	NA
Bracon sp.	England	ASW756	NA
Bracon sp.	England	ASW757	NA
Bracon sp.	England	ASW758	NA
Bracon sp.	Finland	ASW759	NA
Bracon sp.	Finland	ASW764	NA
Bracon sp.	Finland	ASW766	NA
Bracon sp.	Guiana	ASW575	NA
Bracon sp.	Guiana	ASW578	NA

Bracon sp.	Israel	ASW665	NA
Bracon sp.	Israel	ASW668	NA
Bracon sp.	Israel	ASW669	NA
Bracon sp.	Israel	ASW713	NA
Bracon sp.	Israel	ASW716	NA
Bracon sp.	Israel	ASW717	NA
Bracon sp.	Israel	ASW718	NA
Bracon sp.	Israel	ASW719	NA
Bracon sp.	Israel	ASW720	NA
Bracon sp	Israel	ASW741	NA
Bracon sp	Israel	ASW742	NA
Bracon sp	Israel	ASW743	NA
Bracon sp	Israel	ASW745	NA
Bracon sp	Madagascar	ASW708	NA
Bracon sp	Madagascar	ASW709	NA
Bracon sp	Madagascar	ASW699	NA
Bracon sp	Madagascar	ASW700	NA
Bracon sp	Madagascar	ASW702	NA
Bracon sp	Madagascar	ASW703	NA
Bracon sp	Madagascar	ASW704	NA
Bracon sp	Madagascar	ASW706	NA
Bracon sp	Madagascar	ASW707	NA
Bracon sp	Madagascar	NHM670	NA
Bracon sp	Malaysia	ASW554	NA
Bracon sp.	Malaysia	ASW554	NA
Bracon sp.	Malaysia	ASW70	NA
Bracon sp.	Malaysia	ASW70	NA
Bracon sp.	Malaysia	ASW75	NA
Bracon sp.	Malaysia	NA	AJ277504
Bracon sp.	Thailand	ASW71	NA
Bracon sp.	Thailand	ASW75	NA
Bracon sp.	Uganda	ASW598	NA
Bracon sp.	Uganda	ASW600	NA
Bracon sp.	Uganda	ASW604	NA
Bracon sp.	Uganda	ASW605	NA
Bracon sp.	Uganda	ASW606	NA
Bracon sp.	Uganda	ASW607	NA

Bracon sp.	Uganda	ASW610	NA
Bracon sp.	Uganda	ASW631	NA
Bracon sp.	Uganda	ASW649	NA
Bracon sp.	Venezuela	ASW563	NA
Bracon sp.	Zambia	ASW570	NA
Bracon sp.	Zambia	ASW572	NA
Bracon sp.	Australia	FD|2001	AF345617
Bracon sp.	Colombia	indet10	DQ538407
Bracon Striobracon minutator	Turkey	NA	NA
Bracon syntomernus	Uganda	ASW638	NA
Bracon sp.	Thailand	ASW773	NA
Bracon sp.	Brazil	ASW597	NA
Bracon sp.	Israel	ASW744	NA
Bracon sp.	Zambia	ASW569	NA
Bracon sp.	NA	83	NA
Braconella sp.	NA	CNIN185	NA
Braconella sp.	Russia	NL419	NA
Braconella sp.	NA	NA	NA
Bracon sp.	NA	ASW179b	NA
Braconina sp.	Australia	AZR029	NA
Braconinae	Kenya	RAW2006b	DQ538407
Bracon sp.	Malaysia	ASW553	NA
Callibracon limbatus	Brulle	NA	AJ231532
Calobracon bicolor	NA	NA	AJ296057
Campyloneurus sp.	Africa	ASW662	NA
Campyloneurus sp.	Benin	ASW537	NA
Campyloneurus sp.	Madagascar	Jo	NA
Campyloneurus sp	Australia	NA	AJ296047
Chaoilta decorata	NA	NA	AJ231504
Coeloides sordidator	Switzerland	NA	AJ231529
Compsobraconoides sp.	NA	NA	NA
Compsobraconoides sp1	South America	NA	Z97966
Compsobraconoides sp2	South America	NA	AJ231523
Compsobraconoides sp3	South America	NA	AJ231525
Cratobracon sp.	NA	NA	NA
Cratocnema sp.	NA	208	NA
Cratocnema sp.	NA	ASW609	NA

Cratocnema sp.	Taiwan	ASW124	NA
Cratocnema sp.	Uganda	ASW603	NA
Curriea sp.	Africa	ASW661	NA
Curriea sp.	NA	NA	AJ231542
Curriea sp.	Thailand	CNIN188	
Cyclaulax sp1.	South America	NA	AJ231526
Cyclaulax sp2.	South America	NA	AJ231527
Cyclaulax sp3.	South America	NA	AJ231528
Diamblomera sp.	Benin	ASW531	NA
Diamblomera sp.	Ghana	ASW542	NA
Diamblomera sp.	Malaysia	AJ277500	NA
Diamblomera sp.	Uganda	ASW637	NA
Diamblomera sp.	Benin	ASW534	NA
Diganogastra sp.	Colombia	ASWT	NA
Digonogastra sp.	Brazil	ASW281	NA
Digonogastra sp.	Brazil	ASW283	NA
Digonogastra sp.	Colombia	ASW112	NA
Digonogastra sp.	Colombia	ASW145	NA
Digonogastra sp.	Colombia	ASW165b	NA
Digonogastra sp.	Colombia	ASW167	NA
Digonogastra sp.	Colombia	ASW170	NA
Digonogastra sp.	Colombia	ASW173	NA
Digonogastra sp.	country	ASW192a	NA
Digonogastra sp.	Madagascar	ASW134	NA
Digonogastra sp.	Panama	ASW317	NA
Digonogastra sp.	USA	ASW192b	NA
Digonogastra sp.1	South America	NA	AJ231535
Digonogastra sp.2	South America	NA	AJ231536
Digonogastra sp.3	South America	NA	AJ231537
Digonogastra sp.4	South America	NA	AJ231538
Digonogastra sp.5	South America	NA	AJ231539
Digonogastra sp.	South America	NA	AJ296049
Digonogastra sp.	NA	NA	AJ296050
Dioxybracon sp.	Kenya	NA	NA
Doggerella sp.	Madagascar	NA	NA
Dolabraulax sp.	NA	Jo	NA
Euurobracon yokohamae	NA	NA	NA

Euvipio rufa	NA	NA	AJ231505
Fraterarchibracon sp.	Uganda	NA	NA
Fraterarchibracon sp.	NA	NL811	AY296648
New genus 1 sp.	Ghana	ASW551	NA
Glyptomorpha sp.	NA	NA	NA
Glyptomorpha sp.1	NA	NA	AJ231507
Glyptomorpha sp.	Nigeria	NA	AJ296044
Gracilibracon sp.	NA	NA	NA
Gracilibracon sp.	Colombia	NA	AJ231524
Gronaulax sp.	NA	NA	NA
Habrobracon hebetor	NA	NA	NA
Habrobracon hebetor	NA	NA	AJ231498
Habrobracon sp.	Israel	ASW667	NA
Hemibracon sp.	Colombia	ASW184a	NA
Hemibracon sp.	NA	NA	NA
Hybogaster sp.	Malaysia	NA	AJ277504
Indabracon sp.	NA	RDB|2000	NA
Iphiaulax impostor	France	NA	Z97967
Iphiaulax sp.	Benin	ASW532	NA
Iphiaulax sp.	Benin	ASW533	NA
Iphiaulax sp.	Benin	ASW535	NA
Iphiaulax sp.	Benin	ASW537	NA
Iphiaulax sp.	NA	ASW658	NA
Iphiaulax sp.	NA	CNIN183	NA
Iphiaulax sp.	NA	CNIN184	NA
Iphiaulax sp.	Ghana	ASW547	NA
Iphiaulax sp.	Ghana	ASW549	NA
Iphiaulax sp.	Ghana	ASW550	NA
Iphiaulax sp.	Ghana	ASW552	NA
Iphiaulax sp.	Malaysia	NA	NA
Iphiaulax sp.	Australia	NA	NA
Iphiaulax sp.	Thailand	NA	NA
Iphiaulax sp.	Uganda	ASW617	NA
Iphiaulax sp.	Africa	NA	AJ296052
Iphiaulax sp.	NA	NA	NA
Ipobracon sp.	Uganda	04NL1087	NA
Ipobracon sp.	Ghana	ASW543	NA

Ipobracon sp.	Uganda	NL771	AY538267
Ipobracon sp.	Uganda	NL773	AY296642
Ipobracon sp.	Uganda	NL775	AY296641
Ischnobracon sp	NA	CNIN191	NA
Kimavu sp.	Cameroon	ASW685	NA
Kimavu sp.	NA	NA	NA
Kimavu sp.	Uganda	787	NA
Lapicida aquatica	Canada	NA	AJ231512
Lasiophorus sp.	Colombia	NA	AJ231534
Latana keijua	Uganda	NA	NA
Latana sp.	Uganda	NL792	AY296640
Ligulibracon sp.	Australia	ASW156	NA
Lyricibracon sp.	Malaysia	RDB2000	NA
Malagopsis doggeri	Uganda	NA	NA
Megabracon sp.	CostaRica	961	NA
Megacoeloides apricans	Colombia	NA	AJ231521
Megacoeloides psolopterus		NA	NA
Megacoeloides sp.	Colombia	AZR2006	NA
Merinotus sp	Uganda	NA	NA
Merinotus sp	Uganda	NL786	AY296649
Mesobracon sp	Ghana	ASW54	NA
Mesobracon sp	Russia	NA	NA
Mesobracon sp	Uganda	ASW646	NA
Mesobracon sp	West Africa	NL488	NA
Mesobraconoides sp	NA	NA	NA
Mollibracon bimaris	Australia	NA	AJ231519
Monilobracon sp.	SierraLeone	NA	NA
Monilobracon sp.	Uganda	Pupa015	NA
Monilobracon sp.	Uganda	ASW588	NA
Monilobracon sp	Uganda	ASW590	NA
Monilobracon sp	Uganda	ASW591	NA
Monilobracon sp.	NA	NL785	AY296646
Monilobracon sp.	NA	NL832	AY529649
Monocoila sp.	Kenya	NA	AJ231530
Myosoma nyanzaensis	Kenya	NA	AJ231516
Myosoma sp	Columbia	NA	AJ231515
Nedinoschiza sp.	Malaysia	NA	AJ277503

Nedinoschiza sp.	Uganda	ASW643	NA
Nesaulax sp.	NA	NA	NA
Nesaulax sp.	NA	NA	AJ277501
Nesaulax sp.	NA	CNIN192	NA
Nesaulax sp.	Malaysia	NA	AJ231541
Nesaulax sp.2	Malaysia	NA	AJ27750
NHM	Madagascar	723870	NA
NHM	Madagascar	723872	NA
NHM	Madagascar	723897	NA
Nundinella sp.	NA	489	NA
Nundinella sp.	Zambia	ASW571	NA
Odesia sp.	NA	NA	AJ231506
Odontoscapus sp.	Kenya	NA	AJ231503
Pachybracon sp.	Malaysia	NA	AJ277502
Philomacroploea sp.	NA	CNIN197	NA
Physaraia sp.1	Africa	NA	AJ231517
Physaraia sp.	India	NA	AJ296043
Plaxopsis sp.	NA	NA	AJ231533
Plesiobracon sp.	Malaysia	ASW749	NA
Poecilobracon sp.	Papa New Guinea	RDB|2000	AJ296054
Pseudoshirakia sp.	South Korea	BNHM	NA
Pseudovipio sp.	Uganda	ASW636	NA
Pseudovipio sp.1	Kenya	NA	AJ231543
Pseudovipio sp.	Turkey	NA	AJ296055
Pycnobraconoides sp.	Australia	NA	AJ231514
Rhamnura sp.	Uganda	NA	NA
Rhytimorpha sp.	NA	NL485	NA
Rhytimorpha sp.	Ghana	ASW545	NA
Rhytimorpha sp.	Zimbabwe	NA	AJ231508
Rostraulax sp.	Malaysia	NA	NA
Sacirema sp.	NA	NA	NA
Scutibracon hispae	NA	NA	AJ296039
Serraulax sp.	Africa	ASW660	NA
Serraulax sp.	Ghana	ASW538	NA
Serraulax sp.	Ghana	ASW548	NA
Serraulax sp.	Uganda	ASW640	NA
Serraulax sp.	NA	RDB|2000	AJ296051

Shelfordia sp.	NA	NA	AJ277499
Simplicibracon sp.	NA	NA	670590
Simplicibracon sp.	NA	RDB|2000	AJ296040
Simplicibracon sp.	Kenya	RDB|2000	AJ296041
Sobrinarchibracon sp.	NA	1085	NA
Sororarchibracon sp.	Uganda	ASW634	NA
Sororarchibracon sp.	Uganda	ASW644	NA
Sororarchibracon sp.	NA	NL678	AY296645
Sororarchibracon sp.	Uganda	ASW584	NA
Sorroarchibracon sp.	Ghana	ASW544	NA
Sorroarchibracon sp.	Uganda	ASW648	NA
Soter sp.	Africa	ASW652	NA
Soter sp.	Cameroon	ASW683	NA
Soter sp.	Cameroon	ASW684	NA
Soter sp.	Uganda	ASW583	NA
Soter sp.	Uganda	ASW608	NA
Soter sp.1	NA	NA	NA
Spinadesha sp	West Malaysia	.BNHM	NA
Sylvibracon sp.	Uganda	ASW602	NA
Sylvibracon sp.	NA	CNIN194	NA
Sylvibracon sp.	NA	Nl483	NA
Sylvibracon sp.	Uganda	ASW582	NA
Sylvibracon sp.	Uganda	NL772	AY296643
Syntomernus sp.	NA	RDB|2000	AJ302925
Syntomernus sp.	Malaysia	RDB|2000	AJ296042
Trigastrotheca sp.	NA	NA	AJ231518
Trigastrotheca sp.	NA	CNCO	NA
Trigastrotheca sp.	NA	CNIN178	NA
Trigastrotheca sp.	NA	CNIN190	NA
Trigastrotheca sp.	NA	NA	NA
Trispinaria sp.	NA	NA	NA
Tropobracon sp.	NA	NA	AJ231502
Tsavobracon sp.	Uganda	ASW635	NA
Tsavobracon sp.	Uganda	NA	NA
New genus sp.	Benin	ASW522	NA
Vipiellus sp.	Australia	NA	AJ231499
Vipio sp.	NA	NA	NA

Vipio sp.	Ghana	ASW540	NA
Vipio sp.	Israel	ASW585	NA
Vipio sp.	Israel	ASW585	NA
Vipio sp.	China	C2003	AY167648
Vipio sp.	Turkey	RDB|2000	AJ296045
Vipiomorpha ypsilon	NA	608	NA
Virgulibracon sp.	Australia	AJ231520	NA
Zaglyptogastra sp.	Uganda	NL813	AY296647
Zanaopsis sp.	Uganda	ASW155b	NA
Zanzopsis sp.	Uganda	ASW155a	NA
Zanzopsis sp.	Uganda	NA	NA
[outgroups]	NA	NA	NA
Rogadinae	Israel	ASW622	NA
Rogadinae	Kenya	ASW515	NA
Rogadinae	Taiwan	ASW621	NA
Exothecinae	NA	NA	AJ302886
Exothecinae	NA	NA	AJ302887
Exothecinae	UK	RMNH|Jo698	AY935431
Alysiinae	Colombia	ASW558	NA
Alysiinae	Finland	ASW761	NA
Alysiinae	Israel	ASW627	NA
Doryctinae	Africa	ASW653	NA
Doryctinae	Africa	ASW657	NA
Doryctinae	Uganda	ASW615	NA
Doryctinae	Uganda	ASW613	NA
Doryctinae	Uganda	ASW616	NA
Doryctinae	Uganda	ASW630	NA
Doryctinae	Uganda	ASW651	NA
Gnamptodontinae	NA	RAW2006	DQ538405
Gnamptodontinae	UK	NA	Z93662
Gnamptodontinae	Russia	ZISP|Jo89	AY935441
Lysiterminae	Cameroon	ASW689	NA
Opiinae	Africa	ASW659	NA
Opiinae	Brazil	ASW739	NA
Opiinae	Cameroon	ASW692	NA
Opiinae	Colombia	ASW559	NA
Opiinae	Israel	ASW586	NA

Opiinae	Malaysia	ASW556	NA
Opiinae	Malaysia	ASW724	NA
Opiinae	Malaysia	ASW748	NA
Gnamptodontinae	NA	RAW|2006	DQ538411
Gnamptodontinae	NA	RDB|2000	AJ302827
Exothecinae	Russia	NA	AY935433
Opiinae	Kenya	NA	DQ538414
Exothecinae	Russia	NA	AY935432
Exothecinae	Russia	NA	AY935434

∗ NA = Not Available

3.3.2. Extraction

DNA extraction was carried out by taking the middle leg from a large wasp or, in the case of small wasp; the whole wasp was used, preserved in 99% ethanol. Tissue was covered in a master mix of nuclei lysis solution, 0.5 EDTA and proteinase K (Promega), incubated at 55°C overnight. This was followed by sodium acetate/ethanol precipitation and re-suspension in 150µl nuclease free water. The gene fragment examined was approximately 700 bp of the second and third domains (D2-3) of the nuclear 28S DNA. The primers were designed by Belshaw and Quicke (1997) (fwd: 5' AAG AGA GAG TTC AAG AGT ACG TG 3' rev: 5' TAG TTC ACC ATC TTT CGG GTC CC 3'). Polymerase chain (PCR) reactions were carried out in 23µl volumes containing 13.50µl H_2O, 2.0µl buffer, 12µl $MgCl_2$, 8.0µl primer (10mM), 6.0µl dNTP (25mM), 1.2µl *Taq* (5U) and 4µl of DNA template. PCR conditions were: 94°C for 2 min followed by 30 cycles of 94° C for 30s, 47.5°C for 30s, 72°C for 1 min and finally 72°C for 10 min. PCR product was cleaned by using 80% ethanol and sent to the SBS Sequencing Service, Ashworth Laboratories University of Edinburgh for sequencing. The sequences obtained were edited by eye, using sequence navigator (Parker, 1997).

3.3.3. Morphological Data

The dataset included 342 species collectively representing 97 described genera plus 3 putatively undescribed new genera. Morphology was scored at genus level for 92 characters. Characters used are listed and defined in Table 3.2.

Table 3.2. Morpological characters and character states:

Antenna: 0 =longer than forewing; 1 =shorter than forewing.

Antenna: 0 =without annulus; 1 =with pale coloured annulus.

Terminal flagellomere: 0 =not accuminate; 1 = accuminate.

Median flagellomere: 0 =wider than long; 1 =quadrate; 2 =longer than wide. (Ordered.)

Basal flagellomeres: 0 = terete; 1 =apically flared.

Scapus: 0 =shorter ventrally than dorsally; 1 =ventrally produced.

Scapus: 0 = smoothly narrowed basally; 1 = angularly constricted basally.

Scapus: 0 =with no apico-medial area; 1 =with one apico-medial area; 2 =with two apico-medial areas. (Ordered.)

Bulbous scape 0=absent; 1=present

Labio-maxillary complex: 0 =normal; 1 =elongate.

Dorsal clypeal margin: 0 =not carinate; 1 = carinate.

Dorsal clypeal margin: 0 =curved 1 =composed of three straight sections.

Face: 0 =simple; 1 =with a parallel-sided, mid-longitudinal area bordered laterally by a pair of carinae.

Face: 0 =simple; 1 =with a medio-basal triangular area bordered by sulci laterally.

Face: 0 = smooth or irregularly punctatdrugulose; 1 =with fine transverse striae.

Face: 0 =simple; 1 =with mid-logitudinal ridge.

Antennal sockets: 0 =normal; 1 =strongly produced.

Antennal sockets: 0 =without groove running to eye; 1 =with groove.

Eyes: 0 =not or hardly setose; 1 =moderately serose; 2 = densely setose. (Ordered.)

Frons: 0 =smooth or coriaceous; 1 =strongly striate.

Frons: 0 =glabrous except at edge; 1 =moderately setose; 2 =extensively setose. (Ordered.)

Frons: 0 =not impressed except for medial sulcus; 1 =moderately impressed; 2 =strongly impressed. (Ordered.)

Frons: 0 =simple; 1 =with mid-longitudinal carina.

Mesosoma: 0 =smooth and shiny; 1 =extensively sculptured.

Mesosoma: 0 = >1.55 x longer than high; 1 = c1.55 x longer than high.

Mesosoma: 0 =normal; 1 = strongly dorso-ventrally compressed.

Propleural carina: 0 = absent; 1 =present.

Mesoscutum: 0 =largely glabrous; 1 =densely and evenly setose.

Mesoscutum: 0 = notauli weak or absent; 1 =notauli moderately impressed; 2 = notauli strongly impressed.

Mesoscutum: 0 =simple; 1 =with a pair of sub-medial mid-longitudinal grooves anteriorly.

Scutellar sulcus: 0 =crenulate; 1 =smooth.

Scutellar sulcus: 0 =wide; 1 =narrow.

Precoxal suture (sternaulus): 0 =absent; 1 =present.

Mesopleural suture: 0 =smooth; 1 = crenulate.

Metanotum: 0 =simple; 1 =with a complete mid-longitudinal carina.

Propodeum: 0 = smooth; 1 =coarsely sculptured.

Propodeum: 0 =simple; 1 =posterior margin with a pair of posteriorly diverging carinae.

Propodeum: 0= simple; 1 =with a complete, lamelliform mid-longitudinal carina.

Propodeum: 0 =without lateral carinae; 1 =with lateral carinae.

Propodeal spiracle: 0 =round to 2 x taller than wide; 1 =more than 2 x taller than wide. (Ordered.)

Fore wing vein l-SR+M: 0 =straight; 1-3 =increasing degrees of curvature.

Fore wing vein l-SR+M: 0=straight or curving posteriorly after arising from 1-M; 1 =distinctly curving anteriorly after arising from 1-M.

Fore wing vein 1-M: 0 = straight; 1 =moderately curved; 2 =strongly curved. (Ordered.)

Fore wing vein CUa: 0=not at same level as vein 2-CU1;1=same level as vein 2-CU1.

Angle between fore wing veins 1-SR and C + SC + R: 0 = <55"; 1 = >55".

Second submarginal cell of fore wing: 0 =short; 1 =medium; 2 =long. (Ordered.)

Fore wing vein r-m: 0 =not longer than 2-SR 1 =longer than 2-SR.

Number of r-m bullae: 0 =one; 1 =two; 2 =completely unsclerotized. (Ordered.)

Marginal cell of fore wing: 0 =normal; 1 =short, vein SR1 reaching the wing margin less than 0.8 of the way from the apex of the pterostigma to the wing tip.

Subdiscal cell of fore wing: 0 = setose; 1 =with a glabrous patch.

Fore wing vein r length: 0 = >0.55 length of m-cu; 1 = <0.45 length of m-cu.

Fore wing vein cu-a: 0 =far postfurcal; 1 =moderately postfurcal; 2 =interstitial or virtually so; 3 = antefurcal. (Ordered.)

Fore wing vein 3-CU1: 0 =simple; 1 =distinctly expanded posteriorly.

Fore wing vein 2-CU1: 0 =more or less straight; 1 =sigmoid.

Fore wing vein CUlb: 0 =normal; 1 =widened anteriorly (or medially in some *Curriea* where it is wider than the posterior part of 3CU1).

Fore wing veins CUlb and CUla: 0=angled; 1 =forming a continuous curve.

Fore wing vein CUla: 0=without a spur; 1 =with a spur.

Fore wing vein of 2SR+M relative to 1SR+M; 0=<0.5; 1=>0.5.

Apex of hind wing vein C + SC + R 0 =with more than one especially thickened bristle (hamulae); 1 =no or one such bristles.

Hind wing vein lr-m length: 0 =shorter than SC + R1; 1 =equal to SC + R1; 2 =longer than SC + R1. (Ordered.)

Base of hind wing: 0= with a glabrous area; 1 =more or less evenly setose.

Hind wing veins lr-m: 0 =straight; 1 =curved.

Basal lobe of claws: 0 =rounded; 1 =pointed.

Bifurcate claws 0=absent; 1=present.

Apex of fore tibia: 0 =simple; 1 =with a transverse row of chaetobothria-like pegs.

Ventral telotarsal setae in males: 0=not reaching 0.85 ventral length of telotarsus (excluding claw); 1 =extending beyond 0.85 ventral length of telotarsus.

Ratio of lengths of fore tarsus and tibia: 0=<1.3 to 1; 1=1.3-1.5 to 1; 2=1.51-1.7 to 1; 3=>1.7 to 1. (Ordered.)

Ratio of length and maximum depth of fore basitarsus: 0=<5 to 1; 1=5.1-6 to 1; 2=6.1-7 to 1; 3=>7.1 to I. (Ordered.)

Number of coarsely sculptured metasomal tergites: 0 = <6; 1 = 6 or more.

First metasomal tergite: 0 =without dorsal carinae; 1 =with incomplete dorsal carinae; 2 =with complete dorsal carinae; 3 =complete and fused. (Unordered.)

First metasomal tergite: 0 =without dorso-lateral carinae; 1 =with dorso-lateral carinae.

First metasomal tergite: 0 =simple; 1-3 =increasingly well developed mid-longitudinal carinae. (Ordered.)

Metasomal tergites one and two: 0 =moveably jointed; 1 =fused.

Second metasomal tergite: 0 =longer medially than sub-medially or of equal length; 1 =shorter medially than

sub-medially.

Second metasomal tergite: 0 =without a mid-basal area; 1 =with a mid-basal area.

Second metasomal tergite: 0 =without antero-lateral, longitudinal grooves; 1 =with antero-lateral, longitudinal grooves.

Second metasomal tergite: 0 = antero-lateral, longitudinal grooves parallel or diverging posteriorly; 1 = antero-lateral, longitudinal grooves converging posteriorly.

Mid-ventral, anterior metasomal gland 0 = absent; 1 =present as a membranous sac-like structure between the 2nd and 3rd metasomal sternites.

Third metasomal tergite: 0 =simple; 1 =with antero-lateral areas defined posteriorly by a pair of posteriorly diverging grooves.

Metasomal tergites three to five: 0 =simple; 1 =with a sub-posterior, transverse, often crenulate groove.

Intertergal gland between metasomal tergites 7 and 8: 0 =absent or weak; 1 =medium; 2 =large. (Ordered.)

Intertergal gland between metasomal tergites 7 and 8: 0 =unilobed; 1 = bilobed; 2 =paired. (Ordered.)

Tergal gland 0 = absent; 1 =present.

Tergal gland type: 0 = flat pore plate; 1 =depressed pore plate; 2 =flask or tubular gland. (Ordered.)

Tergal gland pore plate shape: 0 =round; 1 =elongate.

Rectal pad shape: 0=round to 1.5 to 1.0; 1 =1.6-3.0 to 1; 2=>3.0 to 1. (Ordered.)

Rectal fusion to the 7th metasomal tergite (males only): 0 =absent; 1 =present.

Ovipositor: 0 =shorter than the metasoma; 1 =longer than the metasoma but less than 1.5 x fore wing length; 2 = >1.5 x fore wing length. (Ordered.)

Ovipositor: 0 =simple; 1 =with a pre-apical dorsal nodus (sometimes lowered leaving just a notch).

Basal ring: 0 = anteriorly transverse; 1 = anteriorly rounded; 2 = anteriorly pointed; 3 = anteriorly produced into a spine-like process. (Ordered.)

Digitus: 0-3 =one to four tooth-like processes respectively. (Ordered.)

Digitus: 0 =triangular; 1 =round.

Digitus: 0=not produced below tooth; 1 =produced below tooth.

Parameral setosity: 0-3 =increasing extents of setose area. (Ordered.)

Parameres: 0 =extending beyond the apex of digitus; 1 =ending level with middle of digitus; 2 =ending before anterior (basal) 0.25 of digitus. (Ordered.)

Volsella: 0 =without a lobe; 1 =with a medial lobe

3.3.4. Sequence Alignment

As most alignment methods need to attribute a cost for a nucleotide substitution versus the insertion of a gap (DeSalle *et al*., 1994; Wheeler, 1994) different parameters often lead to different number of gaps that can also vary in their position for the same sequence. This limits the justification for choosing one set of parameters over the other (Vingron & Waterman, 1994; Kjer, 1995; Doyle & Davis, 1998) that leads to alternative sequence alignments of same data set. Problems have been faced when this is taken to the level of tree construction as different topologies are seen due to different alignments (Wheeler, 1995; Wheeler *et al*., 1995; Soltis *et al*., 1996). As a result secondary structure alignment was done following the model proposed by Gillespie *et al*. (2005) which was based in turn on the chemical structure approach of Kjer (1997) where the length variable regions were further characterized as proposed by Gillespie (2004).

RAA: Regions of ambiguous alignment with not identifiable base pairing.

RSC: Regions of slipped-strand compensation defined by inconsistencies in base-pairing across the alignment.

REC: Regions of expansion and contraction that are RSC present in the apical part of helical regions in hairpin-stem loops.

The coding approach of Zaldivar Riverón *et al*. (2006) was followed that involves excluding and including the RAA and indel information using the 'fragment-level' alignment method (Lee, 2001) but with a modified approach so that potential phylogenetic information can be preserved still keeping the objective criteria.

The part of the alignment that involved fragment-level alignment was named offset while the part that was used to code offset was called pseudomorphology, it includes the unalignable regions that have at least two base changes occurring in two taxa.

3.3.5. Phylogenetic Analysis

Parsimony analyses were performed using PAUP version 4.0b10 (Swofford, 1998) using 100 random additions with tree bisecting reconnection (TBR) branch swapping, holding a maximum one tree at any one time. Relative branch support was assessed by bootstrapping (Felsenstein, 1985) using 500 replicates.

The congruence between molecular and morphological data sets was assessed using the taxonomic retention index (TRI). The taxonomic RI as explained in Hunt *et al*. (2007) '' The taxonomic RI is a measure of how traditionally classified groups, when optimized as a single binary character, are consistent with the phylogeny". The taxonomic RI was calculated by constructing trees with different K values (1-30) for Goloboff's fit. A binary data set was created by representing each species for a genus by 1 and absence of that particular genus by 0 in each column. A column was made for each genus that is represented at least twice in the data set. Each taxon is then treated as a character and RIs were calculated. If the RI value for a taxon is one then it has evolved once on the tree and for any other value it has evolved more than once. The mean of RI values for all the taxa on a tree was taken to get mean RI value for that tree. This mean RI for all the trees with different Goloboff's fit ranging from 1-30 was then plotted in a graph. The peak value in the graph represents the tree with highest RI and it is considered as a best fit to taxonomy.

3.4. Results and Discussion

A combined maximum parsimony analysis including 28S DNA and morphology revealed a phylogenetic tree that was unresolved between Aphrastobraconini and Braconini (fig.3.2) as expected in accordance with the findings of Belshaw *et al*. (2001).

One of the monophyletic groups found was the entirely New World *Compsobraconoides*-group of genera (Quicke, 1997) that is also consistent with previous phylogenetic studies (Quicke, 1988; Belshaw *et al*., 2001). These are medium sized to moderately large wasps and the group contains seven genera, *Compsobracon* Ashmead, *Calobracon* Szépligeti, *Cyclaulax* Cameron, *Compsobraconoides* Quicke, *Cyclaulacidea* Quicke and Delobel, *Gracilibracon* Quicke, *Sacirema* Quicke and *Gozmanycomp* Papp. They show biologies ranging from feeding on concealed Lepidoptera as hosts in the Brazilian cerrado habitat (*Compsobracon*) (Santos *et al.,* 2004), (*Gozmanycomp*) (Papp, 2007) to coleopteran hosts (Bruchidae and Curculionidae) in Central America in palm fruits (*Cyclaulacidea*) (Quicke & Delobel, 1995: Leathers *et. al*., 2005) to adult queens of *Azteca* ants in Peru (*Compsobraconoides*) (Yu & Quicke, 1997) their consistent appearance together in phylogenetic trees support their relationship to be true, that was originally based on unique facial sculpture of paired ridges that run from the antennal sockets to the clypeus (Leathers *et al*., 2005).

The genera comprising the tribe Braconini have individuals that are usually small to medium-sized and show rather more diverse biologies. Their small size may have enabled to invade all possible ecological niches thus making them one of the most successful groups. At genus level the host range varies from highly specialist to ones that are highly generalist found feeding on wide range of hosts. For example, members of the genus *Physaraia* have only been found feeding on gall forming curculionid beetle larva (Donaldson, 1989), *Testudobracon* have been reared from a number of gall forming Diptera larvae while *Bracon variator* Nees, has been recorded from Coleoptera, Lepidoptera, trypetid fly larvae in Asteraceae flower heads, oak galls formed by the cynipid wasps *Biorhiza* and *Andricus*. Among other biological and morphological modifications some members have developed special structural features for their hosts like *Aspidobracon, Hyboteles, Philomacroploea,* and

Physaraia. They have 1st and 2nd metasomal tergites fused to form carapace as they have to pass through hard casings of their host butterfly pupae (in case of *Aspidobracon, Hyboteles, Philomacroploea*) (Quicke, 1987) of the host and/or have modified ovipositors with nodus and serrations (seen in other members like *Soter,* etc) to drill through the dwellings of their

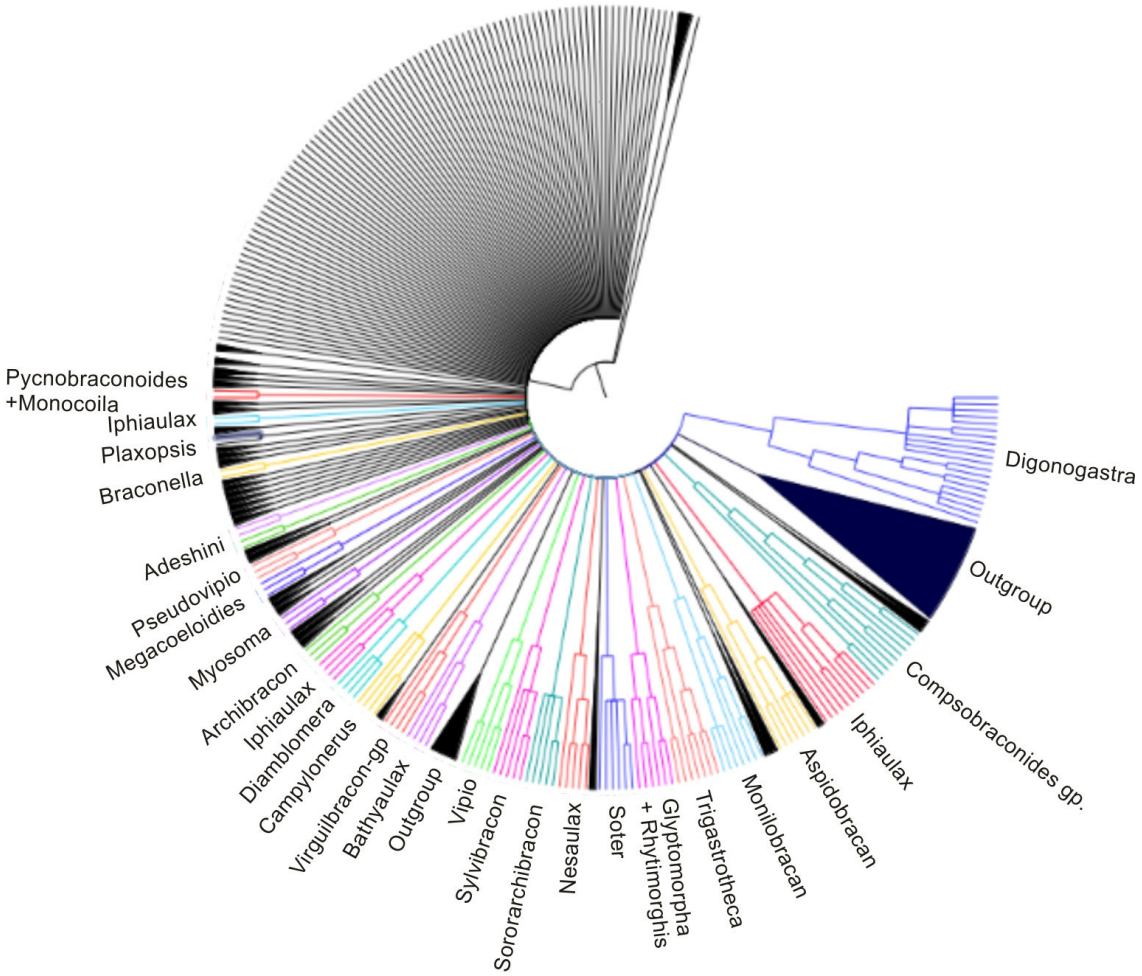

Figure 3.2. Phylogenetic tree of 28S data set using maximum parsimony including RAA's and indel information. Branches in colour show clades with more than 50% bootstrap support.

hosts. The tribe Braconini was based on robust scapus, which is truncate apically and ventrally as long as dorsally or more usually somewhat shorter and by hind wing vein 1r-m shorter than vein SC+R1 (except in *Calcaribracon* and some *Trispinaria*) (Quicke, 1987).

The genus *Braconella* was recovered as monophyletic with more than 50% bootstrap support. The *Myosoma*-group was found to be monophyletic. All the genera in the *Virgulibracon*-group are coming together, as a single clade within Bracionini, confirming the relationship between them and also that they are more closely related to the Braconini rather than to the Euurobraconini. The genus *Physaraia* is next to *Trigastrotheca, Physaraia* is reported to parasitize gall forming weevils and *Trigastrotheca* is found consuming egg, larvae and pupae of acacia ants which inhabit modified *Acacia* thorns thus needing modifications in their metasoma to drill through hard casings of their hosts for egg laying. Also the Aspidobraconina is resolved as monophyletic (a subtribe of Braconini, van Achterberg, 1984) based on 1st and 2nd metasomal tergites fused and differentiated from Physaraiina by having deep and complete transverse sutures (that includes *Aspidobracon, Dioxybracon, Eutropobracon, Hyboteles, Pedinopleura* and *Philomacroploea*). Adeshini (where *Aneuradesha* is found to be the specialist ectoparasitoids of hispine larvae) and members of Aspidobraconina feed within exposed pupae of butterflies and beetles (van Achterberg, 1984b; Quicke, 1987), thus requiring special metasomal modifications, have failed to be recovered as monophyletic.

The Coeloidini *(Coeloides* and *Megacoeloides)*, where *Coeloides* parasitize larvae of various species of Scolytidae, Curculionidae and Buprestidae (pests of coniferous and broadleaved trees) (Kenis, 1997; Pettersson *et al*., 2001; Wang *et al*., 2006), has failed to be recovered as monophyletic. Belshaw *et al*. (2001) found the Coeloidini to be monophyletic and between the tribes Braconini and Aphrastobraconini which considered this situation less clear and putatively based on the plesiomorphic states such as the well developed and densely setose male parameres (Quicke, 1988a). This tribe is based on only two genera out of which *Coeloides* possess the character of the scapus not extending ventrally as with the Braconini, whereas *Megacoeloides* has a ventrally produced scapus as in the vast majority of

Aphrastobraconini thus making the tribe falling in between the other two main tribes. Another group is formed of *Monocoila* and *Pycnobraconides*, that has affinities with Aphrastobraconini.

The Aphrastobraconini (sinus Achterberg, 1993) is based on the status of ventrally produced scapus being an acquired character. The members of Aphrastobraconini are usually parasitoids of wood boring beetles belonging mostly to Cerambycidae, Buprestidae, Scolytidae and Anthribidae in case of *Shelfordia*, but some genera also attack the hosts such as pyralid stem-boring moths that are pests of maize, sorghum, millet and wheat (Quicke & Falco, 1998; Laurenne *et al*., 2000; Quicke *et al.*, 2000; Wang *et al.,* 2003; Quicke & Laurenne, 2005; Wang *et al*., 2009). In the case of the type genus, *Aphrastobracon*, they are the parasitoids of a predatory lepidopterous pest of lac insect (Ayyar, 1926).

The genus *Digonogastra* is resolved as monophyletic. This genus belongs to the tribe Aphrastobraconini. *Digonogastra* Viereck, a completely New World genus that was for a long time wrongly interpreted as Nearctic and Neotropical species of *Iphiaulax,* but the latter is entirely restricted to Old World, is recovered as monophyletic. The Bathyaulacini and Glyptomorphini both include stem borer parasitoids (Quicke & Falco, 1998; Laurenne *et al*., 2000; Quicke *et al*., 2005), though some species of *Bathyaulax* attack wood-boring larvae, thus the relationship supported by their biologies. Glyptomorphini was recoverd monophyletic while Bathyaulacini failed to recover as monophyletic.

The Euurobraconini is not recovered as monophyletic as it has some genera nested within. The genus *Sororarchibracon* was found monophyletic as well as the genera *Sylvibracon* (a member of *Nesaulax* group) and *Soter*, a genus of uncertain placement. *Euurobracon* species are parasitoids of Cerambycidae (Shenefelt, 1975, 1976, 1978). The *Plaxopsis*-group of genera are not recovered as suggesting that they evolved independently of each other although *Plaxopsis* and *Zanzopsis* share fluorescent purple coloration at the lateral

side of metasoma (Quicke, 2005). The *Nesaulax*-group of genera are also not recovered together, again showing paraphyly. The genera *Cratobracon, Shelfordia, Nesaulax, Gronaulax, Rhadinobracon* and *Merinotus* all share long, narrow, parallel sided metasomas which curve ventrally towards the apex (see figs 6 & 9 in Quicke, 1980); this form of metasoma is termed 'merintoid' (Quicke, 1984) but they are not coming together in this tree so needs further investigation.

As the combined analysis has shown some results that were not in agreement with the previous morphological studies it was decided to use an independent measure for the consistency of the data. Goloboff's implied weighting was used to down-weight the poorer performing characters during tree construction to determine whether down weighting the noisy characters improve taxonomic congruence? The molecular data were analyzed with 30 different values of Goloboff fit constant K and calculating the taxonomic RI for each tree.

3.4.1. Effect of Character weighting using Goloboff's method

The Maximum parsimony (MP) trees with different values of the Goloboff's fit criterion K with values 1-30 were run to find out the best possible fit of the data on the tree. Only 28S rDNA sequence data was used as the morphology has been scored at genus level.
The best tree found, the tree at the peak point in the graph (fig. 3.3) between taxonomic RI and Goloboff's constant, was with the K =16 and this is taken as the best fit of the data. In this tree a similar trend to that found in previous combined is seen as tree has failed to divide into two main clades, Braconini and Aphrastobraconini (fig. 3.4). The *Virgulibracon*-group of genera was found to be monophyletic as well as the *Compsobracon*-group of genera. Both these groups of genera have very strongly curved/bent 1-SR+M and no sculpture on tergites. *Physaraia* is no longer recovered in the clade with *Trigastrotheca* but none of these genera are coming monophyletic.

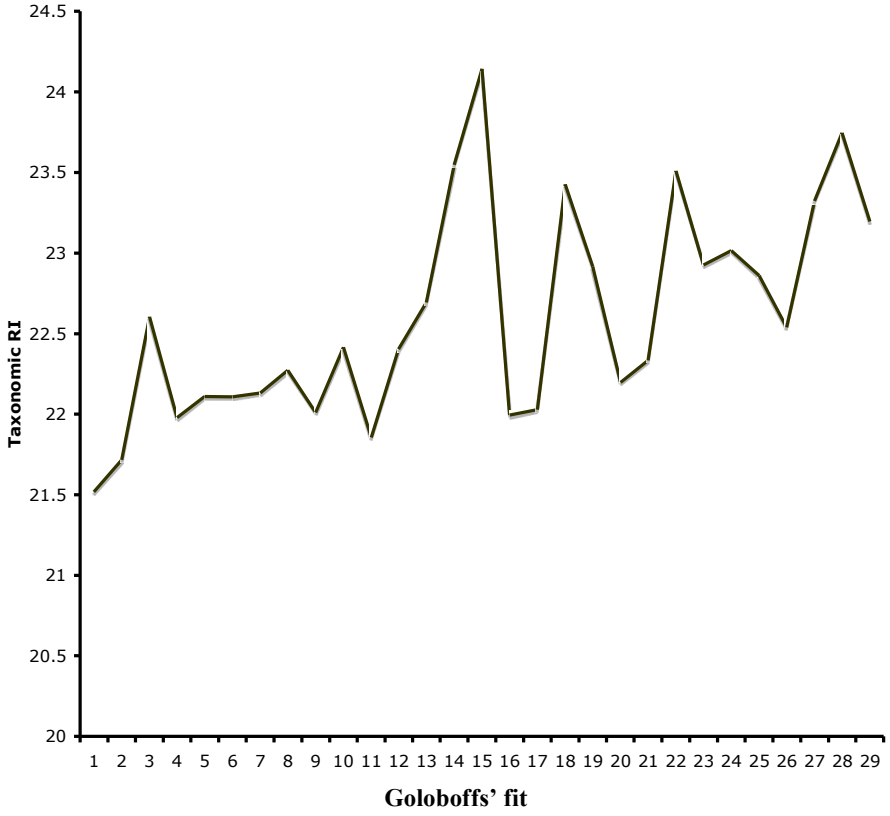

Figure 3.3. Relationship between TRI and Goloboff's fit constant K.

However, most of the morphologically recognized groups within the Aphrastobraconini are recovered as paraphyletic. *Glyptomorpha* and *Rhytimorpha* are coming together retaining the combined tree status and also the Bathyaulacini is recovered monophyletic. *Digonogastra* is recovered monophyletic but *Iphiaulax* is not recovered as monophyletic while *Plaxopsis* has lost its monophyletic status.

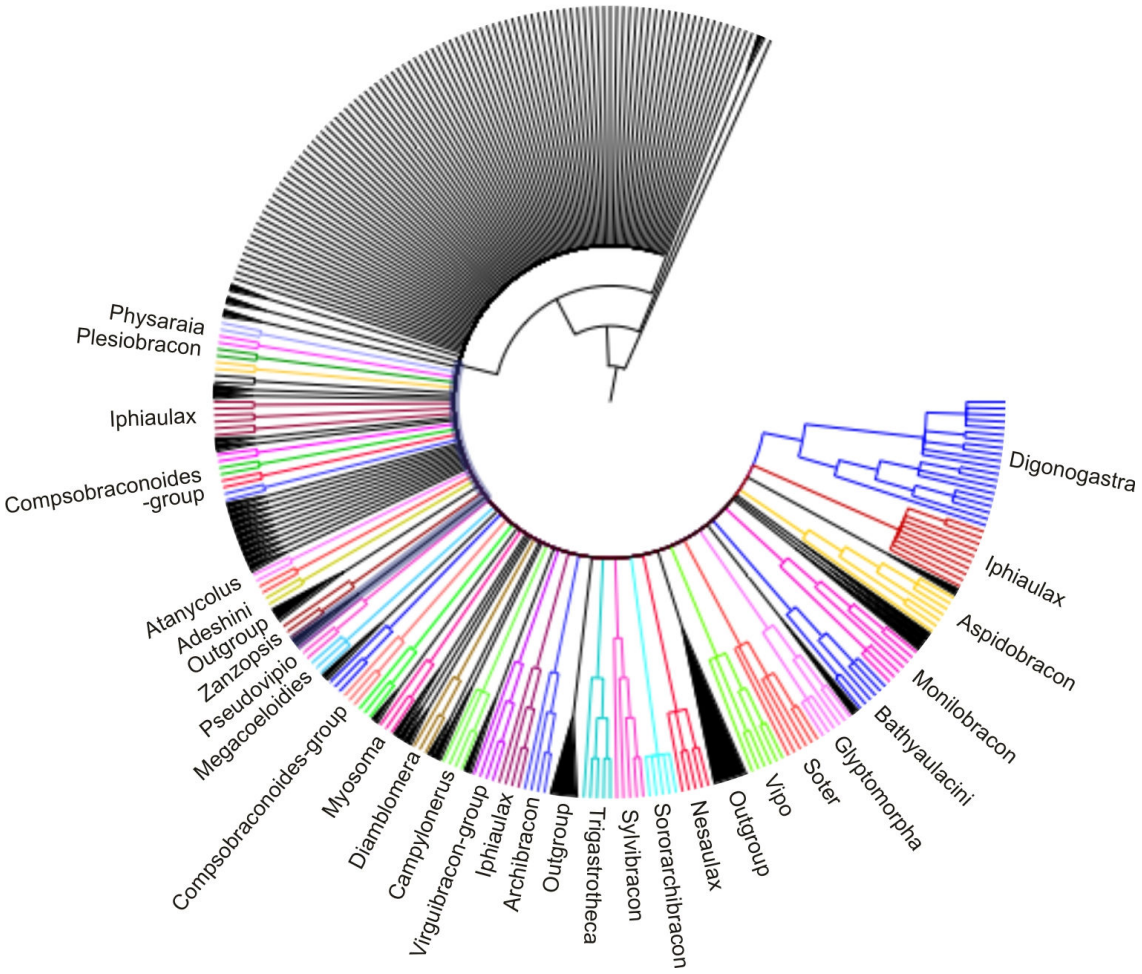

Figure 3.4. Phylogenetic tree of 28S data set using maximum parsimony with Goloboff's constant K=16. Branches in colour show bootstrap support of 50%.

Thus, the results of this independent measure agree that Braconinae is not divided into two main tribes Aphrastobraconini and Braconini. However, some groups as *Compsobracon*-group and *Virgulibracon*-group in Braconini while in case of Aphrastobraconini genus Digonogastra and tribes Glyptomorphini and Bathyaulacini are found to be monophyletic. Thus for the Braconinae phylogeny the Goloboff's method of downweighting the characters does not appear to be very effective.

Chapter 4: Is CO1 effective in resolving the relationship at subfamily level for Braconinae (Hymenoptera: Braconidae)?

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4.1. Abstract

The phylogenetic relationships among 50 genera of the subfamily Braconinae were investigated by utilizing CO1 mtDNA sequence data. The CO1 gene has been given prime importance the past two decades to study phylogenetic relations among different taxa. Two different methods of tree construction were utilized to resolve these relationships. The bootstrap trees obtained from PAUP were even unable to recover monophyletic Braconinae thus confirming that use of CO1alone may not be a good strategy at subfamily studies while it gave good support while recovering many

genera monophyletic thus showing its resolving power at genus and species level. While the Bayesian analysis revealed similar results.

4.2. Introduction

In molecular phylogenetic studies cytochrome oxidase subunit 1 (CO1) is one of the most popular gene fragments as its 5' partition is being used for the "Barcoding of Life" initiative (Hebert *et al*., 2003; Stoeckle, 2003). The universal primers for this gene fragment are very robust and have proved successful for most of animal taxa and also it is considered to possess a greater range of phylogenetic signal than any other mitochondrial gene (Hebert *et al*., 2003). The alignment of the sequences obtained from CO1 is much easier when compared to nuclear genes like 12S rDNA, 16S rDNA, 28S rDNA as it only rarely has indels and therefore can normally be unambiguously aligned by eye (Kitahara *et al*., 2010).

As a result a large number of phylogenetic studies were conducted using CO1, especially among arthropods (Juan *et al.,* 1995; Sperling *et al.,* 1995, 1996, 1999; Lunt *et al.,* 1996; Willett *et al.,* 1997; Knowlton and Weigt, 1998; Sandoval *et al.,* 1998; Wells and Sperling, 1999; Caterino and Sperling, 1999; Dobler and Farrell, 1999; Funk, 1999; Harrison and Crespi, 1999; Moran *et al.,* 1999; Farrell, 2001; Pereira *et al*., 2002; Paquin & Hedin, 2004; Byrne, 2006; Eastwood *et al*., 2006; Ros & Breeuwer, 2007; Jones & Macpherson, 2007). Now with the advances in sequencing technology and with the overcoming of computational difficulties associate with large data sets using a combination of DNA markers it may be possible to recover a stronger phylogenetic signal and resolution at different levels (Hedges & Poling, 1999; Zimmermann *et al.,* 2000; Macey *et.al*., 2001; Kopp & True, 2002; Jordal *et al*., 2002;

Rokas *et al*., 2002; Maddison & Hedin, 2003; Yasuda *et al*., 2006; Smith *et al.,* 2007; Otranto *et al.,* 2008; Michel *et al*., 2008; Campagna *et al.,* 2010; Duncan *et al*., 2010; Wilson, 2010; Herrera & Sánchez, 2010; Schäffer *et al*., 2010). It has been extensively reported that the nuclear genes perform better at deeper level, (e.g. order, family) in the phylogenetic trees, as compared to mitochondrial genes that are found better in resolving shallow relationships (e.g. genus, species). Thus combining nuclear and mitochondrial gene fragment seems likely to offer a good solution.

The combination of different gene fragments has been explored in the Braconidae (Zaldivar *et al*., 2006) as a whole, as well as at subfamily level e.g. Microgastrinae (Mardulyn & Whitfield, 1999; Banks & Whitfield, 2006), Rogadinae (Zaldivar *et al*., 2008), Doryctinae (Zaldivar *et al*., 2008), as well as Ichneumonidae subfamily Diplazontinae (Klopfstein *et al*., 2009). The results from these studies reveal that the use of CO1 is successful for revealing genus level relationships in subfamilies.

In this study we utilize a data set containing 50 genera of Braconinae, representing both main tribes and groups, with genus *Bracon* being represented by 127 specimens. Most of the sequences used were generated during this study but all available (Gene Bank) sequences were also included. Our aim was to achieve as resolved a phylogeny of the Braconinae as possible, especially at genus level, by using both maximum parsimony and Bayesian Markov Chain Monte Carlo (MCMC) methods for analysis. This is the first CO1 based study on the subfamily Braconinae and, although we were unable to entirely achieve our goal, the reasons are discussed and comparison made with the previous phylogenetic trees obtained by use of nuclear large subunit 28rDNA for Braconinae and other subfamilies of Braconidae where CO1 was used for phylogeny reconstruction.

4.3. Materials and Methods

4.3.1. Taxon Sampling

The taxon sampling included 50 genera and 250 species of the subfamily Braconinae. These taxa are distributed among the tribes Adeshini (2 genera, 3 species), Aphrastobraconini (12 genera, 38 species), Braconini (6 genera, 135 species), Bathyaulacini (3 genera, 3 species), Coeloidini (2 genera, 4 species), Euurobraconini (2 genera, 11species), Glyptomorphini (3 genera, 4 species) and genus groups *Atanycolus* group (4 genera, 7 species), *Compsobraconoides* group (4 genera, 11 species), *Merinotus* group (1 genera, 2 species), *Mesobracon* group (2 genera, 3 species), *Myosoma* group (1 genera, 1 species), *Plaxopsis* group (2 genera, 7 species) and *Shelfordia* group (1 genera, 1 species). Besides these tribes and groups 5 unidentified Braconinae were also included.

Twenty outgroup taxa belonging to other cyclostome braconid subfamilies were added to test the monophyly of the Braconinae. These out groups were selected on the basis of previous morphological (Tobias, 1967; Čapek 1970; van Achterberg, 1984), biological (van Achterberg, 1984; Quicke & van Achterberg 1990; Wharton *et al*., 1992; van Achterberg & Quicke 1992) and molecular phylogenetic (Belshaw *et al*., 1998; Dowton *et al*., 2002; Shi *et al*., 2005; Pitz *et al*., 2005; Zaldivar *et al*., 2006) studies conducted in the family Braconidae and include the representatives of the subfamily Alysiinae (4 species), Doryctinae (2 species), Exothecinae (2 species), Gnamptodontinae (3 species), Opiinae (3 species), Rogadinae (5 species) and one unidentified taxon.

All the recovered trees were rooted using *Aleoides ruficornis* (Rogadinae). The taxa examined with the number of specimen used for each taxa are given in table 4.1.

Table 4. 1 List of genera with number of representatives for each genus.

Genera	No. of Representatives	Genera	No. of Representatives
Acrocerilia	**1**	***Odontoscapus***	2
Africadesha	**1**	***Pachybracon***	1
Archibracon	**10**	***Physaraia***	3
Aspidobracon	**1**	***Plaxopsis***	5
Atanycolus	**8**	***Pseudoshirakia***	2
Bathyaulax	**1**	***Rhamnura***	1
Bracon	**125**	***Rhytimorpha***	1
Braconinae	**6**	***Sacirema***	2
Callibracon	**2**	***Serraulax***	2
Campylonerus	**4**	***Shelfordia***	1
Chaoilta	**1**	***Simplicibracon***	1
Coleoides	**2**	***Soter***	3
Compsobracon	**8**	***Spinadesha***	2
Cratobracon	**1**	***Sylvibracon***	3
Cratocnema	**3**	***Synvipio***	1
Curriea	**1**	***Vipio***	1
Cyclauacidea	**1**	***Zaglyptogastra***	1
Diamblomera	**2**	***Zanzopsis***	2
Digonogastra	**12**	***Mesobraconoides***	1
Glyptomorpha	**2**	***Monilobracon***	2
Habrobracon	**2**	***Myosoma***	1
Hemibracon	**2**	***Nundinella***	1
Iphiaulax	**7**	***Odesia***	1
Megacoeloides	**2**		
Merinotus	**3**		
Outgroups		***Gnamptodontinae***	3
Alysiinae	**4**	***Opiinae***	3
Doryctinae	**2**	***Rogadinae***	6
Exothecinae	**2**		

4.3.2. Morphological Data

A set of 92 morphological characters was used at the genus level. The morphology matrix follows Belshaw *et al*. (2001) and Quicke (1988a).

4.3.3. Molecular Data

The molecular data was obtained from the 5' region of the mitochondrial *cytochrome oxidase* subunit 1 (CO1) by using LCO and HCO primers (Folmer *et al*., 1994).

DNA extraction was carried out by taking the middle leg from a large wasp or, in case of small size, the whole wasp was used, preserved in 99% ethanol. The specimens were dried in incubator at 38°C. Then the tissue was incubated at 55°C overnight, covered in a master mix of nuclei lysis solution, 0.5 EDTA and proteinase K. It was followed by sodium acetate/ethanol precipitation and re-suspension in 150µl nuclease free water. Polymerase chain (PCR) reactions were carried out in 23µl volumes containing 18.15µl H_2O, 250µl buffer, 5µl $MgCl_2$, 5µl primer (10mM), 7.5µl dNTP (10mM), 1.2µl Taq (5U) and 4µl of DNA template. PCR conditions were: 94°C for 3 min followed by 40 cycles of 94° C for 30s, 48°C for 38s, 72°C for 1 min and finally 72°C for 10 min. PCR product was cleaned by using 80% ethanol and sent to the SBS Sequencing Service, Ashworth Laboratories University of Edinburgh for sequencing. The sequences obtained were edited by eye, using sequence navigator (Parker, 1997).

4.3.4. Sequence Alignment

Sequence alignment was carried out by eye and confirmed by reference to the amino acid translation using MacClade 4.06 OS X. The first, second and third codon positions were further determined by using: http://www.ncbi.nlm.nih.gov/projects/gorf/.

4.3.5. Phylogenetic Analyses

Maximum parsimony and Bayesian analyses were carried out in order to compare the trees for conflicting clades.

The MP analysis was carried out by using PAUP* version 4.0b10 (Swofford, 1998) with 100 stepwise random additions utilizing heuristic search with tree bisecting reconnection (TBR) branch swapping holding one tree at any one time. Relative branch support was determined by implementing 1000 bootstrap replicates (Felenstein, 1985).

The Bayesian analysis was run for 30 million generations in MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). The analysis consisted of two searches, sampling trees every 1000 generations and using four chains and default priors. The model of evolution selected for the two data sets was the GTR+I+Γ (general time reversible; Lanave *et al*., 1984), which was determined based on the Akaike criterion implemented in MrModeltest version 3.7 (Posada, 2006; Posada & Crandall, 1998). Four different partitions were considered for the analysis, three for the CO1 codon positions and one for morphology. The rate variation among-partition was modelled and parameters were unlinked across all partitions. The burn-in phase was determined by using the programme Tracer (http://beast.bio.ed.ac.uk/Tracer). The tree obtained after discarding burn-in trees and branch lengths were summarized using majority rule

consensus.

4.4. Results

The trees obtained by analyzing the CO1 sequences not only failed to divide the Braconinae into three main tribes i.e. Aphrastobraconini, Braconini and Coeloidini but also could not recover Braconinae as monophyletic.

The maximum parsimony trees with and without third codon positions included are broadly similar in resolving the phylogeny of Braconinae. The main difference was the loss of nine branches with greater than 50% bootstrap support value in the case of excluding third codon positions that were present when all the codon positions were included in the analysis. So overall the inclusion of all the codon positions has behaved slightly better (fig. 4.1, 4.2) than excluding the third codon position (fig. 4.2, 4.4).

The genus *Bracon* was divided largely into three main clades. The first one was the branch with Malaysian material and more than 80% bootstrap value. The second branch was made of the *Bracon* species from England followed by a third clade with six Brazilian species. *Campyloneurus* was divided into two clades with two species each, and *Plaxopsis* was also divided into two clades one with two species and the other with three species. The subtribe Adeshina was found coming next to the clade with genus *Archibracon* that was further divided into four groups of species. There is also a clade of the small tribe Bathyaulacini, erected by Quicke (1988a), with two member genera *Bathyaulax* and *Odesia*.

The main branch that was missing in the analysis that excluded third codon position was the clade with Malaysian material that contained nine *Bracon* individuals. The exclusion of third codon position also failed to recover a clade

containing *Bracon* specimens from England that apparently belonged to the same species. Another clade with *Bracon* from Brazil, comprising six species, was not found in the tree obtained by excluding the third codon position.

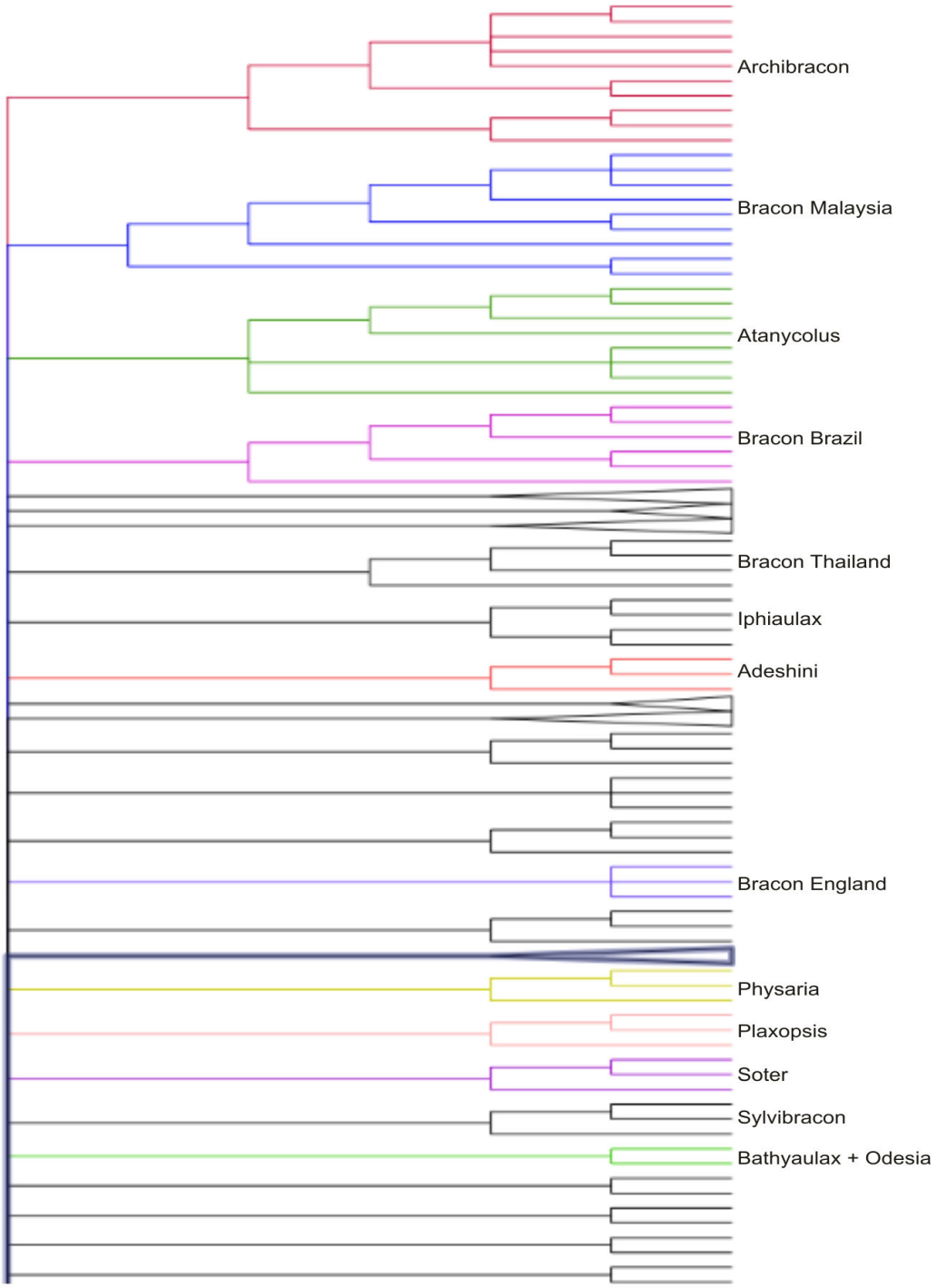

Figure 4.1. Phylogenetic tree of CO1 using maximum parsimony and 1000 bootstrap. Branches with more than 50% bootstrap support value are coloured.

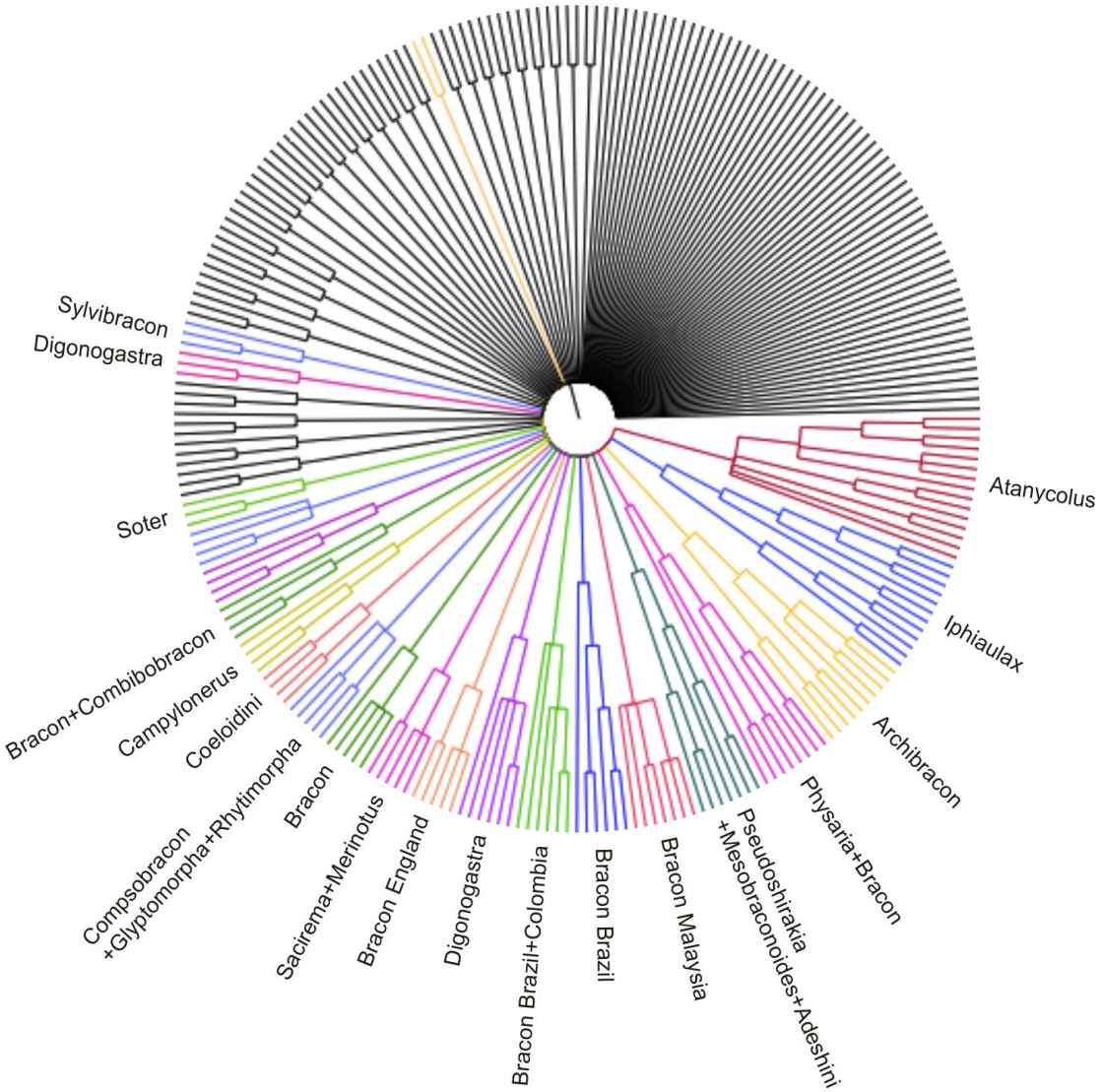

Figure 4.2. Phylogenetic tree with CO1 and morphology using Bayesian analysis. Branches with ≥ 0.95 are shomn in colours.

The exclusion of third codon position (fig 4.3, 4.4) resolved a clade with all the *Campyloneurus* species belonged together that were divided into two clades with two species each when all codon positions were included.

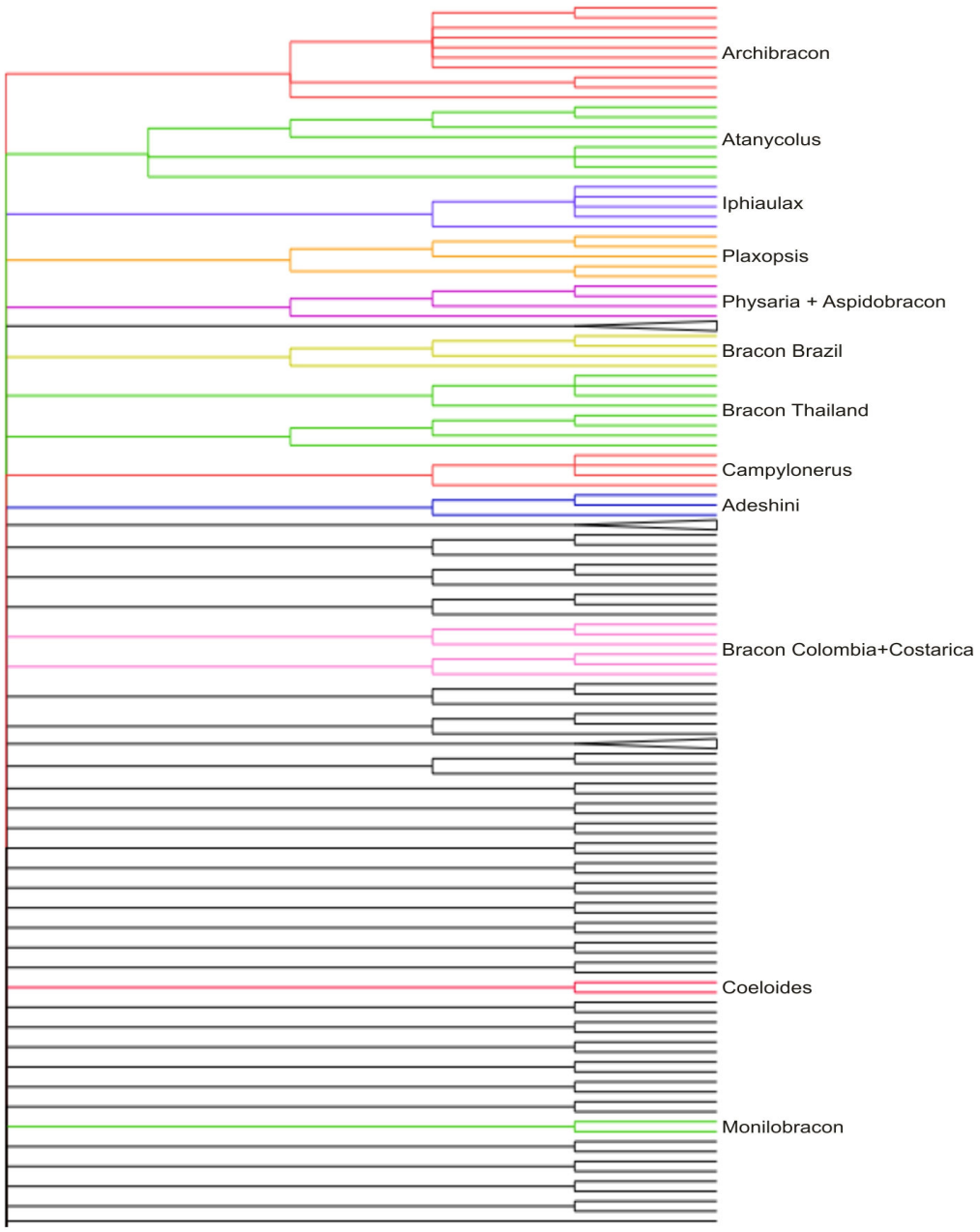

Figure 4.3. Phylogenetic tree of CO1 excluding 3rd codon position using maximum parsimony and 1000 bootstrap. Branches with more than 50% bootstrap support value are coloured.

The same result was found in case of genus *Plaxopsis* where all five species were found in a single clade with third codon position excluded while divided into two clades when all codon positions included.

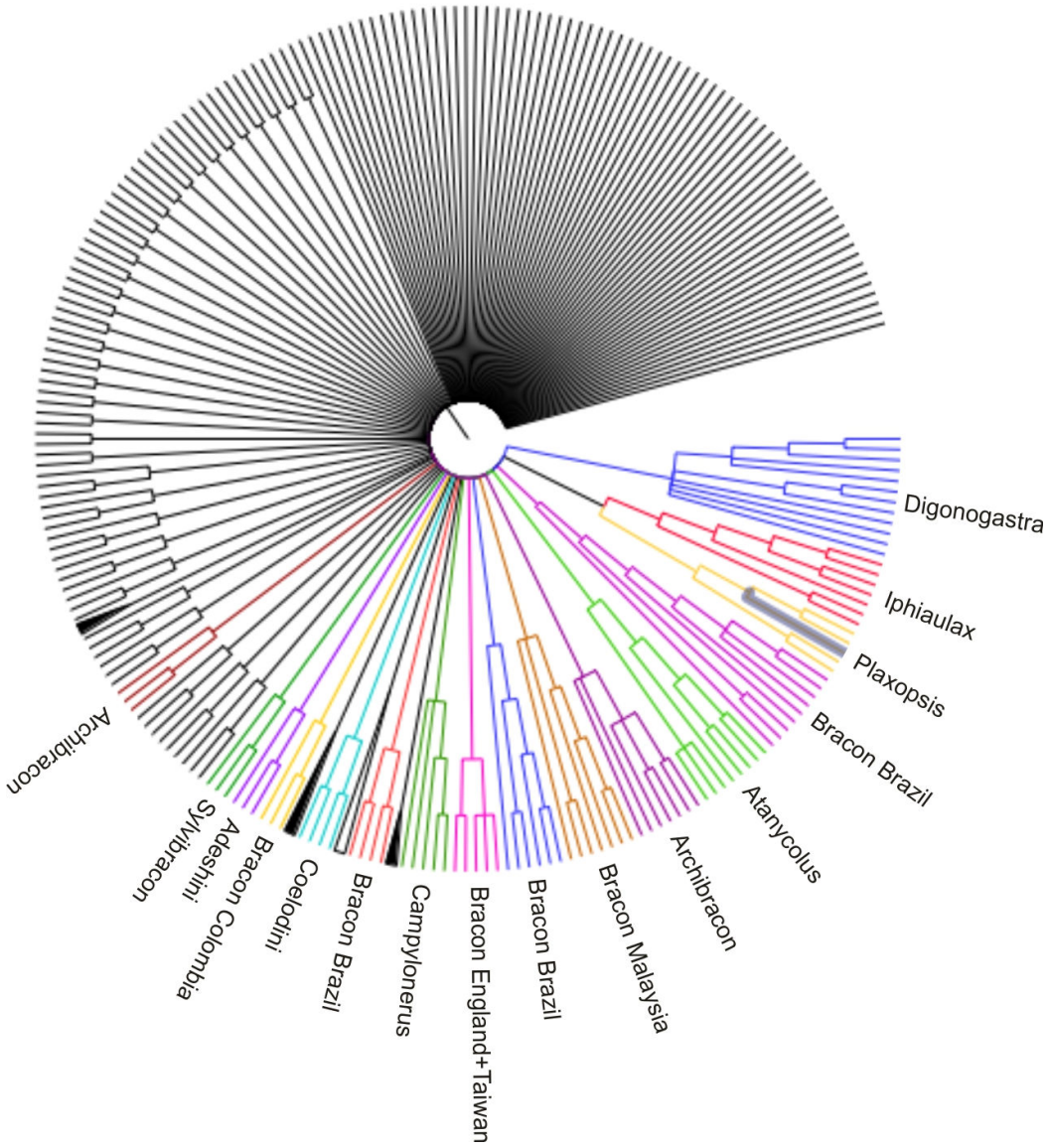

Figure 4.4. Phylogenetic tree of CO1 excluding 3rd codon positionwith Bayesian analysis. Branches with more than 50% bootstrap support value are coloured.

The tree without third codon position resolved five *Iphiaulax* species and two *Monilobracon* species. Both these clades were not found in the tree with all three codon positions. The rest of clades were almost same in both trees, *Archibracon* clade differed in the resolution as exclusion of third codon position shows it to be made of eight individuals while the analysis with all the positions included divides into four groups. Then *Atanycolus* clade came out the same in both analyses. Genus *Aspidobracon* was found in same clade with genus *Physaraia* when third codon position was excluded. The clade with the tribe Bathyaulacini was lost by excluding the third codon position.

4.5. Discussion

Overall the CO1 gene fragment has behaved quite well at the species level and helped to resolve the relationships at genus level especially in the genera *Archibracon*, *Atanycolus* and *Bracon*.

In case of tribe Aphrastobraconini, *Chaoilta* and *Odontoscapus* came together thus showing a small representation of *Atanycolus* group but they were far from the clade with all the *Atanycolus* species. Morphologically this group is supported not only by the highly modified scapus and pedicellus, but also by the single hind wing basal hamulus even in large species, the elongate apico-ventral setae of male 4^{rth} tarsal segment and by male tergal gland structure.

Within the Braconini the genus *Bracon* has its main divisions based largely on localities with the South American species coming together as also did the species from Asia. The position of *Physaraia* with *Aspidobracon* seems justified based on their morphology as both these genera share the modifications of the metasoma with

tergites one and two fused together, an adaption apparently for ovipositor to enter through the hard surfaces to get to their host (van Achterberg, 1984; Quicke, 1988). Members of the tribe Adeshini are also recovered together, both with and without codon positions included in the analysis and thus confirms its status to be raised to a tribe as suggested by Quicke (1988). This may suggest that we need to include more representative taxa from each tribe and subtribe based on traditional classification that may lead us to a better resolved phylogenetic tree.

However, as observed in other studies, CO1 has not been very successful at the higher level (Mardulyn & Whitfield, 1999; Springer *et al*., 2001; Whitfield *et al*., 2002; Michel-Salzat & Whitfield, 2004; Banks & Whitfield, 2006; Klopfstein *et al*., 2010). The main reasons being that the mitochondrial genes are faster evolving and reach saturation rather more quickly than most of the nuclear genes (Banks & Whitfield, 2006; Klopfstein *et al*., 2010). The 3rd codon position of the CO1 gene was found AT biased in previous studies so exhibit homoplasy more frequently on these sites. Also 3rd codon positions show multiple changes that are more variable so likely to achieve saturation more quickly, as a result likely to be too saturated to be phylogenetically useful. Although, 1st and 2nd positions of the CO1 gene fragment have large number of slowly evolving sites that are useful but not enough to resolve relationships higher than species level.

CO1 was classified as good mitochondrial gene for phylogeny, based on studies by Zardoya and Meyer (1996) but still the nucleotide substitution rate looks too fast to resolve the phylogenetic relationship in Braconinae, as was the case for mammalian study by Springer *et al.* (2001).

Thus this gene alone is not a very good choice for phylogenetic signal at the genus and subfamily level for this level of divergence as compared to other

Hymenoptera where it was found useful. It has been used in past in combination with nuclear genes that are considered to have appropriate number of slowly evolving sites to resolve the phylogeny at this taxonomic level. So, may be use of CO1 in combination with some nuclear gene can help to resolve the phylogeny of Braconinae.

Chapter 5: Effects of the NADH 1 mtDNA gene fragment on resolution of the Braconinae (Hymenoptera: Braconidae) phylogenetic tree.

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5.1. Abstract

The NADH1 mtDNA gene fragment has been helpful in the past for resolving phylogenetic trees, where other mitochondrial genes have failed. Here we employ it to help resolve phylogenetic relationship among 32 genera of Braconinae. Both parsimony and Bayesian methods of phylogenetic reconstruction were applied. However, the results show that this particular marker is not good enough to resolve phylogeny of Braconinae on its own at least with this sample of taxa and may need a

better representation of the subfamily through addition of more genera and species within each genus. However, below genus level it was found helpful for dividing *Bracon,* represented by 123 species, into some meaningful groups.

5.2. Introduction

The mitochondrial gene segment NADH 1 plays a vital role in oxidative phosphorylation as it effects the entry enzyme of electron transport chain present in the inner mitochondrial membrane that catalyzes the transfer of electrons from NADH to coenzyme Q (CoQ) (Nakamaru *et al*., 2010). Being a mitochondrial gene, NADH1 performs much better for resolving the external nodes rather than deep phylogenetic relations. So it can best be exploited to study the evolutionary process at genus and species levels.

The gene fragment can further be divided into three codon positions, 1^{st} 2^{nd} and 3^{rd}, where 3^{rd} codon position evolves at a faster rate than the other two, thus it may lead to homoplasy (Nei, 1987; Irwin *et al*., 1991; Kula *et al*., 2006). Also, insect mtDNA shows a bias towards adenine and thymine with an overall tranversional bias for A (-) T transversions (Vest Pederson, 1996; Dowton & Austin, 1997; Smith *et al*., 1999; Belshaw *et al*., 2001; Kula *et al*., 2006).

This gene fragment has been used to resolve the evolutionary relationships of several groups of organisms (Boore & Brown, 1995; Dietrich *et al*., 1997; Moran *et al*., 1999; Smith *et al*., 2002; Leys *et al*., 2003; Salzat & Whitfield 2004; Chang *et al*., 2005; Froufe *et al*., 2005; Hrbek *et al*., 2005; Douglas et al., 2006; Kula *et al*., 2006; Longhorn *et al*., 2007; Phillips *et al*., 2009; Brown & Smith, 2010). In the case of the Braconidae, this gene has been used in phylogenetic studies of the Aphidiinae (Smith

et al., 1999), Microgastrinae (Salzat & Whitfield 2004) and Alysiinae (Kula *et al*., 2006). For the Microgastrinae it was used in combination with three other genes while in the Aphidiinae study, it was combined with 28S rRNA gene, whereas the study of Alysiinae was solely based on NADH1 mtDNA gene fragment.

The study of Smith *et al*. (1999) was based on the work done by Belshaw and Quicke (1997) where the Aphidiinae phylogeny was constructed based on three genes that include nuclear 28S rRNA, elongation factor 1α and a portion of the mitochondrial cytochrome *b* gene. The result of the Belshaw and Quicke (1997) revealed Aphidiinae to be monophyletic with 28S rRNA analysis, polyphyletic with EF-1 α, while it was unresolved with *cyt-b*. In this case only 28S rRNA tree was close to results of previous studies (Mackauer, 1961), but for 28S rRNA results, Belshaw and Quicke were doubtful due to ambiguous alignment resulting from the variable parts (61%) of the sequences. So, in order to overcome these issues of ambiguous alignment in case of 28S rRNA and unresolved *cyt-b* tree, Smith et al. (1999) decided to utilize another mitochondrial gene, NADH1, both alone as well as in combination with 28S rRNA. The trees obtained by use of NADH1 in Smith *et al*., (1999) were close to the results of 28S rRNA found by Belshaw and Quicke (1997). So the NADH 1 seems to be a good choice for a mitochondrial gene fragment to resolve evolutionary relationships in Braconidae.

The previous results that this research has obtained by utilizing nuclear 28S rRNA and mitochondrial cytochrome oxidaze 1 (CO1), also depict a similar story as Belshaw and Quicke (1997). The tree obtained by 28S rRNA plus morphology resolves the subfamily Braconinae monophyletic and divided into three major tribes i.e. Aphrastobraconini, Braconini and Coeloidini, and these results correspond to previous phylogenetic studies (Quicke, 1988; Belshaw *et al*., 2001). MtCO1 gene have

failed to recover the Braconinae monophyletic and has been unable to recover any of the tribes as monophyletic. Following the lead from Smith *et al*., (1999) this chapter is based on NADH1 to resolve the phylogeny of Braconinae. Both parsimony and Bayesian methods were used to construct the phylogenetic trees of Braconinae and the results were compared to see similarities and differences observed between the two methods.

5.3. Materials and Methods

5.3.1. Taxa Sampled

This study included 188 individuals belonging to 32 described genera of the subfamily Braconinae. The list of genera and number of individuals for each genus is given in the table 5.1.

Table 5.1. Genus names and number of individuals representing each genus.

Genus	**No. of Specimens**	**Genus**	**No. of Specimens**
Archibracon	2	*Pachybracon*	1
Aspidobracon	2	*Paranesaulax*	1
Atanycolus	3	*Plesiobracon*	1
Bathyaulax	2	*Pseudovipio*	1
Bracon	123	*Rhytimorpha*	1
Campyloneurus	1	*Serraulax*	3
Composobracon	1	*Sororarchibracon*	3
Compsobraconoides	2	*Soter*	3
Cratocnema	5	*Sylvibracon*	4
Cratobracon	2	*Triapsis*	21
Diamblomera	2	*Tsavobracon*	1
Digonogastra	12	*Zanzopsis*	1
Ficobracon	1		
Habrobracon	1	**Outgroups**	
Hemibracon	2	*Alysiinae*	2
Iphiaulax	4	*Rogadinae*	1
Latana	1	*Doryctinae*	4
Nedinoschiza	1	*Exothecinae*	3
Nundinella	1	*Opiinae*	3

Monophyly of the Braconinae was tested by using five sister subfamilies Alysiinae, Doryctinae, Exothecinae, Opiinae and Rogadinae. Selection of these subfamilies was based on the previous studies on phylogenetics of Braconidae (Quicke & Achterberg, 1990; Belshaw *et al*., 1998; Dowton *et al*., 2002; Shi *et al*., 2005; Pitz *et al*., 2005; Zaldivar *et al*., 2006).

5.3.2. Morphological Data

A set of 92 morphological characters were used at genus level. The morphology matrix follows Belshaw *et al*. (2001) and Quicke (1988).

5.3.3. DNA sequencing

The primers used in this study were designed by Smith *et al.,* (1999) (ND1 F: 5'-GATAAATCAAAWGGKGT-3', ND1R: 5'-CAACCTTTTAGTGATGC-3'). Genomic DNA was extracted from ethanol preserved specimens using nuclei lysis solution, 0.5 EDTA and proteinase K (Promega) with overnight incubation that was followed by sodium acetate/ethanol precipitation and re-suspension in 150µl nuclease free water. The PCR conditions (22 µl reactions) were as follows: each reaction contained 10 µl H_2O, 2µl Buffer, 1.5µl 20µM of each primer, 1.12µl 25mM $MgCl_2$, 0.8µl 10µM dNTP's, 0.1µl Promega *Taq* DNA polymerase and 4µl DNA template. The cycle parameters were as follows: initial denaturation at 94°C for 4 min, denaturation at 94°C (30 s), annealing at 47°C and CR (1min) and extension at 72°C (10min) repeated for 40 cycles. Amplified DNA templates were purified using 80% ethanol. The sequencing primers were the same as the PCR reactions. DNA template

and primers were sent to SBS sequencing service, Ashworth Laboratories University of Edinburgh. The resulting cladograms were edited by eye using sequence navigator (Parker, 1997).

The sequence alignment was carried out by eye and further confirmation by reference to the amino acid translation using MacClade 4.06 for Mac OS X. The first, second and third codon positions were further determined by using http://www.ncbi.nlm.nih.gov/projects/gorf/.

5.3.4. Phylogenetic Analyses

Two different methods, maximum parsimony and Bayesian approach, were used to construct the phylogenetic trees of subfamily Braconinae based on mitochondrial NADH 1 gene fragment.

The maximum parsimony analysis was carried out using heuristic searches with 100 random additions followed by tree bisecting reconnection (TBR) holding one tree any one time in PAUP* 4.0b10 (Swofford, 1998). Bootstrap was used to determine the relative branch support implementing 1000 bootstrap replicates (Felenstein, 1985). The bootstrap values were saved at each node of the tree obtained by majority rule.

The Bayesian analysis was run for 30 million generations in Bioportal (University of Oslo; https://www.bioportal.uio.no/). The resulting trees were sampled for every 1000 generations using four chains and default priors with each analysis consisting of two runs. The model of evolution selected for the two data sets was the GTR+I+Γ (general time reversible; Lanave *et al*., 1984), which was determined based on the Akaike criterion implemented in MrModeltest version 3.7 (Posada, 2006; Posada & Crandall, 1998). Four different partitions were considered for the analysis,

three for the NADH 1 codon positions and one for morphology. The rate variation among-partition was modeled and parameters were unlinked across all partitions. The burn-in phase was determined by using the software programme Tracer (http://beast.bio.ed.ac.uk/Tracer). The tree obtained after discarding burn-in trees and branch lengths were summarized using majority rule consensus.

5.4. Results

The maximum parsimony analysis failed to recover the Braconinae as monophyletic apparently in part due to the characteristics of the mitochondrial gene and partly because of an inappropriate data set (fig. 5.1). However, the genus *Bracon* has been found divided into some meaningful clades.

Aspidobracon and *Plesiobracon* were recovered in the same clade with two *Bracon* species. This was followed by *Bracon* clades. The tree has three main Brazilian *Bracon* species clades where two of these contain solely Brazilian species while one comprises a combination of species from Brazil, Colombia and Malaysia. In addition, there are two more *Bracon* species clades with European material one of which is completely from England while other has individuals from Israel and Finland. Most of these *Bracon* clades have high bootstrap support values. There are two clades with *Digonogastra* species each containing two species of the genus with a bootstrap value of 100. The last main clade is of genus *Soter* with a Doryctinae species that also has a bootstrap value of 100.

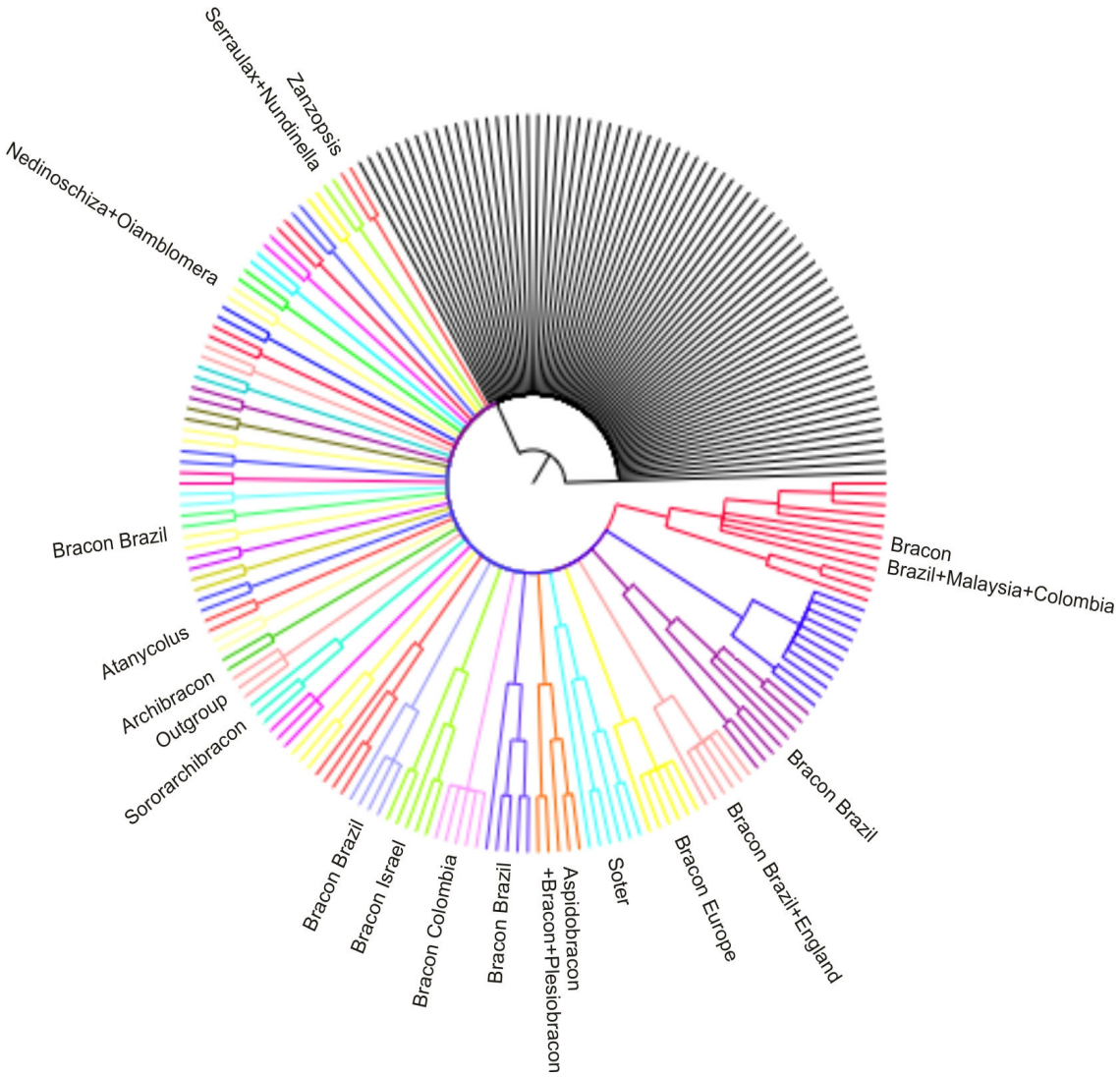

Figure 5.1. Phylogenetic tree of NADH 1 and morphology using maximum parsimony implemented in PAUP. Branches with bootstrap support values ≥ 0.95 are coloured.

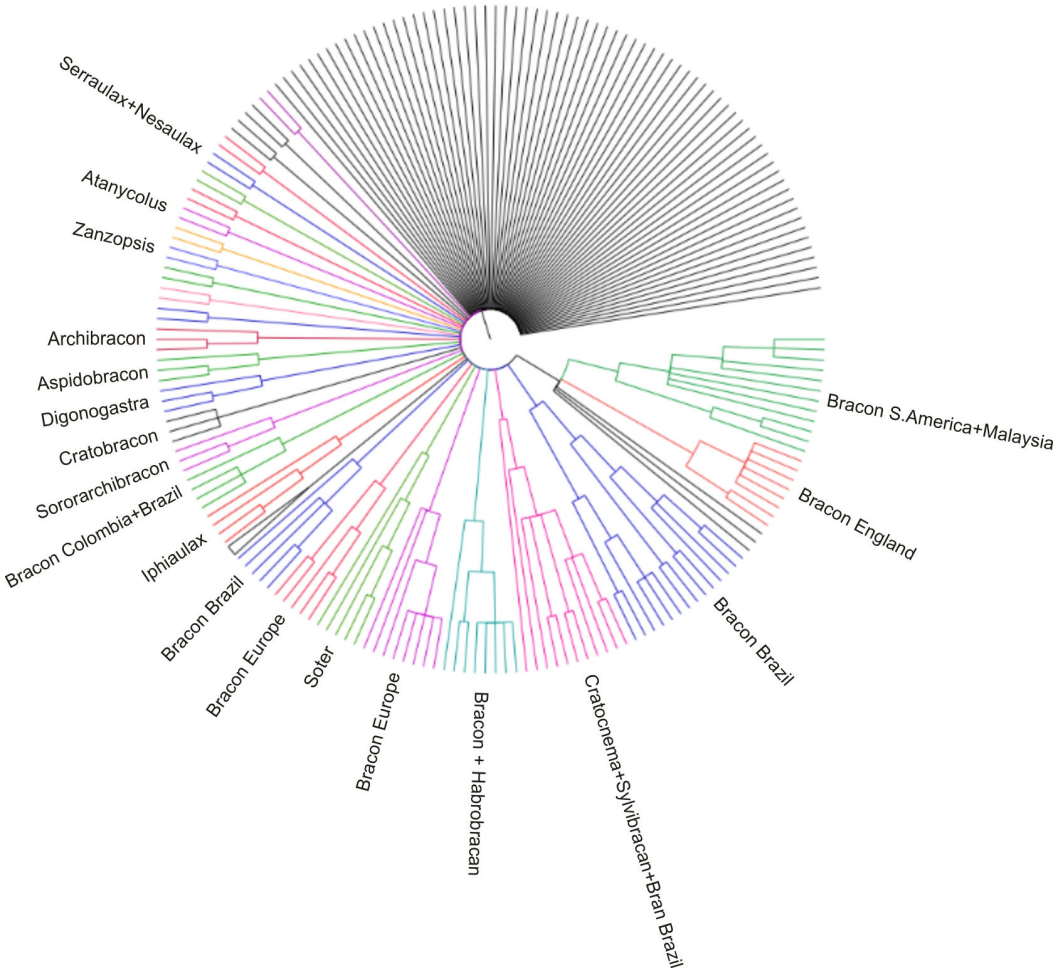

Figure 5.2. Phylogenetic tree of NADH 1 and morphology using Bayesian analysis. Branches with posterior probabilities $\gtrsim$ 0.95 are coloured.

Bayesian analysis (fig. 5.2) revealed Braconinae monophyletic while most of the clades are recovered same as maximum parsimony tree a few other clades were also found. On overall basis Bayesian tree was slightly better than the parsimony tree. The clades *Nundinella+Serraulax+Zanaopsis* and *Diamblomera+Nedinoschiza* showed some relationship of Aphrastobraconini tribe while all others are mainly Braconini except *Archibracon, Atanycolus*, *Iphiaulax* and *Digonogastra* clades.

5.5. Discussion

The trees obtained by both maximum parsimony and Bayesian analysis were not markedly different and showed a high bootstrap support for the recovered clades. The number of recovered clades from both analyses was the same. The main outcome of the study is division of genus *Bracon* in several different clades that were mainly divided into South American and European clades.

The reason for better performance of the gene at resolving inter-species relations compared to inter generic relations is typical of mitochondrial genes as they evolve at rather a fast rate as compared to nuclear genes. We were unable to recover a tree divided into three main tribes the Aphrastobraconini, Braconini and Coeloidini (based on previous phylogenetic studies of Quicke, 1988 & Belshaw *et al*., 2001) that may be due to relatively poor representation of the taxa. This data set can definitely benefit from a good overall sampling of the subfamily Braconinae. Although there has been long standing debate about improving the accuracy of analysis either by increasing the length of gene segment (Rosenberg & Kumar, 2001) or by increasing the number of taxa (Pollock *et al*., 2002) but it has been observed that both have equally good effects. The improvement in phylogenetic accuracy is based partly on the length of the sequence and partly on the divergence between the taxa (Hillis *et al*., 2003) so it is important to have a good representation of the taxa as well as appropriate gene length. The data set in this study was representing only 32 out of 250 genera of Braconinae, so it definitely needs a good representation from each of the three prominent tribes to get a better resolved phylogenetic tree.

Chapter 6: Combined analysis of nuclear (28S rDNA) and mitochondrial (NADH I) genes to resolve phylogenetic problem of Braconinae (Braconidae: Hymenoptera) with the estimation of diversification age.

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6.1. Abstract

The phylogenetic relationships of 18 genera belonging to the subfamily Braconinae were investigated using nuclear 28S rDNA and mitochondrial NADH1. Both separate and simultaneous analyses to revealed the Braconinae monophyletic except the one with NADH1

gene fragment. The tribe Aphrastobraconini was recovered monophyletic in all of these analyses. The genus *Digonogastra* and the *Atanycolus* group of genera were also recovered monophyletic. Node support increased considerably by combining the nuclear and mitochondrial sequences. The age of Braconinae was estimated and found to have originated in the Miocene era based on ultrametric tree obtained by the penalized likelihood method implemented in r8s.

6.2. Introduction

The Braconinae, when compared with all the subfamilies in the family Braconidae, has the largest number of species described so far (Yu *et al*., 2005; Jones *et al*., 2009; Mahmood *et al*., 2010), and therefore it presents us with the difficult task of dividing it into meaningful groups. A few attempts have been made in past (Telenga, 1952; Tobias, 1957; Quicke 1988b, Chisti & Quicke, 1995: Belshaw *et al*., 2001) but failed to give a proper structure to this subfamily. The use of molecular data in addition to morphological characters (Belshaw *et al*., 2001) has divided it into three main tribes Aphrastobraconini, Braconini and Coeloidini where the last one seems to be basal due to its plesiomorphic character (well developed and densely setose male parameres (Quicke, 1988b)) as well as its very derived basal flagellomeres. Results to date, however, are in no way close to being conclusive.

Here, both nuclear and mitochondrial genes have been used individually to resolve tphylogenetic relationships in within the subfamily. The nuclear gene, 28S rDNA large subunit D2-3 region, was quite successful in keeping the above said three tribes with only a few genera of Braconini being recovered among the Aphrastobraconini, and this may be sorted out by improving taxon sampling. The Coeloidini was found to divide the other two tribes while the previously defined groups of genera were also found to come together. But

28S rDNA also has its problems particularly with response to length variable regions (chapter 3). The mitochondrial CO1 and NADH 1 (chapters 4 and 5 respectively) had limitations at the subfamily level as they were good enough to resolve inter- and intra-species relations but failed at inter generic level.

There are many examples where any one particular gene has failed and the combination of genes from different sources of DNA (nuclear and mitochondrial) has produced better results (Gimeno *et al*., 1997; Mardulyn & Whitfield, 1999; Johnson & Clayton, 2000; Dowton *et al*., 2002; Pereira *et al*., 2002; Zaldivar-Riverón *et al*., 2005; Banks & Whitfield, 2006) or helped to improve branch support for some clades. So keeping this in view, this study combines both 28SrDNA (nuclear) and NADH 1 (mitochondrial) genes in an attempt to resolve this phylogenetic problem.

The age of diversification of the Braconinae has never been investigated. One of the reasons could be that there are no existing fossils preserved in Baltic amber, which seems strange, as Braconinae is one of the most diverse groups at present. There is one fossil record that claims to belong to the genus *Bracon*, i.e. *B. cockerelli* (Brues, 1910) whose type specimen is at American Museum of Natural History, but its actual taxonomic affinity is uncertain and it may not actually belong to the Braconinae. So, an attempt is made to estimate divergence times in this subfamily.

The Bayesian Markov Chain Monte Carlo (MCMC) method was employed both for separate and combined analysis of nuclear (28S rDNA) and mitochondrial (NADH1) genes. The resulting trees were used to confirm the monophyly of Braconinae and of its two main tribes i.e. Aphrastobraconini and Braconini. Divergence times of the major clades were also estimated by using Penalized likelihood method (PL; Sanderson, 2002).

6.3. Materials and Methods

6.3.1. Taxon Sampling

A total of 62 species belonging to 18 genera of Braconinae were used in this study. Out of 18 genera, 11 belonged to Aphrastobraconini and 7 to Braconini. The list of taxa and their localities are given in table 6.1.

Table 6.1. List of taxa and their locality.

Specimen	Country	Specimen	Country
Archibracon servillei	Uganda	*Bracon* sp.	Malaysia
Aspidobracon sp.	Malaysia	*Bracon* sp.	Venazuela
Aspidobracon sp.	Malaysia	*Bracon* sp.	Zambia
Atanycolus sp.	USA	*Cratocnema* sp.	Taiwan
Atanycolus sp.	USA	*Cratocnema* sp.	Uganda
Atanycolus sp.	USA	*Diamblomera* sp.	Benin
Bracon sp.	Africa	*Diamblomera* sp.	Uganda
Bracon sp.	Africa	*Digonogastra* sp.	Brazil
Bracon sp.	Brazil	*Digonogastra* sp.	Colombia
Bracon sp.	Brazil	*Digonogastra* sp.	Colombia
Bracon sp.	Brazil	*Digonogastra* sp.	Colombia
Bracon sp.	Brazil	*Digonogastra* sp.	Colombia
Bracon sp.	Brazil	*Digonogastra* sp.	Colombia
Bracon sp.	Brazil	*Digonogastra* sp.	USA
Bracon sp.	Brazil	*Habrobracon* sp.	Israel

Bracon sp.	Brazil	*Habrobracon* sp.	Malaysia
Bracon sp.	England	*Iphiaulax* sp.	Benin
Bracon sp.	England	*Iphiaulax* sp.	Ghana
Bracon sp.	England	*Nedinoschiza* sp.	Uganda
Bracon sp.	England	*Nundinella* sp.	Zambia
Bracon sp.	England	*Plesiobracon* sp.	Malaysia
Bracon sp.	England	*Pseudovipio* sp.	Uganda
Bracon sp.	Finland	*Rhytimorpha* sp.	Ghana
Bracon sp.	Finland	*Serraulax* sp.	Ghana
Bracon sp.	Israel	*Serraulax* sp.	Africa
Bracon sp.	Israel	*Sororarchibracon* sp.	Uganda
Bracon sp.	Israe	*Sororarchibracon* sp.	Uganda
Bracon sp.	Israel	*Synvipio* sp.	Benin
Bracon sp.	Israel	*Tsavobracon* sp.	Uganda
Bracon sp.	Israel	*Zanzopsis* sp.	Uganda
Bracon sp.	Madagascar	*Zanzopsis* sp.	Uganda
Bracon sp.	Madagascar	[Outgroup]	
Bracon sp.	Malaysia	*Doryctinae* sp.	Uganda

A morphological matrix of 92 characters, based on studies of Quicke (1988a) and Belshaw *et al*. (2001), was also used.

6.3.2. Molecular Data

The gene markers used were the D2-3 region of the nuclear 28SrDNA and the mitochondrial NADH1 gene comprised of ~700 and 426 length fragments, respectively. DNA extraction was carried out on alcohol preserved specimens. The protocol for DNA extraction and the primers were the same as that used for 28SrDNA from chapter 3 and for NADH1 from chapter 5.

6.3.3.Sequence Alignment

Sequence alignment for both genes was carried out by eye. The NADH1 sequence alignment was further confirmed by amino acid translation using MacClade. The codon positions were determined using http://www.ncbi.nlm.nih.gov/projects/gorf/. In the case of the 28S rDNA, sequence alignment was based on the secondary structure model of the DNA molecule as was devised by Gillespie *et al.* (2005).

6.3.3. Phylogenetic Analysis

Both separate and simultaneous analyses were carried out for 28S and NADH1 using a mixed model Bayesian MCMC method implemented in MrBayes version 3.1.2 (Ronquist & Huelsenbeck, 2003). Significantly supported clades [i.e. those with posterior probability (PP) $\geq$ 0.95] that were in conflict between the two separate analyses, were used to detect the congruence between the two data sets (Wiens, 1998; Reeder, 2003).

The Bayesian analyses were run for 30 million generations in Bioportal (University of Oslo; https:// www.bioportal.uio.no/). The trees were sampled after every 1000 generations using four chains and default priors in two searches for each analysis. The model of evolution was selected by using the Akaike information criterion implemented in MrModeltest version 2.0 (Posada, 2006; Posada & Crandall, 1998) and was found to be GTR+I+Γ (general time

reversible + invariable + gamma; Lanave *et al*., 1984). Five different partitions were considered for the analyses, one for the 28S region, the three for the NADHI codon positions and last one for the morphology. The rate variation among-partition was modeled and parameters were unlinked across all partitions. The burn-in phase was determined by using the software Tracer (http://beast.bio.ed.ac.uk/Tracer). The tree obtained after discarding burn-in trees and branch lengths were summarized using majority rule consensus.

6.3.4. Divergence dates of clades

Relaxed molecular clock approaches, i.e the semi-parametric penalized likelihood (PL; Sanderson, 2002) was utilized to estimate molecular dating. This PL method utilizes the combination of likelihood and the non-parametric penalty function that was used in nonparametric rate smoothing (NPRS: Sanderson, 1997).

The programme r8s version 1.7 (http://loco.biosci.arizona.edu/r8s/) was used to perform PL method. The range, mean and standard deviation for PL were obtained taking the last 50 trees (with their branch lengths) sampled from each of the two independent MrBayes runs (with 28S and NADH1combined; see above). The truncated Newton algorithm was used, and a cross validation (Sanderson, 2002) was first run on the majority rule consensus tree from the simultaneous Bayesian analysis to determine the optimal value of smoothing. This cross validation helps to determine the smoothing based on the data itself. The smoothing factor helps to reduce the rapidity of change along lineages specially on short branches. The terminal taxa were pruned from the trees in order to avoid near zero branch lengths.

6.4. Results

The phylogenetic tree (fig. 6.1) resulting from the Bayesian analysis of the 28S rDNA gene fragment revealed the Braconinae to be monophyletic and also both the tribes Aphrastobraconini and Braconini were recovered monophyletic. The tribe Braconini was found basal while the Aphrastobraconini seems to be evolved from within the Braconini. In the case of the tribe Braconini, none of the clades were recovered based on the type locality

Figure 6.1. Phylogenetic tree obtained from Bayesian analysis of 28S rDNA. Clades with ≥ 0.95 posterior probabailities are coloured, the genera belonging to the tribe Braconini are shown in red and Aphrastobraconini in blue.

of species as were found with mitochondrial CO1 and NADH 1 in chapter 4 and 5 respectively. The *Atanycolus* group of genera and the genus *Digonogastra* were recovered monophyletic in the tribe Aphrastobraconini. The phylogenetic tree obtained from this analysis had nineteen branches supported with posterior probability of ≥ 0.95.

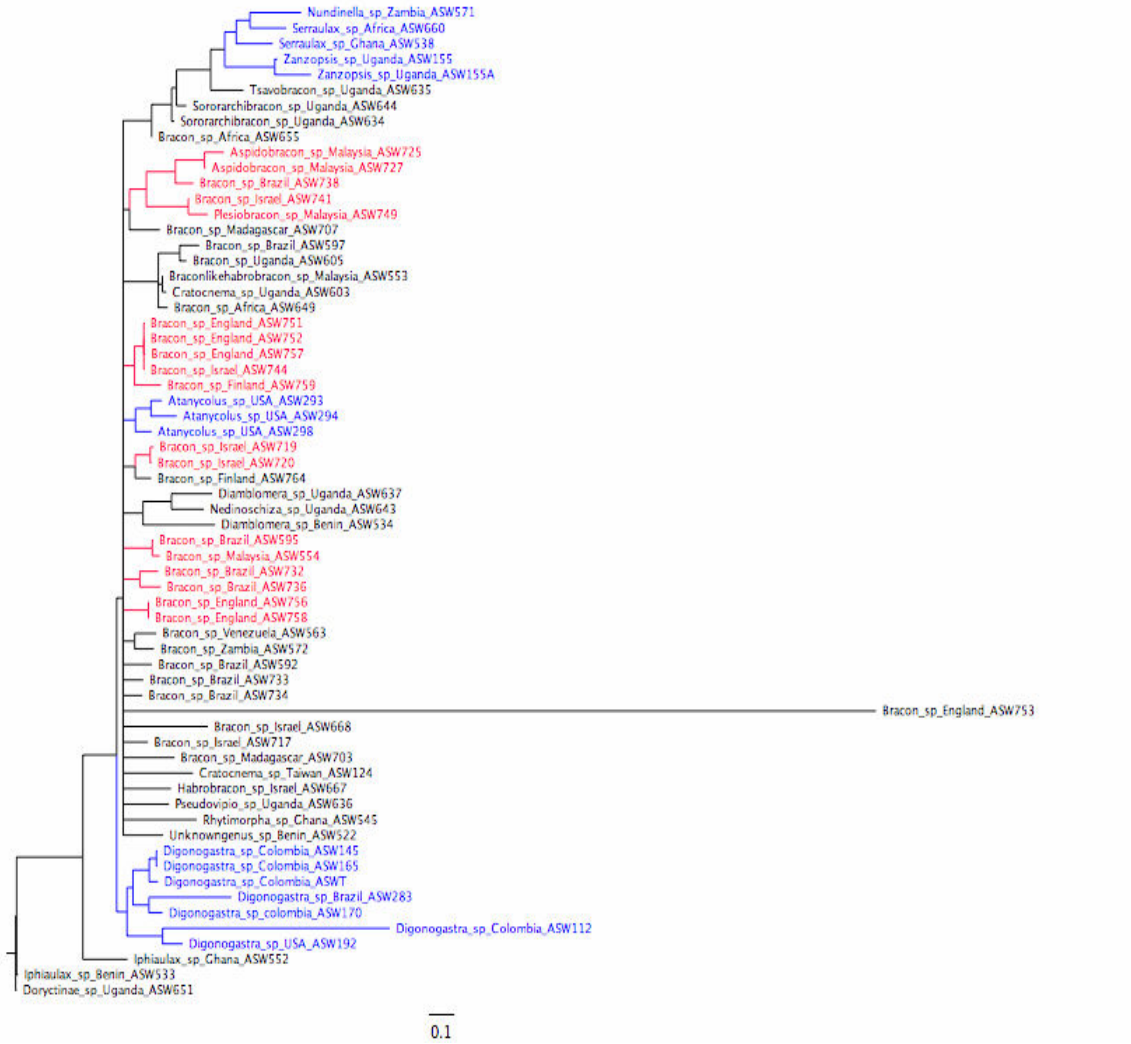

Figure 6.2. Phylogenetic tree obtained from Bayesian analysis of NADH1. Clades with ≥ 0.95 posterior probabailities are coloured, the genera belonging to the tribe Braconini are shown in red and Aphrastobraconini in blue.

The mitochondrial gene NADH1 was unable to recover the subfamily and the tribes monophyletic (fig. 6.2), the main reason being that this gene fragment is graded to belong to the medium region of mitochondrial genome for phylogenetic resolution. Thus, it is not possible to resolve phylogenetic relationship higher than species level. Also the different clades containing the genera belonging to the tribe Aphrastobraconini were coming as basal while the genera belonging to the tribe Braconini were found on terminal branches of the phylogenetic tree thus bringing up the issue of rooting in this subfamily. The genus *Digonogastra* and the *Atanycolus*-group of genera were found monophyletic. Although, this gene fragment was unable to resolve most of the relationships but it had twenty clades with posterior probabilities $\geq$ 0.95.

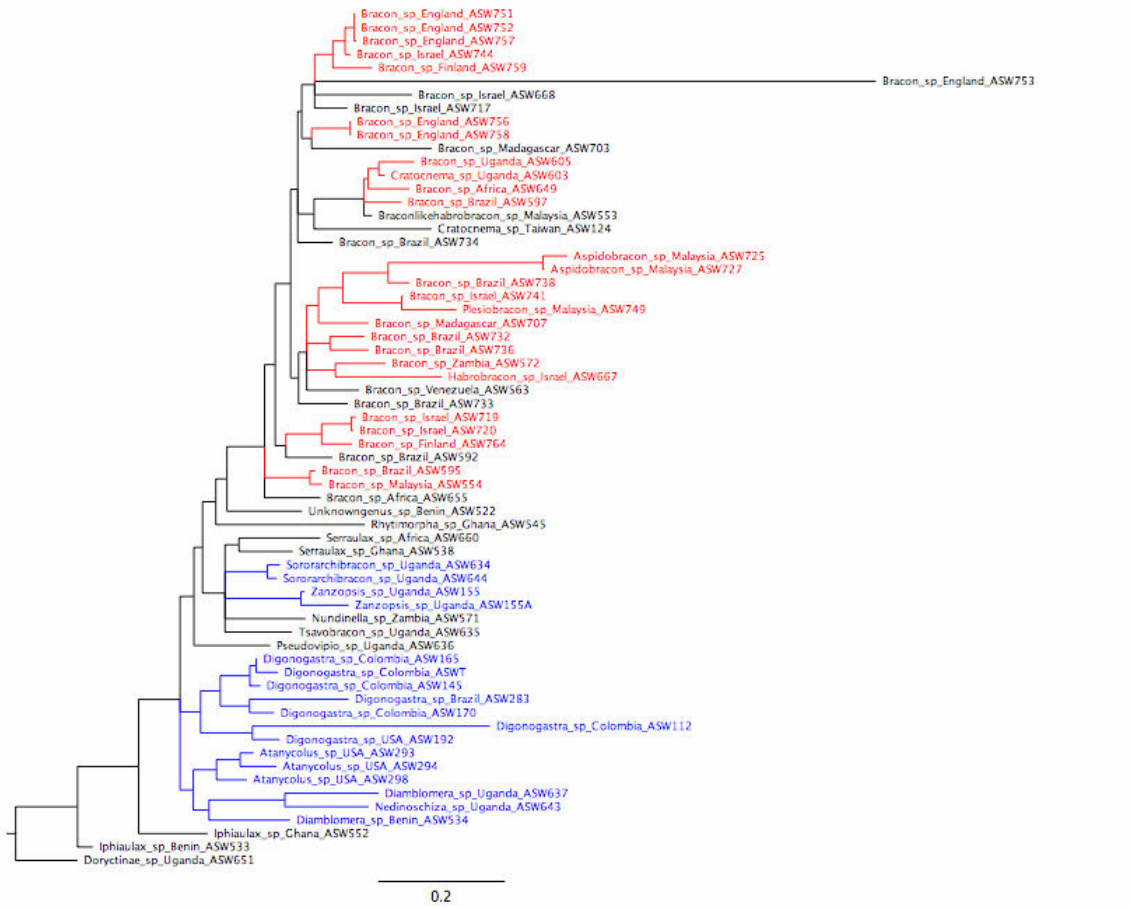

Figure 6.3. Phylogenetic tree obtained from combined Bayesian analysis of 28S and NADH1 genes. Clades with $\geq$ 0.95 posterior probabailities are coloured, the genera belonging to the tribe Braconini are shown in red and Aphrastobraconini in blue.

The phylogenetic tree obtained by the Bayesian analysis of the combined data set (fig. 6.3) revealed the subfamily Braconinae and the tribe Aphrastobraconini along with genus *Digonogastra* and *Atanycolus*-group of genera, monophyletic. In this analysis the Aphrastobraconini seems to be basal while the tribe Braconini looks to be evolved from the tribe Aphrastobraconini. Thus the rooting becomes an obvious issue as the Bayesian analysis of nuclear 28S rDNA alone reveals Braconini basal and Aphrastobraconini evolving later. The main outcome of the analysis is the increase in the support of the clades as the posterior probabilities values have increased markedly. This tree has thirty clades supported with ≥0.95 posterior probability.

6.4.1. Divergence times

The oldest fossil of the cyclostome group *Protorhyssalus goldmani* Basibuyuk, Rasnitsyn, Achterberg, Fitton & Quicke (Basibuyuk *et al.,* 1999) suggests the root node to be at least 93 million years ago (MYA). This was further confirmed by another closely related fossil, *Protorhyssalodes arnaudi* Perrichot, Nel & Quicke with a very similar age and the oldest confirmed cyclostome fossil found to date (Perrichot, Nel & Quicke, 2008). Zaldivar-Riverón *et al*., (2008 a, b) have used *Aivalykus* Nixon (Doryctinae) as their MRCA which was followed in this study as well. The smoothing of 1.0 was used after cross-validation in r8s. The resulting ultrametric tree (fig. 6.4) from PL analysis clearly shows Braconinae to be almost half the age of Doryctinae, which explains why there are no preserved fossils of Braconinae in Baltic amber. The fossil of *Aivalykus dominicanus* Zuparko & Poinar (Zuparko & Poinar, 1997) has an estimated age of 15-20 MYA according to the age of Dominican amber (Kaplan *et al*., 1977: Ritzkwoski, 1997) thus dictating the age of Doryctinae around this period. Also there is evidence that true Doryctinae are found in Baltic amber (personal communication, Quicke).

Figure 6. 4. Ultametric tree obtained from PL analysis in r8s. Tribe Braconini is represented in red and Aphrastobraconini in blue. Node labels represents the age foe the clades.

Braconinae can be suggested to have an estimated age of 9-13 MYA with Aphrastobraconini having age of 7-8MYA and Braconini 8-9MYA. In case of Aphrastobraconini, the genus *Digonogastra* and the *Atanycolus*-group of genera seem to be evolved at about 7 MYA while the rest of the group has an estimated age of 5-6 MYA. In the tribe Braconini, the European *Bracon* appears to be evolving more recently at 5 MYA while

the rest of group have an age 7-7.5MYA.

6.5. Discussion

The use of combined data set has markedly increased the support of the relationships found in both the separate trees i.e. 28S plus morphology, NADH1 plus morphology. All the three data sets have found genus *Digonogastra* and *Atanycolus*-group of genera monophyletic while 28S and combined data sets have also found Braconinae monophyletic. The rooting of the phylogenetic tree was found to be different in all the three data sets thus showing that this subfamily suffers from the uncertainty of the evolutionary course taken by its members. The meaning of monophyly only has its meaning on a rooted tree (Smith, 1994). Although there are a lot of choices available at the subfamily level for the out-group there is no clear indication from the literature as to which would be most appropriate. There is an ongoing debate over the family Braconidae to assign the basal position to cyclostomes or to non-cyclostomes. Also there are subfamilies such as Aphidiinae whose position on the phylogenetic tree of Braconidae is unclear as a few studies place them between cyclostomes and non-cyclostomes while other assign them a basal position to all the other subfamilies (Quicke & van Achterberg, 1990: Van Achterberg & Quicke, 1992; Belshaw *et al*., 1998; Dowton *et al*., 2002; Shi *et al*., 2005; Pitz *et al*., 2005; Zaldivar *et al*., 2006).

One can still make guess about closely related taxa outside but still closely related to the ingroup (Sanderson & Shaffer, 2002). The divergent out groups (Johnson, 2001) and fully saturated sequences with respect to in-group (Wheeler, 1990; Maddison *et al*., 1992) may leave outgroup under the effect of long branch attraction rather than the phylogenetic signal (Smith, 1994). As it may fall next to a long branch of the in-group that seems to be the case for the phylogenetic tree from the combined analysis, thus making Braconini paraphyletic.

This effect can be normalized by addition of more outgroup taxa (Sanderson & Shaffer, 2002). The choice of the outgroup taxa can markedly effect the ingroup topology, even the terminal nodes that are far from the presumed root placement gets effected (Milinkovitch & Lyons-Weiler, 1998; Tarrio *et al*., 2000).

6.5.1. Divergence Times

The fossil record for the subfamily Doryctinae, which is also the MCRA for Braconinae, shows the group to be originated during mid to late Eocene (Zaldivar-Riverón *et al*., 2008a, b). The molecular dating estimates show that the Braconinae has a more recent origin (9-13MYA) than the Doryctinae (15-20MYA). Thus it has been calculated for the Braconinae originated in the Miocene era (23.03-5.33 MYA) (Cox &Moore, 1993; Jim, 2004). The expansion of grasslands and tropical broad-leaved forests to the equatorial belt during the Oligocene resulted in the formation of tropical forests of Africa and South East Asia where the abundance of this subfamily is observed. The Oligocene and Miocene boundary is not clearly set so it seems difficult to exactly assign Braconinae to Miocene era but surely this subfamily has originated with the tropical forests where its maximum presence can be observed (Quicke, 1987; Yu *et al*., 2005; Jones *et al*., 2009; Mahmood *et al*., 2010).

Chapter 7: General Discussion

The results obtained in this thesis have already been discussed in each chapter, therefore in this final discussion a brief overview of the main results is given. Also an insight into how future studies can improve this work, based on the experience gained from this study has been discussed. And finally some general conclusions are given drawn from this thesis.

The work in this thesis was an attempt to resolve the phylogenetic relationship between the genera of the subfamily Braconinae. The hypothesis that was postulated in the start of this thesis was to test the monophyly of the subfamily Braconinae and determine whether it is divided into three main tribes, Aphrastobraconini, Braconini and Coeloidini as suggested by some previous studies (Quicke, 1988a; Belshaw *et al*., 2001). Three different genes, nuclear 28S and mitochondrial CO1 and NADH 1, were used and the results obtained have shown that the nuclear gene has performed much better than the mitochondrial genes in resolving the phylogeny of Braconinae but the results are still far from conclusive. The possible reasons for the non-conclusive results are discussed further in this chapter.

The general discussion is divided into three major divisions, firstly the use and impact of nuclear 28S gene on the phylogeny reconstruction, secondly how the mitochondrial genes CO1 and NADH 1 have performed for this task and finally the combination of both nuclear and mitochondrial genes for phylogeny reconstruction along with the estimation of divergence age in Braconinae.

7.1. Description of two new genera and a new species

Besides the work on phylogenetics of Braconinae a small part of this thesis deals with

the description of two new genera and one new species thus showing diversity of this subfamily. Up until now, the Braconinae consists of 183 valid genera and more than 2,893 valid species (Yu *et al*., 2005; Jones *et al*., 2009; Mahmood *et al*., 2010) and it is not surprising that such a diverse subfamily proposes considerable difficulties in sorting it into meaningful groups.

Two genera *Synvipio* and *Combibobracon* Shaukat & Quicke, and a new species, *Serraulax hassani* Shaukat & Quicke, all from the Afrotropical region are described and illustrated. The genus *Synvipio* is very close to the genus *Vipio* but differs from it due to lack of a well developed midlongitudinal carinae and well developed dorsal carina on the 1st metasomal tergite, as it is striate with no distinct carina and other characters specified to it such as fused clypeal hair pencils are absent. The second genus *Combibobracon* keys close to genus *Liomorpha* but has a coarse propodeum, while it differs from *Vipio* due to lack of the pair of clypeal hair pencils. The new species of genus *Serraulax*, *S. hassani*, is close to *S. orientalis* but differs in midlongitudinal length of the 3rd metasomal tergite the two species plus color of pterostigma, legs and wings.

7.2. Efficacy of 28S rDNA Nuclear gene for the phylogeny of Braconinae

The phylogenetic tree obtained by utilizing the 28S rDNA, in chapter 3, has been found not very far from the traditional views about the phylogeny of the subfamily Braconinae. This nuclear gene has not been able to divide the Braconinae into two main tribes, the Aphrastobraconini and Braconini with the Coeloidini (*Coeloides*) in the middle. The *Compsobraconoides* group of genera was found monophyletic. *Braconella* was not found to be monophyletic. *Braconella* is a largely Afrotropical genus, that are small wasps and only genus of Braconinae with a toothed hind femur (Quicke, 1986). Within this tribe, several

genus groups were recovered including *Virgulibracon*-group of genera and *Myosoma*-group coming monophyletic while genus *Bracon* was found paraphyletic. On the other hand genera *Digonogastra, Sororarchibracon, Soter, Vipio* and *Pseudovipio,* belonging to the tribe Aphrastobraconini, were found monophyletic. The Euurobraconini was not recovered as monophyletic. The tribe Euurobraconini was erected by Ashmead (1900) and Quicke (1987) further added more genera based on the 3-4 digital teeth, reduced sculpture, anteriorly orientated hypoclypeus, a distinct mid-logitudinal carina on the first metasomal tergite. However, most of the characters linking these genera are plesiomorphic thus affecting the monophyly of this tribe. So, it can be said that the paraphyletic condition of this tribe found in this study is not unusual. The tribe Glyptomorphini was based on the blunt terminal flagellomeres by Tobias (1957) as well as setose parameral processes of the male genitalia while Bathyaulacini (erected by Quicke (1988)) was separated from Glyptomorphini due to characters like face with a medio-basal triangular area bordered by sulci, moderately short marginal cell of fore wing and presence of elongate rectal pads except fro genus *Odesia*, Glyptomorphini and Bathyaulacini were recovered monophyletic. Both of these tribes were also recovered monophyletic in study of Quicke (1988) and Chisti & Quicke (1995). These results were further confirmed by using the Goloboff's weighting, in order to down weight the poor performing characters. Both the trees were unable to recover Braconinae monophyletic but the tree without Goloboff's criteria behaved slightly better than the one where it was used.

One of the reasons for poor performance of Goloboff's criteria could be that the tree used with this weighting criteria, was only DNA tree while in case of the first tree morphology was added to the DNA to construct the phylogenetic tree. Addition of morphological characters has been reported to improve the resolution of a molecular phylogenetic tree and increase branch support (Banks & Whitfield, 2006). This nuclear gene

has performed rather well for the phylogeny of the Braconinae. Also, the combined molecular and morphological tree has the modified alignment of 28sr DNA based on the secondary structure and the modification of Zaldivar-Riverón *et al*. (2006). Thus this tree includes more phylogenetic information as an attempt has been made to include the unalignable regions of the sequences.

7.3. Efficacy of CO1 and NADH1 Mitochondrial genes for resolving the phylogeny of Braconinae

Chapter 4 and 5 were based on mitochondrial genes, CO1 and NADH1, respectively. Both these genes have performed poorly to recover the Braconinae monophyletic. The main result obtained from these genes was the division of the genus *Bracon* into groups largely based on their geographic region of origin. The main divisions for the genus *Bracon* were found to be into South American and Asian clades in case of CO1 and South American and European clades in case of NADH1. Both these genes have recovered good species level resolution for genus *Archibracon*, *Atanycolus* and *Iphiaulax.* Thus proving that mitochondrial genes behave better on species level in a phylogenetic tree.

One of the reasons behind the lower resolution is the lack of data for this level of phylogenetic study. There has been a debate for a long time about increasing the length of the gene or number of taxa. The outcome of the work on this issue suggests that both of these factors have equally good impact on the resolution of the phylogenetic tree. Thus a reasonable amount of data representation is necessary to achieve good results.

The mitochondrial genes used in this study belong to both good (CO1) and moderate (NADH1) classes of resolution power, for phylogenetics, as defined by Zardoya and Meyer (1996). So the argument that they may not be a good choice for phylogenetic studies can

easily be rejected. Another reason for the failure of CO1 could be the saturation at its 3rd codon position as this saturation may coincide with the age of the clades thus the signal gets lost, although it has been successfully utilized for higher levels in Hymenoptera. This saturation may not be good enough for the divergence level of the clades in the Braconinae.

7.4. Efficacy of combined use of Nuclear (28Sr DNA) and Mitochondrial (NADH 1) genes for resolving the phylogeny of Braconinae.

The results of the combined use of the both nuclear and mitochondrial genes have proved successful to divide Braconinae into the two main tribes, the Aphrastobraconini and Braconini. It shows Aphrastobraconini to be basal to the Braconini, which is opposite to the results of the 28S rDNA gene. As in that case Aphrastobraconini was coming as the evolved group while Braconini was coming as basal. So this presents us with the issue of rooting the phylogenetic tree as both the trees with single gene and combined gene analysis were rooted but differently, so the problem is which of the rooting is correct, if any. But definitely the combined result agrees with the traditional classification of the Braconinae. This combined study definitely needs more taxa representation as only 16 genera out of 183 described so far (Yu *et al*., 2005; Jones *et al*., 2009; Mahmood *et al*., 2010). The inclusion of more taxa can further enlighten the classification of Braconinae as to confirm the status of the traditional morphology based genus groups.

7.5. Future Work

The results of this thesis clearly identify the areas that can be improved in future studies to get a more comprehensive phylogeny of Braconinae. Following are some points that can

be considered before planning the future studies.

- More genera need to be added to the combined data set that will help to organize the tribes, subtribes and genus groups that can be achieved by increasing the number of specimens sequenced. All this needs combined efforts of the different research groups at international level.
- The study can definitely benefit from addition of more genes that are more suitable for the kind of divergence observed in this group at the subfamily level e.g. 18S rDNA, CAD (carbamoyl-phosphate sythetase-asparate transcarbamoylase-dihydroorotase), CPSase (carbamoylphosphate synthetase), ACC (acetyl-coenzyme A carboxylase).
- A set of individually designed primers targeting the specific parts of a gene effective for this particular subfamily can definitely increase the output sequences.
- A more comprehensive data set based on morphological characters (at species level) can help to resolve some questionable relations that can help to classify the subfamily in a better way.
- This study can benefit from sequencing of the complete mitochondrial genome in order to get much better resolution and insight.
- More work should be done to resolve the rooting issue of the Braconinae.

7.6. Conclusions

- The subfamily Braconinae is monophyletic, based on DNA as was strongly suspected from morphology.
- The subfamily Braconinae can be divided into two main tribes the Aphrastobraconini and the Braconini.

- These two tribes Aphrastobraconini and Braconini are monophyletic.
- The Genus *Bracon* is not monophyletic but can be divided into groups based on geography.
- The tribe Adeshini can be reduced to the level of subtribe in the tribe Braconini.
- The *Compsobraconides*-group of genera is monophyletic.
- The *Virgulibracon*-group of genera, based on Australian material, are monophyletic.
- The genera (*Physaraia* and *Aspidobracon* in Braconini and *Bathyaulax* and *Odesia* in Aphrastobraconini) with modified abdomens are closely related.

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